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DOCTORAL DISSERTATION

Preclinical platform for the translational research of the abuse potential of novel drug candidates

Doctoral dissertation submitted to obtain the degree of
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List of Abbreviations

AAALAC	Association for Assessment and Accreditation of Laboratory Animal Care
AAPM	American Academy of Pain Medicine
AMP	d-Amphetamine
AMPA	α-Amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid
APS	American Pain Society
ASAM	American Society of Addiction Medicine
ADHD	Attention Deficit Hyperactivity Disorder
ADME	Absorption/Distribution/Metabolism/Excretion
ALS	Alanine amino transferase
APA	American Psychiatric Association
APAC	Abuse Potential Advisory Committee
AST	Aspartate amino transferase
AUC	Area under the curve
AVMA	American Veterinary Medical Association
BBB	Blood brain barrier
BC	Before Christ
b.i.d.	bis in die (twice daily)
BRIC	Brasil, Russia, India, China
BST	British Society of Toxicology
BT	Body temperature
BW(G)	Body weight (gain)
cAMP	cyclic Adenosine monophosphate
CAS	Chemical Abstracts Service
CB1	Cannabinoid receptor 1
CCALC	Cross-Company Abuse Liability consortium
CDC	Centers for Disease Control and Prevention
C_0	Maximum (or peak) concentration that a drug achieves after intravenous drug administration
C_{eff} Hu	Human efficacious dose, expressed as C_{max}
C_{max}	Maximum (or peak) concentration that a drug achieves after a drug administration

CNS	Central Nervous System
CPDD	College on Problems of Drug Dependence
CPP	Conditioned Place Preference
CREB	cAMP response element-binding protein
CRF	Corticotropin Releasing Factor
CRO	Contract Research Organisation
CSA	Controlled Substances Act
CSS	Controlled Substances Staff
D1	Dopamine 1 receptor
DAT	Dopamine transporter
DDL	Drug Discrimination Learning
DEA	Drug Enforcement Administration
DIA	Drug Information Association
DMT	N,N-dimethyltryptamine (hallucinogen)
DNA	Desoxyribonucleic acid
DOI	*R*(−)-2,5-dimethoxy-4-iodoamphetamine (hallucinogen)
DOM	2,5-Dimethoxy-4-methylamphetamine
DSM	Diagnostic and Statistical Manual of Mental Disorders
EBPS	European Behavioural Pharmacology Society
ECNP	European College of Neuropsychopharmacology
ED_{50}	Effective dose for 50% of a group
EMA	European Medicines Agency
ER	Emergency Room
EU	European Union
FDA	Food and Drug Administration
FR	Fixed Ratio of Reinforcement
FRF	Sum of the responses made on either lever before the first reinforcement occurs
GABA	Gamma-AminoButyric Acid
GI	Gastrointestinal
GLP	Good Laboratory Practices
GnRH	Gonadotropin-releasing hormone
GPCR	G protein-coupled receptor
HA UN	Health Authorities of the United Nations

HPLC	High Performance Liquid Chromatography
HT	Hydroxytryptamine
ICH	International Conference on Harmonisation
IP	Intraperitoneal
IT	Information Technology
I.U.	International Unit
IUPAC	International Union of Pure and Applied Chemistry
IV	Intravenous
IV SA	Intravenous Self-administration
IWT	Government agency for Innovation by Science and Technology
Karg	Knowledge database for addiction related genes
KEGG	Kyoto encyclopedia of genes and genomes
LC- MS/MS	Liquid chromatography-tandem Mass spectrometry
LD_{50}	Median lethal dose
LSD	Lysergic acid diethylamide (hallucinogen)
MAPK	Mitogen-activated protein kinase
MDMA	3,4-methylenedioxy-N-methylamphetamine (Ecstasy)
MoA	Mechanism of Action
MPH	Methylphenidate
mRNA	messenger Ribonucleic acid
MS-access	Microsoft access
MSDS	Material Safety Data Sheet
N.Aacc.	Nucleus accumbens
NAM	Negative Allosteric Modulator
NAN	NMDA receptor Antagonist Neurotoxicity
NDA	New Drug Application
NE	Norepinephrin
NIAAA	National Institute on Alcohol Abuse and Alcoholism
Nm	Nanomolar
NMDA	N-Methyl-D-aspartate
NME	New Molecular Entity
NIDA	National Institute on Drug Abuse
NSDUH	National Survey on Drug Use and Health
OECD	Organisation for Economic Co-operation and Development

OD	Overdose
ONDCP	White House Office of National Drug Control Policy
OPRM	mu Opioid receptor gene
PAL	Preclinical Abuse Liability
PAM	Positive Allosteric Modulator
PFC	PreFrontal Cortex
PU	Polyurethane
QA	Quality Assurance
q.d.	quaque die (once daily)
R&D	Research & Development
RD	Repeated dose
REMS	Risk Evaluation Mitigation Strategy
SAMSHA	Substance Abuse and Mental Health Services Administration
SC	Subcutaneous
SD	Sprague-Dawley
SfN	Society for NeuroScience
SOP	Standard Operating Procedure
SPF	Specific Pathogen Free
SPS	Safety Pharmacology Society
SQL	Structured Query Language
SSRI	Selective Serotonin Reuptake Inhibitor
$T_{1/2}$	Half-life
THC	Tetrahydrocannabinol
TK	Toxicokinetic(s)
T_{max}	Time at which the C_{max} is observed
TO	Time-out
TRF	Tamper-resistant formulation
UN	United Nations
U.S.	United States (of America)
VAB	Vascular Access Button
VTA	Ventral Tegmental Area
WD	Withdrawal
WHO	World Health Organization

Tables and figures

I

Introduction on abuse liability of prescription drugs: mechanism of action and regulatory consequences

I.1. Setting the scene for abuse of prescription drugs

Since the dawn of history, mankind has been taking addictive substances to escape from reality. The earliest written notes go back to the ancient Mesopotamian culture around 3000 BC, where the alcohol in beer was discovered as an enjoyable way to pass the day. This resulted in a widespread addiction, accountable for many early deaths (Damerow, 2012). Narcotics like opium on the other hand were known all over the ancient cultures from 4000-5000 BC onwards, as useful agents to relief pain and to create pleasant feelings (Aggrawal, 1995). As for religious purposes, chewing coca leaves, containing cocaine, was an accepted ritual by the Inca's as early as 3000 BC, to allow contact with the spirits (Karch, 2005). A longstanding Andean tradition of ritual killing embraced the use of alcohol and cocaine during the months preceding the child's sacrifice. This was recently demonstrated by Wilson (Fig. I.1).

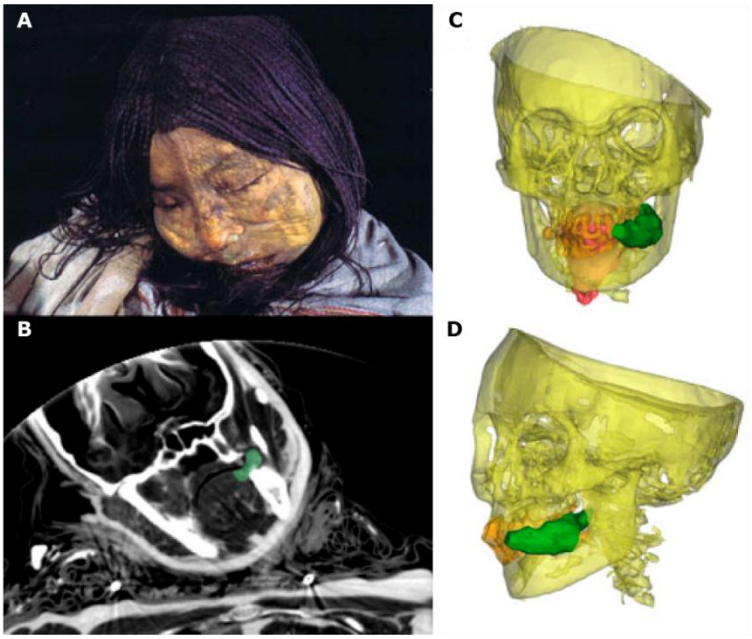
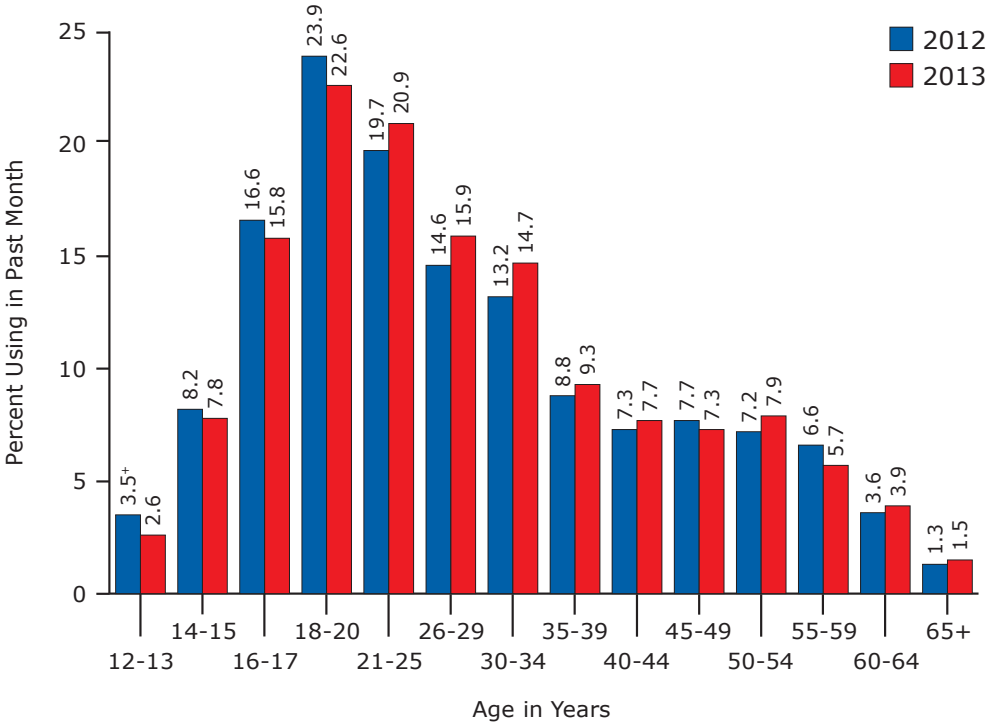


Fig. I.1. The Maiden's coca quid shown: (*A*) within the cheek in an anterior photograph of the Maiden's face; (*B*) Axial radiograph of the interior of the mouth shows the coca quid (green) held between the teeth; (*C* and *D*) Three-dimensional visualizations of the cranium (yellow), teeth (orange), tongue (red), and coca quid (green). Taken with permission from Wilson (Wilson, 2013).

Nowadays the use of illicit drugs (natural or chemical substances inducing feelings of euphoria and/or hallucinations) is widespread amongst all classes of society and through all age groups worldwide, although it appears to be most frequent in youth (16 to 29 years) (Fig. I.2). Well-known examples of classical illicit drugs include opioids (*example*: heroin), stimulants (*example*: cocaine), and hallucinogens (*example*: LSD).

Past Month Illicit Drug Use among Persons Aged 12 or Older, by Age: 2012 and 2013



+Difference between this estimate and the 2013 estimate is statistically significant at the .05 level.

Fig. I.2. Age distribution of illicit drug use (epidemiological data: yearly national survey of approximately 67500 persons of the civilian, nonintitutionalized population in the U.S.). Illicit drugs included marijuana/hashish, cocaine (including crack), heroin, hallucinogens, inhalants, or prescription-type psychotherapeutics used non-medically. Illicit drug use is relatively low at ages 12-13, but increases through the teenage years, reaching a peak between the ages of 18 and 20. Prevalence rates decline fairly rapidly among young adults in their 20s, and remain fairly stable from age 30 to age 44. Following a slight decline in the late 40s and early 50s, illegal drug use drops after age 55. (SAMSHA, 2013).

Geographically, opioids are most commonly abused in Asia and Europe, which is most probably due to the historical cultivation of Papaver plants through the ages. Cocaine counts for more than 50% of the abused drugs in South America (Fig. I.3, data based upon treatment request). In Africa and Oceania, cannabis is the most favorable drug of abuse. The North American population abuses primarily cocaine (33%), followed by cannabis (23%), opioids (21%) and amphetamines (18%).

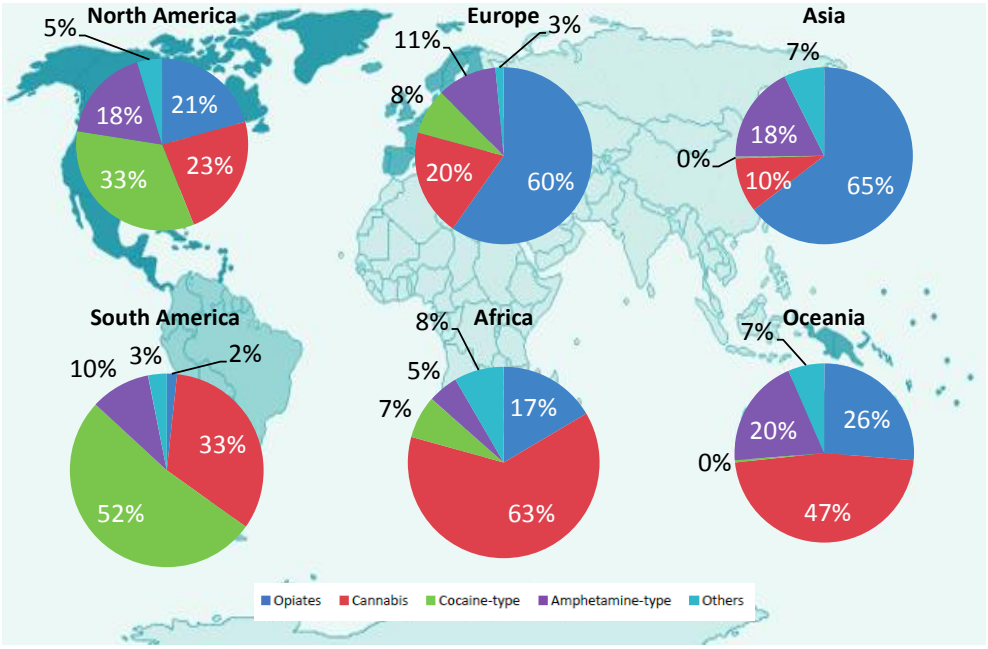


Fig. I.3. Worldwide use of illicit drugs [data based upon the number of problematic drug users (aged 15-64 years in 2007) who were registered for treatment]. The geographical differences of types of drugs being used is most probably historical and based upon the availability of the initial source (*amongst others*: herbal plants: Papaver, Coca, Cannabis). World Drug report (2009).

"Natural" but illegal products as paddo's (mushrooms containing the hallucinogenic psilocybine) and bufo's (toads releasing bufotenine via the skin) are also being used to create a different level of consciousness (Fig. I.4).

Fig. I.4. Natural illegal products: specific mushrooms and toads are known for their hallucinogenic properties if swallowed or licked at, respectively.

However a new trend has become fashionable: legally prescribed drugs, developed to cure a medical need, are now more and more being employed in nonmedical situations to create feelings associated with euphoria or feel-good, to enhance concentration or to enlarge stamina. Medical drugs that pass the blood-brain-barrier and, depending on the dose, actuate or interfere with the brain circuitry, can demonstrate such effects. These types of drugs cover various chemical classes including pain killers, stimulants, sedatives, antidepressants, cardiovascular drugs, and asthma medications (SPS, 2010). Unfortunately it is often assumed that prescription drugs are much safer than the classically known illicit drugs like heroin and cocaine. Received from friends or relatives, people initially try the medicinal drugs at the prescribed dose. As they experience the centrally derived effects, higher and more frequent doses will be taken, insidiously leading to substance abuse or addiction. Therefore prescription drugs are being considered illicit drugs in the context of misuse.

This increasing and sustained nonmedical use of prescription drugs is worrisome, in particular in young people (12-24 year) in the (Western) world. In the U.S. prescription drugs are second to marijuana as the drug of choice in substance abuse for today's teens (White House ONDCP, 2014) and include, amongst others, stimulants (Adderal®), pain killers (Vicodin®), and anti-coughs (dextromethorphan) (Fig. I.5).

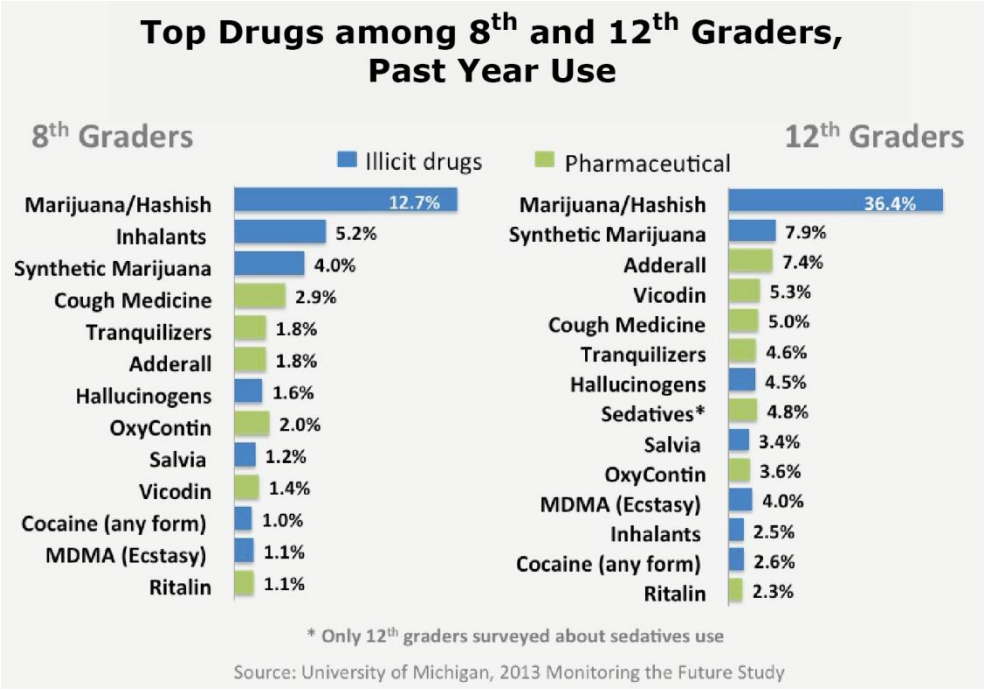
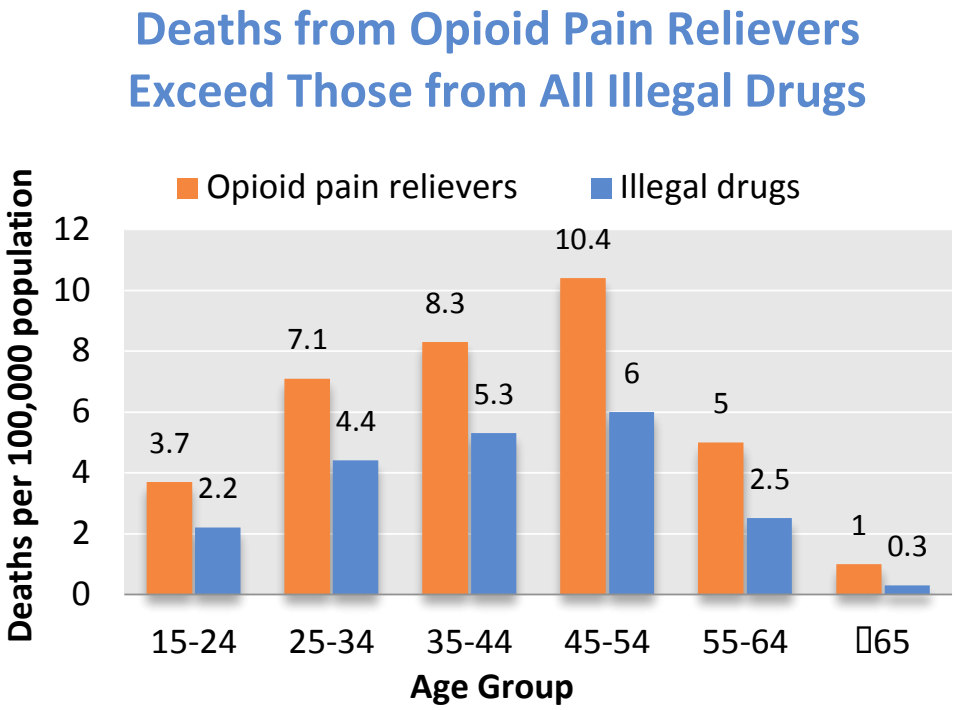


Fig. I.5. Top drugs use by adolescents in the U.S. Of the most commonly drugs used by 8th (13-14 years of age) to 12th-graders (17-18 years of age), prescription drugs are prominently present. NIH, 2013.

Moreover, the lethality induced by misuse of prescription drugs is now much higher than the mortality rate associated with the use of illegal drugs. Data from the Centers for Disease Control and Prevention (CDC) demonstrated that 22.134 (or 58%) of the 38.329 drug overdose deaths in 2010 were related to pharmaceuticals, of which 16.651 (or 75%) involved prescription opioid analgesics or prescription painkillers and 6.497 (or 30%) involved benzodiazepines (CDC, 2014). Deaths associated with excessive use of or addiction to pain killers included, *amongst others*, paracetamol, the combination acetaminophen/hydrocodone, oxycodone, salicylate, fentanyl and morphine (Fig. I.6, CDC, 2014). Also for intranasal administration of methylphenidate (prescribed for treatment of ADHD) at least 3 death cases in teens have been reported in the nineties (Morton, 2000).



Source: CDC, Morbidity and Mortality Weekly Report, 60(43): 1489, 2011.

Fig. I.6. Overview of the incidence of deaths caused by opioid pain killers per age group (intentional deaths or overdose). CDC, 2014 (data from 2012).

These reports on the inappropriate use of medicines have raised an increasing awareness on the risks related to non-medical use of prescription drugs, and in particular to their potential to develop abuse or addiction in humans.

In order to further understand the mechanism of addiction and the insult of substances of abuse, first some terms will be defined.

I.2. Definitions of addiction/abuse potential

The term addiction is a widely used term, comprising both behavioural addiction such as compulsive gambling and stealing, and dependence on CNS-active substances like cocaine, opioids, alcohol and prescription drugs. The clinical definitions as listed below are specifically directed at the latter category, *i.e.* the addiction to CNS-active substances.

The Diagnostic and Statistical Manual of Mental Disorders (DSM-IV) of the American Psychiatric Association (APA, 2000) uses the term "substance use disorders" to characterize illnesses associated with drug use. There are two broad categories: substance dependence and substance abuse. Both are associated with a maladaptive pattern of substance use that leads to clinically significant impairment.

Substance Dependence is defined as ”a chronically relapsing disorder that is characterized by a compulsion to seek and take drug or stimulus, continued use despite harmful consequences, with loss of control in limiting intake, and emergence of a negative emotional state (e.g. dysphoria, anxiety, irritability) when access to the drug or stimulus is prevented”. The DSM does not use the term addiction. (APA, 2000; Koob, 2008a; Koob, 2010).

The term Substance Abuse only includes the harmful consequences of repeated use and can thus not be used as a synonym for use or misuse of substances.

Remark: To avoid confusion on the used terminology in the DSM-IV, the recently released DSM-V (APA, 2013) has combined the categories of substance dependence and substance abuse into one single term “substance use disorder”.

Several organizations have slightly adopted this primary description. According to the consensus document on definitions related to the use of opioids for the treatment of pain [AAPM-American Academy of Pain Medicine, APS-American Pain Society, and the ASAM-American Society of Addiction Medicine, 2001), addiction is defined as “a primary, chronic, neurobiologic disease, with genetic, psychosocial, and environmental factors influencing its development and

manifestations. It is characterized by behaviors that include one or more of the following: impaired control over drug use, compulsive use, continued use despite harm, and craving".

The National Institute on Drug Abuse (NIDA) defines addiction as "a chronic, relapsing disease characterized by compulsive drug seeking and use despite harmful consequences as well as neurochemical and molecular changes in the brain". NIDA's use of the term addiction roughly corresponds to the DSM definition of substance dependence, but favors the term "addiction" rather than dependence, to avoid confusion with the condition of physical dependence (NIDA, 2012).

Physical dependence is defined as a state of adaptation that is manifested by a drug class specific withdrawal syndrome that can be produced by abrupt cessation, rapid dose reduction, decreasing blood level of the drug, and/or administration of an antagonist (AAPM, 2001). Many drugs, which are not associated with drug abuse, are known to develop physical dependence upon cessation of (chronic) dosing. Examples hereof include corticosteroids, antidepressants, and cardiovascular medicines (beta blockers, alpha-2 adrenergic drugs). For these drugs, the treatment needs careful tapering in order to avoid an abstinence syndrome. This withdrawal or abstinence syndrome includes signs of psychic and physical nature that are characteristic for each drug type (Eddy, 1965) and may involve anxiety, sweating, restlessness, gastro-interstinal dysfunction and so on.

Thus physical dependence is *not* equivalent to dependence or addiction and may occur with the chronic use of any substance, legal or illegal, even when taken as prescribed (NIDA, 2012). However, physical dependence can lead to craving for the drug to relieve the withdrawal symptoms.

Other terms that are often mentioned in literature on addiction are tolerance and sensitization. Tolerance is defined as a decrease in response to a repeated dose of a drug substance.

Metabolic or pharmacokinetic tolerance is an adaptation of the body by biological changes to the presence of the substance, to ensure a more rapid elimination of that substance from the body. An example hereof is the metabolic tolerance to alcohol. Repeated dose administration induces a higher production of the liver enzyme alcohol dehydrogenase, allowing a faster metabolism of alcohol, as such adding to the clearance of alcohol from the body. This metabolic tolerance does not contribute to drug dependence or to withdrawal symptoms.

Functional or pharmacodynamic tolerance reveals an aspect of neuroadaptation, manifested by a decrease in sensitivity of the central nervous system to a drug substance (EMA, 2006) due to changes at the synaptic level (Littleton, 1983). A well-known example hereof is the functional tolerance to alcohol: the response to alcohol (sensitivity) in the central nervous system is reduced, resulting in an attenuated effect. To experience a similar effect, the alcohol dose needs to be increased (NIAAAS, 1995). These neuronal adaptations are protective mechanisms and occur to compensate for the increased alcohol concentration in the brain with chronic alcoholism.

Functional tolerance may contribute to the development of dependence but is not regarded as a robust marker.

Sensitization to active substances is defined as an increased effect of the substance following repeated doses (or the opposite of tolerance). In particular behavioural sensitization or psychomotor sensitization can be observed in animals treated with stimulants, as evidenced by enhanced locomotor activity (Wise, 1993). For these types of drugs, an increase in the mesolimbic dopamine transmission, associated with the behavioural sensitization could be demonstrated (Kalivas, 1993; Stewart, 1993; Vanderschuren, 2009). These drug-induced neuro-adaptations can lead to incentive salience (motivational wanting), hence contributing to the relapse to drug seeking behavior. This is also called the incentive sensitization theory (Robinson, 2008; Steketee, 2011).

Psychic (Psychological) dependence is defined as "a condition in which a drug produces 'a feeling of satisfaction and a psychic drive that require periodic or continuous administration of the drug to produce pleasure or to avoid

discomfort' (Eddy, 1965). To date, this term is no longer employed and replaced by substance dependence.

Abuse potential of a CNS-active substance comprises a complex of alerts that are indicative of possible physical and/or psychological dependence of this substance for humans.

Misuse indicates the use of prescription drugs in a non-medical environment.

In this manuscript, addiction refers to the clinical state of substance abuse, whereas for preclinical investigation the term abuse potential of a CNS-active substance is being employed.

I.3. Misuse of prescription drugs: implications for Pharma

The incremental non-medical use of prescription drugs and their concomitant risk for dependence behaviour has created an increased concern on the abuse potential of CNS-active drug candidates by both the Pharma Industry and the drug licensing authorities. The latter translated their responsibility towards the protection of human health into more stringent guidances, which were released during the past recent years [the EMA guideline (EMA, 2006); the ICH guideline (ICH, 2009), the FDA draft guidance (FDA, 2010) and the subsequent FDA's decision tree (Bonson, 2011)]. These guidances are discussed in detail under section I.4. Regulatory framework.

For the Pharma Industry a major area of research was pushed forward, in particular within the preclinical safety evaluation area where drug abuse liability testing of all compounds in development (including their major CNS-active metabolites), exerting an activity in the brain (regardless of therapeutic indication) has become mandatory, according to the regulatory requirements of drug licensing authorities on testing of abuse potential.

To date four behavioral animal tests are being employed to prepare a Preclinical Drug Abuse Liability Assessment, each investigating various aspects of abuse potential.

The non-precipitated withdrawal test studies the physical dependence potential of a CNS-active drug, that might occur when drug treatment is abruptly terminated.

The conceivably discriminative properties of a test compound towards the known effects of psycho-active reference drugs are determined in a drug discrimination learning model.

Measures of direct and indirect reinforcing properties and of rewarding assets are investigated through an intravenous self-administration and/or a conditioned place preference model.

According to the regulatory requirements of drug licensing authorities, the rat is considered the preferred species to perform drug abuse liability studies (EMA, 2006; ICH, 2009; FDA, 2010).

All tests also need to be performed in compliance with the GLP regulations (OECD, 1998).

The translational approach of this preclinical abuse liability testing involves a thorough understanding and knowledge of the (neuro)pharmacology and the toxicological profile of the test compound and of the psycho-active reference drugs. Indeed, the selection of a proper dose range in terms of preclinically testing a several-fold of the human efficacious dose (C_{eff} Hu), and the correct choice of a known psycho-active reference drug relevant to the receptor binding profile of the test compound, are the key of a successful assessment acceptable for the regulatory authorities. The complexity of testing new CNS-active compounds in development for possible abuse potential often lies within the off-target pharmaco-toxicological profile, as the mechanism of action of the majority of these New Molecular Entities (NMEs) is distinct from that of the known classes of abused drugs, defined by the regulatory authorities worldwide (Table I.1).

SPECIFIC CLASSES OF ACTIVE SUBSTANCES	
FDA	**EMA**
Opioids	Opioids
Stimulants	Stimulants
Depressants	Sedatives and anxiolytics
Cannabinoids	Cannabinoids
Hallucinogens	NMDA-antagonists
	Nicotine-like active substances

Table I.1: Classification of substances of abuse according to the EMA (2006) and FDA (2010) guidances on abuse potential.

This implies that the construct of the preclinical abuse liability assessment, in particular with regard to the relevance of the reference drug versus the NME is not straight forward and might even impose more studies with different reference drugs.

The requirements of drug licensing authorities on testing abuse potential have been outlined rather vaguely in the various guidances, thus allowing a case-by-case approach for each NME. Nevertheless, a well-considered communication is needed between the sponsor (pharmaceutical company) and the authorities, to receive agreement on the proposed types of studies, the selected dose range and the choice of reference drugs in the preclinical studies.

This Abuse Liability Assessment aims, through the translation of robust and predictive preclinical data on the abuse potential of new medicines with a novel mechanism of action, to protect patients whilst being treated with these medicines, and to discourage healthy users from using these prescription drugs in nonmedical situations (off-label and creative use). The latter involves support from the chemists and formulation experts, to modify the product form into a final commercial product, using abuse-deterrent formulations which are less suitable to misuse. It also implies a demand for support from the Regulatory staff to eventually prepare for scheduling of the final product based upon its abuse potential properties, thus influencing the prescription procedures and subsequently leading to a restricted availability of the drug product.

The timelines to propose and to perform these preclinical studies are mostly situated after Phase IIA, when proof of concept is confirmed and an efficacious human dose is determined. Preclinical abuse liability data need to be preferably available at the end of phase IIB, in order to discuss, at the end-of-phase 2 meeting with the FDA, the possible need of a human liability study to be conducted in phase III. Finally, with regard to the biological relevance of the outcome of the preclinical tests, a broad expertise is needed to thoroughly evaluate the findings, in view of both scientific and regulatory perspective, as well as in-depth knowledge of the scheduling strategy of similar comparators on the market.

I.4. Regulatory framework

I.4.1. Regulatory guidances on the investigation of abuse potential

The most stringent regulatory documents intended to provide guidance on the investigation of abuse potential of new CNS-active molecular entities comprise the EMA guidance (2006), the international ICH M3(R2) guideline (2009) and the FDA guidance (2010).

The EMA Guideline on "The Non-Clinical Investigation of the Dependence Potential of Medicinal Products" specifically focuses on the non-clinical assessment, using a two-tiered approach to investigate the dependence potential of new CNS active substances (Fig. I.7).

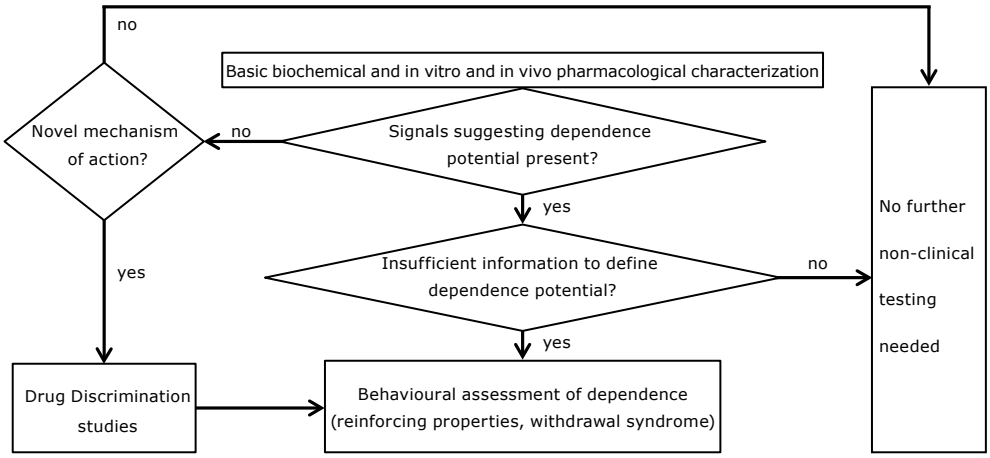


Fig. I.7. Decision tree for executing nonclinical tests to determine the dependence potential of CNS-active compounds. These tests include the investigation of the physical dependence (withdrawal syndrome), the discriminative stimulus properties (Drug Discrimination studies) and the reinforcing properties of new drug candidates. From the EMA guideline (2006).

The first tier comprises *in-vitro* and *in-vivo* pharmacological investigations, primarily to explore if the compound crosses the blood-brain barrier (BBB), and if there is interaction with the specific receptors known to be involved in the development of abuse (*amongst others*, dopamine- or opioid receptors). The functional receptor occupancy is also studied in vivo through dose- and time-

occupancy relationships after systemic administration in rats. Other neuropharmacological tests might involve microdialysis to determine the chemical components of the fluid in the extracellular space of tissues, or models to investigate motor activity (*example*: rotarod test), pain nociception (*example*: hot plate test) or head twitch (*example*: DOI test for studying the hallucinogenic activity at the 5HT2A receptors). For a drug candidate, this fundamental information is classically obtained during the phase prior to its selection and further entrance into Drug Development.

If these data indicate an alert for possible abuse potential, the second tier will be initiated, comprising behavioural pharmacology studies to further investigate abuse liability.

A similar stepwise approach is suggested by the FDA in its "Guidance for Industry: Assessment of Abuse Potential of Drugs; Draft Guidance (2010)" (Fig.I.8).

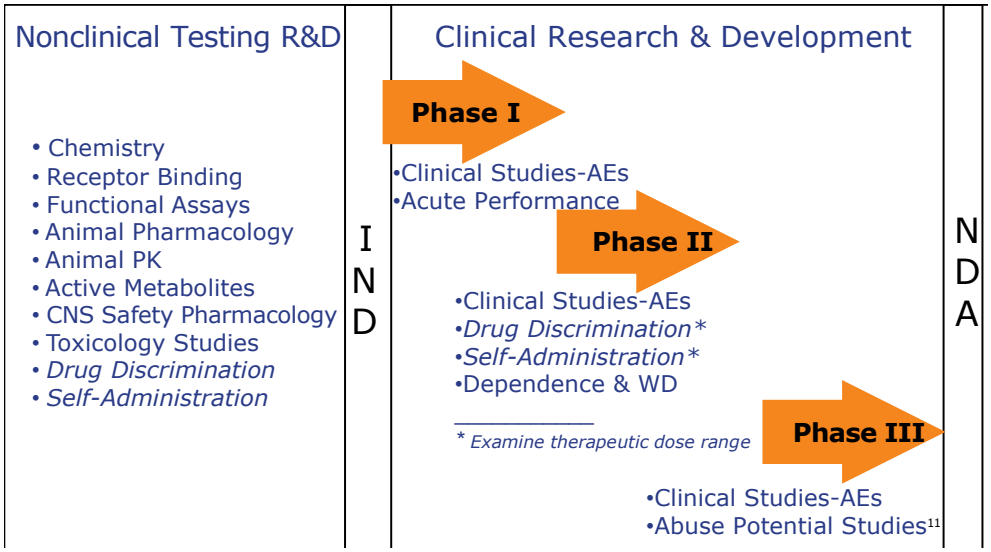


Fig. I.8. U.S. stepwise approach for testing the abuse potential of new CNS-active drug candidates. From Dr. M. Klein (2011).

However, this guidance covers besides nonclinical, also the clinical and post-marketing aspects of an abuse potential assessment, including advice on the

design and conduct of appropriate studies and proposal for scheduling. In addition, a FDA Draft Decision Tree for Assessment of Abuse Potential was made public in 2011 (Bonson, 2011). This document presents an overview of the decision points, the type of preclinical studies to perform and timelines hereof within the Drug Development plan of NMEs as visualised in the Roadmap (Fig. I.9).

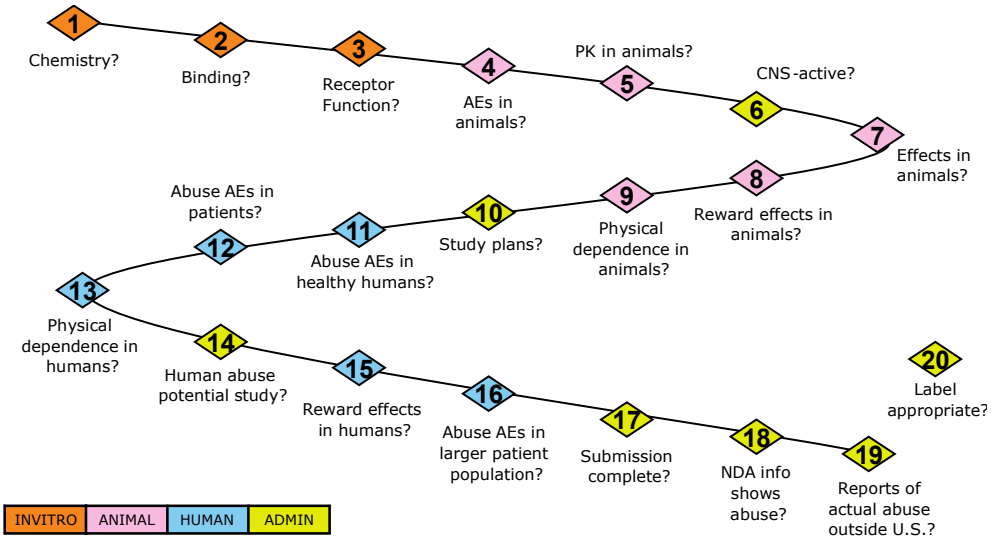


Fig. I.9. Abuse potential Assessment Decision Tree: Roadmap to assess the investigation of abuse potential of new CNS-active drug candidates. From Dr. M. Klein (2011).

Lastly, the International Conference on Harmonisation (ICH) of Technical Requirements for Registration of Pharmaceuticals for Human Use released a guidance in 2009 in which was stated that "the evaluation of abuse liability should be considered for drugs that are distributed into the brain and produce central nervous system activity, regardless of therapeutic indication".

The guidances slightly differ in their definitions and focus: in Europe (EMA, 2006), dependence potential of an active substance is defined as the propensity, as a consequence of its pharmacological effects on physiological or psychological functions, to give rise to a need for repeated doses of the active substance to "feel good" or to avoid "feeling bad." According to the FDA guidance, abuse potential refers to CNS-active drugs that are being used in nonmedical situations for the positive psychoactive effects they produce, such as a pleasant state

(euphoria) or the relief of distress. The EMA guidance is thus relying more on the protection of the patient when using drugs with an abuse potential within a medical environment, whilst the FDA is more focused on the nonmedical use of therapeutic drugs in a healthy population.

I.4.2. Regulatory framework for scheduling CNS-active drugs with abuse potential

I.4.2.1. Global framework

The global regulatory framework is complex, as often countries have implemented the requirements regarding the control of drugs with abuse potential differently.

The World Health Organization (WHO) and the Health Authorities of the United Nations (UN) follow the international conventions of 1961 (Single Convention on Narcotic Drugs) and of 1971 (Convention of Psychotropic Substances), allowing the participating countries to pass laws to enforce the provisions as stated in these conventions through national laws. The European Union (EU) does not have its own scheduling system and typically follows the recommendations as stated in these conventions and in the WHO guidelines.

The original conventions (1961, 1971) provide four schedules for both narcotic and psychotropic substances. The Single Convention's schedules of narcotic drugs range from most restrictive to least restrictive, in this order: Schedule IV (which is a stricter subset of schedule I), Schedule I, Schedule II, Schedule III (Table I.2).

Schedule IV	Stricter subset of schedule I The drug, which is already in Schedule I, is particularly liable to abuse and to produce ill effects, and such liability is not offset by substantial therapeutic advantages
Schedule I	The substance is liable to similar abuse and productiv e of similar ill effects as the drugs already in Schedule I or Schedule II, or is convertible into a drug
Schedule II	The substance is liable to similar abuse and productiv e of similar ill effects as the drugs already in Schedule I or Schedule II, or is convertible into a drug
Schedule III	The preparation, because of the substances which it contains, is not liable to abuse and cannot produce ill effects; and the drug therein is not readily recoverable

Table I.2. Scheduling of drugs with abuse potential according to the Single Convention on Narcotic Drugs (1961).

In 2002, a European Parliament report informally described the international Schedules as follows (EU Parliament, 2002): Schedule I includes drugs claimed to create a serious risk to public health, whose therapeutic value is not currently acknowledged by the Commission on Narcotic Drugs. It includes synthetic psychedelics such as LSD in addition to natural psychedelics like N,N-dimethyltryptamine (DMT) isomers. MDMA, commonly known as Ecstasy, also falls under this category. Schedule II includes stimulants of the amphetamine type, deemed to have limited therapeutic value, as well as some analgesics such as morphine. Dronabinol, which is a tetrahydrocannabinol (THC) isomer, is also included. Schedule III includes barbiturate products (with fast or average effects, and object of serious abuse even though useful therapeutically), flunitrazepam and some analgesics like buprenorphine. Schedule IV includes some weaker barbiturates (like phenobarbital), benzodiazepines (except flunitrazepam), and some weaker stimulants. More information can be found in the "Yellow list" of Narcotic Drugs under International Control, prepared by the International Narcotics Control Board (INCB) in 2011.

I.4.2.2. Scheduling drugs with abuse potential in the U.S.

In the United Sates (U.S.), the framework for abuse control is regulated by the Drug Enforcement Administration (DEA), a U.S. federal law enforcement agency under the U.S. Department of Justice, which presides over the observance of the Controlled Substances Act (CSA) (DEA, 2014). The DEA agency evaluates the

scientific evidence relevant to scheduling of a new CNS-active drug candidate, based upon an eight-factor analysis (Table 3).

1.	Actual or relative potential for abuse
2.	Scientific evidence of pharmacological effects
3.	State of the current scientific knowledge concerning the substance
4.	Historical and current pattern of abuse
5.	Scope, duration, and significance of abuse
6.	Potential or actual risk to public health
7.	Potential of the substance to produce psychic or physiological dependence liability
8.	Whether the substance comprises a precursor of a substance already controlled.

Table. I.3. Components of the eight-factor analysis as specified in the U.S. Food and Drug Code of Federal Regulations, Title 21, 811 (c). (Calderon, 2014).

This analysis is then included in the New Drug Application (NDA) file of the drug candidate that was previously submitted to the FDA. The NDA file is further evaluated by the FDA's Controlled Substances Staff who uses the DEA's 8-factor analysis to assist them in their final scheduling decision.

In the U.S. the various schedules implement different levels of control over substances with abuse potential, with schedule I having the most control while schedule V has the least (Table I.4).

Schedule I	• The drug or other substance has a high potential for abuse. • The drug or other substance has no currently accepted medical use in treatment in the United States. • There is a lack of accepted safety for use of the drug or other substance under medical supervision.
Schedule II	• The drug or other substance has a high potential for abuse. • The drug or other substance has a currently accepted medical use in treatment in the United States or a currently accepted medical use with severe restrictions. • Abuse of the drug or other substances may lead to severe psychological or physical dependence.
Schedule III	• The drug or other substance has a potential for abuse less than the drugs or other substances in schedules I and II. • The drug or other substance has a currently accepted medical use in treatment in the United States. • Abuse of the drug or other substance may lead to moderate or low physical dependence or high psychological dependence.
Schedule IV	• The drug or other substance has a low potential for abuse relative to the drugs or other substances in schedule III. • The drug or other substance has a currently accepted medical use in treatment in the United States. • Abuse of the drug or other substance may lead to limited physical dependence or psychological dependence relative to the drugs or other substances in schedule III.
Schedule V	• The drug or other substance has a low potential for abuse relative to the drugs or other substances in schedule IV. • The drug or other substance has a currently accepted medical use in treatment in the United States. • Abuse of the drug or other substance may lead to limited physical dependence or psychological dependence relative to the drugs or other substances in schedule IV.

Table I.4. U.S. scheduling of drugs based upon their potential for abuse. (FDA, 1970).

Schedule CI, the most restrictive schedule, contains substances which have no medicinal use and for which prescriptions cannot be written. Examples hereof include LSD and ecstacy.

Examples of substances controlled according the other schedules include cocaine and MPH (schedule CII); the dissociative anaesthetic ketamine (schedule CIII); the combination acetaminophen/proxyphene as painkiller (schedule CIV); and the anticonvulsant pregabalin (schedule CV).

I.4.2.3. Scheduling drugs with abuse potential in the EU

The EU scheduling system is different from that of the U.S. If a compound is listed in the UN Conventions, the EU procedure will follow the UN conventions. Individual countries can schedule a new psychoactive/narcotic substance if it is not listed on the UN Conventions. The length of time to schedule a substance and levels of scheduling varies between nations.

I.4.2.4. Canada, the BRIC countries and Japan

These countries either follow the 1961 and 1971 conventions or have these translated in their own legislations.

I.4.3. Implications of scheduled drugs for the Pharma Industry

Overall, the time needed to add a new substance to the UN conventions- or the U.S. scheduling system is extremely prohibitive to the timelines for launching a compound on the market and can be as long as 16 months once the submitted file has been approved by the FDA but before the scheduling is final (Fig. I.10). This delay has its consequences with respect to patent protection and subsequent shelf life of the molecule.

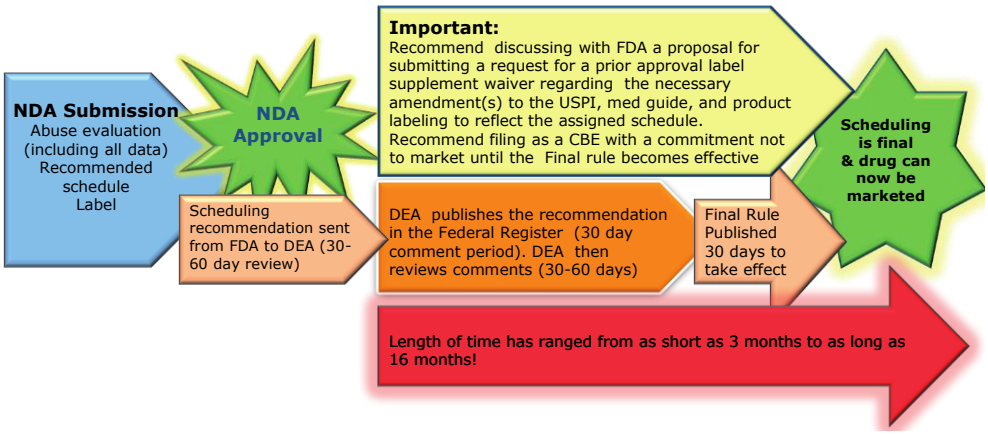


Fig. I.10. Scheduling Procedure in the U.S. after NDA submission involves a prolonged timeframe resulting in a delayed launch of the product on the market. USPI: United States Prescribing Information (FDA labelling); CBE: changes being effected (type of labeling supplement that can be sent to FDA for any change to a product that has a moderate potential to have an adverse effect on the safety or effectiveness of the product).

A marketed scheduled drug has also an impact on the business model, as these types of drugs are subject to restrictive use within a controlled environment (*i.e.* prescriptions: only by specialists or in hospitals), driven by a firm Risk Evaluation Mitigation Strategy (REMS), that needs to ensure that the benefit of the product outweighs the risk. Such a REMS may include a medication guide and/or patient package insert, a communication plan (clinician - patient), and elements to assure safe use (*example*: patient monitoring). It also includes registered follow-ups during post-vigilance up to seven years after approval.

I.5. Development of substance abuse and addiction

Substance abuse and addiction do not appear as a sudden condition, but rather develop following a cascade of emotional, behavioural, neurological and pathophysiological responses (Wise, 2014). However, the experience of one single, but strong positive reinforcement might be sufficient to initiate repetitive drug taking, leading to a sustained drug use. This latter can result in compulsive drug-taking behavior, a component of addiction (Koob, 2010; Wise, 2014). This process may occur in particularly in vulnerable persons or in individuals at periods during which the psychological balance is disrupted (*examples*: in cases of death, redundancy, divorce).

How a single drug administration can evolve to true drug addiction is subject of current research in the field of drug abuse, and comprises genetic, epigenetic, cellular and molecular research of the neurobiology to better understand this disease (Koob, 2010). A brief overview of these areas of research is given in the next sections, and illustrated each with some examples of current trends being followed and/or employed.

I.5.1. Neurocircuitry of substance abuse and addiction

The mesocorticolimbic dopaminergic system in the brain is the crucial neuronal pathway involved in substance abuse and addiction through reward/antireward and emotional memory. The most significant anatomical sites involved are the nucleus accumbens (N.Acc.), the ventral tegmental area (VTA), the amygdala and the prefrontal cortex (PFC) (Koob, 1992; Nestler, 2005; Camí, 2003) (Fig. I.11).

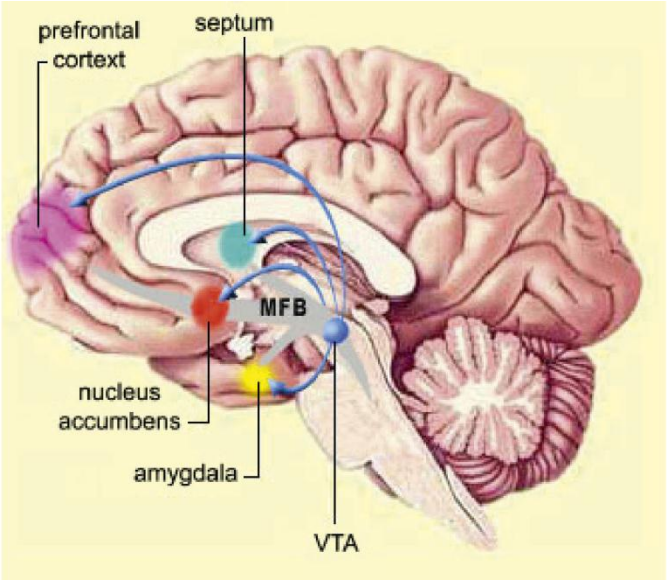


Fig. I.11. The mesocorticolimbic dopaminergic pathway involved in drug abuse includes several neuroanatomical sites. MFB: median forebrain bundle; VTA: ventral tegmental area.

The *mesolimbic or reward pathway* is an important neuronal network, responsible for pleasurable effects. Primary rewards or reinforcers resulting in feelings of pleasure include eating and sexual contact, necessary to survive. Secondary rewards or reinforcers include, *for example*, monetary rewards and emotional satisfaction after a winning game, and are learned from experience.

The primary neurotransmitter involved in the mesolimbic or reward pathway is dopamine, which embraces about 80% of the catecholamines present in the brain. Other neuromodulators or neurotransmitters defined to play a major role in the development of substance abuse and drug-induced addiction via direct or indirect interference in the mesocorticolimbic dopaminergic system include opioid peptides (enkephalins, dynorphin), gamma-aminobutyric acid (GABA), serotonine, and endocannabinoids. The stress-system neurotransmitters CRF, norepinephrine, neuropeptide Y and nociceptin are also involved in the mechanism (Koob, 2005 and 2011) (Fig. I.12).

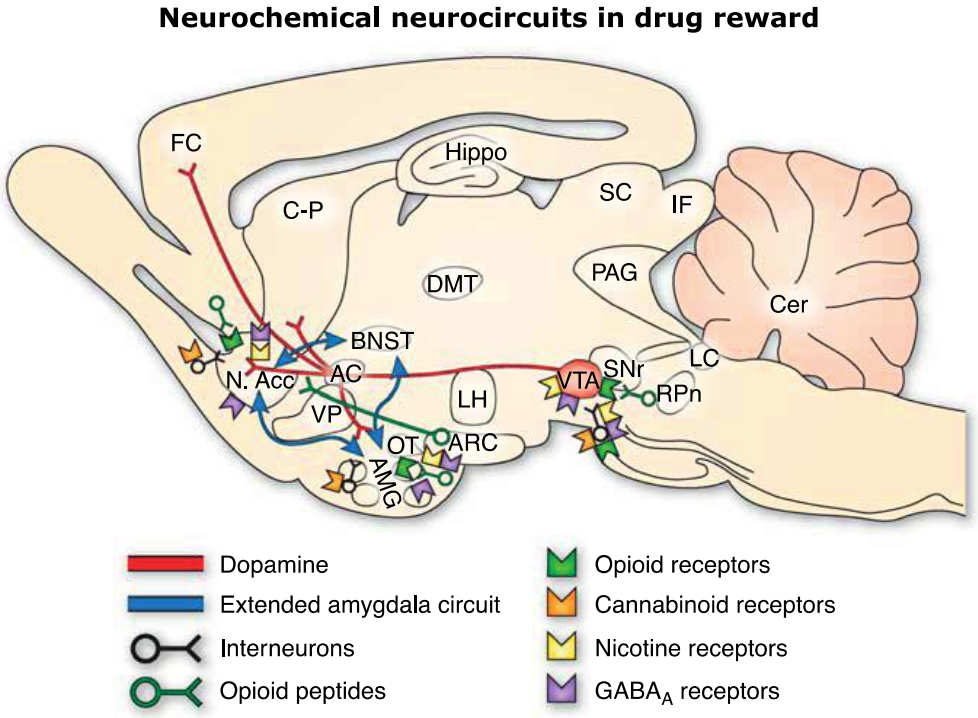


Fig. I.12. Sagittal section through a representative rodent brain illustrating the pathways and receptor systems involved in drug reward. FC: frontal cortex; Hippo: hippocampus; C-P: caudate-putamen; SC: superior colliculus; IF: inferior colliculus; DMT: dorsomedial thalamus; PAG: periaqueductal gray; BNST: bed nucleus of the stria terminalis; N.Acc.: nucleus accumbens; AC: anterior commissure; VP: ventral pallidum ; LH: lateral hypothalamus; VTA: ventral tegmental area ; SNr: substantia nigra pars reticulata; RPn: reticular pontine nucleus; LC, locus coeruleus ; ARC: arcuate nucleus; OT: olfactory tract; AMG: amygdala; Cer: cerebellum. Taken with permission from Koob (2010).

Dopaminergic neurons from the VTA projecting to the nucleus accumbens, the olfactory tubercle (ventral striatum) and parts of the limbic system increase the extracellular dopamine level in the nucleus accumbens and amygdala whenever an unexpected reward is met. The mesolimbic pathway is also modulated after acute exposure to all drugs of abuse (Feltenstein, 2008; Koob, 2010), increasing the extracellular dopamine concentration, and as such accountable for the acute reinforcing or pleasurable effects of drugs of abuse (Rouge-Pont, 2002) (Fig. I.13).

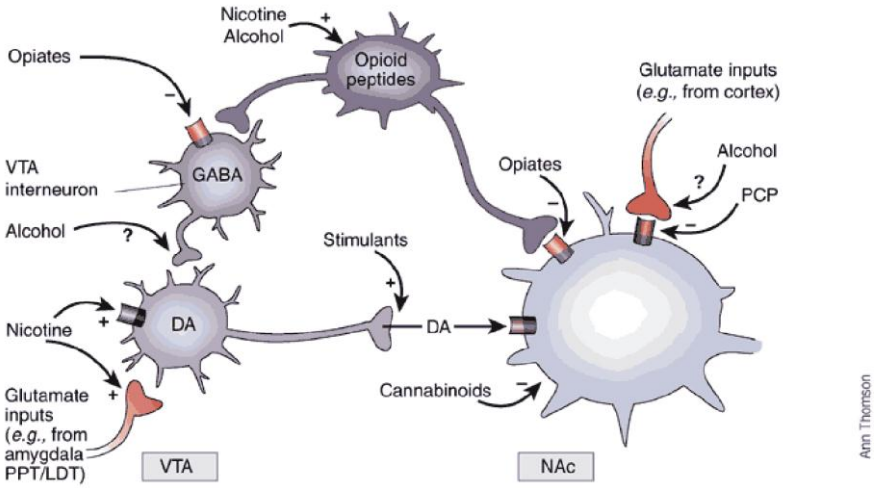


Fig. I.13. Acute actions of drugs of abuse on the VTA-N.Acc. via direct (*example*: stimulants) or indirect (*example*: opioids) increases of the extracellular dopamine levels. PPT/LDT, peduncular pontine tegmentum/lateral dorsal tegmentum. PCP: phencyclidine. Taken with permission from Nestler (Nestler, 2005).

The increase in extracellular dopamine levels in the nucleus accumbens can be directly or indirectly affected. A direct effect on the dopamine concentration, as happens with the intake of cocaine and amphetamine, occurs through dopaminergic transmission in the nucleus accumbens. Also nicotine acts directly via activation of the VTA dopamine neurons through stimulation of the nicotinic cholinergic receptor. Cannabinoids activate CB1 receptors on glutamatergic and GABAergic nerve terminals in the nucleus accumbens, and on neurons of the nucleus accumbens distinctively, as such increasing the extracellular dopamine levels directly (Nestler, 2005).

Indirect increases of extracellular dopamine result via opioid peptide activation and via $GABA_A$ modulation when exposed to opioids and alcohol (Vallone, 2000, Koob, 2008a; Koob, 2011).

Dopamine-synthetizing neurons involved in the *mesocortical pathway* are situated in the VTA, projecting to the dorsolateral prefrontal cortex (PFC). This area regulates, *amongst others*, cognitive function, motivation and emotional response. High concentrations of dopamine modulate mechanisms of learning and memory, enabling the brain to remember how to replicate a reward. On the

contrary, a negative experience will decrease the dopamine concentration, urging the brain to avoid this in future (anti-reward circuitry).

The mesocortical pathway is modulated during chronic exposure to psycho-active drugs by inducing a glutamate dysregulation (from PFC and basolateral amygdala to the nucleus accumbens core) and dopamine neurotransmission in the dorsal striatum, thus inducing morphological changes in the PFC (orbitofrontal, medial prefrontal, prelimbic/cingulate) and the basolateral amygdala. (Nestler, 2005; Koob, 2010; Feltenstein, 2008) (Fig. I.14).

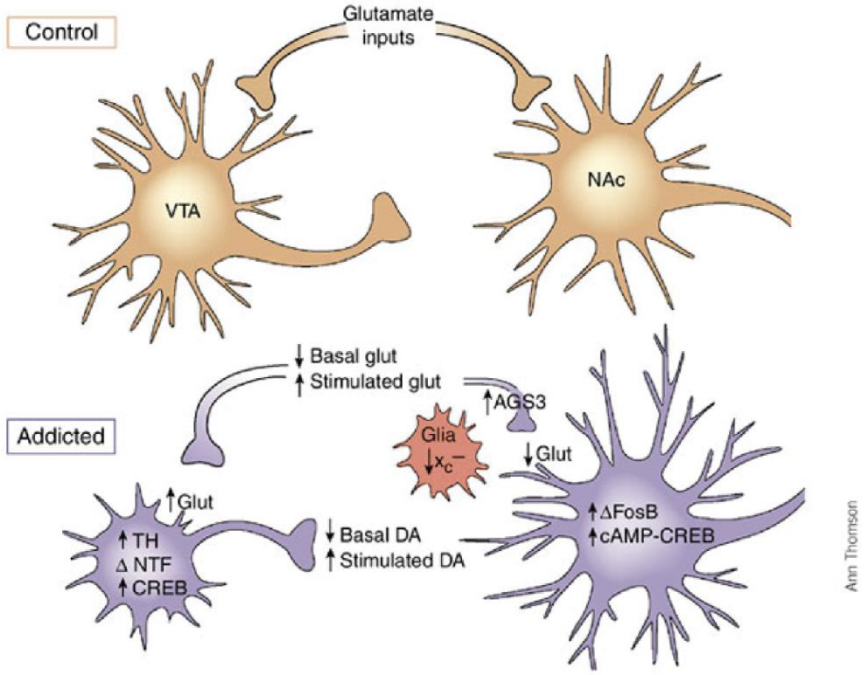


Fig. I.14. The glutamate dysregulation after chronic actions of drugs of abuse on the VTA-Nucleus Accumbens (N.Acc.). Control: under normal conditions, a VTA neuron innervates a N.Acc. neuron with glutamatergic inputs to the VTA and N.Acc. neurons. Addicted: Chronic drug administration induces tyrosine hydroxylase (TH) and increases AMPA glutamatergic responses (Glut) in the VTA (glutamate receptors). The VTA dopamine neurons are also being decreased in size. In the N.Acc., all drugs of abuse induce the transcription factor ΔFosB. Not all drugs of abuse induce CREB activity in this region (via upregulation of the cAMP pathway). Additionally, stimulants decrease AMPA glutamatergic responses in N.Acc. neurons, possibly by regulation of glutamate receptors, resulting in complex changes in glutamatergic innervation of the N.Acc. Taken with permission from Nestler (2005).

Substance abuse and drug-induced addiction have been considered a way of "toxic learning" that negatively affects the motivation for primary rewards (or the liking effect) whilst it strengthens the motivation to take drugs to boost dopamine (the wanting effect) (Hyman, 2005). However, addiction is not only a learned association, and to date the incentive sensitization theory is endorsed, stating that a pathological motivation (wanting or incentive salience) arises from sensitization of brain circuits caused by repeated exposure to drugs of abuse (Robinson, 2008).

I.5.2. Molecular mechanisms of neuroadaptation to drugs of abuse

At the postsynaptic molecular level, three different mechanisms are involved through which psycho-active drugs can activate the mesolimbic system (Camí, 2003; Furst, 2013) (Fig. I.15).

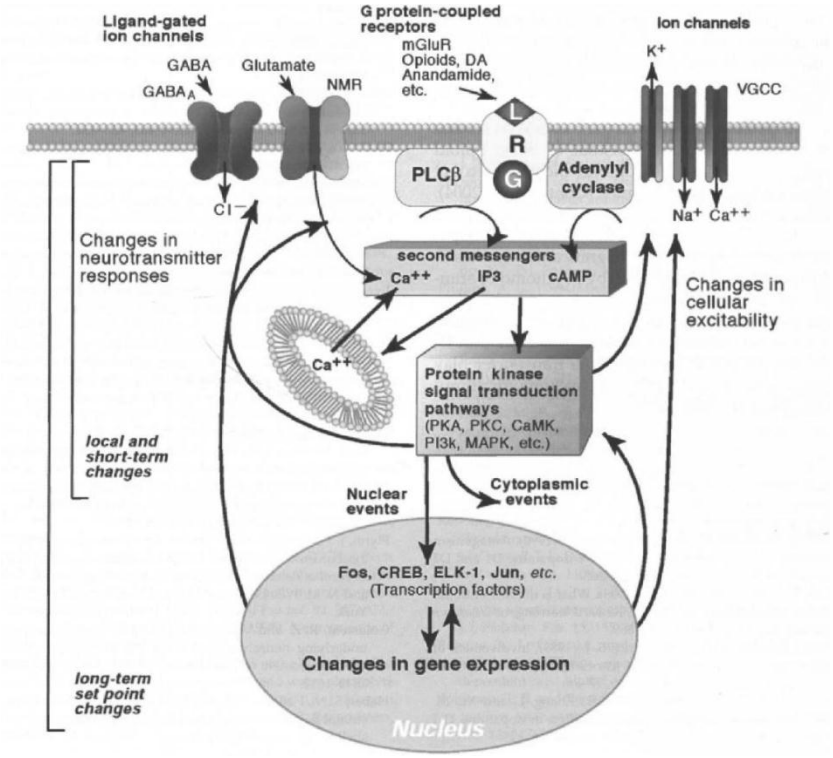


Fig. I.15. The cascade of molecular neuroadaptation involved in drug-induced addiction. Ligand gated ion channels (examples: ionotropic $GABA_A$ and glutamate NMDA receptors) and opioid, dopamine and endocannabinoid metabotropic receptors are shown. Taken with permission from Koob, 2008b.

The first or metabotropic mechanism involves G-protein-coupled receptors (GPCR) like, *amongst others*, the dopamine D2 receptors, opioid receptors (μ, κ, δ) and cannabinoid CB1 receptors, thereby mediating a slow synaptic transmission.

Examples of drugs acting via the metabotropic cascade are opioids, cannabinoids, hallucinogens and cocaine (Camí, 2003), involving dopamine receptors, receptors for neuropeptides, epinephrine, norepinephrine, endocannabinoids, and serotonin receptors. The cascade of activating or inhibiting enzymes like adenylyl cyclase or phospholipase-C are initiated by the α, β and γ subunits of these GPCRs, resulting in the synthesis of second messengers such as cyclic adenosine monophosphate (cAMP), cyclic guanosine monophosphate, inositol triphosphate, and diacylglycerol. Chronic exposure to cocaine (acting on G-protein-coupled D2 receptors) and opioids (acting on G-protein-coupled opioid receptors) *for example*, increases the levels of adenyl cyclase and protein kinase A, thereby increasing the expression of cAMP-responsive element–binding protein (CREB) in the nucleus accumbens, and of ΔFosB (Vallone, 2000; Li, 2008; Koob, 2011). This will lead to long-term functional changes in neurotransmission and in changes of the gene expression of proteins involved in signal transduction, thus inducing altered neuronal responses and clinical symptoms like dysphoria (Koob, 2008b, 2011).

Endocannabinoids act via the G-protein-coupled cannabinoid CB1 receptors, inhibiting the second messenger cAMP and Ca^{++} levels; chronic exposure also alters the expression of the transcription factors (reduction of phosphorylated CREB).

Secondly, the ionotropic mechanism involves direct binding to postsynaptic ionotropic receptors, thereby activating the ligand-gated ion channels, and as such mediating fast synaptic transmission. Ionotropic receptors include, *amongst others*, nicotinic cholinergic receptors (containing a Na^+ channel), $GABA_A$ receptors (containing a Cl^- channel), and NMDA sensitive glutamate receptors (containing Ca^{++} and Na^+channels). The β and γ subunits of the GPCReceptors can also directly regulate Ca^{++}-, Na^+-, and K^+-ion channels by

activating protein kinase transducers, resulting in a phosphorylation of the ion channels (Camí, 2003).

Examples of drugs acting via this mechanism include nicotine, benzodiazepines, barbiturates and alcohol (Camí, 2003) and ketamine, through activation of the ionotropic nicotinic acetylcholine receptors, $GABA_A$ receptors, NMDA glutamate receptors and AMPA receptors (Philibin, 2011).

A third, indirect mechanism is the activation of the mesolimbic system through inhibition of transporters like the dopamine transporter (DAT), resulting in an increase of dopamine at the synaptic level. Drugs like methylphenidate and cocaine are known to act via this mechanism (Koob, 2010, Teuns, 2014).

I.5.3. Pathological state of human addiction

The human pathological state of addiction is characterized by a change in the motivation for taking drugs, during which an individual, driven by impulsivity (*examples*: the wish to be part of a group or taking opioids to relieve pain), initially experiences the positive effects of reinforcement (euphoria), later on evolving to negative reinforcement behavior (motivational wanting or incentive salience), which is characterized by compulsivity (drug seeking and drug taking behavior). These stages are characterized by sequential neuroadaptations (Koob, 2010). To describe these processes, a three-stage model has been proposed by Koob and LeMoal (1997) (Fig. I.16).

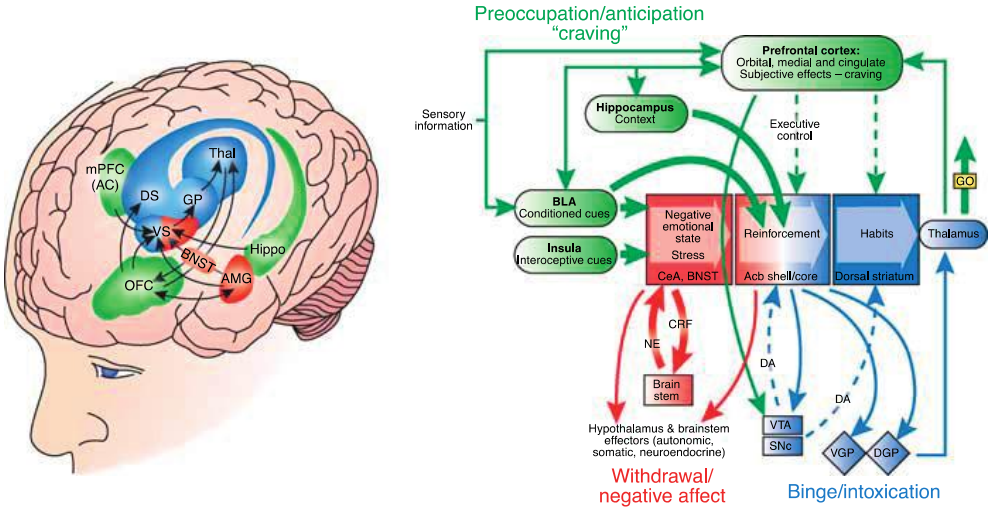


Fig I.16. The three stages of the addiction cycle: 1) Binge intoxication; 2) Withdrawal or negative effect stage; 3) Craving stage. AC: anterior cingulate; Acb: nucleus accumbens; AMG: amygdala; BLA: basolateral amygdala; BNST: bed nucleus of the stria terminalis; CRF: corticotropin-releasing factor; DA: dopamine; DGP: dorsal globus pallidus; DS: dorsal striatum; GP: globus pallidus; Hippo: hippocampus; mPFC: medial prefrontal cortex; NE: norepinephrine; OFC: orbitofrontal cortex; SNc: substantia nigra pars compacta; Thal: thalamus; VGP: ventral globus pallidus; VS: ventral striatum. Taken with permission from Koob (2010).

The earliest stage or binge intoxication includes dopamine and opioid peptides primarily involved in the acute and positive rewarding effects of drugs of abuse. These two neurotransmitters mediate the dopamine concentration in the nucleus accumbens and in the central nucleus of the amygdala, but the anatomical sites in which they are released differs for the various drugs of abuse (Koob, 2010). For stimulants and nicotine, the initial start of drug reward lays within the dopamine release in the nucleus accumbens, whereas opiates mediate via the nucleus accumbens and the VTA. Alcohol involves $GABA_A$ systems in the nucleus accumbens and the amygdala.

To date it is assumed that the nucleus accumbens plays a critical role for the acute reinforcing effects of drugs, with the central nucleus of the amygdala and the ventral palladium supporting these early manifestations (Koob, 2010).

The second stage is called the withdrawal or negative effect stage. Decreases in the dopaminergic and serotonergic systems in the mesolimbic system and the nucleus accumbens, respectively, occur during acute withdrawal for all classes of drugs of abuse (Rossetti, 1992; Weiss, 1992). These effects cause an emotional dysregulation, often characterized by increased stress and anxiety effects.

The last stage called craving stage involves drug seeking and drug taking behavior. This has been recently linked to the glutamatergic pathway from the PFC to the nucleus accumbens core, the dopamine projection from the VTA to the medial PFC, and the GABA projection from the nucleus accumbens to the ventral pallidum (Kalivas, 2008; Feltenstein, 2008; Koob, 2010).

The cascade of the described neuroadaptations in different brain regions, induced by chronic drug exposure, will finally result in neuroplastic changes affecting the prefrontal functioning like, *for example,* the decision making function.

I.5.4. Genetics, epigenetics and environmental factors

Genetic factors are known to, at least partially, add to the development of drug addiction. Data from literature cite numbers of 40 to 60% being accountable for the vulnerability to drug addiction due to the impact of genetic factors (Li, 2008), by directly affecting the gene function and/or gene expression.

Investigation of genetic relationship to addiction is mostly studied in human twin trials. Goldman (2005) analyzed data from national and international surveys on homozygote and heterozygote twin pairs and demonstrated that the heritability factor (varying from 0 to 1) on dependence or abuse of 10 addictive agents ranged from 0.39 (for hallucinogens) to 0.72 (for cocaine) (Fig. I.17).

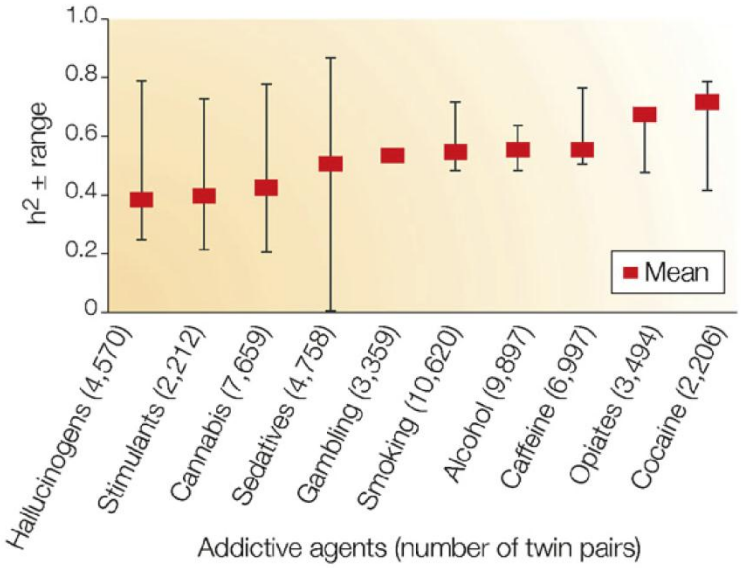


Fig. I.17. Heritability (h^2: weighted mean and range) of 10 addictive disorders. Data were derived from national surveys in large, carefully characterized cohorts of adult twins, including epidemiologically ascertained cohorts (U.S. and Australia). Between brackets: sum of monozygote and dizygote twin pairs included in the surveys. Taken with permission from Goldman (2005).

Li (2008) published a list of 1500 human genes, related to addiction. They identified five molecular pathways that were shared for the four investigated drugs of abuse (alcohol, cocaine, nicotine and opioids) and mostly linked to addictive behavior. These pathways were 1) the neuroactive ligand-receptor interaction (including, *amongst others*, GPCRs, cannabinoids, ion channels); 2) the GnRH signal transduction pathway [via feedback loops through CREB and calcium/calmodulin-dependent protein kinase II (CaMKII)]; 3) mitogen-activated protein kinase (MAPK) signaling pathway; 4) the neuronal gap junction; and 5) the long-term potentiation pathway (Fig. I.18).

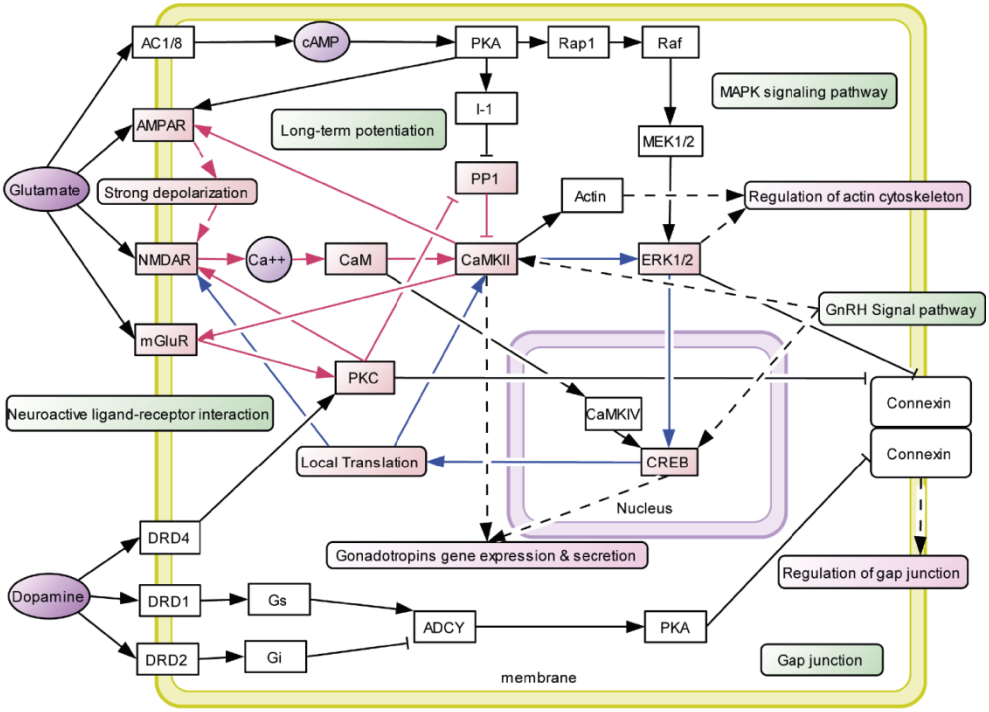


Fig. I.18. Hypothetical Common Molecular Network for Drug Addiction based on the common pathways and protein interaction data. Addiction-related genes: white boxes; neurotransmitters and secondary massagers: purple; the 5 common pathways: green boxes. Related functional modules: carmine boxes. Fast positive feedback loops: red lines; slow feedback loops: blue lines. Taken with permission from Li, 2008.

The "long-term potentiation" pathway has been linked to addiction-induced adaptations in glutamatergic transmission and synaptic plasticity and is also considered a key molecular circuit underling the memory system. The "MAPK signaling pathway" is another example, as it has a role in regulating synaptic plasticity related to long-lasting changes in both memory function and addictive properties, in particular after chronic exposure to opioids (Christie, 2008). Cocaine also activates this MAPK cascade (Renthal, 2009).

Another example of the impact of genetic factors involved in the maintenance of addiction was shown in a NIDA-sponsored study to test the efficacy of naltrexone-treatment in alcohol-dependent patients (Oslin, 2003). Individuals treated with naltrexone, showed a lower relapse of alcohol consumption if they

were identified with the genotype Asp40 in the opioid gene OPRM1, compared to those who were homozygous for the Asn40 variant (Fig. I.19).

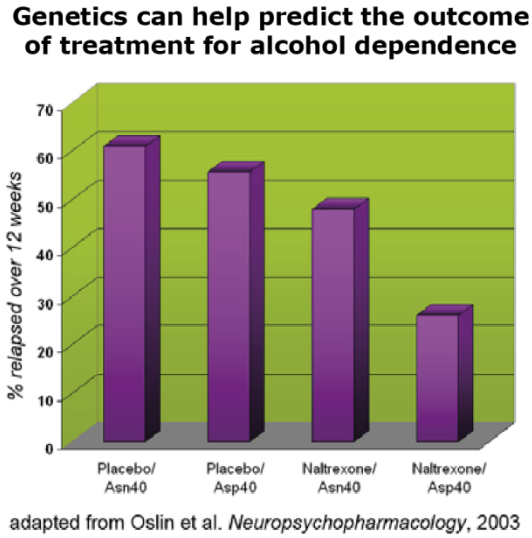


Fig. I.19. Incidence of relapse to alcohol after treatment with naloxone, based upon genotyping of the opioid gene OPRM1. The relapse of alcohol consumption over 3 months was much lower in individuals treated with naloxone and identified with the genotype Asp40. NIDA, 2008.

These examples illustrate the importance of further genetic research to determine functional genes and pathways involved in substance abuse and addiction, and to add to the personalized treatment hereof.

Besides the genetics, epigenetics are also considered of significance to the susceptibility to develop addiction. Epigenetics are defined as factors modifying the gene activity without changing the DNA sequence or, at a molecular level, as the functionally relevant changes to the genome that do not involve a change in the nucleotide sequence on specific gene promoters. At this molecular level, histone acetylation, DNA methylation, or phosphorylation can change the transcriptional activity of a gene through alterations of the chromatin structure (Furst, 2013; Renthal, 2008). *For example*, acute exposure to cocaine increases histone H4 acetylation on the proximal gene promoters of the cFos and Fosb

genes, whereas acute ethanol reduces histone acetylation by increasing histone deacetylase (HDAC) activity (Fig. I.20).

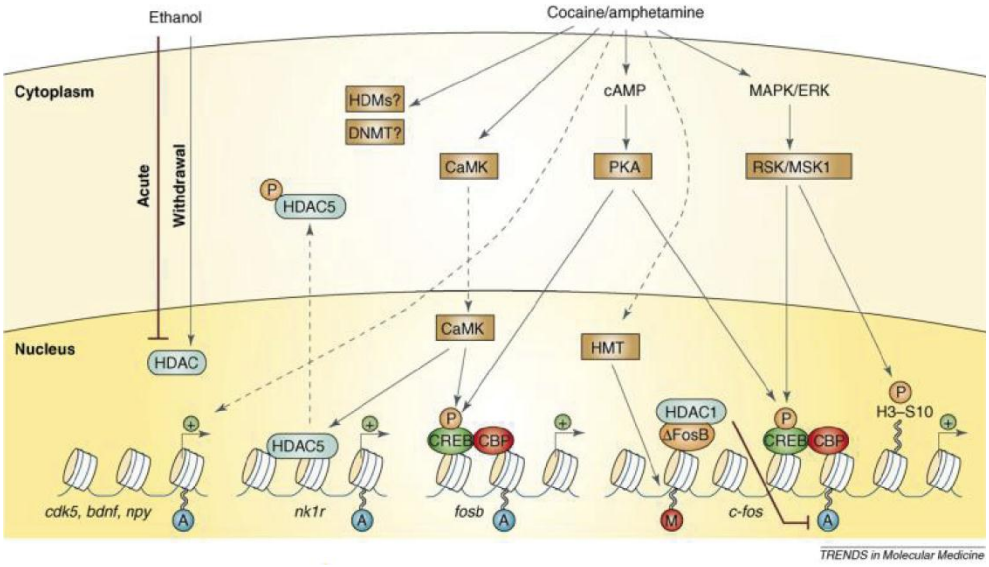


Fig. I.20. Regulation of chromatin remodeling by drugs of abuse. Example shown for stimulants (cocaine and amphetamine) and ethanol. Renthal, 2008. Taken with permission of Nestler.

Stress and a regular exposure to drugs are other examples of epigenetic factors that can alter both gene expression and gene function (NIDA, 2008; Koob, 2010; Furst, 2013) through, *amongst others*, an alteration of the mRNA expression of CRF (stress-system neurotransmitter) in the hypothalamus (paraventricular nucleus), increased CRF-like immunoreactivity in the locus ceruleus (LC), and increased CRF receptor levels in the locus ceruleus and raphe nuclei (Sinha, 2008).

Besides the genetic and epigenetic factors, environmental influences also play a role in the development of human substance abuse and addiction. The environmental factors to which an individual is exposed to, include, *amongst others*, cultural background, social situation, religion, economic and financial status. They can trigger to the first or occasional intake of addictive drugs in vulnerable individuals and as such add to the development of substance abuse.

Various research studies have looked into the genetics, epigenetics and environmental factors playing a role in addiction (Uhl, 1999; Goeders, 2003; Hemby, 2006; Nestler, 2005; Rhodes, 2005; Ron, 2005; Simmons, 2008; Sinha, 2008; NIDA, 2009; Epps, 2012; Romieu, 2014). However within Drug Development, the preclinical investigation of the abuse potential of medicinal drug candidates based upon genetic and epigenetic factors is still an open field for future research.

In conclusion, the research of genomics and of epigenetic mechanisms, combined with the molecular insights in the neurobiology, are promising to disentangle how addiction originates. Further research of environmental factors influencing the vulnerability of persons to substance abuse will add to better understand the pitfalls associated with relapse. Finally this knowledge might also provide the Pharma Industry with information for the development of drugs to treat substance abuse.

I.6. Available literature on substance abuse, abuse potential research and related animal models

During the past decades, numerous articles have been published on abuse potential and related research (11.429 citations in PubMed). The majority of these publications deals with the classical substances of abuse like opioids, stimulants, depressants, cannabinoids and hallucinogens, and includes data of preclinical and clinical research, and publications on possible pathways/brain structures related to the processes of abuse and dependence of these compounds.

Within the domain of preclinical research, various animal models are available to study different aspects of abuse potential. Basic research on the physical dependence potential of psycho-active drugs revealed an overwhelming body of literature related to withdrawal models (7.911 citations). In general, these models study the appearance of withdrawal symptoms after the cessation of chronic drug exposure; however, the possible benefits of treatment on apparent withdrawal symptoms (precipitated withdrawal) have also been described for, *amongst others*, opioids (using naloxone), benzodiazepines (with flumazenil as antagonist) and alcohol, for which various molecules have been investigated (Bozarth, 1987; Brady, 1987; File, 1990; Camí, 2003; Uzbay, 2012).

Reinforcement and rewarding have been investigated in great detail for CNS-active substances, using several preclinical models. The reinforcing properties of drugs (drug-seeking behavior) can be determined either directly (self-administration paradigms) or via indirect models, measuring the rewarding properties due to secondary conditioning (such as conditioned place preference paradigm). Investigation of the discriminative properties of drugs, *for instance* determined in the DDL (Drug Discrimination Learning) test is another common field of research, as it is assumed that substances of abuse have at least detectable discriminative properties.

Within these paradigms, particular drugs of abuse such as cocaine and opioids have been extensively studied (Table I.5).

Citations of	
FDA	**EMA**
Cocaine and	Opioids and
- withdrawal: 1920 citations	- withdrawal: 6857 citations
- reinforcement: 3167 citations	- reinforcement: 2141citations
- drug discrimination: 744 citations	- drug discrimination 745 citations
- conditioned place preference: 873 citations	- conditioned place preference: 871 citations

Table I.5. Number of citations related to abuse potential research for the main cited models in combination with either cocaine or opioids. (from PubMed, 2014).

Based upon these models and the data of the existing research, four animal models have been recommended by the drug licensing authorities worldwide to assess the abuse potential of CNS-active drug candidates (prescription drugs). They are enumerated in the guidances (EMA, 2006; ICH, 2009; FDA, 2010; Bonson, 2011) and include:

1) the non-precipitated Withdrawal test to investigate the physical dependence potential,

2) the Drug Discrimination Learning test to evaluate discriminative stimulus properties, including drug profiling and functional resemblance to known psycho-active reference drugs,

3) the conditioned place preference test and 4) the intravenous self-administration model to determine rewarding and reinforcing properties.

To employ these models in a Drug Development mode with its typical aspects and criteria regarding traceability and reproducibility (GLP-compliance), to fulfill the regulatory requirements and to consolidate or improve the predictive power of the outcomes, adaptations and/or modifications of the models as used in fundamental research were needed. Important aspects of these essential modifications were related to the following:

1. The requirements of the drug licensing authorities aim for comparison of the human efficacious dose with the dose range tested in animals, established through determination of the ratio of the peak plasma exposures (C_{max}), as part of the assessment of the biological relevance of the findings. Procedures on toxicokinetic profiling of test compounds in the abuse liability studies needed to be specifically designed and included in order to fulfill this requirement, relying upon scientifically based evidence.

2. To determine the abuse potential of CNS-active new molecular entities (NMEs) with a new mechanism of action, a correct selection of an appropriate dose range of the NME and a proper choice of the psycho-active reference compound or a scheduled comparator relevant to the mechanism of action of the new drug candidate are significant considerations that need to be taken into account.

In order to overcome these issues, a major part of the Ph.D. thesis dealt with experiments related to the optimization and standardization of the four different behavioural assets, recommended by the drug licensing authorities to be used in the characterization of the abuse potential of CNS-active drug candidates (prescription drugs) (Chapters IV to VII). As an end result these models should comply with all the requirements that are mandatory within a Drug Development environment.

To date the Pharma Industry puts a lot of effort on the harmonization of the protocols of the four proposed models through, *amongst others*, establishing cross-company pharmaceutical consortia like the CCALC (Cross-Company Abuse Liability Consortium) and its PAL (Preclinical Abuse Liability) subgroup, which are both acknowledged by the authorities. By means of face-to-face Dialogue Sessions between these consortia and FDA (2008, 2010, next session forseen in 2015), the Pharma Industry aims to discuss both the scientific value and the regulatory requirements related to these models with the drug licensing authorities. During the Dialogue Session in November 2010, several topics related to the preclinical part (section IV C) of the FDA guidance on Abuse potential (released in January 2010) were discussed, including, *amongst others*, the "Discussion of 'best practice' parameters for behavioral models" (Andy Mead

& Greet Teuns), in which questions on the dose selection of a drug candidate, the choice of a positive control/comparator, self-administratoin schedules and the use of other models besides the four models mentioned to study abuse potential of drug candidates were discussed.

An other effort of the Preclinical Cross-Company Abuse Liability Consortium (PAL) to furhter harmonize on the use of the proposed preclinical models for abuse liability testing is the current publication of a book "Preclinical Assessment of Abuse Potential for New Pharmaceuticals" in which, *amongst others*, these four preclinical tests as employed in a Drug Development environment are described (*example*: Chapter V of this book: Assessing Physical Dependence, by Greet Teuns, 2014).

All these initiatives are undertaken to clarify on the best practice to investigate abuse potential of CNS-active compounds in Drug Development, which are meant to enter the market as prescription drugs.

In conclusion, the profound review of the different models available in literature to study abuse potential of -mainly- psycho-active drugs, has led to further investigation of the methodologies and the predictive validity hereof for testing abuse liability of prescription drugs, taking the requirements of the drug licensing authorities into account.

The data described in this Ph.D. on the predictability of a translational approach for drug abuse liability testing in Drug Development aim to implement high-quality methods to properly study abuse potential of new CNS-active drug candidates within a Drug Development environment. The personal added value in these efforts described in this manuscript is also meant to be part of the valorization at industry level.

II

Objectives of this thesis

II.1. Introduction

The objective of this Ph.D. project was to develop a preclinical platform of neurobehavioural tests and data minding that should allow to evaluate the abuse potential of novel drug candidates with CNS-active properties. The assessment of the outcome of this research (Preclinical Abuse Liability Assessment) is of major importance to predict the risk-benefit of drug-induced abuse versus the medical need of these prescription drugs, but also adds to assess the possible risk of misuse of these products in nonmedical situations once they are marketed.

Five major critical points had to be taken into account. Firstly, with regard to the scientific basis of this research, one must bear in mind that these novel drug candidates most often function via novel mechanisms of action of which the activity in humans is not always well known. Therefore it is uncertain whether those novel drug candidates could directly or indirectly interfere with those neurological pathways known to be involved in the development of abuse and drug dependence. Also the off-target pathways of the drug candidates need to be considered thoroughly, as these might also (partially) interact with the neurocircuitries of abuse development. These considerations will be fundamental to define a correct preclinical Abuse Liability Assessment, representing the investigation of the eventual abuse properties of novel drug candidates. In particular the complexity of novel mechanisms, possibly combined with modulation of off-target pathways might give rise to the selection of more than one reference drug to test the drug candidate against in the various preclinical models used in drug abuse research.

Secondly, the preclinical abuse liability studies must comply with the regulatory requirements of the drug-licensing authorities. Four types of test models are requested by the guidances worldwide (EMA, 2006; ICH, 2009; FDA, 2010), being the non-precipitated withdrawal test, the drug discrimination learning test, the intravenous self-administration test and the conditioned place preference test. However, additional compound safety information through other parameters is mandatory within these test models such as, *for example*, the use of a dose range of the compound which includes a multiple of the human

efficacious dose and the inclusion of toxicokinetic parameters of the doses applied.

As a consequence, a third point of consideration is the biological relevance of the outcome of the various studies and hence the translational research of the abuse potential of the novel drug candidates. Indeed, when a drug candidate is being tested at various doses, the dose-response curve might differ between the study models. If the dose range is not correctly determined, false negative results might be obtained, in particular when (very) low or (very) high doses are selected to be tested. A known example hereof is the bell-shaped dose-response curve in the intravenous self-administration studies that has been observed with a variety of drugs (Van Ree, 1999; Piazza, 2000; Broadbear, 2004; Peana, 2010). When higher and increasing concentrations of a drug (*for example*: morphine) are presented in this model, much lower responses will be obtained, as an attempt to maintain a level of reinforcement which will not result in an overdose or in experiencing severe adverse effects. Another example can be present in the conditioned place preference model, where, for some drugs (*for example*: cocaine) a shift from drug-induced preference towards a drug-induced aversion might become apparent at very high doses (Bardo, 1995, Mueller, 2011). Thus the biological relevance and hence the correct prediction must be considered in view of, *amongst others*, the doses tested.

Fourthly, the models had to be set up fulfilling the demands of the GLP-guidances. This implies extra efforts with regard to the traceability and reproducibility of the test data, like, *for example*, inclusion of audit trails in automated programs, in case specific adaptations during the course of the study are needed.

Fifthly the requirements concerning animal welfare might also induce a delicate exercise/balance between the accomplishments hereof on the one hand and the limitations to fulfill these needs due to the high specificity of the test models on the other hand. An example hereof is the single housing of animals and the appropriate cage enrichment in an intravenous self-administration model due to the specific surgical technique employed (i.e. the vascular access button), which prohibits group housing.

To fulfill the above critical points, which are characteristic when working within a pharmaceutical research environment, a battery of existing paradigms used in addiction research needed to be modified to become applicable in Drug Development and compliant with both the drug licensing authority guidances and the GLP requirements; and to enhance the predictability of a translational approach of the potential abuse properties of these new drug candidates (prescription drugs).

In order to achieve this objective, the steps described in the following sections were completed.

II.2. Research of model adaptations: Chapters III to VII

The test battery comprised the four neurobehavioural study models as stated in the guidances worldwide (EMA, 2006; FDA, 2010), which are each investigating different aspects of drug abuse:

1) the (non-precipitated) Withdrawal test (WD) was designed to investigate the physical dependence potential of a CNS-active compound in view of a positive reference drug relevant to the drug candidate

2) the Drug Discrimination Learning test (DDL) evaluated discriminative stimulus properties of new drug candidates, resulting in drug profiling and functional resemblance to different known psycho-active reference drugs chosen according to the receptor binding profile and the pharmaco-toxicological properties of the drug candidate

3) determination of the rewarding properties and as such indirect measurements of the reinforcing properties of CNS-active NMEs via the Conditioned Place Preference test (CPP)

4) direct measurements of reinforcing properties of drugs in development were investigated via the Intravenous Self-Administration model (IV SA).

II.2.1. Investigation of critical variables in the four studies to develop robust and scientifically valid animal models for testing abuse liability

Fundamental investigation was carried out on the different, critical variables which might impact the outcome of the test results within each of the test designs, and modified on a scientific basis if necessary to contribute to validity of the test models. Examples hereof included, *amongst others*, the following:

Criteria in the WD model:

- length of withdrawal period
- proper selection of time points to observe behavioural parameters during the repeated dose phase and during the withdrawal period

- impact of sham treatment during the withdrawal period

Criteria in the DDL model:

- dose-response of the test compound and of the reference drug
- design of the toxicokinetic procedure
- sham treatment when different administration routes were used for drug compound and reference drug
- investigation of morphological brain changes after long-term administration of known psycho-active drugs (post-mortem data)

Criteria in the CPP model:

- length of the conditioning phase (number of drug-pairings)
- determination of the biological relevance of the outcome through additional analyses of the data

Criteria in IV SA model:

- development of proper technical systems enabling long-term studies as the IV SA model (vascular access button, surgical procedure, choice of appropriate indwelling catheters)
- identification and sequence of the 5 phases in IV SA study design (C-S-C-TA-C; C: reference drug, S: saline, TA: test substance)
- dose-response curve of several psycho-active drugs
- procedure to execute the additional toxicokinetic phase

Modifications of and/or adaptations to each investigated variable within a specified study model were implemented whenever appropriate and employed in the further subsequent studies.

II.2.2. Compliance of the models with regard to regulatory and GLP requirements

The study designs had to comply with the current regulatory guidances of the drug licensing authorities on abuse liability testing with regard to, *amongst others*:

- choice of species
- specific requisites on the design relevant to the investigation of novel drug candidates
- choice of appropriate dose ranges applicable in the test models in view of, *amongst others*, the intrinsic pharmacological properties of the test compound, its toxicological profile, and its formulation properties
- methodology to measure plasma exposure of both the test compound and reference drugs in the four test models

The models also had to meet the GLP (Good Laboratory Practices) principles in terms of reproducibility and traceability. To comply with this requirement, specifications relative to GLP were implemented with regard to, *amongst others*, the IT technology.

II.2.3. Translational research

To determine the abuse potential of new CNS-active molecular entities (NMEs) with a novel mechanism of action, considerable knowledge of the pharmacology, toxicity and kinetics is needed to enable the choice of the correct tests for the preclinical Abuse Liability Assessment, the selection of an appropriate dose range of the test compound and the proper choice of the psycho-active reference compound(s) or a scheduled comparator.

Moreover, the interpretation of these scientific data collected from the four preclinical standardized tests as described in Chapters IV to VII and the biological relevance hereof with regard to the translational research is complex, as within Drug Development these NMEs typically aim for new mechanisms of action, not necessarily involving receptors and neurotransmitters known to play a role in abuse. Thus a reliable prediction of the drug-induced abuse potential of new CNS-active molecular entities (NMEs) involves profoundly considering the characteristics of both test compound and reference drug, and a correct methodoly to support the interpretation of data obtained. An example of the latter was the implementation of an extended analysis of the data in the Conditioned Place Preference model.

II.2.4. Animal welfare

The study designs had to be ethically acceptable. A great deal of attention was paid to the animal welfare and comfort in these types of neurobehavioural studies. This was evidenced by, *amongst others*, the serious efforts that were made in modifying the technique used in intravenous self-administration studies through the implementation of a new vascular access button, to enhance the animal's comfort. Finally ethical protocols were written for each study type, describing in detail the outline and possible impact of pain and distress on animal welfare. These protocols were approved by the internal Ethics Committee for animal testing.

II.3. Proof of concept: Validation of the four models: Chapter VIII

In this chapter, presented as a published article, the face validity of the implemented models was investigated, using methylphenidate, a known CII scheduled drug of abuse. The biological relevance of the results and the translational approach towards human were discussed.

In conclusion, this project aimed to cover a broad scope with regard to research, translational approach and valorization for industry concerning the investigation of the abuse potential of new CNS-active drug candidates. Therefore considerable knowledge of and expertise within Drug Development (pharmacology, toxicology, comprehension of the clinical research, regulatory issues and formulation/final product forms) are inevitable to enable the development of a scientifically valid and compliant platform for preclinical abuse liability testing.

With the implementation of a standardized platform, necessary to design and execute the optimal preclinical package to achieve a reliable Abuse Liability Assessment of new CNS-active drug candidates with novel mechanisms of action (prescription drugs), a high predictability of the investigated abuse potential and its translation to men should be obtained.

III

Materials and Methods: general aspects

The research phase of this project comprised 26 rat studies in total which were performed in an AAALAC-accredited (Association for Assessment and Accreditation of Laboratory Animal Care) testing facility. To set up a standard design for each of the four required test models that are mentioned in the international guidances (non-Precipitated Withdrawal, Drug Discrimination Learning, Intravenous Self-Administration and Conditioned Place Preference; EMA, 2006; ICH, 2009; FDA, 2010), several variables, possibly influencing the outcome of these types of models, were identified and investigated. For this purpose 21 mechanistic studies were designed and executed (Table III.1). After this phase, a validation study was performed for each modified or adapted type of model, to demonstrate the scientific validity and the face validity of the outcome, and to verify the GLP-compliance. In that event methylphenidate, a known drug of abuse was employed. The full description and outcome of these four validation studies can be read in Chapter VIII (Teuns, 2014).

During the course of this project, 1 study was executed to support the development of drug candidate.

Ethical protocols were written and approved by the Ethics Committee for each type of study.

	WD	DDL	IVSA	CPP	**Total**
Mechanistic studies	4	5	6	6	21
Validation study	1	1	1	1	4
Drug Development studies	0	0	0	1	1
Total	5	6	7	8	26

Table III.1. Enumeration of the various studies performed during the course of the project.

Within this Materials and Methods chapter, general topics or conditions that were similar in all models are described. Study-type specifics are incorporated in detail in the methods of the individual studies discussed in Chapters IV to VII.

III.1. Laboratory animals and husbandry

According to the policy of the Johnson & Johnson Family of Companies, animals for laboratory research were used to the minimum extent necessary to assess the current research.

The guidances of the regulatory authorities endorse the use of rats for preclinical abuse liability testing. Therefore young Specific Pathogen Free (SPF) Sprague-Dawley (Crl: CD®) rats were ordered externally. These were males of 6 to 9 weeks old upon arrival. Female rats used in CPP studies were re-allocated from our internal stock. All rats had an acclimatisation period of at least 5 days before being used in any experimental procedure. For some studies, in particular for the DDL and IV SA mechanistic studies, rats from previous experiments were re-used. In these cases, a washout period of minimum 10 days was respected if tests with active compounds were to be initiated.

All rats were housed separately in transparent polysulphone home cages (floor area: 940 cm^2) filled with bedding material (Corn Cob size 12, Eurocob, France), a wire-mesh lid on top and suspended in wheeled racks (Fig.III.1).

Fig. III.1. Home cages of the rats in the four tests.

Various forms of cage enrichment were tested (Chapter VI) and/or provided (Wooden blocks and Sizzle nest: in all four test designs; transparent polycarbonate tunnels: provided in all test designs but the IV SA model). There was a 12/12 light/dark cycle and illumination did not exceed 300 lux in order not to initiate retinal damage in these albino rats. The test rooms were air-conditioned with their own supply of filtered fresh air. The standardized test conditions for temperature (20 - 23 °C), relative humidity (40 - 70%) and illumination (300 lux) were regularly controlled and recorded.

Rats were given free access to water. Food [R/M-H pelleted maintenance diet, Ssniff (Soest, Germany)] was provided ad libitum in the Withdrawal (WD) and the Conditioned Place Preference (CPP) studies but was restricted to 20 grams per day in the Self-Administration test (IV SA) to maintain a stable body weight. For the Drug Discrimination Learning test (DDL), which is primarily based upon food rewards offered via sugar pellets (45 mg dustless precision pellets, Bioserv, U.S.) during the training- and test sessions, rats were fed according to the following schedule: a daily food supply of 9 g was presented in the home cages after training on Monday to Thursday (training days). On Fridays (test days) 18 g was made available in the home cages after test sessions. During the weekends when neither training nor test sessions were scheduled, 16 g was provided on Saturdays and 13 g on Sundays.

In all studies, rats were checked at least once daily for general health, abnormal behaviour or unusual appearance, untoward clinical signs, toxic or pharmacological response, moribund state or mortality. The conducted experiments were in accordance with the European (European Convention, 1986, 2007) and Belgian guidelines (Belgian Law, 1991), and with the principles of euthanasia as stated in the Report of the American Veterinary Medical Association Panel (AVMA, 2001).

III.2. Drugs and dose rationale

The psycho-active drugs of abuse used in the mechanistic studies included cocaine, morphine, fentanyl, diazepam, chlordiazepoxide, ethanol, 2,5-Dimethoxy-4-methylamphetamine (DOM), ketamine and LSD. During the validation studies, rats were exposed to d-amphetamine and methylphenidate. The study performed to support a NME in Early Drug Development included fentanyl as the reference drug.

The drugs were stored at room temperature in a closed and labelled container, protected from (white) light). Most reference drugs were formulated as aseptic, watery-based solutions but diazepam was formulated as an aqueous suspension in 0.5% w/v Methocel (Hydroxypropyl methylcellulose). The pH, density and osmolality were measured (including homogeneity for prepared suspensions) and the concentration in and the stability of the different formulations was determined, using (high performance) liquid chromatography [(HP)LC]. The results all complied with the concentration and stability acceptance criteria (expressed as percentage against the nominal concentration) for solutions and for suspensions (85-115%).

The volumes to be administered were adapted daily for changes in body weight of the rats. Daily dosage was performed at the same time each day throughout the studies as much as possible. For each day of dosing a new bottle of a formulation was used.

Detailed information on the reference drugs and additional components is further outlined in the next sections.

III.2.1. Cocaine

Cocaine (methyl (3S,4R)-3-benzoyloxy-8-methyl-8-azabicyclo[3.2.1]octane-4-carboxylate is an alkaloid ester that easily crosses the blood-brain barrier. It acts as a D2 agonist/dopamine-serotonine-norepinephrin transport inhibitor. Cocaine is scheduled for its abuse potential as CII in the U.S. (DEA, 2014) and worldwide (Convention on Psychotropic Substances, 1971).

Cocaine hydrochloride (CAS-number: 52-26-6; conversion factor = 1.128) was used and is further referred to as cocaine.

In the withdrawal study 1.5 mg cocaine was dissolved in 1 ml NaCl 0.9% for *intraperitoneal* (IP) dose administration at 15 mg/kg (10 ml/kg) (Kalivas, 1988; Kimmel, 2003).

The *subcutaneous* (SC) dosages of 10 or 15 mg/kg cocaine used in the studies (DDL, CPP, TK study) were selected based upon data from literature (Mayer, 1993; Tzschentke, 2007) and from internal experiments (see Chapter VII CPP). For SC use, cocaine was formulated at 1, 2 or 3 mg/ml. The volumes administered were 10, 5 and 5 ml/kg, respectively. In the toxicokinetic study cocaine was formulated at 10 mg/ml for SC administration at 1 ml/kg.

The *intravenous* dose of cocaine used in the IV SA studies was based upon numerous literature (NIDA, 1988; Piazza, 2000; Xi, 2005). The IV LD_{50} of cocaine in rats is 17.5 mg/kg (Cordell, 1993). Cocaine was formulated at 6 and 12 mg/ml to obtain IV infusions of 0.48 (~0.5) and 0.96 (~1) mg/kg , respectively (volumes of 0.08 ml/kg/infusion).

III.2.2. Morphine

Morphine is a narcotic analgesic (μ-opioid agonist) (Goodsell, 2005), scheduled for its abuse potential as CII. Morphine hydrochloride (CAS-number: 52-26-6; conversion factor = 1.128) was used and is further referred to as morphine.

Morphine, administered subcutaneously (SC), has a T_{max} of 50 - 90 minutes (CPS, 1994) and the SC LD_{50} is 480 mg/kg (MSDS, 2014).

Morphine was formulated at 5, 10, 20 or 25 or 30 mg/ml for *subcutaneous* (SC) use at 2 or 4 ml/kg.

The subcutaneous doses of 10 and 100 mg/kg used in the non-precipitated withdrawal studies were selected based upon literature (Martin, 1963; Houshyar, 2003) and internal data. The top dose of 100 mg/kg was only administered after

previously dosing with increasing dosages (10, 20, 40, 60 and 80 mg/kg) to avoid lethality due to morphine administration.

Morphine was also formulated for *intravenous* use (IV SA) at 1.5 mg/ml. The selected IV dose was 0.3 mg/kg per infusion (Van Ree, 1999), presented as 0.2 ml/kg/infusion.

III.2.3. Fentanyl

Fentanyl (*N*-(1-(2-phenylethyl)-4-piperidinyl)-*N*-phenylpropanamide) is a lipophylic narcotic analgesic (μ-opioid agonist) and scheduled for its abuse potential as CII. Its potency is 100x that of morphine (Janssen, 2013; Flacke, 1985). Because the narcotic and analgesic effects of fentanyl last for only a very short time, addiction is known to occur very quickly. Clinically its main adverse feature is respiratory depression (Nilsson, 1982).

Fentanyl citrate, further referred to as fentanyl, was used in the studies.

The *subcutaneous* dose of 0.02 mg/kg fentanyl was selected based upon internal expertise (Colpaert, 1976), and adapted for use in Sprague-Dawley rats. The aseptic aqueous solution contained 0.02 mg/ml and was administered at 1 ml/kg (DDL, CPP, TK study).

The *intravenous* dosages of fentanyl of 0.001 and 0.0025 mg/kg/infusion (IV SA) were selected based upon data from literature (Van Ree, 1978, 1999; Morgan, 2002). Fentanyl has a half-life of 2 minutes after IV administration, and the IV LD_{50} is 2.3 milligrams per kilogram in rats (Galloway, 2012). The calculated relative IV ED_{50} in IV SA studies is 0.0025 mg/kg/infusion (Van Ree, 1999).

Fentanyl was formulated at 0.01 and 0.025 mg/ml for volume administration of 0.1 ml/kg/infusion.

III.2.4. Diazepam

Diazepam is a highly lipid-soluble benzodiazepine derivative and scheduled for its abuse potential as CIV. It exerts anxiolytic, sedative, muscle-relaxant,

anticonvulsant and amnestic effects. Most of these effects are thought to result from a facilitation of the action of gamma aminobutyric acid (GABA), an inhibitory neurotransmitter in the central nervous system. Diazepam is rapidly absorbed after oral administration and has a fast onset of action. Continual daily doses of diazepam will quickly build up to a high concentration in the body (mainly in adipose tissue), which will be far in excess of the actual dose for any given day.

The dose selection was based upon literature data (IPCS Inchem; 1998; Martijena, 1996; Kunchandy, 1986). Diazepam has a $T_{1/2}$ of 1.42 hours in the rat (Bachmann, 1996) and the oral LD_{50} ranges from 249 to 2.075 mg/kg.

Diazepam (Valium®; CAS-number: 439-14-5; conversion factor = 1.00) was formulated at 0.2, 2 and 20 mg/ml for *oral* dosing at 2, 20 and 200 mg/kg, respectively (withdrawal study). The volume administered was 10 ml/kg.

III.2.5. Ethanol

Ethanol is the principal psychoactive constituent in alcoholic beverages, with depressant effects on the central nervous system. It has a complex mode of action and affects multiple systems in the brain; most notably ethanol acts as an agonist to the $GABA_A$ receptors. Ethanol is an unscheduled drug. The LD_{50} in rats ranges from 7060 to 9000 mg/kg when dosed orally.

Ethanol, (CAS-number: 64-17-5; Merck lot number: K40869283; conversion factor = 1.000) was administered *orally* at 1500 mg/kg (withdrawal study), based upon literature data (Mohsen, 1996; File, 1991; Meert, 1994), and formulated as an aqueous solution at 150 mg/ml for an oral volume administration of 10 ml/kg.

In the toxicokinetic study, ethanol was formulated at 1900 mg/ml for dosing *intraperitoneally* at 1500 mg/kg (1 ml/kg).

III.2.6. D-amphetamine

Dextroamphetamine (d-Amphetamine, (2S)-1-phenylpropan-2-amine) is the dextrorotatory stereoisomer of amphetamine and is a slightly polar, weak base with lipophilic properties. D-amphetamine is a CII scheduled drug.

In the present rat studies (withdrawal, DDL and CPP) a *subcutaneous* dose of 0.8 mg/kg AMP was selected based upon internal experimental and literature data (Spyraki, 1982; Levi, 2012). The LD_{50} is 180 mg/kg when administered subcutaneously in the rat (Drug Information Portal, 2014; Warren, 1945).

D-amphetamine sulphate (CAS Nr 51-63-8, Fagron, Belgium), further referred to as d-amphetamine, was formulated at 0.16 mg/ml and the *subcutaneously* administered dose volume was 5 ml/kg.

In the toxicokinetic study, d-amphetamine was formulated at 0.8 mg/ml for subcutaneous dosing at 0.8 mg/kg. The volume was 1 ml/kg.

In the IV SA study d-amphetamine was presented at 0.06 mg/kg/infusion (Carroll, 1997). Its LD_{50} when administered IV to rats is 30 mg/kg (RTECS, 2013). Aseptic, aqueous solutions at a concentration of 0.3 mg/ml were prepared and the *intravenous* dose volume was 0.2 ml/kg/infusion.

III.2.7. DOM: 2,5-Dimethoxy-4-methylamphetamine

DOM is a substituted amphetamine, also known as STP (Serenity, Tranquility, and Peace). It has hallucinogenic properties and is as such scheduled as CI. DOM is a selective 5-HT2A, 5-HT2B, and 5-HT2C receptor partial agonist.

DOM was administered *intraperitoneally* (IP) at 0.63 mg/kg (DDL). The dose selection was based upon internal communication. DOM was formulated at 0.063 mg/ml and administered at a volume of 10 ml/kg. In the toxicokinetic study, DOM was formulated at 0.63 mg/ml for IP administration of 0.63 mg/kg (1 ml/kg).

III.2.8. Ketamine

Ketamine is a non-selective NMDA-antagonist and scheduled as CIII.

The dose of 10 mg/kg (DDL) was based upon internal communication. Ketamine was formulated at 1 mg/ml and *intraperitoneally* administered at a volume of 10 ml/kg.

In the toxicokinetic study, ketamine was formulated at 10 mg/ml for IP administration of 10 mg/kg (1 ml/kg).

III.2.9. Chlordiazepoxide

Chlordiazepoxide is a benzodiazepine mainly used as an anxiolytic. It is a CIII scheduled drug.

In the presented studies (DDL), chlordiazepoxide was administered *intraperitoneally* (IP) at 5 mg/kg. This dose was selected based upon internal communication and formulated as an aseptic, 10% hydroxypropyl-beta-cyclodextrin based aqueous solution at 0.5 mg/ml (10 ml/kg).

In the toxicokinetic study, chlordiazepoxide was formulated at 5 mg/ml for IP administration of 5 mg/kg (1 ml/kg).

III.2.10. LSD

Lysergic acid diethylamide (LSD) is a hallucinogenic and as such scheduled as CI.

LSD was formulated at 0.016 mg/ml (10 ml/kg) for *intraperitoneally* (IP) dose administration at 0.16 mg/kg (DDL). The dose selection was based upon internal communication.

In the toxicokinetic study, LSD was formulated at 0.16 mg/ml for IP administration of 0.16 mg/kg (1 ml/kg).

LSD was also administered *intravenously* (IV SA) at a dose of 0.1 mg/kg/infusion (0.5 mg/ml with a volume of 0.2 ml/kg per infusion). The IV LD_{50} in rats is 16.5 mg/kg (Passie, 2008).

III.2.11. Methylphenidate

Methylphenidate is a piperidine derivative (methyl 2-phenyl-2-(piperidin-2-yl) acetate) and acts as a dopamine transport inhibitor. Methylphenidate is

commercially available as a hydrochloride salt (Concerta®, Ritalin®) for treatment of ADHD. It is a CII scheduled drug.

Methylphenidate hydrochloride (CAS Nr. 298-59-9, Noramco) was formulated at 0.25, 0.5 and 1.0 mg/ml for *oral* dosages of 2.5, 5 and 10 mg/kg (administered at a dose volume of 10 ml/kg) (withdrawal, DDL, CPP), based upon literature data (Kollins, 2001; Botly, 2008; Wooters, 2011). The oral LD_{50} of Methylphenidate in the rat is 350 mg/kg (Sigma-Aldrich, 2006).

In the IV SA study methylphenidate was presented at 0.05, 0.1, 0.5 or 1 mg/kg/infusion (Kollins, 2001; Botly, 2008). Aseptic, aqueous solutions at concentrations of 0.25, 0.5, 2.5 and 5 mg/ml were prepared and the *intravenous* dose volume was 0.2 ml/kg/infusion. The IV LD_{50} of methylphenidate is 48 to 50 mg/kg (Johnson Matthey, 2006; Separham, 2011).

III.2.12. Nicotine

Nicotine is an alkaloid that, in low concentrations (an average of about 1 mg of absorbed nicotine), acts as a stimulant and is the main factor responsible for the dependence-forming properties of tobacco smoking. It is an unscheduled drug.

In the toxicokinetic study, L-nicotine was formulated at 0.6 mg/ml for *subcutaneous* administration of 0.6 mg/kg (1 ml/kg).

The subcutaneous LD_{50} in the rat is 25 mg/kg (Farmakologiya i Toksikologiya, 1984).

III.2.13. WIN55,212-2

WIN55,212-2 is a chemical described as an aminoalkylindole derivative, which produces effects similar to those of cannabinoids such as tetrahydrocannabinol (THC) but with an entirely different chemical structure. WIN55,212-2 is a full agonist at the CB1 cannabinoid receptor. This compound is not marketed and thus not scheduled.

WIN55,212-2 was administered *subcutaneously* at 0.15 and 0.3 mg/kg (DDL). The dose selection was based upon data from literature (De Vry, 2002). It was formulated at 0.06 and 0.03 mg/ml, respectively and administered at 5 ml/kg.

In the toxicokinetic study, WIN55,212-2 was formulated at 0.3 mg/ml for SC administration of 0.6 mg/kg (1 ml/kg).

III.2.14. Escitalopram

Escitalopram is the S(+) enantiomer of citalopram. Escitalopram selectively blocks the reuptake of 5-HT (SSRI) but with no effect on the reuptake of norepinephrin (NE) and dopamine (DA). It is an unscheduled drug.

The commercial product Seroplex® [20 mg escitalopram/ml solution (as 25.551 mg escitalopram oxalate/ml)] was diluted with NaCl 0.9% to 4 mg escitalopram/ml. The pH and density of the diluted solution were measured. The concentration in and the stability of escitalopram in the prepared solution were not determined.

Escitalopram was tested *orally* in rats (DDL) at 40 mg/kg (4 mg/ml; 10 ml/kg).

The NOEL in rats is 40 mg/kg/day (OR) (Lundbeck, 2012).

III.2.15. Ro15-4513

Ro15-4513 (Roche compound) is a benzodiazepine receptor partial inverse agonist, initially developed to treat alcoholism. The *oral* dose of 1.5 mg/kg was based upon data from literature (Becker, 1989). The formulation was an aqueous solution with 40% Hydroxypropyl-β-cyclodextrin containing 0.15 mg/ml. The volume administered was 10 ml/kg.

III.2.16. SKF 82958

SKF 82958, an experimental D1 agonist was used in the toxicokinetic study at 0.1 mg/kg. It was dissolved at 0.1 mg/ml for *intraperitoneal* dose administration at 10 ml/kg.

III.2.17. Propofol

Propofol is a non-barbiturate sedative that was used to test the intravenous catheter patency testing in the IV SA model. It is on the market as a sterile, non-pyrogenic emulsion containing 10 mg/ml of propofol suitable for intravenous administration (Diprivan®). It is not scheduled as a drug of abuse. Propofol was applied as a fast *intravenous* bolus at 5 mg/kg, corresponding to 0.5 ml/Diprivan®/kg/bolus. The chosen dose was based on literature data (Simons, 1991; Larsson, 1994; Naguib, 2003) and on a previously conducted experiment on proper dose selection of propofol (Chapter VI).

III.2.18. Heparin solution

An aseptically prepared 50 I.U. heparin/ml solution (NaCl 0.9%) was used for daily flushing (0.2 ml/rat) of the *intravenous* catheters of the IV SA rats.

III.2.19. Ketalar®/Dormicum®/saline solution

An aseptic mixture of Ketalar®/Dormicum®/saline was prepared at a ratio of 3/1.5/5.5 to use as a short-acting *intravenously* administered anaesthetic for patency testing in the IV SA model at 0.2 ml IV per rat.

III.2.20. NaCl 0.9%

Commercially available NaCl 0.9% (Kela Pharma, Belgium) was used whenever appropriate.

III.3. Equipment

Body temperature recordings were executed in the withdrawal studies with a Plexx thermometer [Temperature reader (DAS 7007S)]. The rats were implanted with a programmed temperature transponder (IPTT-300), enabling data retrieval of the peripheral body temperature without disturbing the animals (Fig. III.2).

Fig. III.2. A validated probe thermometer (Plexx thermometer) with implantable transponders for reading the peripheral body temperature without disturbing the animals.

For the DDL test operant conditioning chambers (Skinner boxes: modular test chamber ENV-007, MED Associates Inc; floor area: 750 cm^2) with 2 response levers and equipped with a pellet dispenser (ENV-203-45, MED Associates Inc.) were used (Fig. III.3). The test chambers were placed in a steel soundproof box with light and ventilation.

Fig. III.3. Operant chamber for DDL testing.

There were 2 sets of 6 boxes, each connected with one IT system (Fig. III.4).

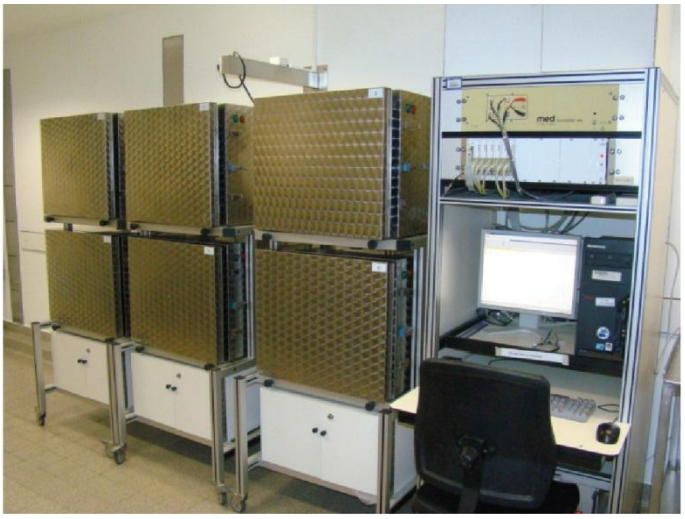


Fig. III.4. Setup of one system used in the DDL: 6 boxes are connected with 1 IT system.

The conditioned place preference apparatus (MED Associates Inc.) consisted of a test box divided into two separate compartments [21 x 21 x 68 cm (width x height x length)] interconnected by a short grey tunnel section (so-called neutral compartment). The compartments had different coloured sidewalls (black and white) with differently textured floors (smooth metal horizontal rods or wire mesh) in order to provide both tactile and visual environmental cues (Fig. III.5).

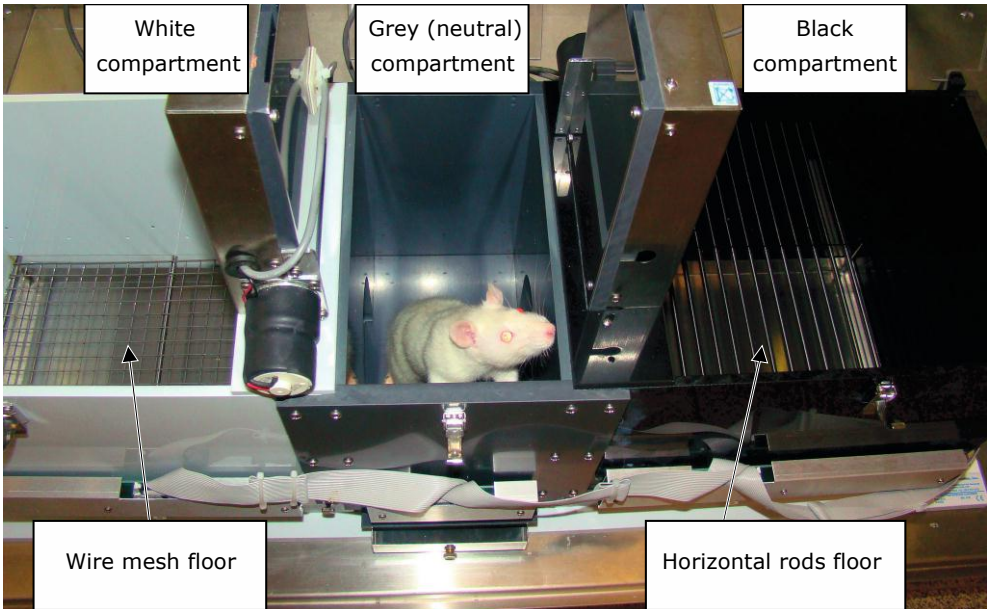


Fig. III.5. The conditioned place preference apparatus (MED Associates Inc.).

The test box was housed in a sound-proof box to prevent any audible cues disrupting the conditioning (Fig. III.6).

Fig. III.6. The CPP apparatus is placed in soundproof box, and connected with the IT system.

For the IV SA test operant conditioning chambers (Skinner boxes: modular test chamber ENV-007, MED Associates Inc, USA) with 2 respond levers (of which

the left one was made inactive) were used. The chambers were put in a wooden box with light and ventilation. An automated syringe pump system (Model PHM-100-3.3 and model PHM-100-20; MED Associates Inc, USA) was configured to intravenously deliver the appropriate drug amounts via short infusions, with each infusion lasting between two and five seconds (Fig.III.7).

Fig. III.7. Operant chamber used for IV SA testing.

III.4. Statistics

The mechanistic studies did not all contain statistical analysis due to the sometimes small number of animals employed. If included then statistical tests were performed at a two-sided significance level of 5 percent.

III.4.1. Withdrawal test

In the withdrawal tests statistics were performed on body temperature, body weight and food consumption. The significances were computed by Mann-Whitney-U (Siegel, 1988).

III.4.2. Drug discrimination learning test

In the drug discrimination learning test a post-hoc Dunnett test (Dunnett, 1955) was performed to investigate all pairwise comparisons of each dose group to the first dose administration of the control formulation (referred to as C1). All DDL-specific parameters (description: see Chapter V.1. Introduction) recorded during the study were analyzed statistically. A repeated ANOVA was adopted on latency data and on the total number of responses to check whether there is evidence of any difference among dose groups while accounting for the within-animal effects.

III.4.3. Intravenous self-administration test

In the self-administration tests a repeated ANOVA (Verbeke, 2000) with group as explanatory factor was fitted to the data. Posthoc, pairwise comparisons versus control were evaluated using Tukey's method (Hochberg, 1987). Statistical analyses were performed on the mean of the last 3 infusion data per animal per group per phase. The same animals were followed over phases 1 to 5 (Chapter VI). To account for the within-animal variability in comparison across different phases, a mixed effects model was fitted with a random animal effect (Verbeke, 2000).

III.4.4. Conditioned place preference test

The number of rats showing a drug-induced conditioned place preference were analysed using the Fisher Exact probability test (Siegel, 1988). ANOVA models (Neter, 1990) were fitted to the time spent in the drug-paired chamber at

pretest as well as at post-test with group and colour (white and black) of the drug-paired chamber as explanatory factors (Dela Cruz, 2009). A repeated ANOVA was adopted on latency data and on the total number of responses to check whether there was evidence of any difference among dose groups while accounting for the within-animal effects.

All statistical calculations and graphs were performed using the SAS (SAS® 9.2 Language) and the R systems (R Core Team, 2013), which are software programming languages and software environments for statistical computing and graphics.

III.5. Toxicokinetics

If included in studies, then toxicokinetic parameters were followed up in satellite animals (Withdrawal test) or in the animals of the respective studies after completion of the main experiment (CPP, DDL, IV SA).

A volume of 0.3 ml blood was taken from the tail vein for each sampling time point and put on K3EDTA. Bioanalytical analysis was performed on the plasma with a qualified research LC-MS/MS method.

The peak plasma exposure (C_{max}) and the peak plasma exposure time (T_{max}) were calculated.

IV

Withdrawal test in rats: optimizing the model for implementation in Drug Development

IV.1. Introduction

Withdrawal symptoms related to abrupt cessation of, primarily, opioids, stimulants and alcohol have been widely investigated (NIDA, 1984; McKetin, 2000; Christy, 2008; Zahr, 2008) because they are largely related to physical dependence and known to add to the relapse of drug addictive (drug seeking) behaviour. The term "cold-turkey" is frequently used in this context, referring to the aversive withdrawal effects which might include dysphoria, insomnia, shivering, fever, nausea, diarrhea, and delirium tremens (Hartney, 2011). As such basic research on possible drug candidates, prohibiting or interrupting these symptoms has been encouraged throughout the years. Of several behavioural models, the precipitated withdrawal test in particular is being employed to investigate whether occurring withdrawal symptoms can be more rapidly induced by administrating an antagonist after a (sub)chronic treatment of a drug of abuse. The most classical examples hereof include the use of naloxone after opioid administration (heroin, morphine, ocycodone, …) (Brady, 1987; Camí, 2003) or flumazenil for benzodiazepines testing (FDA, 2010; Linseman, 1977; Lewanowitsch, 2006; Martin, 1995). In this respect, we have conducted a mechanistic study (study number TOX9650) in which we tested Ro15-4513, a weak benzodiazepine inverse agonist and a competitive $GABA_A$ antagonist, in chronically ethanol (1500 mg/kg, OR) treated rats. We investigated the potential precipitation after a single Ro15-4513 dose (1.5. mg/kg, OR) as well as possible regression of the withdrawal effects of ethanol by daily administration of Ro15-4513 after discontinuation of ethanol treatment (see IV.2.3. and IV.3.3).

As the precipitated withdrawal test can only be performed if a specific antagonist of the test substance is available, this model is less convenient for the investigation of the physical dependence potential of new NMEs with a novel mechanism of action, and more frequently used to investigate the affinity for a specific (well-known) receptor. An example hereof is the research of the antagonistic effects of Ro15-4513 on the $GABA_A$ receptor.

Within Drug Development the physical dependence potential of new CNS-active molecular entities needs to be investigated according to the requirements of the drug-licensing authorities.

To date the non-precipitated withdrawal test in the naive rat is a mandatory study to study physical dependence and part of the Abuse Liability Assessment of a CNS-active drug candidate (EMA, 2006; ICH, 2009; FDA, 2010).

In general, this test includes a repeated dose phase (RD) with 3 different dosages, ranging from a pharmacologically active dose up to a toxic dose which is a multiple of the human efficacious dose (expressed as C_{max}), followed by a withdrawal period during which possible signs related to abstinence of the drug in the body might appear.

Along with the requirements of the drug-licensing authorities, the implementation of the non-precipitated withdrawal model as a standard test also required compliance with the GLP guidelines. Thirdly, the possibility of a flexible design in view of the pharmacotoxicological and kinetic characteristics of a test substance had to be maintained feasible. Indeed, for testing a new CNS-active drug candidate, many factors have to be taken into account to ensure a proper study design and hence a correct interpretation of the results, including, *amongst others*, the proper selection of a reference drug relevant to the drug candidate, the length of both the repeated dose phase and the withdrawal period relevant to steady state and elimination of the test substance, and the setting of the observational time points during these stages in view of the exposure-effect curve. Therefore the methodology was optimized and several variables, possibly influencing the outcome, were investigated, using different known psycho-active substances. In total 4 mechanistic studies were performed, identified as study numbers TOX8955, TOX9320, TOX9650, and TOX10109. These studies are reported in this chapter.

Finally a validation study was conducted with methylphenidate, a CII scheduled drug of abuse, to demonstrate the scientific validity and the face validity of the outcome, and to verify the GLP-compliance before the model was approved for use in Drug Development. This study is described in Chapter VIII.

IV.2. Materials and Methods: optimization of the methodology of the non-precipitated withdrawal test for use in Drug Development

IV.2.1. Non-precipitated Withdrawal Test with morphine (study ID number TOX8955)

The purpose of this study was 1) to determine whether a predefined 21-day subcutaneous (SC) repeated dose administration of morphine up to 100 mg/kg (q.d.) was sufficient to induce withdrawal effects in male rats; 2) to investigate if the length of a 7-day withdrawal period was appropriate to encompass all withdrawal effects; 3) to investigate whether repeated SC administration of a fixed low dose of 10 mg/kg morphine would be subject to withdrawal after cessation or to sensitisation without leading to withdrawal effects afterwards; 4) to investigate if stimuli associated with drug administration (i.e. SC injection) act as a conditioned stimulus which elicits a compensatory response to drug administrations.

Twenty male rats were divided into 4 groups of 5 males each via a body weight based stratified randomisation procedure. One group (C-group) received the control solution (NaCl 0.9%); one group (L-group) was administered 10 mg/kg morphine, and two groups (M- group and H-group) were similarly dosed at 100 mg/kg morphine. The low dose of 10 mg/kg morphine (L-group) was administered subcutaneously from Day 0 (first dosing day) till Day 20 (last dosing day). The top dose of 100 mg/kg (M- and H-groups) was reached on the 13^{th} day of repeated dosing, after increasing daily doses to avoid lethality due to high morphine dose administration (Table IV.1) and was further maintained for 7 days up to day 20.

Day	C-group Control group NaCl 0.9% 2 ml/kg/day	L-group Dose morphine-HCl (mg/kg/day) 2 ml/kg/day	M-group, H group Dose morphine-HCl (mg/kg/day) *2 ml/kg/day or **4 ml/kg/day
0,1	0	10	10*
2,3	0	10	20*
4,5	0	10	40*
6, 7, 8	0	10	60*
9, 10, 11	0	10	80**
12 - 20	0	10	100**

Table IV.1. Escalating dose administration of morphine (SC) during the repeated dose phase (Day 0 to Day 20) of the non-precipitated withdrawal test in order to avoid lethality due to the high morphine dose administration.

After 21 days of repeated SC dose administration, rats of the L-, M- and H-group were abruptly devoid of morphine and a withdrawal period of 7 successive days was initiated. The H-group received daily SC saline injections (2 ml/kg) during this period (Days 21 to 27) to mimic the previous dose treatment, whilst the M-group was left untreated during withdrawal. The C- control group followed the same dose- and withdrawal regimen as outlined for the L- and M morphine groups.

A number of specific behavioural observations alerting for possible physical dependence potential in the non-precipitated withdrawal model was determined (Table IV.2), including some observations that are known to be induced by withdrawal of some psycho-active drugs (*example*: wet dog shakes at opioid withdrawal). The incidence, severity and duration of these observations were recorded.

Observations	Scores	
Lethality	P,D,I or -	Preterminally Killed, Dead, Incidental Dead or Absent
Sniffing	+/-	Present / Absent
Abnormal Licking	+/-	Present / Absent
Abnormal preening	+/-	Present / Absent
Grooming	+/-	Present / Absent
Wet dog shakes	+/-	Present / Absent
Twitches	0/4	0: no; 1: slight; 2: mild; 3: obvious; 4: severe
Tremors	0/4	0: no; 1: slight; 2: mild; 3: obvious; 4: severe Specific Tremor : X if present
Convulsions	+/-	Present / Absent
Specific Convulsions:		X if present
- Clonic-type convulsions		Asymmetrical; Running excitement; Champing; Popcorn; Asphyxial
- Tonic-type convulsions		Opisthotonus; Emprosthotonus
- Miscellaneous-type convulsions		Rock and roll; Sitting-up; Praying
Locomotor activity	0/4	0: no; 1: slight; 2: mild; 3: obvious; 4: severe
- Abnormal biting	+/-	Present/Absent
- Restlessness	+/-	Present/Absent
- Writhing Body carriage	+/-	Present/Absent
- Straub Tail	0/4	0: flat; 1: horizontal stretched; 2: 30° upwards; 3: 60° upwards; 4: 90° upwards
Dyspnoea Arousal	+/-	Present/Absent
- Excitation	0/4	0: no; 1: slight; 2: mild; 3: obvious; 4: severe
- Jumping	+/-	Present/Absent
- Sedation	0/4	0: no; 1: slight; 2: mild; 3: obvious; 4: severe
- Alertness	+/-	Present/Absent
Narrowing palpebral fissure	0/4	0: completely closed; 1: narrowed; no reaction to stimuli; 2: narrowed; half-closed eyelids; 3: slightly narrowed; open after stimulus; 4: open
Defecation	-2/2	-2: no feces; -1: decreased feces; 0: normal; 1: increased feces 2: diarrhea
Lacrimation	+/-	Present/Absent
Catalepsy	+/-	Present/Absent
Abnormal gait		
- Ataxia	0/4	0: no; 1: slight; 2: mild; 3: obvious; 4: severe
- Tiptoe gait	+/-	Present/Absent
- Shuffling movements	+/-	Presen/Absent
Salivation	+/-	Present/Absent

Table IV.2. List of specific behavioural observations with the concomitant scores and/or grades.

The behavioural observations, body temperature, body weight and food uptake were measured prior to the start of the study, during specified days of the

repeated dose phase and daily during the withdrawal period as outlined in Table IV.3.

	T_{-1} (-1h)/T0	1h	3h	5h
Day -1	BT_{-24}			
	$BObs_{-24}$			
	BW_{-24}			
Day 0	BT_{-1}	BT_1		BT_5
	$BObs_{-1}$	$BObs_1$		
	BW_{-1}			
Day 12	BT_{-1}	BT_1		BT_5
	$BObs_{-1}$	$BObs_1$		
	BW_{-1}			BW_5
	FC_{-1}			FC_5
Day 19	BT_{-1}	BT_1		BT_5
	$BObs_{-1}$	$BObs_1$		
	BW_{-1}			BW_5
	FC_{-1}			FC_5
Day 20	BT_{-1}	BT_1		BT_5
	$BObs_{-1}$	$BObs_1$		
	BW_{-1}			BW_5
	FC_{-1}			FC_5
Day 21	BT_0	BT_1	BT3	BT_5
	$BObs_0$	$BObs_1$	BObs3	$BObs_5$
	BW_0	BW_1	BW3	BW_5
	FC_0			FC_5
Day 22	BT_0		BT3	
	$BObs_0$		BObs3	
	BW_0		BW3	
	FC_0		FC3	
Day 23	BT_0		BT3	
	$BObs_0$		BObs3	
	BW_0		BW3	
	FC_0		FC3	
Day 24, 25, 26, 27	BT_0			
	$BObs_0$			
	BW_0			
	FC_0			

Table IV.3. Outline of the measurements in the non-precipitated withdrawal test with morphine on various days (prior to start, during the repeated dose phase and during withdrawal) at specific time-points. BT: Body temperature; BObs: Behavioural Observations; BW: Body Weight; FC: Food Consumption; h: hour(s); T_{-24}: 24 h prior to start of dosing; T_{-1}: time point prior to dosing; T_0:0h: 24 h after last daily drug treatment/actual hour defined on Day 21.

General clinical observations were performed at least once a day from Day -1 (day before start of dosing) onwards until the end of the in-vivo phase (Day 27), for signs of ill health, abnormal behaviour or unusual appearance, occurrence of untoward clinical effects and manifestations of toxic and pharmacological response, moribund state and mortality.

On Days -1, 0, 12, 19, 20 of the repeated dose phase and during the withdrawal period the general observations were performed after the behavioural observations had been recorded.

At the end of the study, all animals were killed with use of CO_2 and discarded without examination. Only the 2 animals found dead during the study underwent a macroscopic examination.

IV.2.2. Non-precipitated Withdrawal Test with diazepam (study ID number TOX9320)

The purpose of this study was 1) to determine whether a 21-day oral repeated dose administration of diazepam at 2, 20 and 200 mg/kg induced detectable withdrawal effects in male rats upon cessation; 2) to investigate if a 1-month withdrawal period was appropriate to encompass all withdrawal effects.

Twenty male rats were divided into 4 groups of 5 males each via a body weight based stratified randomisation procedure. One group (V-group) received the vehicle solution (0.5 % w/v Methocel in demineralised water); three groups (L-, M- and H-group) were orally dosed at 2, 20 or 200 mg/kg diazepam (Methocel suspensions), respectively, during 21 days, followed by a withdrawal period that was initially set at 4 weeks but was extended to 6 weeks. The end of the in-vivo phase was defined as Day 63.

Care was taken to avoid noise in the animal room, as noise during diazepam treatment might produce specific changes in 5-HT tone in the hippocampal brain region and hence modify the pattern of changes found during diazepam withdrawal (Fernandes, 1994).

One day before starting the study (Day -1), body temperature and behavioural observations were registered. On Days 0 (first day of dosing), 14 and 20 of the repeated dose phase, body temperature and behavioural observations were recorded in each group prior to daily dosing and after 1 and 5 hours post-dose. Body weight was registered only prior to daily dosing. All measurements were also executed during the withdrawal phase, from Day 21 up to Day 27, and on Days 28, 35, 42, 49, 56 and 63, at 1 and 5 hours after the previous dosing time point defined as 0 hour.

General clinical observations were performed at least once a day from Day -1 onwards until the end of the in-vivo phase (Day 63). On Days -1, 0, 14, 20 of the RD phase and during the withdrawal period the general observations were performed after the behavioural observations had been recorded.

At the end of the study, all animals were killed with use of CO_2 and discarded without examination.

IV.2.3. Non-precipitated Withdrawal Test with ethanol combined with precipitated Withdrawal test using Ro15-4513 (study ID number TOX9650)

The purpose of this study was 1) to determine and assess the potential withdrawal effects of ethanol upon cessation of a 21-day repeated dose oral (via gavage) administration; 2) to investigate whether these potential withdrawal effects could be precipitated by administering a single dose of Ro15-4513 shortly after the last ethanol dose; 3) to investigate if Ro15-4513 could counterbalance the withdrawal effects when dosed daily during the 7-day withdrawal period.

Twenty male rats were divided into 4 groups of 5 males each via a body weight based stratified randomisation procedure. One group (C-group) received the control solution (NaCl 0.9%); in the other three groups (groups A, B and D) 1500 mg/kg ethanol was administered orally from Day 0 till Day 20. After 21 days of repeated oral dose administration, rats were abruptly devoid of ethanol (groups A, B, D) or water (group C) and a withdrawal period of 7 successive days was initiated. The A- and the C-groups followed the non-precipitated withdrawal pattern whereas the B-group received 1.5 mg/kg Ro15-4513 OR at 2

hours after the last ethanol treatment on Day 20 before the withdrawal period was started on Day 21 (precipitated withdrawal model). The D-group was treated daily with 1.5 mg/kg Ro15-4513 OR during the withdrawal period (Days 21 to 27).

Body temperature and behavioural observations were recorded in each group prior to daily dosing and after 0.5 and 3 hours post-dose on Days 0 (first day of dosing), 14, 19 and 20 of the repeated dose phase. Body weight and food uptake were measured on these days prior to daily dosing; food uptake was also measured after 3 hours. All these parameters were also registered during the withdrawal phase (Day 21 up to Day 27) at the same time points.

General clinical observations were performed at least once a day from Day -1 onwards until the end of the in-vivo phase. On Days -1, 0, 14, 19 and 20 of the RD phase and during the withdrawal period the general observations were performed after the behavioural observations had been recorded.

At the end of the study, all animals were killed with use of CO_2 and discarded without examination.

IV.2.4. Non-precipitated Withdrawal Test with cocaine (study ID number TOX10109)

The goal of the study was 1) to determine the behavioural profile of cocaine after acute or repeated intraperitoneal (IP) administration at 15 mg/kg; 2) to assess the withdrawal symptoms upon abrupt cessation; 3) to assess the kinetic profile of cocaine after IP administration.

Thirty-five male rats were divided into 7 groups of 5 males each via a body weight based stratified randomisation procedure. Four groups were dosed with 15 mg/kg cocaine IP: group B received a single dose of cocaine; Groups E and G were treated with cocaine for 10 and 23 successive days, respectively, without a subsequent withdrawal period. Group H was dosed with cocaine during 10 days, followed by a withdrawal period of 13 days. Three concurrent control groups (A, D and F-groups) received the control solution (NaCl 0.9%) at the same dosing regimen as the B, E and G cocaine groups, respectively. There was no extra

control group next to the H group, as the D- control group could serve for comparison to the H-group as well. The outline of the dosing groups is presented in Fig. IV.1.

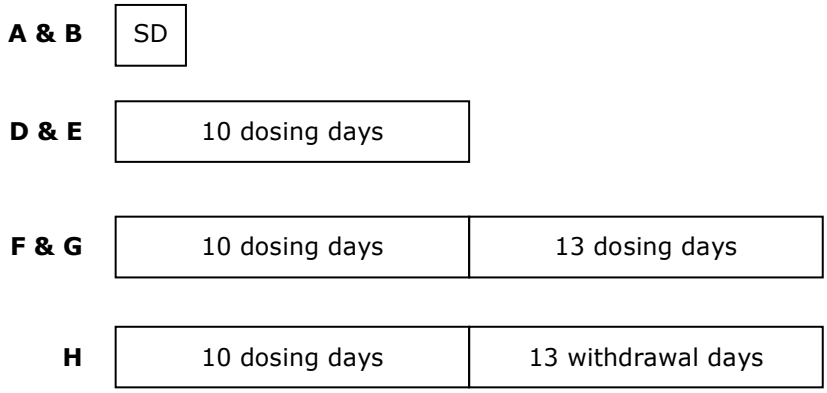


Fig. IV.1. Outline of the non-precipitated withdrawal study with cocaine. Groups B, E, G and H received cocaine whereas groups A, D and F were dosed with NaCl 0.9%. SD: single dose. In addition, a 13-day withdrawal period was added to the H-group.

General clinical observations, behavioural observations, body weight, and food uptake were recorded as presented in Table IV.4.

Clinical observations	Days 0 and 9: at 0, 0.5, 1, 2, 4, 6, 8 and after 24h dosing
	Day 22: at 0, 0.5, 1, 2, 4, 6, 8 and after 24h dosing
	Other days: before and after dosing
Behavioural observations	Days 1, 8, 10, 11, 14, 21
Body weight	Days 0 and 9
	Day 10 (prior to dosing and after 6h)
	Day 11 (prior to dosing and after 6h)
	Days 12 and 22
Food consumption	Day 1
	Day 10 (prior to dosing and after 6h)
	Day 11 (prior to dosing and after 6h)
	Days 12 and 22

Table IV.4. Outline of the measured parameters included in the non-precipitated withdrawal study with cocaine.

Kinetic analyses were performed on plasma samples collected from the cocaine dosed groups E, G and H on Days 0, 9 and 22, at 0.25, 2 and 6 h post-dose (first three rats of these groups) and at 0.08, 0.5 and 4 h post-dose (last two rats of these groups).

Overall remark: studies were performed sequentially; all variables tested were hence implemented incrementally and as such retested in each following study. This added to the reproducibility of these variables on the outcome of the individual studies.

IV.3. Results

IV.3.1. Non-precipitated Withdrawal Test with morphine (study ID number TOX8955)

An overall summary of the findings is given in Table IV.5.

Morphine-HCl; 21d RD; 7d WD 5M/group (4 groups)	RD phase 10 mg eq./kg	WD phase	RD phase 100 mg eq./kg	WD phase	RD phase 100 mg eq./kg	WD phase Saline 0.9%
Mortality [a]	0/5	0/5	1/5 (Day 0)	0/5	1/5 (Day 0)	0/5
Body temperature	I (+1, +5h)	N	I (+1, +5h)	D (Days 22, 23)	I (+1, +5h)	N
Behavioural observations[a]		-		-		-
- restlessness	P (+1h)	A	P (+1h)	A	P (+1h)	A
- abnormal licking (pica)	P (+1h)	1/5	P (+1h)	A	P (+1h)	A
- sedation	P (+1h)	A	P (+1h)	A	P (+1h)	A
- less alertness	P (+1h)	-	P (+1h)	-	P (+1h)	-
- higher alertness	-	P (Day 22)	-	P (Day 22)	-	P (Day 22)
- excitation (only when stimulated or touched)	P (+1h)	-	P (+1h)	-	P (+1h)	-
- excitation		P		P		P
- jumping	-	A	-	A	-	A
- twitches	P (+1h)	A	P (+1h)	A	A	A
- catalepsy	P (+1h)	A	P (+1h)	A	P (+1h)	A
- salivation	P (+1h)	A	P (+1h)	A	P (+1h)	A
- wet dog shakes	P (+1h)	P	P (+1h)	P	P (+1h)	P
- grooming	N	P	P (-1h)	P	P (-1h)	P
- locomotor activity	-	N	-	A (Day 21)	-	A (Day 21)
	-	I (Day 22)	-	I (Day 22)	-	I (Day 22)
General clinical observations[a]					-	-
- excitability	P	-	P	-	P	-
- excessive salivation	P	-	P	-	P	-
- compulsive behaviour	P	-	P	-	P	-
- decreased general activity	P	-	P	-	P	-
- absence of eye blinking	P	-	P	-	P	-
- soft faeces	P	-	P	-	P	-
- small amount of faeces	-	N	-	N	-	P(1/4)
- catalepsy	P(1/5)	-	P	-	P	-
- audible respiration	P(1/5)	-	P	-	P	-
- piloerection	-	N	-	P(1/4)	-	P(1/4)
- hypertonia	-	N	-	P	-	P
- aggression	-	N	-	P(1/4)	-	P(1/4)
Body weight/ Weight gain	D+/D++	D+/D+	D++/D++	D++/D++	D++/D++	D++/D++
Food consumption	D++	D++	D++	D++	D++	D++
Post-mortem data (RD phase)[a]	N	-	1/5	_	1/5	_
- discoloured liver			P		N	
- congested lungs			N		P	
- distension						
urinary bladder			P		N	
caecum			P		N	
stomach			N		P	
- red-coloured subcutis (injection site)			N		P	

Legend: [a]: number of rats showing the observation/total number.
N: Normal P: Present A: Absent D: Decrease I: Increase +: Mild ++: Marked +++: Severe

Table IV.5. Summary of the findings in the non-precipitated withdrawal study with morphine.

Subcutaneous repeated dose administration of either a fixed dose of 10 mg/kg morphine (L-group) or escalating doses up to 100 mg/kg (M and H groups) resulted in findings characteristic for subchronic morphine administration and included restlessness, abnormal licking (pica), compulsive behaviour, sedation, less alertness, absence of eye blinking, excitation (only when stimulated or touched), jumping, twitches, catalepsy and salivation. These observations were recorded after 1 hour post-dose. At 3 to 4 hours after daily dosing animals experienced a general excitation phase (without stimulation or touching the animals). Wet dog shakes were seen in both 100 mg/kg dosed groups at the end of the repeated dose phase prior to daily dosing. A drug-related increase in body temperature was recorded in all groups after 1 and 5 hours post-dose and soft faeces were noted in a few animals. Audible respiration was recorded once in one male of the 10 mg/kg dosed group and on occasion in several 100 mg/kg dosed animals from the second week of dosing onwards.

In the 100 mg/kg M-group one male was found dead on Day 0 (first day of dosing) shortly after SC injection of 10 mg/kg. Post-mortem macroscopic evaluation revealed a moderately dark discolouration of the liver, a moderate distension of the urinary bladder and a slightly distended caecum. In the 100 mg/kg H-group one male was found dead on Day 4 of the repeated dose phase, after SC injection of 40 mg/kg. At autopsy all lung lobes were slightly congested and the stomach was slightly distended. The subcutis at the site of injection was red-coloured.

Morphine is known to induce urinary retention that might result in distension of the urinary bladder. The prolonged gastric emptying effect of morphine accompanied by the increased food uptake during the dosing days might have caused the gastric and caecal distension. In addition, aspiration of food (due to extensive pica) might have induced lung congestion in the dead animal.

There was a drug and dose related decrease in body weight and in weight gain (Table IV.6). However, body weights were 3 to 7% higher in the 10 and 100 mg/kg morphine dosed groups, respectively, when recorded after 5 h post-dose and compared to the data prior to daily dosing, and in comparison with the control group.

EXPERIMENT : TOX - 8955
Abuse Liability Testing in the Rat: Non-precipitated Withdrawal Test
Morphine Hydrocloride - SC - RAT - RD Phase Body weight data

	Day 12	Day 12	Day 19	Day 19	Day 20	Day 20
	0h	+5h	0h	+5h	0h	+5h
Control						
Mean	307,0	305,0	351,0	352,0	355,0	356,8
Standard Error	8,3	9,7	11,2	11,0	11,2	12,0
Low						
Mean	269,0*	277,0	302,0*	313,0	307,0*	316,2
Standard Error	5,6	7,4	6,5	7,0	6,0	7,0
Medium						
Mean	256,0*	264,8	270,0*	284,3	270,0*	289,5
Standard Error	4,6	6,0	1,9	5,3	1,9	4,4
High						
Mean	263,0*	271,5	286,0*	300,5	287, 0*	304,0
Standard Error	8,3	9,4	7,2	6,7	6,3	6,4

Significance (Days 12, 19 and 20 at 0h) computed versus the Control group by Mann-Whitney U test (two-tailed): * p <.05 ** p <.01 *** p <.001

Table IV.6. Repeated dose phase with morphine: mean body weights (g) recorded prior to dosing (0h) and at 5h post-dose. Dose administration: Low: 10 mg/kg morphine; Medium and High: 100 mg/kg morphine from Day 12 onwards.

A marked decrease in food consumption was recorded, although food uptake during the ongoing day(s) [measured at 5h post dosing versus 0h (prior to dosing)] appeared to be higher in the 100 mg/kg morphine groups when compared to the control group.

Upon abrupt cessation of 10 or 100 mg/kg there were some clinical observations which were similar to those noticed at 3 to 4 hours after daily dosing, i.e. slightly increased activity and alertness, excitation, grooming and wet dog shakes. Effects were most pronounced or present on the second day of the withdrawal phase. Additional clinical observations in the previously 100 mg/kg dosed groups included piloerection, hypertonia and aggression (Table IV.5).

The body weight and food uptake evolution did not indicate signs of withdrawal in the 10 mg/kg dosed group but there was a decrease in body weight and weight gain in the 100 mg/kg dosed groups during the first 3 days; this was however more pronounced in the group that did not receive NaCl 0.9% during withdrawal (Table IV.7).

	Dosage Group (mg eq./ kg) Males			
Week/Day	**Control**	**Low:10**	**Med.:100**	**High:100**
3/21	362	314 *	269 *	285 *
	(11.5)	(6.2)	(3.0)	(6.2)
4/22	366	312 *	249 *	268 *
	(11.9)	(7.2)	(1.8)	(8.3)
4/23	373	324 *	258 *	272 *
	(12.0)	(7.2)	(3.9)	(8.0)
4/24	380	331 *	274 *	285 *
	(12.5)	(7.0)	(5.9)	(7.5)
4/25	385	339 *	286 *	296 *
	(11.6)	(7.8)	(6.1)	(7.7)
4/26	391	346 *	294 *	307 *
	(12.9)	(8.3)	(6.7)	(6.8)
4/27	396	352	301 *	312 *
	(13.4)	(8.5)	(8.0)	(7.6)

Significance computed versus the Control group by Mann-Whitney U test (two-tailed):
* p <.05 ** p <.01 *** p <.001 Standard error is shown between brackets

Table IV.7. Mean body weights (g) during the withdrawal phase of the non-precipitated withdrawal study with morphine.

Food uptake was markedly lower in both 100 mg/kg groups during the first two days of withdrawal as evidenced in Table IV.8.

	Dosage Group (mg eq./ kg) Males			
Week/Day	**Control**	**Low:10**	**Med.:100**	**High:100**
4/22	32	26	16 *	18 *
	(0.8)	(1.9)	(1.3)	(1.2)
4/23	35	32	23 *	23 *
	(1.6)	(1.5)	(1.4)	(1.7)
4/24	34	31 *	32	31
	(1.0)	(0.7)	(2.8)	(1.5)
4/25	35	33	37	31
	(0.9)	(1.7)	(3.8)	(1.7)
4/26	34	32	37	33
	(1.0)	(0.7)	(5.8)	(2.1)
4/27	32	32	35	33
	(0.6)	(1.1)	(4.3)	(2.3)
Total:	**202**	**186**	**178**	**168 ***

Significance computed versus the Control group by Mann-Whitney U test (two-tailed):
* p <.05 ** p <.01 *** p <.001 Standard error is shown between brackets

Table IV.8. Daily and total food uptake during withdrawal in the non-precipitated withdrawal test with morphine.

Body temperatures of the 10 mg/kg group remained very stable throughout the first day of withdrawal and during the further withdrawal period. The 100 mg/kg group without any treatment during the withdrawal period experienced a slightly lower body temperature during the second day of withdrawal. This was not seen in the 100 mg/kg group which received daily SC NaCl 0.9% injections during the withdrawal phase (Fig. IV.2).

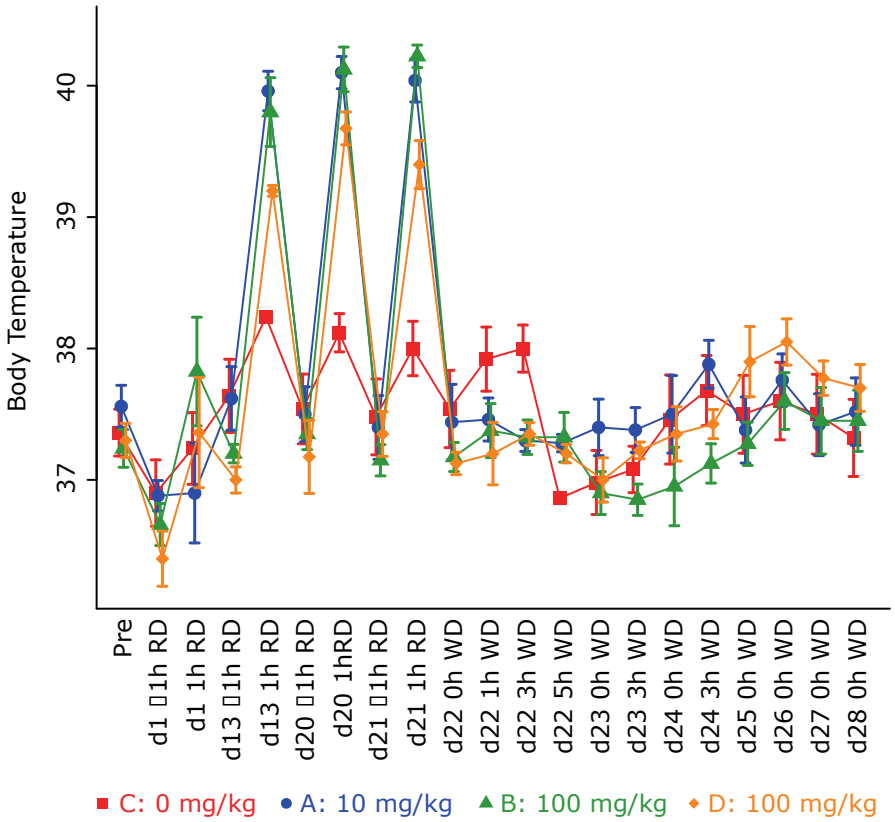


Fig. IV.2. Overall graph of mean body temperatures recorded during the repeated dose phase and the withdrawal period of the non-precipitated withdrawal study of morphine. A drug-related increase in body temperature was noticed after daily dosing in all morphine dosed groups (5h time point not uploaded in this graph). During the first days of withdrawal a slightly lower body temperature was recorded in the 100 mg/kg group that did not receive daily SC NaCl 0.9% injections during withdrawal.

IV.3.2. Non-precipitated Withdrawal Test with diazepam (study ID number TOX9320)

A list of the general clinical observations noticed in the different groups is given in Table IV.9.

Repeated dose (RD) administration of diazepam at 2 mg/kg/day for 3 weeks induced compulsive behaviour (nibbling on food pellets), dose-related narrowing of the palpebral fissure and restlessness in most rats during several days of the RD phase. One animal also displayed abnormal licking on one day during the first dosing week.

At 20 mg/kg/day, sedation, a decreased general activity, narrowing of the palpebral fissure, ataxia and tiptoe gait were noticed on several days throughout the RD phase (generally at 1 hour post-dose on Days 0, 14 and 20).

Additionally at 200 mg/kg/day, catalepsy, absence of alertness, and narrowing of the palpebral fissure (autonomic response) were seen on several days (at 1 and/or 5 hours post-dose on Days 0, 14 and/or 20).

Dosage Group (mg / kg):	Vehicle X / N	Low: 2 mg/kg X / N	Med.: 20 mg/kg X / N	High: 200 mg/kg X / N
Ataxia	0 / 5	0 / 5	5 / 5**	5 / 5**
Compulsive behaviour	0 / 5	4 / 5*	5 / 5**	5 / 5**
Decreased general activity	0 / 5	0 / 5	5 / 5**	5 / 5**
Audible respiration	0 / 5	0 / 5	0 / 5	1 / 5
Catalepsy	0 / 5	0 / 5	0 / 5	3 / 5
Tiptoe gait	0 / 5	0 / 5	1 / 5	5 / 5**
Restlessness	0 / 5	3 / 5	5 / 5**	5 / 5**
Abnormal licking	0 / 5	1 / 5	2 / 5	1 / 5
Hyperreactive to touch	0 / 5	0 / 5	2 / 5	1 / 5

Table IV.9. Enumeration of the incidence of clinical observations during the repeated dose phase of diazepam at 2, 20, 200: dose in mg/kg/day. X: Number of positive animals; N: Total number of animals. Significance computed by Fisher Exact test (right-sided); p*<0.05; p**<0.01.

At 20 mg/kg, a dose-related decrease in body temperature at 1 hour after daily dosing and in body weight gain was recorded. This change in body weight evolution, showing a decreased weight gain after two weeks of dosing (Table

IV.10) was in agreement with the published results of a diazepam study in the rat (Grimm, 1983).

	Dosage Group (mg/kg) Males			
Week/Day	Vehicle	Low:2	Med.:20	High:200
2/14	112	110	103	99
	(2.4)	(5.5)	(4.7)	(2.2)
3/20	145	143	138	135
	(4.4)	(9.8)	(7.3)	(2.9)

Table IV.10. Mean body weight gain recorded during the repeated dose phase of the non-precipitated withdrawal test with diazepam at 2, 20 and 200 mg/kg. Standard errors are given between brackets.

The decreased body weight gain at 200 mg/kg was accompanied by a minimally decreased body weight and a drug-related decrease in body temperature at 1 hour after daily dosing was recorded (Fig. IV.3).

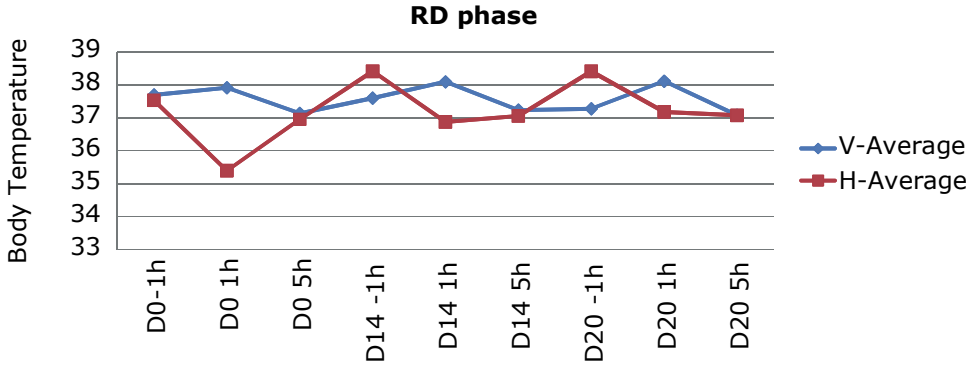


Fig. IV.3. Mean body temperatures during the repeated dose phase of the H (200 mg/kg) group versus V (vehicle).

Clinical manifestations including motor impairment, sedation-related signs and a decreased body weight gain are common effects reported following treatment with diazepam. Occasionally, paradoxical excitation (restlessness) can occur as well (Martijena, 1985; Kunchandy, 1986).

During the withdrawal phase, most repeated dosing effects were not present anymore (except for a tendency to a higher body temperature in the 200 mg/kg/day dosed group on the third and fourth day of withdrawal (Fig. IV.4).

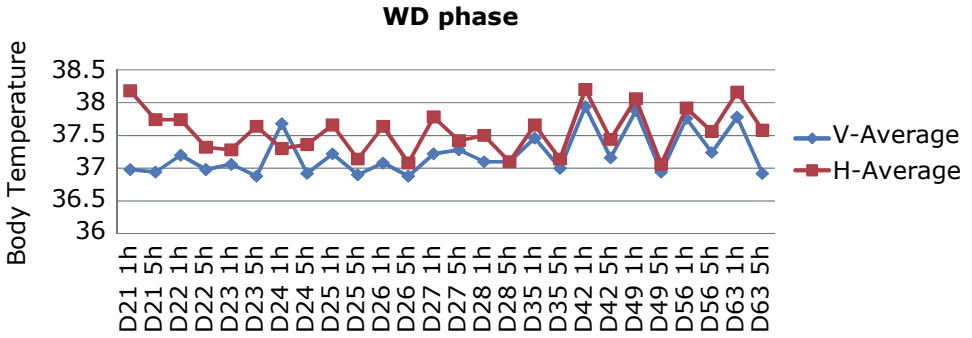


Fig. IV.4. Mean body temperatures of the H (200 mg/kg) group and the V(vehicle) group during the withdrawal phase.

Possible withdrawal effects at 2 mg/kg/day were reflected by restlessness and increased incidence of sniffing, noticed on occasion during the first two weeks and increased alertness throughout the withdrawal phase. Abnormal preening and wet dog shakes were observed once in one rat during the 5th week of the withdrawal phase. All animals were excitable, and this feature remained present up till the 5th week of withdrawal.

In the 20 and 200 mg/kg/day dosed group, restlessness, sniffing, increased locomotor activity, abnormal preening, compulsive behaviour, increased alertness and wet dog shakes were present in the first or second week of withdrawal, although abnormal preening, increased locomotor acitivity and wet dog shakes were also noted in some animals during the 3rd, 4th or 5th treatment-free weeks. Excitability, narrowing of the palpebral fissure (one male previously dosed at 200 mg/kg) and hyperreactivity to touch were additionally observed and remained present till the end of the 6-week withdrawal phase in some males. Body weight loss or decreased body weight gain were noted in both groups up to the 3rd week of withdrawal. From the 2nd week onwards a tendency of increased food uptake was noted in the 200 mg/kg group, which led to recovery of the body weight changes in this group after 4 weeks of withdrawal.

IV.3.3. Non-precipitated Withdrawal Test with ethanol combined with precipitated Withdrawal test using Ro15-4513 (study ID number TOX9650)

During the RD phase no adverse behavioural or clinical observations were noticed in whatever group. Body weight and food uptake increased in a similar way as in the control group.

In the non-precipitated withdrawal paradigm (group A) no adverse clinical effects were noticed during the 7-day withdrawal period. Food uptake was slightly (8%) decreased on the first day of withdrawal, but increased to control levels at the end of the study. Body weights and weight gain were not adversely affected.

A single dose of Ro15-4513 administered at 2 hours after the last ethanol treatment (group B) initiated a decrease in body weight gain (13%, non-significant) and food uptake (20%, $p<0.05$) after 24 hours (versus control), in contrast to the other groups where no effects on these parameters were recorded after the last ethanol treatment day (Table IV.11).

When Ro15-4513 was administered daily during the 7-day withdrawal period (group D), increases in alertness (Table IV.12), in sniffing and in defecation were observed after 0.5 and 3h post dose.

Body weight (24 hours after the last ethanol dose administration)														
Dosage	**Animal**	**1**	**2**	**3**	**4**	**5**	**N**	**MN**	**SD**	**P**		**MN**	**SD**	**P**
Control	0	373	395	412	402	397	5	396	14			96	21	
Group A	20	354	392	419	366	419	5	390	30	0.841		94	19	0.841
Group B	40	385	401	343	410	357	5	379	29	0.421		80	26	0.222
Group D	60	407	365	407	394	394	5	393	17	0.690		95	11	0.841
Food uptake (24 hours after the last ethanol dose administration)														
Dosage	**Animal**	**1**	**2**	**3**	**4**	**5**	**N**	**MN**	**SD**	**P**		**MN**	**SD**	**P**
Control	0	51	53	64	65	65	5	60	7			609	44	
Group A	20	52	53	59	53	58	5	55	3	0.421		583	44	0.421
Group B	40	49	48	47	53	44	5	48	3	0.024	*	559	33	0.095
Group D	60	60	49	56	58	59	5	56	4	0.421		604	38	0.690

Table. IV.11. Body weight and food uptake measured at 24 hours after the last ethanol dose administration (1500 mg/kg OR). Control and Group A: non-precipitated withdrawal design; Group B: precipitated withdrawal design with Ro15-4513 single dose (1.5 mg/kg OR) shortly after last ethanol dose; Group D: 21 day-treatment with ethanol followed by Ro15-4513 daily treatment (1.5 mg/kg OR) during 7-day withdrawal period; N: number of rats/group; MN: mean value/group; SD: standard deviation; P: p-value (Mann-Whitney-U), significance *p<0.05.

Alertness	D20	D21	D22	D23	D24	D25	D26	D27
	0.5h 3h	0.5h 3h	0.5h 3h	3h	3h	3h	3h	3h
Control	0/5 0/5	0/5 0/5	1/5 0/5	0/5	0/5	1/5	1/5	1/5
Group A	1/5 0/5	1/5 0/5	0/5 1/5	1/5	1/5	2/5	0/5	2/5
Group B	0/5 2/5	0/5 0/5	2/5 0/5	0/5	0/5	1/5	0/5	1/5
Group D	2/5 0/5	4/5* 0/5*	2/5 1/5	2/5	2/5	5/5*	4/5	1/5

Table IV.12. Incidence of alertness during the 7-day withdrawal period following ethanol treatment. D20: last day of ethanol treatment at 0h; D21-D27: withdrawal period (WD); Control and Group A: non-precipitated withdrawal; Group B: precipitated withdrawal with Ro15-4513 (1.5 mg/kg OR); Group D: 21 day-treatment with ethanol (1500 mg/kg OR) followed by Ro15-4513 daily treatment (1.5 mg/kg OR) during 7-days; X/N: number of rats with increased alertness/group (n=5). Significance computed by Fisher Exact test (right-sided); *<0.05.

IV.3.4. Non-precipitated Withdrawal Test with cocaine (study ID number TOX10109)

Behavioural observations recorded during the repeated dose phase included increased sniffing, restlessness, arousal, excitation, jumping, head shaking, stereotyped behaviour and an increased startle response. A significant ($p<0.05$-0.01) decrease in body weight gain or body weight loss was recorded (Table IV.12).

	Males Dosage Group (mg eq/ kg)						
Week/Day	**F**	**A**	**D**	**G:15**	**E:15**	**H:15**	**B:15**
1/1	5	7	-23 **	-12 *	-8	-6	-23 **
	(10.5)	(1.4)	(3.1)	(1.7)	(1.2)	(3.4)	(3.1)
2/9	54	51	-	16 **	20 *	16 *	-
	(13.0)	(4.4)		(2.5)	(2.4)	(6.1)	
2/10	50	-	-	11 **	-	15 *	-
	(11.8)			(3.9)		(7.1)	
2/11	53	-	-	18 **	-	14 *	-
	(11.0)			(3.5)		(7.5)	
2/12	58	-	-	18 **	-	20 *	-
	(13.8)			(5.1)		(7.9)	
2/14	68	-	-	22 **	-	26 *	-
	(14.1)			(4.1)		(9.4)	
3/15	70	-	-	21 *	-	25 *	-
	(14.4)			(6.1)		(9.3)	
3/16	78	-	-	32 *	-	32 *	-
	(13.6)			(5.5)		(9.4)	
4/22	103	-	-	46 **	-	54 *	-
	(12.7)			(5.0)		(10.8)	

Significance level computed with Fisher Exact probability test (two-tailed):
* p < .05 ** p < .01 *** p < .001
(Significance computed versus the F dosage group)
X: Number of animals dead or sacrificed at stated period N: Total number of animals S: Significance

Table IV.13. Body weight data after the single dose or repeated dose phase in the cocaine groups (G, E, H, B). Control groups: F, A, D.

Food uptake significantly decreased ($p<0.05$) after 10 days of dosing (group G) and remained lower ($p<0.01$) after 23 dosing days. During the withdrawal period no adverse clinical effects were present, apart from a slightly higher body temperature in the early morning. Body weights increased at a normal rate from the third treatment-free day onwards and food uptake (group H) reached values comparable to those of the control group (group F), as daytime food consumption was increased during withdrawal.

Toxicokinetic investigation indicated similar plasma exposures after single and repeated daily dosing of cocaine (15 mg/kg IP) during 10 and 23 days (Table IV.14).

Group	G			H		
Day	0	9	22	0	9	22
C_{max} (ng/ml)	906	1010	818	834	1020	<BQL
T_{max} (h)	0.5	0.08	0.25	0.5	0.5	-
$AUC_{0\text{-}6h}$ (ng.h/ml)	1050	1160	854	809	1270	<BQL

Table IV.14. Plasma exposure levels were similar after both single and repeated dosing with cocaine. Group G: levels after 1, 10 and 23 daily dosages of cocaine (15 mg/kg IP); group H: levels after 1 and 10 daily dosages and after a 2-week withdrawal period. <BQL: below quantification level.

Peak plasma concentrations were observed within the first 30 minutes after dosing after both single and repeated exposure to cocaine 15 mg/kg IP; thereafter a gradual decline in plasma exposure occurred within the next 6 hours (Fig. IV.5). Exposure was no longer measurable after the two weeks withdrawal period. Brain tissue concentrations and plasma concentrations sampled at necropsy (24 hours after the last exposure for group G and after the 13-day withdrawal for group H) were all below the limit of quantification.

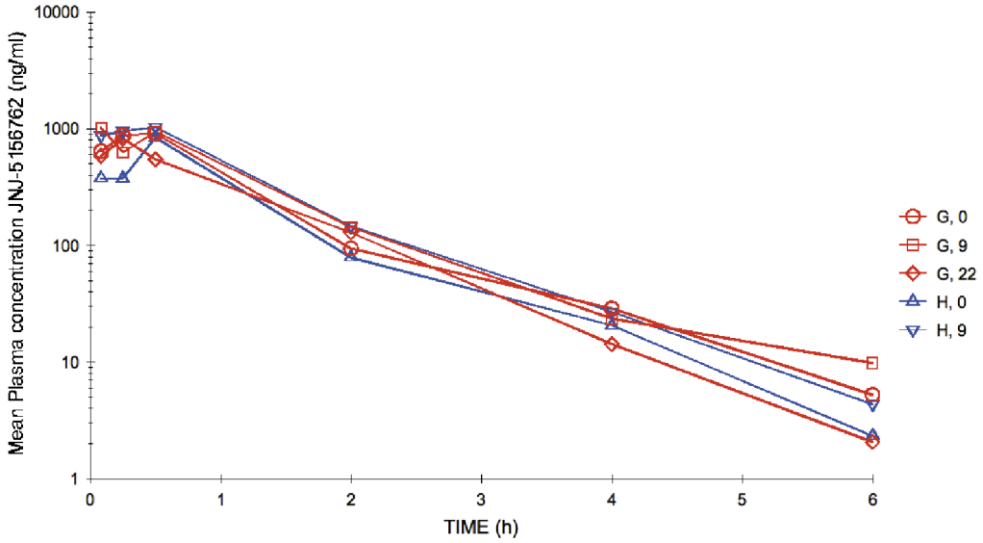


Fig. IV.5. Mean plasma concentrations vs time profiles on Days 0 (single dose), Day 9 (RD) and day 22 (RD) of cocaine administration.

IV.4. Discussion

IV.4.1. Results of the four mechanistic studies

In order to optimize the non-precipitated withdrawal model for use in Drug Development, four mechanistic studies and one validation study were conducted, using different reference drugs to investigate the variables that could impact on the results.

When morphine was tested at a fixed daily SC 10 mg/kg dose for 21 days, no clear withdrawal symptoms could be generated after abrupt cessation, but escalating doses up to 100 mg/kg, a dose that was further administered for 7 days and followed by treatment discontinuation, resulted in the withdrawal effects known for morphine. The effects were most pronounced during the first 3 days of the 7-day withdrawal period, indicating that the length of this treatment-free period was sufficiently long for testing morphine in this paradigm. These data also showed the importance of both the dose selection and the chosen duration of the repeated dose phase.

The withdrawal symptoms were less pronounced when rats were administered SC injections of NaCl 0.9% during the withdrawal phase, demonstrating the compensatory response to the previously drug administrations during the repeated dose phase. Although this specific finding is proven not to be drug related, but conditioned, we recommend to not mimic drug administration during the withdrawal phase in order not to suppress or disguise minor or discrete drug related withdrawal effects.

Abrupt cessation of a 21-day repeated dose phase with diazepam up to 200 mg/kg resulted in withdrawal effects known for this compound (Martijena, 1996). They were most pronounced or present during the first two weeks of the withdrawal period, although some observations were also noticed during the third week of withdrawal or remained present until the end of the 6-week withdrawal period. These results demonstrated that a 21-day repeated dose phase was sufficiently long to induce withdrawal effects for this class of compounds, but that the classical 7-day withdrawal period was not adequate to

embrace the withdrawal effects of substances like diazepam, as these developed slowly and were perceived rather subtle.

Ethanol effects in rats are mainly characterized by aberrant social behaviour like, *for example*, decreases in social grooming and sniffing, and approaching to other rats. This was not investigated in the combined non-precipitated and precipitated withdrawal study with ethanol as rats were housed individually. The selected dose of 1500 mg/kg ethanol did not induce adverse effects, nor were withdrawal effects noticed. This was in line with other publications on ethanol treatment in rats (Kuzmin, 2012). However, single or repeated administration of Ro15-4513 enhanced known ethanol withdrawal symptoms (Wood, 1989; Kuzmin, 2012), inherent in the precipitated withdrawal design. These precipitated signs might be driven by its increased binding at the α4 $GABA_A$ subunits in the cerebellum, induced by sustained ethanol treatment (Cagetti, 2003). Ro15-4513, a $GABA_A$ receptor inverse agonist will thus act as a positive $GABA_A$ modulator after cessation of ethanol treatment (Sanna, 2003), hence elucidating its acute effects as shown in this study. Whether this compound can be a truly an ethanol antagonist in human is not proven as alcohol exerts its activity not only via $GABA_A$ modulation but also via 5-HT3, nicotinic and NMDA receptors (Dopico, 2009).

Discontinuation from IP cocaine treatment resulted in a normalization of the behavioural symptoms, probably caused by a rapid decrease in dopaminergic neuronal activity (Fung, 1994). It was demonstrated that cocaine is eliminated from the body very rapidly and that no accumulation occurs.

IV.4.2. Investigation of the variables to optimize the design of a non-precipitated withdrawal model for use in Drug Development

In the 4 conducted mechanistic studies we clearly demonstrated that the length of the repeated dose phase must be sufficiently long in order to obtain adaptation to the test compound by the body during repeated drug administration as part of the homeostatic process, thus allowing the animal to develop a neuroadaptive response. Sufficient exposure to the drug also includes adequate receptor occupancy and/or brain penetration. For a drug candidate, this fundamental information is obtained during the phase prior to its selection

and during further entrance into Drug Development. Indeed, within Drug Development, receptor selectivity of the drug candidate is investigated through functional in-vitro assays for its agonist or antagonistic activity and for possible interaction with other targets. The binding profile of a drug candidate is evaluated on different receptor-, ion channel- and transporter targets. Further, assays are designed to evaluate whether a drug candidate occupies the functional receptor in vivo through dose- and time-occupancy relationships after systemic administration in rats. Besides these primary pharmacology data, the results of general pharmacological models (sleep-wake architecture, specific drug target related CNS models, models related to additional body functions (autonomic nervous system, GI system, …) are also studied to ensure a proper dose selection with sufficient exposure in the brain. The excipients used to formulate the drug candidate as a (micro)suspension or solution, the frequency of dosing (q.d., b.i.d., continuous IV infusion, …) and the route of administration are also determining brain- and peak plasma exposures. Therefore, the pharmacokinetic profile of the most stringent formulations and routes has to be investigated and taken into account as well to allow a correct, compound driven design of the non-precipitated withdrawal test.

With regard to the doses used in these tests, a proper selection of the test dose range is not only based on the data of the above mentioned *in-vitro* and *in-vivo* pharmacological studies, but very importantly, also on the toxicological and concomitantly toxicokinetic profile of the test compound, as the off-target toxicity is often triggering the selected dosages The results of the toxicological tolerance studies, which are designed to determine the maximum tolerated single dose; the repeated dose toxicity studies, that investigate possible effects due to repeatedly administrating the test compound (*amongst others* accumulation, induction, tolerance, target organ toxicity, …); and the Modified Irwin's study (Teuns, 2005a,b,c), in which the neurofunctional integrity in the rat is explored after single doses, add to a scientifically based selection of various test dosages in the non-precipitated withdrawal design, of which the low dose lays within the range of the pharmacologically active dose, and where the high dose is situated within the toxic range. According to the requirements of the drug-licensing authorities, this high dose needs to cover a multiple of the human efficacious dose, expressed as C_{max}.

We have opted to set the RD phase at 21 successive days, to ensure a steady state level of the plasma exposures during the repeated dose phase.

This 21-day repeated dose design was proven relevant and scientifically valid and therefore used as a standard in the non-precipitated withdrawal test.

The appearance of withdrawal signs during the withdrawal phase represents the elimination of the active form of the drug from the body and is independent of any particular mechanism. The toxicokinetic profile of the test compound determined during the former studies in Drug Development is one of the key factors to rely upon for determining the length of the WD phase. The maximum or peak plasma concentration (C_{max}) and the subsequent T_{max}, and the half-life ($T_{1/2}$) after single and repeated doses have to be taken into account to ensure a sufficiently long withdrawal period. The physico-chemical properties of the drug might also influence the ADME profile (Absorption/Distribution/Metabolism/ Excretion), of which in particular the elimination phase is of importance during this withdrawal phase. This was easily illustrated with morphine, a hydrophilic compound (Wells, 2004) that is rapidly metabolised and eliminated via the urine. In the first study, the withdrawal effects of morphine, were followed during 7 days but were most pronounced during the first 3 days of withdrawal. The same applied to ethanol which is also a hydrophilic compound. On the other hand, diazepam, a lipophylic compound, is extensively (re)distributed into the tissue, metabolized into active metabolites and excreted via the urine only after 1 to 3 days (Klotz, 1976). Abrupt cessation of subchronic treatment of diazepam led to subtle withdrawal signs which were most pronounced, but not limited to 2 weeks after stopping the treatment. The withdrawal period was extended to 6 weeks during which some withdrawal effects remained present up to the end of this phase.

In the fourth study with cocaine, most withdrawal symptoms were recorded during the first 3 days of a 13-day withdrawal period. The structure of cocaine contains both hydrophilic and lipophilic pockets, enabling to easily cross the blood-brain barrier but also allowing a fast breakdown and elimination process (Sharma, 2009).

In a standard design we have chosen to set the withdrawal phase at 7 days. This 7-day withdrawal design was proven relevant and scientifically valid and therefore used as a standard in the non-precipitated withdrawal test. However, this period can be extended based upon the pharmacotoxicological and kinetic profile of the test compound.

Another key to a successful non-precipitated withdrawal study is the appropriate choice of the positive control (a psycho-active drug of abuse as reference drug or a scheduled comparator of the same therapeutic class). Considerable expertise on the pharmacology and on the toxicology of both the drug compound and the various psycho-active reference drugs or comparator drug is needed, because the choice for using a particular reference drug must be of relevance in view of the test compound's profile and/or therapeutic class. This must be defined by assessing the receptor binding profile, the pharmacological action and the toxicological effects of both drug compound and positive control.

The above mechanistic studies were conducted with known drugs of abuse (morphine, diazepam, ethanol, cocaine), to verify the well-established withdrawal profile of these drugs at the dose range tested. This objective was met for all four studies. As such these psycho-active drugs were considered appropriate to use as positive control in GLP withdrawal studies.

Specific behavioural observations alerting for possible physical dependence potential in the non-precipitated withdrawal model were included and the incidence, severity and duration of these observations were monitored during the repeated dose phase on particular days at various daily time points to obtain baseline values for comparison during the withdrawal period. The selected days and the time points supported the examination of rats after a single and repeated (steady state) dose, and at time points representing an exposure-effect curve based upon C_{max}. In some cases, when there is a dose-effect response but no exposure-effect relationship, these factors must be taken into account as well for setting the appropriate time points. This might be the case when investigating pro-drugs or test compounds that trigger a molecularly based cascade within a certain pathway (*example*: positive or negative allosteric modulators).

During the withdrawal phase the behavioural observations (incidence, severity and duration) were examined daily, at the various time points as defined during the repeated dose phase.

Mortality and general clinical observations (*example*: pale mucosa) were also recorded daily during the repeated dose phase and during the withdrawal period. It was considered appropriate to include several daily time points to observe the animals for those general signs of toxicity that were not included in the list of behavioural observations. The general observations were executed after the assessment of the behavioural observations.

Apart from the behavioural observations, the body temperature is another sensitive marker to detect possible signs of physical dependence and is recorded throughout the RD phase and the withdrawal period, usually at several time points per day. The introduction of a probe thermometer (Plexx thermometer) to measure the peripheral body temperature via subcutaneously implanted chips allowed recordings without additional manipulation of the rats, which might otherwise interfere with the withdrawal signs.

Body weight and food uptake were measured at various daily time points on specified days during both the repeated dose phase and the withdrawal period of the study. The daily time points are chosen based upon the toxicological and kinetic profile of the drug candidate. The so-called daytime measures of body weight and food uptake recorded in the repeated dose phase prior to dosing and at 5 hours post-dose or during the withdrawal period at 5 hours after the time point correlating with previous dosing time in the RD phase, were considered of relevance for investigating possible effects on the diurnal rhythm (as for stimulants) or when compounds with a biphasic exposure profile were investigated (like opioids). The 24-hour measures of body weight and food uptake recorded at 24 hours post-dose, prior to the next daily dose during the RD phase or every 24 hours during WD at the time points correlating with the previous dosing time are deemed necessary to investigate the possible influence of the nocturnal nature of the rats on these parameters. Finally body weight gain is also calculated appropriately.

The various routes of administration (oral, intravenous (bolus or infusion), subcutaneous, intraperitoneal, …) that are commonly employed within preclinical Drug Development were not an issue to apply in this type of study. In the four mechanistic studies the subcutaneous (morphine), oral (diazepam, ethanol) and intraperitoneal (cocaine) routes were selected. In addition, solutions as well as suspensions could be administered when dosed orally.
However osmotic pumps should not be employed in this particular design. These pumps are usually uploaded for a time period of one week in general and do not allow full and abrupt cessation of drug administration unless they are surgically removed. Surgical removal of the osmotic pumps and possible anaesthetics after the repeated dose phase would affect the subsequent withdrawal period (as evidenced by the recovery needed after surgery) and are therefore not recommended in this type of study.

The possible impact of conditioning via the manipulation of daily treatment with the drug candidate (learned association of treatment procedure and effect of the drug) on the incidence and severity rate of the withdrawal symptoms was investigated in the first mechanistic study with morphine. In that study, two groups subcutaneously dosed at 100 mg/kg/day were abruptly devoid from morphine after 21 days of daily treatment. One group received subcutaneous saline injections during the days of the withdrawal period to mimic former drug treatment and as such to evaluate the possible effect of conditioned stimuli (i.e. SC injections) on the withdrawal symptoms. This sham treatment resulted in less clear withdrawal signs as evidenced by absence of the lowered body temperature and a tampered body weight gain compared to the withdrawal symptoms in the other 100 mg/kg morphine group which received no saline injections during withdrawal.

We do not recommend to mimic drug administration during the withdrawal phase in order not to suppress or disguise minor or discrete drug related withdrawal effects.

It was decided to include the investigation of toxicokinetic parameters of the test compound (T_{max}, C_{max}, $T_{1/2}$) through separate satellite groups of male rats (3/dose) which are dosed according to the main study design. A day-curve is

executed after the first daily dose (= single dose exposure) and after the last daily dose (= repeated dose exposure). Time points for blood sampling are selected based upon the kinetic profile of the test compound, determined during previous toxicity studies or based upon literature data. A 24-hour sample (corresponding to the first day of the withdrawal period) is always included, and depending on the test compound's kinetic profile, more samples can be taken during the withdrawal phase to support possible withdrawal symptoms with exposure levels measured during this period.

IV.4.3. Data management of a non-precipitated withdrawal model for use in Drug Development and GLP compliance

A new IT system was build that included a system capable of capturing various time points per day on which specific parameters (body weight, body temperature, food uptake, behavioural observations) were measured/recorded. It was also made possible to differentiate within a specific behavioural observation (*example*: Convulsions - clonic – head). Care was taken to ensure easy input of electronic data and flexible reporting hereof. IT validation of this system included an inventory of the user requirements, execution of the various test scripts and a validation report.

For IT implementation of the PLEXX thermometer an interface was built and temperature reading using subcutaneously implanted temperature chips and a detection probe was validated.

The study design and all the necessary equipment and/or tools were made GLP-compliant. This included the writing and subsequent approval of Standard Operating Procedures (SOP) for both the in-vivo procedures and the IT procedures, the technical validation of the PLEXX thermometer, and the validation of the newly developed IT system to record all measurements according to the GLP requirements (state of the art: traceable and reproducible data). This objective was fully met.

After investigating the above mentioned variables, a definitive study design was validated through the non-precipitated withdrawal test in the rat for testing

methylphenidate, a known CII scheduled drug, with d-amphetamine as reference drug. This is reported in Chapter VIII.

IV.4.4. Other considerations

It was investigated whether this type of study could be integrated in a standard 1-month toxicity study, in order to reduce the number of animals needed. Based upon careful evaluation of the non-precipitated withdrawal model and the classical 1-month toxicity design, it was concluded that the assessment of physical dependence cannot be incorporated in toxicity studies because behavioural studies as the non-precipitated withdrawal study require an environment in which the animals should not be disturbed by the various actions as performed in toxicity studies (*example:* manipulations for blood sampling, urine sampling, ..).

In addition, care was taken to limit the number of persons entering the room in which the behavioural studies were performed, to avoid disturbance of the behaviour of the rats.

Invasive methods as telemetry for continuous monitoring of the internal blood pressure, heart rate and temperature as possible symptoms of withdrawal have been described in literature (Moser, 2011b; Froger, 2011). Froger (2011) described this procedure to study the withdrawal effects of morphine and chlordiazepoxide in rats.

Based upon literature and on the results of studies performed externally, it was decided that the use of surgically prepared telemetered animals to study blood pressure and heart rate was not as critical in detecting major withdrawal symptoms indicative of physical dependence, as were the careful and punctual recordings of behavioural observations, body temperature, body weight and food uptake if executed as described above (i.e. at several daily time points and for the observations in terms of incidence, severity and duration).

The use of 24-hour camera monitoring can be of added value, in particular during the last days of repeated dose phase and the first days of withdrawal

period, to evaluate possible effects on behaviour and on diurnal/nocturnal rhythm (as the rat is a nocturnal animal).

All scientific employees were trained to perform this type of study and Study Directors were trained to set up the correct design, follow up on the in-vivo part and to report properly.

IV.5. Conclusions

Preclinical investigation of the physical dependence potential of new molecular entities (NMEs), exerting an activity in the brain, is part of the Abuse Liability Assessment and a mandatory test contributing to the safety evaluation of a CNS-active drug candidate within Drug Development (EMA, 2006; ICH, 2009; FDA, 2010).

The non-precipitated withdrawal test in the rat studies the physical dependence potential and is proven highly predictive when the design is carefully considered: preferably this test is conducted as a stand-alone study, since the manipulations and procedures commonly executed in the classical 1-month repeated dose studies might give rise to stress-related behavioural changes that can disguise or interfere with the drug-induced behaviour and –withdrawal symptoms. The length of the repeated dose phase needs to be sufficient in duration to obtain neuroadaptive responses form the body to the test substance, but does usually not exceed three weeks. Appropriate doses have to be selected to ensure sufficient plasma exposures and/or brain penetration during the repeated dose phase. In addition, the high dose must demonstrate exposure levels that are multiples of human efficacious dose (C_{eff} Hu). If feasible, the clinical administration route is employed. The effects of subchronic administration of various doses of the test substance may differ in nature, duration and severity from sometimes subtle changes in behaviour or autonomic processes that might occur during the withdrawal period. The staff carrying out these observations throughout the 2 subsequent phases must be trained thoroughly to distinguish from these discrete signs of withdrawal.

The choice of a relevant reference drug (positive control) requires extensive knowledge of both the test substance and the psycho-active drugs available, in view of the pharmacological or therapeutic class of the test substance.

The non-precipitated withdrawal study needs to be conducted in compliance with the GLP regulations (OECD, 1998), as the study is part of the safety evaluation of a test substance. Given the broad dose range that can usually be tested in this type of study, the timing for conducting this test may vary from very early

on in drug development to identify possible alerts, up to the end of phase 2, when the human efficacious dose (expressed as C_{max}) is known. However, when the dose range selected for testing in a very early stage of drug development does not include a dose demonstrating a plasma exposure that is a multiple of the C_{eff} Hu, this early conducted study does not comply with the requirements of the drug licensing authorities and will need to be repeated.

Finally, if the rat, as the preferred species for testing physical dependence potential, would not be appropriate for a testing a particular test substance, other laboratory animals can be used. Consequently a rationale for the choice of the other species selected needs to be written in accordance with the requirements of the regulatory authorities.

Drug-induced withdrawal may appear upon cessation of prescription drugs that do not necessarily develop abuse potential, but might nevertheless lead to craving to overcome the negative effects upon discontinuation of the medical treatment. In other cases the physical dependence of the new molecular entities will be indicative of abuse potential, in particular when positive signals of psychological dependence are also present. The data will thus provide safety information relevant to the patient and to the health care professional, but also to the drug-licensing authorities who will schedule a new molecular entity with abuse potential properties to protect healthy subjects from using these substances in non-medical situations (FDA, 2010; DEA, 2014). In any case the risk/benefit of the drug candidate must be considered carefully in view of the aimed therapeutic indication.

Overall, considerable scientific knowledge and behavioural expertise is needed to perform the non-precipitated withdrawal study and to ensure the translational approach of this highly predictive test in view of physical dependence testing as a possible indication of the development of abuse potential in humans.

V

Drug Discrimination Learning test in rats: a sensitive method to profile the discriminative properties of novel drug candidates

V.1. Introduction

The Drug Discrimination Learning test (DDL) was primarily designed to evaluate the discriminative stimulus properties of a known psycho-active drug at a particular dose from non-stimulus effects of a control substance as saline (Jarbe, 1989). In most cases, a food reinforcement is employed. The DDL was also used to gain information on the dose-response of these psycho-active drugs, or to test the stimulus effects of different drugs of abuse of a similar class (NIDA, 1991).

In 1975 Colpaert found that candidate drugs did not discriminate for a particular psycho-active drug, if the stimulus effects were induced through interactions with a central receptor and/or neurotransmitter different from that of the substituted psycho-active drug. This was denoted as a cue of a pharmacological class because of the specificity of the pharmacological properties (receptor profile) of these drugs (NIDA, 1991), and allowed researchers to distinguish stimulus effects from one drug class to another. Examples hereof include, *amongst others*, opioids (mu opioid receptors), nicotine (nicotinic acetylcholine receptors), and cannabinoids (CB1 receptors). As such, the DDL became a useful behavioural tool in the characterization of the pharmacological characteristics of CNS-compounds (Meert, 1990).

To date, the drug-licensing authorities request the investigation of the discriminative properties of new drugs in development, based upon the possible interactions or effects on neurotransmitter systems known to be involved in the abuse potential of substances of abuse (Bonson, 2011). These systems include dopamine and serotonin receptors and transporters, norepinephrine-, gamma-aminobutyric acid (GABA)-, nicotinic acetylcholine-, opioid-, N-methyl-D-aspartate (NMDA)- and cannabinoid receptors (EMA, 2006; FDA, 2010).

In view of abuse liability testing, the Drug Discrimination Learning test is thus considered a sensitive test to profile the stimulus effects of new CNS-active drug candidates (stimulus generalization) relative to those of known psycho-active drugs in view of their receptor binding profile (functional resemblance) or interaction with new neurotransmitters/brain pathways. This stimulus

generalization procedure can be executed using a variety of known substances of abuse, which display their existing intrinsic stimulus effects via different receptors.

If the drug candidate has no pharmacological similarity with known drugs of abuse by acting via the above mentioned neurotransmitters, a comparator drug approved for the same disease condition can be considered. Besides, the drug candidate needs to be tested at a dose range of which the highest dose represents a multiple of the human efficacious dose (expressed as C_{max}) (EMA, 2006; ICH, 2009; FDA, 2010).

In order to fulfill these requirements in a well-founded, scientifically validated and GLP-compliant way, the fundamental design of the DDL model needed to be adapted and optimized. We founded the basis of our research on the publication of Meert (1990) on a two- lever food-reinforced DDL procedure which included a food reward training, a drug discrimination training with a psycho-active drug and a final test phase.

During the food reward training, animals were learned to press levers (daily 15-minute sessions), and each lever press was followed by access to a sugar pellet (4.5 mg). Training was conducted for both left and right lever press food rewarding. Once the system of lever press-food reward was established, animals were further trained to press levers for several times before a food reward was made available, up to 10 lever presses for 1 food reward (so-called "fixed ratio of reinforcement 10" or FR10). When the animals achieved stable levels of lever pressing using a FR10 schedule at the two levers on successive days, they were allowed to enter into the drug discrimination training.

This subsequent drug discrimination training (15-minute daily sessions) implied that administration of a known psycho-active drug (so-called "training drug") was linked to a selected lever for obtaining a food reward using a FR10 schedule. On the other hand, when animals were administered saline, selecting the opposite lever was rewarded with a food pellet. Half of the number of animals was trained to select the left lever associated with drug administration whereas the other half was trained to select the right lever associated with drug

administration. As such discrimination between saline and a training drug, based upon the subjective effects of the training drug as experienced by the animal, could be established, and was made visible through the correct levers presses for food rewarding (food-reinforcement).

During the final test phase, the training drug or saline was replaced by single escalating doses of the drug candidate (including a control/vehicle substitution without active compound). If the animals experienced the drug candidate as having comparable effects as the training drug, animals were selecting the drug lever; subsequent bar pressing of this drug lever was rewarded with food pellets (FR10 schedule). If the animals did not associate the cueing effects with the training drug or if no effects were present, the saline lever was selected and subsequent pressing on that lever was reinforced (FR10 schedule).

The sequence of daily administrating either the training drug (D) or saline (S) throughout the training and test phase was based upon an 8-week alternating sequence schedule, i.e., 1) D-S-S-D-S, S-D-D-S-S, S-D-S-D-D, D-S-D-S-D followed by 2) S-D-D-S-S, D-S-D-S-D, D-S-S-D-D, S-D-S-D-S (Fig. V.1). Animals with odd identification numbers started the drug discrimination training with the first sequence, whereas training of the even-numbered animals started according to the second sequence.

Fig V.1. The 8-week sequence schedule in the drug discrimination training phase and test phase.

Sessions performed during the three different phases all lasted for 15 minutes and were conducted under non-extinction conditions (no limitation on the number of reinforcements).

The parameters that were investigated in this DDL paradigm included the following (Colpaert, 1987; Meert, 1990; NIDA, 1991; Young, 2009):

- The FRF value: this is the sum of the responses made on either lever before the first reinforcement (i.e. rewarding through the first food pellet at a FR10 schedule) occurs. It is a measure for the accuracy of the choice of response. The FRF needs to be lower than 15.

- The selected lever (drug or saline) is the first lever on which the rat performed 10 responses (first reinforcement).
- The number of responses on the saline lever during the 15-minute session.
- The number of responses on the drug lever during the 15-minute session.
- The overall number of responses (response rate): this equals the number of responses on the saline lever plus the number of responses on the drug lever.
- The percentage of the selected lever which is the frequency (recalculated to %) an animal chooses the selected lever compared to the total number of responses on both levers. That percentage needs to exceed 85%.
- The latency time represents the number of seconds before an animal receives its first food pellet. The maximum latency value is 900 seconds (15 minutes) in case an animal is unable to perform a FR10 reinforcement (i.e 10 lever presses to receive a food reward), thus covering the total duration of a session.

Outside the test sessions, the following variables were also recorded:

- The latency in the last saline training session.
- The latency in the last training drug session.

- The total number of responses in the last saline training session. This number has to be more than 100.
- The total number of responses in the last drug training session. This number has to be more than 100.

The selection criteria for acceptance from the training phase into the final test phase included a sustained FR10, >100 responses on the drug lever (if the training drug was administered) or the saline lever (if saline was administered) during at least 10 successive training sessions, a FRF<15, and a percentage of responses on the selected lever [versus the total number of responses on both levers during a 15-minute session] of >85%.

The above described basic model was employed as a standard design and several variables, possibly influencing the outcome, were identified and investigated. In total 4 mechanistic studies were performed [identified as study identification numbers TOX9488, TOX9491, TOX9493 and TOX10140] and reported in this chapter. The latter study (TOX10140) involved a non-scheduled drug for testing (escitalopram), to investigate the possible use of a comparator to support future DDL testing of drug candidates of the same therapeutic class for which no scheduled drugs are available.

In addition, a single dose toxicokinetic study was executed in the rat to verify the maximum plasma exposure (C_{max}) and the time interval needed to reach the peak plasma exposure (T_{max}) of eleven psycho-active drugs (study ID number TOX10306). These data are also discussed in this chapter.

Finally, a validation study was conducted with methylphenidate, a CII scheduled drug of abuse, to demonstrate the scientific validity and the face validity of the outcome, and to verify the GLP-compliance before the model was approved for use in drug development. This study (study ID number TOX10344) is described in Chapter VIII.

V.2. Materials and Methods: optimization of the methodology of the Drug Discrimination Learning test for use in Drug Development

V.2.1. Optimisation of training cues

Eleven different training cues were set up according to the above described procedure and included cocaine, fentanyl, nicotine, ethanol, chlordiazepoxide, LSD, DOM, ketamine, a D1-agonist (SKF 82958), d-amphetamine, and a CB1 agonist (WIN55-212-2).

The dose, the route of administration and the time interval between administration and the entrance into the operant chamber were initially based on stimulus generalization/antagonism pharmacological models formerly executed *in-house* (Colpaert, 1975; Meert, 1990; Colpaert, 1999) or on literature data (WIN55,212-2) (Panagis, 2008).

All cues were established in the male Sprague-Dawley rat. Animals which were trained for 1 cue (*for example a* group of animals trained to discriminate cocaine from saline) were not allowed to be tested via a cross-over paradigm between the different training cues to exclude carryover effects of training drugs.

V.2.2. Drug Discrimination Learning tests of JNJ-X (a psychotrophic drug candidate) with cocaine (study ID number TOX9488), ketamine (study ID number TOX9493), and 2,5-Dimethoxy-4-methylamphetamine (DOM) (study ID number TOX9491) as training drugs

The purpose of these three separate studies was to evaluate JNJ-X for its subjective effects profile relative to that produced by the known pharmacological drugs of abuse (cocaine, ketamine and DOM). These cues were selected because JNJ-X interacts with the 5-HT_{2A} receptor and the norepinephrine (NE) transporter, and inhibits the NMDA receptor to varying degrees, while minimally potentiating GABA current.

JNJ-X was tested in male Sprague-Dawley rats (minimum of 8 rats per cue) at 3 escalating doses (50, 150 and 300 mg/kg), administrated orally at 10 ml/kg

[suspensions with Methocel (hydroxypropyl methylcellulose 0.5% w/v) in water]. The pH, homogeneity and density of the suspensions were measured and the concentration in and the stability of JNJ-X in the prepared formulations were determined at 1 and 7 days after preparation using quantitative determinations (HPLC).

Cocaine (10 mg/kg SC), ketamine (10 mg/kg IP) and DOM (0.63 mg/kg IP) were dissolved in saline (NaCl 0.9%) at 1, 1 and 0.063 mg/ml, respectively, for administration at 10 ml/kg.

The test was executed as described in V.1. and the FRF value, the selected lever (drug or saline), the number of responses on the saline lever; the number of responses on the drug lever; the overall number of responses (response rate); the percentage of the selected lever; and the latency time; the latency in the last saline training session and in the last training drug session; the total number of responses in the last saline training session and in the last drug training session were recorded.

The first test day at which the vehicle solution (Methocel) was tested for stimulus generalisation to the training drug was defined as Day 0, followed by substitutions on Day 7 (low dose or 50 mg/kg JNJ-X), 14 (mid dose or 150 mg/kg JNJ-X), and 21 (high dose or 300 mg/kg JNJ-X). On Day 28 the vehicle solution (Methocel) was tested again.

At the end of the study, animals were re-allocated to the DDL stock and further maintained under training with cocaine, ketamine or DOM according to the training schedule.

V.2.3. Drug Discrimination Test of escitalopram with cocaine, ketamine and chlordiazepoxide as training drugs (study ID number TOX10140)

The purpose of this study was to determine and assess the evaluation of escitalopram, a SSRI, for its subjective stimulus effects profile relative to that produced by known psychoactive drugs of abuse (cocaine, ketamine and chlordiazepoxide).

Escitalopram was tested in male Sprague-Dawley rats (minimum 8 rats per cue) at 40 mg/kg, administrated orally at 10 ml/kg. The control solution in the study was saline (NaCl 0.9%) from a commercial batch.

Aseptic, watery solutions of cocaine (10 mg/kg), ketamine (10 mg/kg) and chlordiazepoxide (5 mg/kg) were employed for SC, IP and IP administration, respectively, at 10 ml/kg.

The test was executed as described in V.1. and the FRF value; the selected lever (drug or saline), the number of responses on the saline lever; the number of responses on the drug lever; the overall number of responses (response rate); the percentage of the selected lever; and the latency time; the latency in the last saline training session and in the last training drug session; the total number of responses in the last saline training session and in the last drug training session were recorded.

The test day at which the control solution (saline) was tested for stimulus generalisation to the training drug was defined as Day 0, followed by substitution by escitalopram at 40 mg/kg (Day 7).

After completion of the DDL phases a toxicokinetic section was included for the determination of plasma exposure levels of escitalopram after a single oral administration at the proposed dose concentration of 40 mg/kg.

The assignment of the animals was as follows:

- 3 animals per cue received the training drug(s) 24h before blood sampling
- 3 animals per cue received saline 24h prior to blood sampling

Cue	Number of rats	dosing 24h prior to blood sampling
Cocaine	3 rats	cocaine
	3 rats	Solvent (NaCl 0.9%)
Ketamine	3 rats	Ketamine
	3 rats	Solvent (NaCl 0.9%)
Chlordiazepoxide	3 rats	Chlordiazepoxide
	3 rats	Solvent (NaCl 0.9%)

Table V.1. Procedure for a single dose administration of escitalopram to study toxicokinetics. Of the six rats per group, 3 were dosed with the active compound 24 hours prior to blood sampling whereas the other 3 rats were administered solvent.

After the last blood sampling for toxicokinetic purposes all animals were re-allocated to the DDL stock and further maintained under training with cocaine, ketamine or DOM according to the training schedule.

V.2.4. Single dose toxicokinetic study of eleven psycho-active drugs (Study ID number TOX10306)

The purpose of this study was to determine the T_{max} and C_{max} of eleven psycho-active drugs (formulated as outlined in Table V.2.) after administration to 6 male Sprague-Dawley rats per drug.

Compound	Dosage (mg/kg)	Concentration (mg/ml)	Solvent used	Route of administration	Volume administered
Cocaine	10	10	NaCl 0.9%	SC	1
d-amphetamine	0.8	0.8	NaCl 0.9%	SC	1
Nicotine	0.6	0.6	Pyrogen free water HCl to pH adjustment; NaCl to isotonicity	SC	1
Fentanyl	0.02	0.02	Pyrogen free water ; mannitol to isotonicity	SC	1
WIN55,212-2	0.3	0.3	Solutol/water (1:9), mannitol to isotonicity	SC	1
DOM (2,5-dimethoxy-4-methylamphetamine)	0.63	0.63	Pyrogen free water NaCl to isotonicity	IP	1
Chlordiazepoxide	5	5	10% HP-β-CD + 3H2T NaOH to pH = 6.0 NaCl to isotonicity	IP	1
Ethanol	1500	1900 (δ = 0.789 g/ccm)	NaCl 0.9%	IP	1
Ketamine	10	10	NaCl 0.9%	IP	1
SKF 82958	0.1	0.1	Pyrogen free water + 2H2T NaOH to pH = 4; NaCl to isotonicity	IP	1
lysergic acid diethylamide -LSD	0.16	0.16	10% HP-β-CD + Pyrogen free water; NaCl to isotonicity	IP	1

Table V.2. Enumeration of eleven psycho-active drugs used in the toxicokinetic study. δ: density; HP-β-CD: hydroxypropyl-β-cyclodextrin; H_2T: tartaric acid; SC: subcutaneous; IP: intraperitoneal.

Blood samples were collected at 5, 15, 30, 60, 120, and 240 minutes post-dose (pooled samples) and for each reference drug the C_{max} and T_{max} were determined. Deviant results in exposure from the ketamine group obtained from the first trial required a repeat of this part.

Blood sampling for toxicokinetic determination was performed as outlined in Table V.3.

Blood sampling	
Sampling site	Tail vein
Pooled samples	Each group: 3 rats/sampling time point: - first 3 rats sampled at 5' - 30' - 2h - last 3 rats sampled at 15' - 1h - 4h
Amount	0.3 ml on EDTA
Precautions	Protected from light / kept on melting ice (blood/plasma)
Plasma preparation	
Centrifugation	Started within 1 h after blood sampling
Centrifugation conditions	5°C, 1500 x g, ± 10 min
Precautions	Protected from light / kept on melting ice (blood/plasma)
Storage	In the freezer within 1 h after start of centrifugation
Bioanalytical analysis	
Method	Qualified research LC-MS/MS
Toxicokinetic data analysis	
Software TK parameters	Watson and Microsoft Excel C_{max}, T_{max} were deduced from the pooled data in Excel

Table V.3. Outline of the methodology for blood sampling and subsequent analysis. 5', 15', 30': 5, 15 and 30 minutes after dosing; 1h, 2h, 4h: 1, 2 and 4 hours after dosing; h: hour; x *g*: times gravity (*g* = relative centrifugal force).

For all compounds but DOM and LSD the bioanalysis was performed at Janssen R&D, Raritan, U.S. For DOM and LSD the Bioanalysis was performed at Janssen R&D, Beerse, Belgium.

Assay performance was verified before sample analysis was started. Assay qualification data were generated during sample analysis.

At the end of the study, all surviving animals were killed and discarded.

Overall remark: studies were performed sequentially as outlined in this section; all variables tested were hence implemented incrementally and as such retested in each following study. This added to the reproducibility of these variables on the outcome of the individual studies.

V.3. Results

V.3.1. Optimisation of training cues

The drug discriminative properties of ten out of eleven training cues could be established and were considered stable. The face validity was proven after substitution with test compounds tested within a pharmacological environment (Drug Discovery: investigation of 30 test compounds for stimulus generalization towards these psycho-active drugs; internal communication). The WIN55,212-2 cue could not be established for drug discrimination training (TOX10344) as the FRF did not stabilize below the critical value of 15. This training drug was omitted from that study and not further tested. For the fentanyl cue the dose was adapted from the originally described dose of 0.04 (Colpaert, 1975) to 0.02 mg/kg, as the dose-response in Sprague-Dawley rat was experimentally found to be below that of the rat strain used in the previous pharmacological models (Wistar Wiga rat).

Overall, the food reward training phase preceding the drug discrimination phase with the psycho-active drugs took about 4 weeks in general, whereas a drug discrimination training phase typically lasted for 4 to 10 weeks, depending on the psycho-active drug chosen for training. Of the training drugs tested, cocaine was by far the easiest to establish, whereas animals failed to discriminate WIN55,212-2, a CB1 agonist, from saline in this paradigm.

V.3.2. Drug Discrimination Learning tests of JNJ-X (a psychotrophic drug) with cocaine (study ID number TOX9488), ketamine (study ID number TOX9493), and 2,5-Dimethoxy-4-methylamphetamine (DOM) cocaine (study ID number TOX9491) as training drugs

Of the eleven rats selected for stimulus generalization towards cocaine, one rat was excluded from further testing after the first substitution with the vehicle (Methocel) because the criteria were not met on two successive days prior to substitution. A second animal was excluded from testing with the mid dose because of failure to meet the criteria on one day prior to testing this dose. Retesting was performed for substitution with vehicle (1 male), low dose (2 males), mid dose (1 male), high dose (2 males) (Table V.4).

Dose (mg/kg)	Subject	D/S	FRF	Latency (sec)	Drug Resp.	Saline Resp.	Sum Resp.	% on Select Lever	Latency in last ... (sec)		Responses in last ...		Session Date
									Saline	Drug	Saline	Drug	
0,00000	6577	S	10	15	0	1350	1350	100,00	41	15	1292	1209	09-Oct-2009
0,00000	6577	S	10	36	0	1361	1361	100	46	67	1272	481	06-Nov-2009
50,00000	6577	S	10	29	0	1031	1031	100	16	27	1331	1331	16-Oct-2009
150,00000	6577	S	10	42	0	909	909	100	28	33	1191	1062	23-Oct-2009
300,00000	6577	S	10	36	0	912	912	100	17	64	1350	1291	30-Oct-2009
0,00000	6592	S	10	25	0	1472	1472	100	19	29	1487	1085	13-Nov-2009
0,00000	6592	S	10	14	3	1561	1564	99,8	25	21	1362	1600	09-Oct-2009
50,00000	6592	S	10	11	0	1522	1522	100	41	24	1588	812	20-Nov-2009
150,00000	6592	S	10	19	3	1391	1394	99,78	38	12	1414	1470	23-Oct-2009
300,00000	6592	S	11	29	1	1301	1302	99,92	48	43	1538	1419	30-Oct-2009
0,00000	6601	S	10	14	0	1602	1602	100	14	19	1706	1508	09-Oct-2009
0,00000	6601	S	10	35	0	1733	1733	100	18	24	1766	1422	06-Nov-2009
50,00000	6601	S	11	22	1	1365	1366	99,92	37	37	1727	1800	16-Oct-2009
150,00000	6601	S	10	39	0	1286	1286	100	20	30	1566	1380	23-Oct-2009
300,00000	6601	S	10	22	0	1242	1242	100	19	33	1821	1661	30-Oct-2009
0,00000	6603	S	11	59	1	1982	1983	99,94	25	44	1903	1024	09-Oct-2009
0,00000	6603	S	10	34	0	2070	2070	100	30	32	1910	915	06-Nov-2009
50,00000	6603	S	10	55	0	1617	1617	100	76	39	1885	860	16-Oct-2009
150,00000	6603	D	10	23	742	183	925	80,21	47	48	1982	1008	13-Nov-2009
150,00000	6603	D	10	31	744	73	817	91,06	76	60	1800	495	23-Oct-2009
300,00000	6603	S	10	38	1	573	574	99,82	62	56	2109	653	30-Oct-2009
300,00000	6603	D	10	34	890	0	890	100	50	44	1670	813	20-Nov-2009
0,00000	6605	S	10	18	0	1174	1174	100	31	25	1117	573	09-Oct-2009
50,00000	6605	-	0	0	0	0	0		71	47	116	230	16-Oct-2009
0,00000	6617	S	10	37	0	1903	1903	100	16	14	1772	1837	09-Oct-2009
0,00000	6617	S	10	31	0	1860	1860	100	22	16	1882	2081	06-Nov-2009
50,00000	6617	S	10	50	0	1543	1543	100	45	24	1791	1943	16-Oct-2009
150,00000	6617	S	10	43	0	1690	1690	100	20	29	1973	2004	23-Oct-2009
300,00000	6617	S	10	22	0	1677	1677	100	9	34	2076	1853	30-Oct-2009
0,00000	6618	S	10	27	0	1257	1257	100	34	40	1200	827	09-Oct-2009
0,00000	6618	S	10	49	0	1135	1135	100	19	15	1026	1166	06-Nov-2009
50,00000	6618	S	11	32	1	1063	1064	99,9	62	32	1190	805	16-Oct-2009
150,00000	6618	S	10	39	0	918	918	100	38	27	976	947	23-Oct-2009
300,00000	6618	S	10	33	0	861	861	100	43	21	986	1064	30-Oct-2009
50,00000	6623	S	10	37	16	1523	1539	98,96	49	14	1546	1748	16-Oct-2009
300,00000	6623	S	10	21	0	870	870	100	18	23	1290	1477	30-Oct-2009
0,00000	6630	S	10	50	0	1703	1703	100	25	16	1734	1761	09-Oct-2009
0,00000	6630	S	10	53	0	1546	1546	100	52	17	1692	1785	06-Nov-2009
50,00000	6630	S	10	27	0	1427	1427	100	38	25	1693	1793	16-Oct-2009
150,00000	6630	S	10	33	0	1487	1487	100	53	37	1659	1742	23-Oct-2009
300,00000	6630	S	10	33	0	1140	1140	100	21	16	1905	1970	30-Oct-2009
0,00000	6634	S	10	25	1	959	960	99,89	29	6	730	1247	09-Oct-2009
0,00000	6634	S	11	23	1	1103	1104	99,9	17	10	1073	1291	06-Nov-2009
50,00000	6634	S	11	49	112	311	423	73,52	37	13	1022	1261	16-Oct-2009
50,00000	6634	S	11	30	1	1070	1071	99,9	62	15	816	1132	13-Nov-2009
150,00000	6634	S	11	29	8	520	528	98,48	34	21	895	1376	23-Oct-2009
300,00000	6634	S	13	49	3	504	507	99,4	58	4	681	1462	30-Oct-2009
0,00000	6730	S	10	71	0	1965	1965	100	33	57	2033	764	06-Nov-2009
0,00000	6730	S	10	24	35	2210	2245	98,44	34	31	2100	1791	09-Oct-2009
50,00000	6730	D	10	37	1641	83	1724	95,18	35	43	2267	1274	16-Oct-2009
150,00000	6730	D	10	28	2110	0	2110	100	30	37	2194	1500	23-Oct-2009
300,00000	6730	S	10	17	0	2041	2041	100	54	51	2031	735	20-Nov-2009
300,00000	6730	S	10	26	0	1790	1790	100	30	39	2140	1520	30-Oct-2009

Table V.4. Individual rat data for the substitution with JNJ-X [represented in column "Dose (mg/kg)"] in rats trained with cocaine as reference drug (drug=10 mg/kg cocaine). Subject: animal number; D: selected drug lever; S: selected saline lever; Resp.: response

Substitution of the cocaine cue with JNJ-X demonstrated a partial generalization at the low (1 rat out of 10 or 10%), mid (2 rats out of 9 or 22%) and high (1 rat out of 10 or 10%) dose (Fig. V.2)

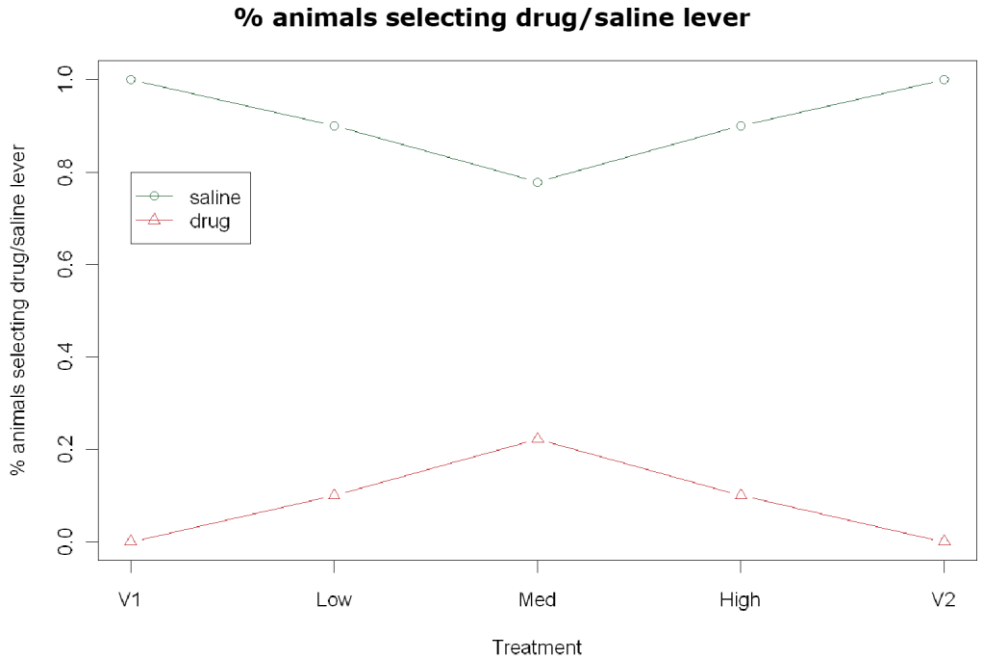
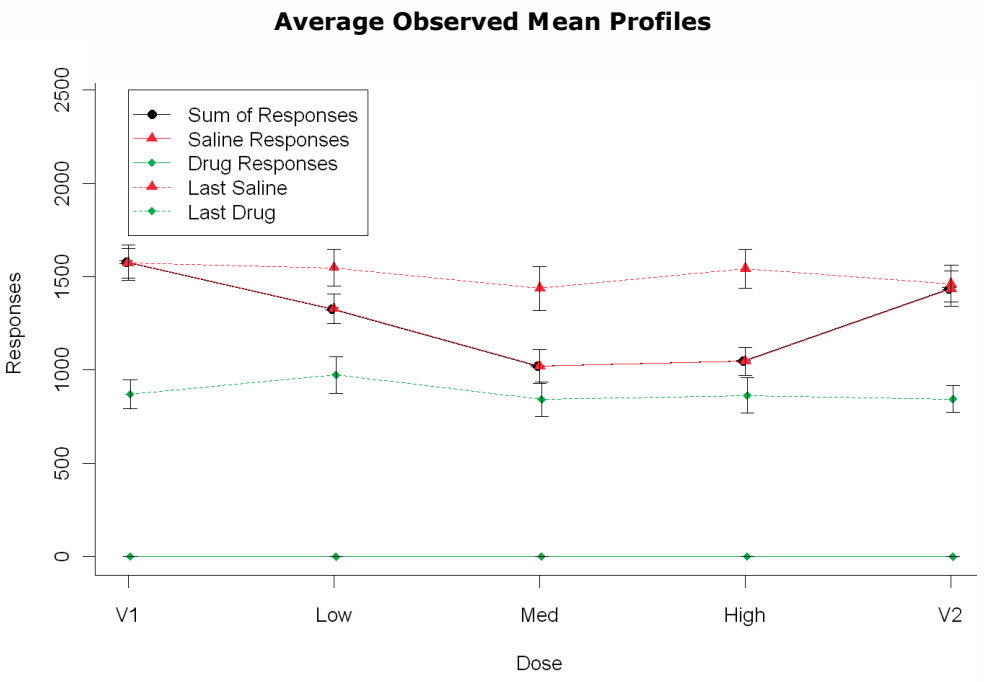


Fig. V.2. Percentage of animals selecting the saline and cocaine levers across the different JNJ-X dose groups. Partial generalisation to cocaine was noticed at all dosages of JNJ-X. V1, V2: vehicle (Methocel).

JNJ-X did not generalize to ketamine up to 300 mg/kg, the highest dose tested. The total number of responses in the JNJ-X groups (that equaled the number of saline responses during the test sessions) was smaller than those in the vehicle groups, and also lower than the number of responses in the last saline training sessions prior to substitution with the low, medium and high JNJ-X dose (Fig.V.3). A repeated ANOVA analysis indicated a highly significant compound effect ($pval<0.0001$). Post-hoc Dunnett tests on the total number of responses revealed significant differences versus the first vehicle group for all dose groups (Fig. V.3). On average, these values were smaller in the JNJ-X groups than in the vehicle group. A comparison of the saline responses in the test sessions with the ones in the last saline training sessions revealed significant differences in the

low, medium and high dose groups with p-values respectively equal to 0.0025, 0.0003 and <0.0001.

These data are indicative of a suppressive effect of the rats activity level by the test compound.



Group	Estimate	StdErr	DF	tValue	Probt	Adjustment	Adjp
High	-249.58	54.7725	43	-4.56	<.0001	Dunnett-Hsu	0.0002
Low	-193.00	54.7725	43	-3.52	0.0010	Dunnett-Hsu	0.0038
Med	-205.99	56.2956	43	-3.66	0.0007	Dunnett-Hsu	0.0026
V2	-18.2500	54.7725	43	-0.33	0.7406	Dunnett-Hsu	0.9919

Fig. V.3. Average observed profile for the total number of responses, the number of saline responses and the number of drug (JNJ-X) responses in the test session, as well as the number of saline responses in the last saline training session and the number of drug responses in the last drug training (ketamine) session (with one standard error of the mean Error Bars). Statistics performed with Pairwise Comparisons versus V1 using Dunnett's test.

JNJ-X did not generalize to DOM up to the highest dose tested. Statistical analyses demonstrated significant (pval=0.0004) drug-related trends for increased latencies in the low and mid JNJ-X dose groups (Fig. V.4) and a decreased total number of responses in all JNJ-X groups compared to the vehicle group. The changes in these variables were indicative of the known suppression of the rats' activity level by the compound.

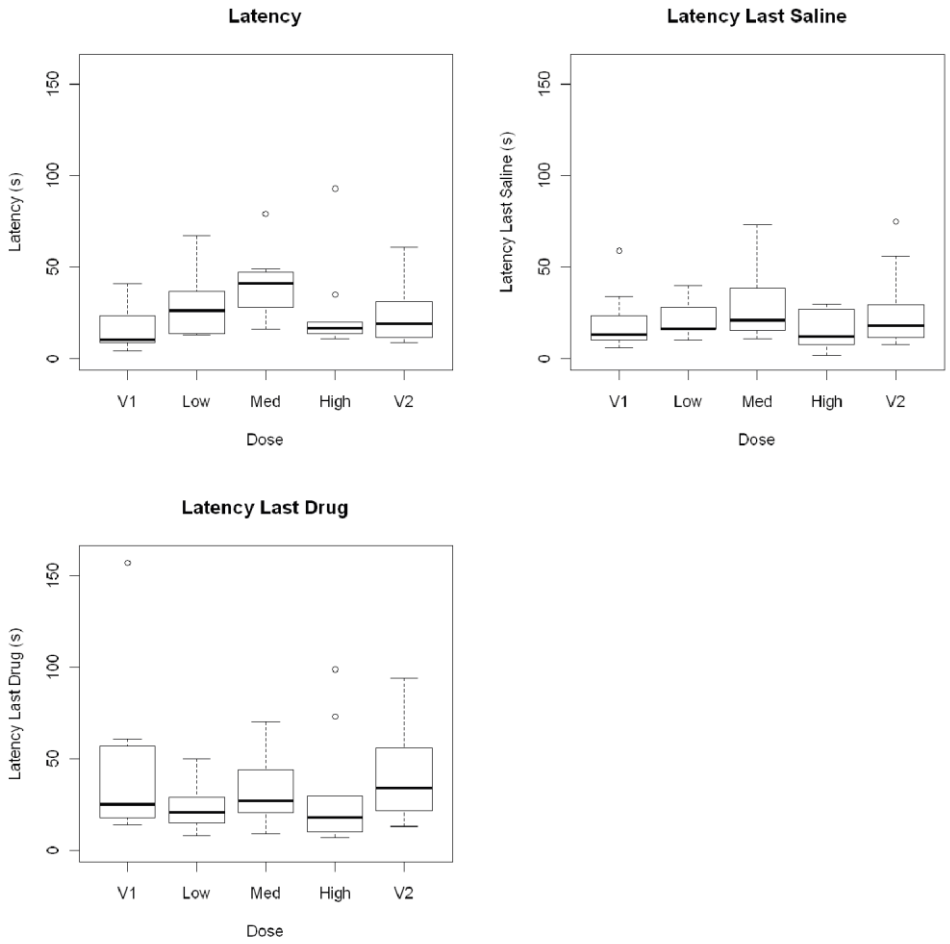


Fig. V.4. Box-Whisker plots for the latency in the test sessions, the last saline training session and the last drug training (DOM) session. A significant increase in latency was recorded in the low and mid JNJ-dose groups (Latency plot). Statistics on the Latencies in the Test Session: Anova.

The measurements and analyses of the test compound formulations (JNJ-X, unpublished confidential data) and of the training drugs (Table V.5) complied with the aim of the study.

	Cocaine	Ketamine	Dom
Nominal concentration (mg eq./ml)	1	1	0.063
pH	5.68	5.22	5.79
Density (g/ccm)	1.005	1.005	1.004
Osmolality (Osmol/kg)	0.3	0.277	0.29
HPLC analysis after 5 days	104%	103%	103%
after 5 days (1dKT)	103%	102%	102%
after 3 weeks	103%	101%	103%
after 4 weeks	102%	101%	105%
after 2 months	103%	102%	104%

Table V.5. Measurements and analysis of the training drug solutions. HPLC: high performance liquid analysis with ultraviolet detection at 232 (cocaine), 230 (ketamine) or 292 (DOM) nm. Samples were diluted in Water/Acetonitrile (50/50,v/v) (cocaine and ketamine) or in methanol (DOM). A volume of 10 (cocaine, DOM) or 15 (ketamine) µl was injected on an Xterra RP18 column at 35 ºC and the flow was 2 ml/min. For cocaine and ketamine a linear gradient was used, starting at 100% Ammonium Acetate (0.1% w/v in water) and 0% Acetonitrile, and finishing after 10 minutes with 10% Ammonium Acetate (0.1% w/v in water) and 90% Acetonitrile, followed by a linear gradient finishing after 2 minutes with 100% Acetonitrile and a purge time of 3 minutes with 100 % Acetonitrile. For DOM a linear gradient was used, starting at 100% 0.01M Tetrabutylammonium hydrogen sulfate (=TBAHS) in water and 0% Acetonitrile, and finishing after 10 minutes with 0% 0.01M TBAHS in water and 100% Acetonitrile. This was followed by a purge time of 5 minutes with 100 % Acetonitrile.

V.3.3. Drug Discrimination Test of escitalopram with cocaine, ketamine and chlordiazepoxide as training drugs (study ID number TOX10140)

Escitalopram did not generalize to the cocaine cue (Table V.6) nor to the chlordiazepoxide cue (Table V.7) or the ketamine cue (Table 5.8) at a single oral dose of 40 mg/kg.

Dose (mg/ kg)	Rat ID	D/S	FRF	Latency (sec)	Drug Resp.	Saline Resp.	Sum Resp.	% on Select Lever	Latency in last ... (sec)		Responses in last ...	
									Saline	Drug	Saline	Drug
0	6928	S	10	9	0	1422	1422	100,00	18	18	939	1019
40	6928	S	12	22	5	980	985	99,49	18	24	1552	1598
0	6929	S	10	9	0	2270	2270	100	10	30	2193	2019
40	6929	S	10	22	0	1738	1738	100	19	35	2022	1446
0	6933	S	10	13	0	2056	2056	100	15	37	2047	1913
0	6937	S	10	6	0	1710	1710	100	6	34	1746	1489
40	6937	S	10	58	0	1366	1366	100	15	22	1668	1317
0	6939	S	10	3	0	2900	2900	100	4	13	2991	1038
40	6939	S	10	91	2	1720	1722	99,88	15	33	2824	2483
0	6942	S	11	16	7	1783	1790	99,6	13	14	1814	1574
40	6942	S	19	69	25	858	883	97,16	19	23	1754	1439
0	6943	S	10	13	0	2471	2471	100	18	13	2499	1777
40	6943	S	10	47	34	865	899	96,21	25	25	2081	1561
0	6944	S	10	18	0	1745	1745	100	52	20	1682	1601
0	6962	S	11	11	3	1490	1493	99,79	11	26	1358	1855
40	6962	S	11	35	21	944	965	97,82	27	32	1458	1677
40	6999	S	10	26	0	1402	1402	100	25	24	2083	1833
0	7001	S	10	6	4	1766	1770	99,77	8	4	1660	1555
40	7001	S	10	18	1	1581	1582	99,93	11	12	1653	1568

Table V.6. Individual data showing the absence of stimulus generalisation of escitalopram [represented in column "Dose (mg/kg)"] to cocaine.

Dose (mg/kg)	Rat ID	D/S	FRF	Latency (sec)	Drug Resp.	Saline Resp.	Sum Resp.	% on Select Lever	Latency in last ... (sec)		Responses in last ...	
									Saline	Drug	Saline	Drug
0	6932	S	10	17	0	2191	2191	100,00	8	2	2290	2476
40	6932	S	10	35	0	1461	1461	100	20	7	2189	2662
0	6965	S	10	22	0	2087	2087	100	23	9	2117	2266
40	6965	S	10	51	2	1020	1022	99,8	25	13	1947	2156
0	6969	S	10	9	0	2148	2148	100	36	4	1888	2304
40	6969	S	10	26	2	1108	1110	99,81	11	17	1924	1959
0	6970	S	10	22	3	2099	2102	99,85	15	16	2155	1981
40	6970	S	10	73	2	912	914	99,78	23	36	2091	1990
0	6972	S	10	42	1	1870	1871	99,94	23	12	1841	2057
0	6973	S	10	8	0	1673	1673	100	10	7	1639	1820
40	6973	S	10	82	0	1110	1110	100	22	11	1662	1893
0	6974	S	11	14	1	1494	1495	99,93	14	2	1701	1655
40	6974	S	13	73	3	1178	1181	99,74	19	11	1577	1783
0	6976	S	11	39	2	1543	1545	99,87	32	20	1760	1913
40	6976	S	11	110	1	340	341	99,7	51	24	1447	1854
0	6977	S	10	28	0	2480	2480	100	8	4	2400	2659
40	6977	S	10	34	10	1190	1200	99,16	40	10	2534	2261
0	6978	S	10	15	8	1789	1797	99,55	16	4	1786	2060
40	6978	S	12	52	3	760	763	99,6	18	9	1595	2121
0	6979	S	10	13	0	1849	1849	100	7	12	1872	1852
40	6979	S	10	52	0	1140	1140	100	12	30	1760	1662
0	7004	S	10	42	0	1291	1291	100	15	8	1502	1723
40	7004	S	10	113	4	302	306	98,69	26	18	1710	1911

Table V.7. Individual data showing the absence of stimulus generalisation of escitalopram [represented in column "Dose (mg/kg)"] to chlordiazepoxide.

Dose (mg/kg)	Rat ID	D/S	FRF	Latency (sec)	Drug Resp.	Saline Resp.	Sum Resp.	% on Select Lever	Latency in last ... (sec)		Responses in last ...	
									Saline	Drug	Saline	Drug
0	6930	S	10	4	0	1720	1720	100,00	3	6	1697	1692
40	6930	S	10	29	0	1097	1097	100	8	29	1648	1643
0	6946	S	10	19	0	1702	1702	100	22	43	1530	1479
40	6946	S	10	53	0	1344	1344	100	24	49	1615	1304
0	6947	S	10	3	0	2688	2688	100	4	11	2528	1654
40	6947	S	10	55	0	1190	1190	100	7	67	2631	1558
0	6954	S	10	6	0	2203	2203	100	9	9	2133	2035
40	6954	S	10	41	0	1410	1410	100	16	29	2042	1919
0	6955	S	10	10	0	2890	2890	100	6	2	2873	2006
40	6955	S	10	19	24	1887	1911	98,74	7	12	2814	2051
0	6956	S	10	34	1	1315	1316	99,92	16	10	1310	1818
40	6956	S	10	50	2	730	732	99,72	29	11	1242	1638
0	6957	S	10	2	0	1920	1920	100	3	4	1921	1520
40	6957	S	10	14	0	1492	1492	100	5	52	1900	1330
0	6960	S	10	5	0	2380	2380	100	12	6	2286	2091
40	6960	S	15	71	17	1440	1457	98,83	19	11	2002	2103
0	6963	S	10	5	0	1681	1681	100	4	12	1489	1997
40	6963	S	10	30	0	1120	1120	100	8	47	1693	1726
0	6987	S	10	3	0	2492	2492	100	5	11	2259	2092
40	6987	S	13	32	3	1601	1604	99,81	11	40	2314	1995
0	7000	S	10	7	0	2204	2204	100	14	35	1850	1069
40	7000	S	10	124	1	957	958	99,89	8	50	2244	1147
0	7005	S	10	8	0	1771	1771	100	6	7	1811	1388
40	7005	S	11	23	5	1361	1366	99,63	9	10	1990	1442

Table V.8. Individual data showing the absence of stimulus generalisation of escitalopram [represented in column "Dose (mg/kg)"] to ketamine.

At the selected dose of 40 mg/kg escitalopram, toxicokinetic parameters revealed a T_{max} of 1.5h, independent of training drug cue and the training treatment 24 hours before dosing (training drug or saline), and consistent with the assumed T_{max} of 1.5h (Table V.9).

Sex	Males					
Dose escitalopram (m/kg)	40					
Group	A		B		C	
Cue	cocaine		chlordiazepoxide		ketamine	
Training treatment Day 10	cocaine	saline	chlordiaze-poxide	saline	ketamine	saline
C_{max} (ng/ml)	556	342	431	434	441	236[2)]
T_{max} (h)	0.5-1.5	1.5-4	0.26-1.5	1.5-4	1.5-4	0.68, 1.5
AUC_{0-6h} (ng.h/ml)	2015	1574	1534	1564	1894	1089[2)]
AUC_{0-inf} (ng.h/ml)	-	2504[1)]	1826	2192[2)]	-	-

[1)] n=1; [2)] n=2

Table V.9. Mean (n=3) preclinical toxicokinetic data of escitalopram after a single oral dose of 40 mg/kg.

The dose of 40 mg/kg escitalopram chosen in the present drug discrimination paradigm was based upon published data (Lundbeck, 2010) in which the NOEL in rats (13-week study) was set at 40 mg/kg/day OR. At this dose level the C_{max} plasma levels of escitalopram were 1076 nM or 445,92 ng/ml (males) to 1383 nM or 573,14 ng/ml (females) nM (Molecular Weight: 414,42), i.e. approximately 8-11 fold the human exposure of 131 nM or 54,29 ng/ml (following repeated dosing at the maximum recommended dose of 20 mg/day). The present 40 mg/kg dose in male rats reached similar plasma exposures, resulting in a rat/Hu plasma ratio of 6 to 10, thus fulfilling the regulatory requirement that rat plasma exposures be at least 2-3 times the efficacious human exposure (therapeutic range).

V.3.4. Single dose toxicokinetic study of eleven psycho-active drugs (Study ID number TOX10306)

To determine the time-exposure profile of the different available psycho-active drugs at the stated dosages, the C_{max} and T_{max} were determined after a single parenteral dose. The results are displayed in Table V.10.

Generic name	Dosage (mg/kg)	RoA	Sampling time points (minutes)						Time interval from administration to start session (minutes)
			5	15	30	60	120	240	
			Plasma exposure (ng/ml)						
Cocaine	10	SC	33	128	156	336	**430**	161	30
D-amphetamine	0.8	SC	122	**196**	136	103	51,6	16,3	30
Nicotine	0.6	SC	**145**	128	128	113	55,8	12,4	15
Fentanyl	0.02	SC	0,727	1,5	**2,78**	2,61	1,41	0,457	30
WIN55,212-2	0.3	SC	4,85	17,6	**26,2**	24,5	12,7	9,85	15
DOM (2,5-dimethoxy-4-methylamphetamine)	0.63	IP	1,89	3,68	3,09	6,4	**9,32**	1,66	15
Chlordiazepoxide	5	IP	164	**2100**	1000	1780	1280	753	15
Ethanol	1500	IP	511000	**567000**	507000	353000	291000	BQL	15
Ketamine (first group)	10	IP	29,9	**462**	47,7	187	19,5	8,16	15
Ketamine (second group)			**440**	324	370	138	90,7	26,3	
lysergic acid diethylamide (LSD)	0.16	IP	47,4	50,4	**92,7**	47,7	4,73	12,0	15
SKF 82958	0.1	IP	**3,38**	3,13	3,04	1,26	1,47	0.555	15

Table V.10. Kinetic data of the 11 training drugs (TOX10306). Data in bold indicate the maximum plasma concentration. RoA: route of administration; BQL: below the limit of quantification.

V.4. Discussion

IV.4.1. Results of the four mechanistic studies and the toxicokinetic study

In order to optimize the Drug Discrimination Learning model for use in Drug Development, four mechanistic studies, a toxicokinetic study and one validation study were conducted, using different reference drugs to investigate the variables that could impact on the results.

JNJ-X, a drug candidate with interaction at the 5-HT_{2A} and the NMDA receptor, and the norepinephrine (NE) transporter was tested for stimulus generalisation to three different known psycho-active drugs, based upon its receptor profile and on the preclinical effects that were demonstrated in earlier pharmacological and toxicity studies. The small and partial generalisation to cocaine (10 to 22% at the various dosages tested) was considered an alert, as interpreted by the defined biological significance of the outcome of a positive or negative reinforcement, based upon the drug response after treatment at various dosages as follows: less than 25% of rats with a positive effect: alert; 25–70% of rats with a positive effect: signal; and more than 70% of rats with a positive effect: full generalization (Horton, 2013). These data imply that JNJ-X needed to be further tested in an IV SA study with cocaine as reference drug (Chapter VI: see study ID number TOX10046).

The study in which escitalopram (a marketed drug often used as a comparator in clinical studies to test antidepressants) was employed, demonstrated no discriminative properties of this drug at the chosen dose. Indeed, escitalopram, a SSRI, is a non-scheduled CNS-active drug to treat major depressive disorder and generalized anxiety disorder in adults. As of to date, the marketed prescription drugs, intended to treat any form of depression do not fall under the Controlled Substance Act (Klausmeier, 1993), although they do exert an activity in the brain and sometimes display psychological effects. An example hereof is bupropion (Zyban®), a NE/DA reuptake inhibitor, of which euphoric effects have been reported (Evans, 2014).

With regard to the toxicokinetic study it was assumed that there was a clear correlation between maximal exposure and maximal drug effect for the investigated psycho-active drugs. This investigative study allowed possible fine-tuning on the proper design for each of the psycho-active drugs used for training [i.e. correct timing for placing the animals into the operant chamber for testing (at C_{max} / T_{max})]. The originally selected time-interval between the drug administration and entrance into the operant chamber was considered relevant as evidenced by the positive outcome of the mechanistic studies, but for cocaine a maximal exposure level was determined round 60 and 120 minutes after SC dosing at 10 mg/kg, in contrast to the predefined interval between dosing and entrance into the operant chambers which was originally set at 30 minutes. The same holds true for DOM, where 2- to 3-fold higher exposure levels were measured at 1 and 2 hours post-dose, respectively, compared to the levels measured after the classical 15 minutes before starting the sessions. This might be adapted in the DDL design in the future.

The time interval between dose administration and the entrance into the operant chambers usually equals the duration to reach the respective T_{max} of the administered drug candidates. Within Drug Development, toxicokinetic data of a test compound, comprising C_{max}, T_{max} and $T_{1/2}$, are known in a very early stage and can thus be employed without performing an extra kinetic study.

V.4.2. Investigation of the variables to optimize the design of a Drug Discrimination Learning model for use in Drug Development

The different mechanistic studies were executed in order to establish an optimized model for the investigation of potentially discriminative properties of new CNS-active compounds in Drug Development, also complying with the regulatory and GLP requirements. The variables described in the next sections were thoroughly considered and implemented.

V.4.2.1. Retest data

On occasion a fully trained rat did not meet the mandatory acceptance criteria [*i.e.* sustained FR10, >100 responses on the drug lever (if the training drug was administered) or the saline lever (if saline was administered), a FRF<15, and a percentage of responses on the selected lever (versus the total number of

responses on both levers during a 15-minute session) of >85%] on one of the three days (Tuesday, Wednesday or Thursday) preceding the substitution day (Friday) in an ongoing study (test phase). According to the standard design this rat was then not allowed to test for substitution in that week. The possibility to substitute in a later phase (after the last substitution with control/vehicle was performed) was considered and included in the standard design. Retesting of individual rats for a certain test dose was then performed on Day 35, one week after the last substitution in the escalation design (Control/Vehicle, Low dose, Mid dose, High dose and Control/Vehicle). *For example*, if a particular rat was excluded from testing of the mid dose on Friday (test day) because it failed to meet the acceptance criteria on the Wednesday before, this rat was allowed to substitute for the mid dose in the week after all dosages were tested, on the condition that the acceptance criteria were met on the three preceding days prior to the retest date.

During a full test phase, a maximum of 2 failures during the training days preceeding any of the substitution days was allowed for retesting. However, when the acceptance criteria were not met on 2 successive days prior to the substitution on Friday (*example*: on Tuesday and Wednesday), no further testing was allowed and the rat was omitted from the study.

If on the test day itself (Friday) the criteria were not met during substitution with a particular dose of the test compound (*example*: an FRF value of 17), the results of this test day for this rat were considered a "failure". However, in this case retesting with the appropriate test dose was not permitted and the results were reported as such.

V.4.2.2. Dose-effect versus exposure-effect

The mechanism of action, the pharmacological outcome, and possible side-effects that might become present at high doses of a test compound always need to be taken into account to ensure a scientifically valuable and realistic design.

If, for some test compounds, a dose-effect response but no exposure-effect relationship is present, the study design should be adapted accordingly to look

at the differences. An extra substitution can then be included for the highest dose with entrance into the operant chambers when the maximal clinical effect is present, next to the classical substitution with entrance of the animals into the operant chambers at the time of maximum plasma exposure (C_{max} / T_{max}).

This response in time might be of importance for those compounds that have a narrow pharmacotoxicological margin within a chosen dose range (*example*: muscle relaxants), for compounds that influence the consciousness (*example*: severe sedation at the highest dose) or mood (*example*: anti-anxiety compounds that influence the animal's interest to press levers for reward conditions), for prodrugs or for compounds that have an indirect mechanism of action (*example*: allosteric modulators).

V.4.2.3. Sham treatment in view of administrating test compounds and training drugs

The test compounds are administered according to their proposed clinical route, which is mostly the oral route, whereas the training drugs are classically administered subcutaneously or intraperitoneally. Therefore sham treatment, similar to the administration route of the test compound was performed at its "minus T_{max}" ($-T_{max}$ is defined as the time interval between dose administration and entrance into the operant chamber, similar to the time needed to reach the highest plasma exposure after a dose administration) during the four weekdays preceding the substitutions on Fridays, to avoid conditioning differences due to the different routes of administration. Consequently, on the substitution days (Friday), sham treatment similar to the administration route of the training drug was performed at the $-T_{max}$ of the training drug.

V.4.2.4. Sequence of the substitutions during the test phase

The first substitution for stimulus generalisation to the training drug started with the control/vehicle formulation (Day 0), followed by substitution with a low dose of the test compound (Day 7), a mid dose (Day 14), a high dose (Day 21) and again the control/vehicle formulation (Day 28). The last control/vehicle substitution verified the saline lever response expected after administration of inactive excipients.

If an active or unknown vehicle should be used to formulate the test compound, this vehicle is included as a separate group, and the first and the last substitutions will then consist of water or saline as control solutions.

The sequence of the substitutions as described (C/V →low → mid → high → C/V) was preferred over a Latin Square model, since it not only allows careful and stepwise monitoring of the potentially increasing toxicity at higher doses, but also requires less of the active compound to be formulated (one fixed dose per substitution versus all prepared doses in a Latin Square design).

V.4.2.5. Toxicokinetic parameters

How and when toxicokinetic (TK) parameters should be included into this type of study was considered thoroughly. Within the classical DDL model these parameters are usually not included, as the discriminative properties of psycho-active drugs investigated in fundamental research are clearly discernible. However for new CNS-active drug candidates, with a novel mechanism of action often differing from any activity within pathways involved in abuse potential, it is crucial to demonstrate measurable plasma concentrations in order to avoid false negative results.

It was decided to add this phase at the end of the study, usually 1 week after the last substitution or the retest(s) had taken place in order to assess full washout of the compound from the body. Animals were thus further trained in a way that, on the day of dosing for TK purposes (low dose and high dose) and subsequent blood sampling, 50% of the selected animals (6 rats per dose) had received the training drug 24 hours prior to blood sampling whereas the other 50% had received saline (NaCl 0.9%) 24 hours prior to blood sampling. The choice for this design was based upon the possible drug-drug interaction (training drug - test compound) that might be present during the testing phase. This design was followed in the validation study and the results hereof can be read in Chapter VIII.

V.4.2.6. Statistics

Statistics were properly designed and implemented. Descriptive statistics were performed on all parameters of interest, *i.e.* the FRF value; the selected lever

(drug or saline), the number of responses on the saline lever; the number of responses on the drug lever; the overall number of responses (response rate); the percentage of the selected lever; and the latency time.

Outside the test sessions, following variables of the preceding training sessions were also recorded and analyzed statistically: the latency in the last saline training session and in the last training drug session; the total number of responses in the last saline training session and in the last drug training session.

V.4.2.7. Outline of the final test design

The final design of a full DDL model per training drug is outlined in Fig. V.5. Test compounds in an early or late phase of development can be tested for substitution of any of the training drugs available *in-house* (stimulus generalization procedure). The proper choice for a training drug is dependent on the intrinsic properties of the test compound (i.e. receptor binding profile, pharmacotoxicological characteristics) and not always limited to only 1 cue. This was clearly shown in the studies that were conducted with one drug candidate JNJ-X and three different training drugs (JNJ-X: substitution towards cocaine, ketamine and DOM). It was also proven that full drug discrimination testing of a drug candidate towards several training drugs can be executed within one single study (TOX10140: escitalopram: substitution towards cocaine, ketamine and chlordiazepoxide). This latter design was considered the standard and as such implemented.

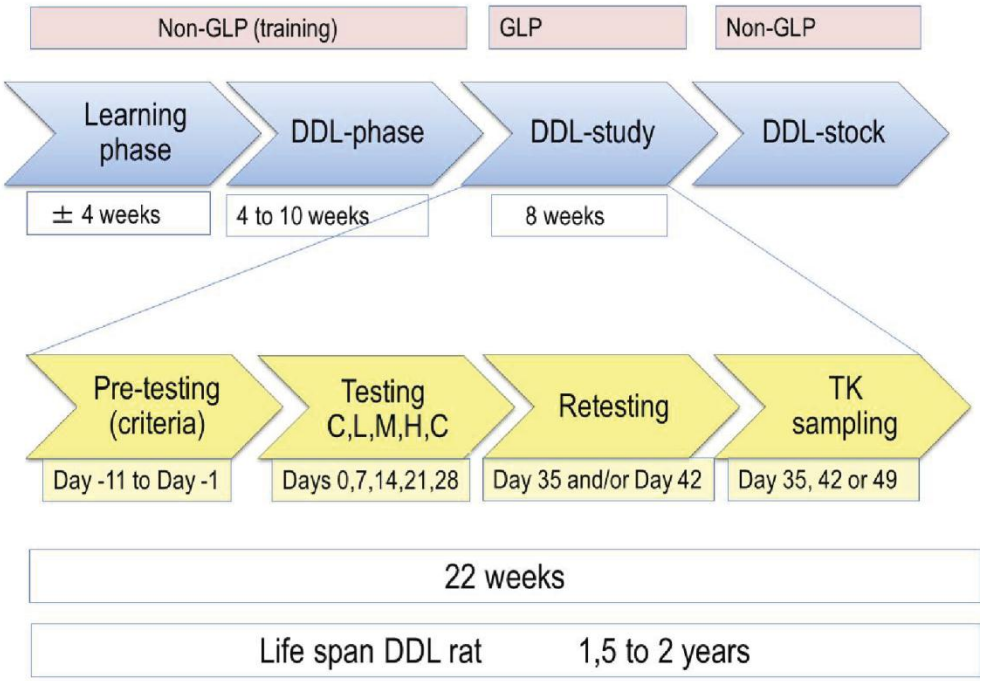


Fig. V.5. Outline of a full Drug Discrimination Learning study. The training phases preceding the study and the maintenance of the stock-animals are not under GLP regulation.

V.4.3. Data management of a Drug Discrimination Learning model for use in Drug Development and GLP compliance

The GLP-compliant IT package included the development, validation and implementation of a newly built IT system and concomitantly developed tools to perform the standard study design and to facilitate the daily procedures, according to the GLP requirements (state of the art: traceable and reproducible data).

IT validation included an inventory of the user requirements, execution of the various test scripts and a validation report. The newly built IT system allowed the management of the animals involved in DDL studies through an inventory of the animals entering in this type of studies. For inclusion and read-out of the various parameters in the daily sessions, macros (for daily use) were developed to subscribe the test phase. An automatically created file was developed to ensure the correct daily administration of the training drugs and the test

compound. Concerning data management the system included data import, storage of the electronically raw data, and data security. All systems were provided with audit trail to ensure GLP-compliance (traceability). Finally a dose-response table, a dose-response curve and dose-response charts were made available according to the requirements of the test facility and of the GLP requirements.

To make the commercially available system from MED Associates Inc. GLP-compliant, their specific hardware and concomitant software were reviewed. In addition a specific method was developed for calibration of the hardware (MED-PC from MED Associates Inc.) and registration hereof. Software modifications were discussed with the vendor (MED Associates Inc.) and implemented to create a GLP-compliant environment (with regard to traceability).

A pre-test period of 11 days was introduced in the final study model to enable inclusion of the individual rat training data, proving the "ready for testing" criteria of the trained individual animals and as such acceptance of the animals into the test phase. This procedure was deemed necessary for GLP compliance purposes in view of the traceability of the animal's history.

The study design and all the necessary equipment and/or tools were made GLP-compliant. This included the writing and subsequent approval of Standard Operating Procedures (SOP) for both the in-vivo procedures and the IT procedures, and the validation of the newly developed IT system to record all measurements according to the GLP requirements (state of the art: traceable and reproducible data). This objective was fully met.

After investigating the above mentioned variables, the definitive study design was validated through the Drug Discrimination Learning test in the rat for testing methylphenidate, a known CII scheduled drug, with d-amphetamine as reference drug. This can be read in Chapter VIII.

V.4.4. Other considerations

V.4.4.1. Postmortem evaluation of the brain

The life span of the rats that were trained in-house with the psycho-active training drugs mostly exceeded 1.5 to 2 years. Thereafter the rats were killed and post-mortem evaluation of the different brain sections was executed.

Microscopic evaluation was performed to detect potential morphological brain effects due to chronic exposure to the training drugs and possible interaction hereof with candidate drugs. In particular the presence of Olney's lesions [also known as NMDA receptor antagonist neurotoxicity (NAN)] in the retrosplenial cortex/posterior cingulate(d) gyrus (Fig. V.6) after exposure to ketamine was investigated in detail.

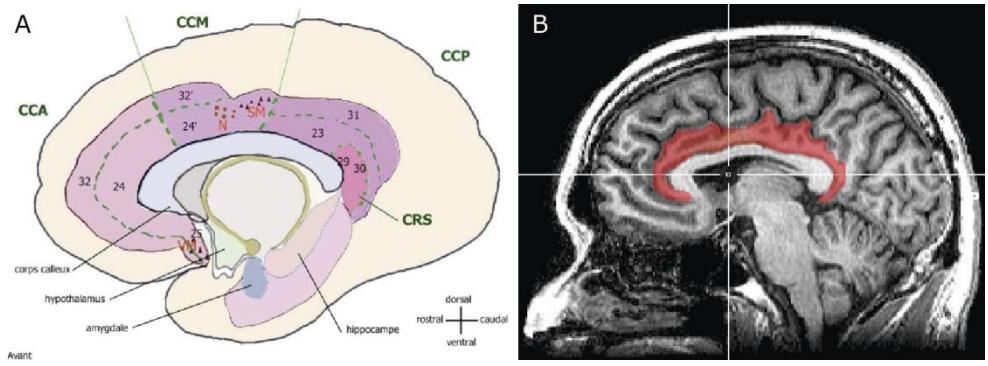


Fig. V.6. Section of the brain. A: schematic view: CRS: retrosplenial cortex; CPP: posterior cingulate cortex, CCM: medial cingulate cortex; CCA: anterior cingulate cortex. B: Sagittal MRI slice: red-highlight indicates the location of the cingulate cortex. Olney's lesions (small vacuoles) can be detected in the retrosplenial cortex and posterior cingulate after exposure to ketamine.

It was demonstrated that the chosen dosages of the different psycho-active drugs did not give rise to morphological brain tissue changes.

V.4.4.2. Postmortem evaluation of other tissues and organs

Other organs or tissues were investigated whenever appropriate. The liver was investigated microscopically to explore possible effects of the psycho-active drugs on the metabolic interaction (enzyme induction) or possible morphological

effects that might have an impact on the drug candidate's effect. Apart from centrilobular hepatocyte degeneration in some animals chronically treated with the training drug d-amphetamine, no histopathological changes were found in the liver of rats chronically exposed to the various psychoactive drugs. Microscopic examination of the kidneys, investigated in most animals of all cues but WIN55-212 and SKF 82958, revealed renal tubular vacuolation. The relevance of these liver and kidney findings could not be determined.

Some lesions due to the repetitive and chronic subcutaneous administration of training drugs led to the development of malignant fibrous tumors in the subcutaneous tissues at the site of injection. (Table V.11). Rats treated with WIN 55,212-2 were not examined as this cue was not maintained.

Histopathological reports were provided.

Compound	SC dose (mg/kg)	Histological finding	Incidence (X/N)
Cocaine	10	fibrous-type tumors at the injection site	5/26
d-amphetamine	0.8	subcutaneous/mammary region sarcomas near the site of injection	10/23
Nicotine	0.6	fibrous tumors (fibrosarcoma, fibroma or Malignant Fibrous Histiocytoma) in the subcutaneous tissues	6/22
Fentanyl	0.02	malignant fibrous tumors in the subcutaneous tissue	10/24
WIN55,212-2	0.3	NA	NA

Table V.11. Incidence of subcutaneous findings after chronic exposure to cocaine, d-amphetamine, nicotine and fentanyl. Rats treated with WIN 55,212-2 were not examined. X/N: incidence of affected rats on total number of animals; NA: not applicable.

V.5. Conclusions

The Drug Discrimination Learning (DDL) test is part of the Abuse Liability Assessment of CNS-active drug candidates in Drug Development. The choice of a relevant cue to test for stimulus generalisation needs detailed knowledge of the characteristics of both the test substance and the psycho-active drugs available, in view of the pharmacological or therapeutic class of the drug candidate. Indeed, as these novel drug candidates predominantly operate via new mechanisms of action, small alerts in either their receptor binding profile (regarding the neurotransmitters and subsequent receptors involved in abuse potential) or clinical observations recorded in previously conducted pharmacological and/or toxicity studies (*examples*: sedation or increased alertness) will be subject to further investigation of possible, sometimes discrete, discriminate stimulus properties. This will imply DDL studies in which often more than one cue will need to be tested. Therefore the staff conducting these complex studies must be trained thoroughly.

To comply with the requirements of the drug-licensing authorities (EMA, 2006; ICH, 2009; FDA, 2010), the originally described DDL design was adopted and several additional variables were investigated to accomplish a standardized, validated and GLP-compliant method. Examples hereof include proper criteria for the possibility to retest a certain test dose, sham treatment to avoid conditioning effects due to the chosen clinical route of the drug candidate versus the common injectable (IP or SC) route of the psycho-active training drugs, and careful consideration of possible responses due to active excipients used as vehicle. Using the clinical route and the feasibility to test different types of formulations in this model [*examples*: solutions or (micro-) suspensions] add to the opportunity to use a broad test dose range. This allows performing the studies in both early development to detect possible alerts, as well as in late development, when the known human efficacious dose can effectively determine the dose range to test (*i.e.* selection of a high dose that is a multiple of the C_{eff} Hu).

Differently from the basic DDL model, the dose-effect versus the exposure-effect was also taken into account in the standardized DDL design for use in Drug Development, thus ensuring proper investigation of possibly present but delayed

discriminative properties of new drug candidates (*examples*: prodrugs and allosteric modulators).

A major challenge involved in the DDL studies comprised the design of the toxicokinetic phase. This requirement is not only deemed necessary to demonstrate the validity of the chosen dose range, but also adds to detect possible drug-drug interactions and the interpretation hereof in view of the safety evaluation of the drug candidate.

The present DDL model was made GLP-compliant, through implementation of major modifications regarding the IT system and the traceability of the animal's history (OECD, 1998).

The DDL studies are classically executed in the rat as this is the preferred species forced by the regulatory authorities. If another species should be needed to test a particular test substance (*example*: when the toxicokinetic parameters reveal no or very low plasma or brain exposures in the rat compared to other species), a rationale needs to be written in accordance with the requirements of the regulatory authorities.

Lastly, if none of the receptors involved in abuse potential is targeted by the drug candidate, a marketed comparator drug of the same therapeutic class can be used for further investigation and comparison.

Overall, the currently described DDL model is proven highly predictive for detection of abuse potential properties of drug candidates when the design is carefully considered. Therefore substantial scientific knowledge and pharmacotoxicological expertise is needed to design the DDL study and to ensure the translational approach of the pharmacologically selective discrimination stimulus effects in the rat to subjective and discriminative stimulus effects in humans.

VI

Intravenous self-administration test in rats: development of a robust and reliable paradigm

VI.1. Introduction

The intravenous self-administration (IV SA) model has been widely assessed in numerous labs over the past 50 years, to determine the neurobiological mechanisms of abused drugs, to study the different behavioural responses present in human drug abuse, and to assess the abuse liability of psychoactive drugs (Richardson, 1996; Arnold, 1997; Thomsen, 2005). The latter is determined by direct investigation of the reinforcing properties of these drugs. Reinforcement is a term used to describe the relationship between the behaviour (i.e. drug taking) and the consequences of that behaviour (i.e. drug effect) (Bozarth, 1987). A positive reinforcement changes the environment by adding a stimulus that increases the likelihood of the behaviour to recur in the future. Examples hereof include food, recreational drugs, direct stimulation of pleasure centres in the brain and conditioned reinforcers such as money. Negative reinforcement changes the environment by removing an aversive stimulus through which an increase in the frequency of a specific behaviour will occur. An experiment often used in this context is the electric shock model, where the electrical shock is used as an aversive stimulus, functioning as a negative reinforcement. By pressing a lever this stimulus will be removed; hence an increase in lever presses will become apparent.

In fundamental research on the reinforcing properties of known psycho-active drugs like*, in particular*, cocaine and heroin, two specific procedures have been extensively used (Arnold, 1997). The first procedure employs a fixed ratio reinforcement schedule (FR), which implies a fixed number of lever presses before an intravenous dose is released via self-administration. Classical examples hereof include FR5 or FR10 schedules. This method allows high and stable rates of responding.

The second procedure is the Progressive Ratio reinforcement schedule, which compels increasing lever presses from the animals before an intravenous dose is released via self-administration. This procedure enables to define the so-called breakpoint, at which an animal will no longer press the required amount of lever presses to obtain an intravenous dose. This breaking point is considered a measure for compulsivity (Richardson, 1996).

To date, the intravenous self-administration (IV SA) model in the rat, using a fixed ratio schedule of reinforcement (FR) is being requested by the drug-licensing authorities to investigate the abuse potential (through determination of the reinforcement properties) of new CNS-active molecular entities. This classical IV SA animal model for abuse liability testing supports the positive reinforcement procedure, and is been considered very predictive. As such this model is postulated as the "gold standard" for investigating the reinforcing properties of drug substances.

The basis of this IV SA rat model includes 1) a surgical procedure providing rats with an IV indwelling catheter; 2) a training phase during which the animals are trained to IV self-administer a psycho-active drug at a fixed ratio of reinforcement (usually FR10); and 3) the full study, during which the test compound is presented for IV self-administration.

The three phases were thoroughly investigated and/or modified when appropriate for implementation in Drug Development. Major efforts were made on the improvement of the technology and the methodology to enhance the survival rate and animal welfare. Different techniques and variables possibly influencing the outcome of the studies were identified and explored to verify the impact hereof on the design and the requested methodology, using different known psycho-active substances. Along with the requirements of the drug-licensing authorities, compliance with the GLP guidelines was also taken into account. In total 6 mechanistic studies were performed, identified as study identification (ID) numbers TOX10046, TOX10139, TOX10141, TOX10211, TOX10287, and TOX10412, to set up a standard but tailor-made design of an IV SA test in the rat for abuse liability testing of drug candidates in Drug Development. The proposed general outline and the deriving modifications as performed in these studies are described in this chapter. The order in which the experiments were conducted reflects the steps that were followed, starting from a basic design up to a fully outlined study.

A validation study with methylphenidate, a CII scheduled drug of abuse, was conducted to demonstrate the scientific validity and the face validity of the outcome, and to verify the GLP-compliance before the model was approved for

use in Drug Development (study ID number TOX10335). The results of this study can be read in Chapter VIII.

VI.2. Materials and Methods: optimization of the methodology of the intravenous self-administration (IV SA) test for use in Drug Development

VI.2.1. Surgery and concomitant technical features

VI.2.1.1. Surgical procedures

Two different surgical procedures were tested, based upon literature and our internal expertise with indwelling catheter techniques. The first procedure implied an IV indwelling catheter via the jugular vein up to the entrance of the left atrium. This technique is most widely used as evidenced by numerous publications (Thomsen, 2005; Lynch, 2010) and by personal communication with pharmaceutical companies and contract labs (Gauvin, 2007; Nawreen, 2013).

In the second procedure an indwelling IV catheter was secured in the v. femoralis with the top at kidney height (close to the bifurcation to the renal veins). This is a common technique used in continuous infusion studies at Janssen R&D.

The surgical procedures were executed "at secundum artem" and written down in a Standard Operating Procedure (SOP). Animals were treated post-surgically with Baytril® 2.5% during 10 -12 days. Flushing was performed daily post-surgery, during training and during final tests with 50 I.U. heparin/ml NaCl 0.9% solution (0.2 ml/rat).

VI.2.1.2. Development of a modified Vascular Access Button

To ensure a proper connection of the internal IV catheter and the external tether, the majority of labs use an external port in combination with a harness.

The commercially available vascular access buttons (VAB), commonly used in the IV SA model as a fixing medium of internal catheter to external tether, are not comfortable to the rat because of their size and weight, limiting the movements of the rats in the cages (Fig.VI.1).

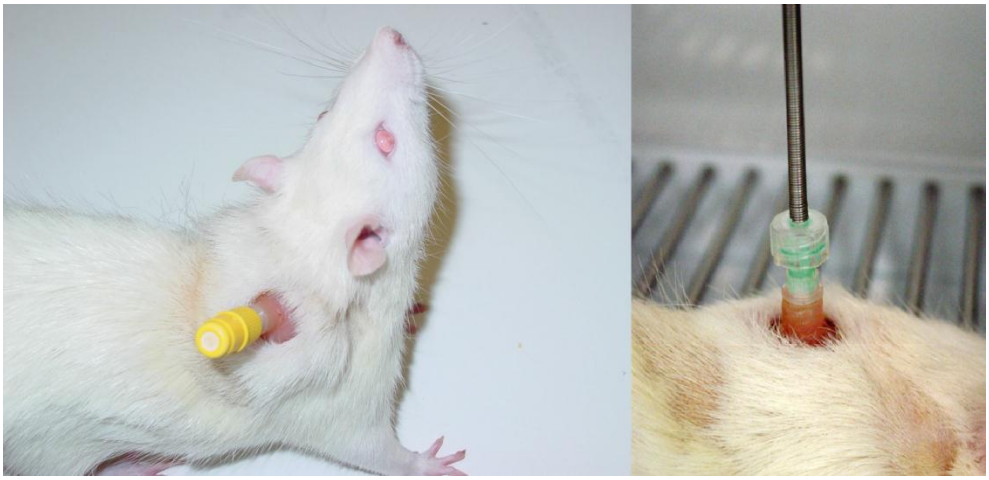


Fig. VI.1. A classical VAB can be as high as 2.7 cm, hampering the rats in freely moving.

Because of the large size of the available VABs, these buttons also need to be combined with the use of a harness (Fig. VI.2), to protect the animals from biting at this external connection, which otherwise leads to either repair/re-surgery or elimination of the animals.

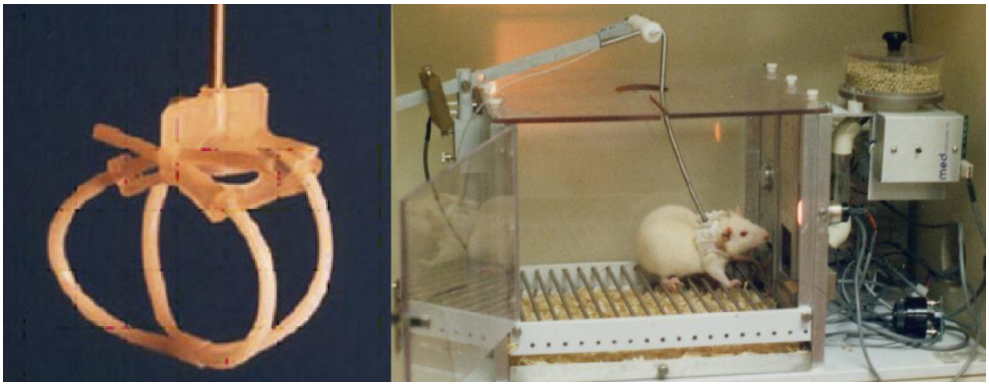


Fig. VI.2. Use of a harness in an IV SA model.

However this harness causes irritation to the animal, is often bitten through and does not add to the animal's comfort.

Therefore this technology, using a large VAB in combination with a harness, was considered not endorsing the ethical animal welfare standard at Janssen R&D. The need for a commercial system that could be used in long-term rat IV SA

studies, comfortable to the rat and applicable in a GLP environment, resulted in a close collaboration with an external company (Instech) for the development of a new vascular access button, usable without a harness.

VI.2.2. Training phase and subsequent test phase

During the training phase the surgically prepared rats were trained to press lever to receive a psycho-active reference drug via a short IV infusion (within 2 to 5 seconds). Initially an infusion is administered after each press (FR1: fixed ratio of reinforcement: one lever press initiated one IV infusion of the psycho-active drug), with a time out (TO: seconds between two successive infusions) of 5 seconds. This allowed the rats to become familiar with the pressing procedure (also conditioned through light stimulus whenever the lever appeared out of the wall for pressing) and with the effects of the drug. When a stable drug response was obtained during three successive days, the FR1 was increased to FR3. From this FR onwards, the time out period was gradually increased up to 20 seconds. Reaching stable responses at three successive days with a minimum of 5 responses (IV infusions) per session enabled a progressive increase of the FR3 up to FR10 (TO=20 seconds).

The fixed ratio of FR10 in IV SA studies is a requirement from the drug-licensing authorities (FDA at the dialogue session, 2010) and as such needed to be implemented in IV SA Drug Development studies.

The sessions lasted for 1 hour and were conducted under extinction conditions (limitation on the number of reinforcements) defined by the toxicity profile of the drug. If the maximum number of infusions, which could be selected as n=20 or n=50, was reached before the end of the 1-hour session, animals were removed from the operant chamber and placed back in their home cage.

Once a stable lever press at FR10 was obtained, the rats were considered ready for testing.

For the test phase we investigated a study design consisting of five successive phases. Each phase lasted for 5 working days during which animals were placed in the operant chambers and presented for IV self-administration over 1-hour

daily sessions. During the first, third and fifth phase, all animals were presented the selected reference drug to which they were habituated for IV self-administration. The second phase allowed these animals to self-administer saline. During the fourth phase, animals were divided into four groups for IV self-administration of the control/vehicle, a low, mid and high dose of the test compound.

VI.2.3. Experiments

VI.2.3.1. Mechanistic IV self-administration Test with two cocaine cues (study ID number TOX10139)

The purpose of this study was to determine and assess the reinforcing properties of cocaine-HCl at a low (0,5 mg/kg/infusion) and a high dose (1 mg/kg/infusion) in the self-administration paradigm.

Cocaine was dissolved in saline (NaCl 0.9%) at 6 and 12 mg/ml for IV self-administration of 0.08 ml/kg/infusion. Male Sprague-Dawley rats (n=19), surgically prepared with an indwelling jugular catheter and trained for cocaine IV self-administration, were presented a maximum number of infusions of n=50 per daily session in a fixed ratio schedule of reinforcement of FR=10 during two test phases of 5 days each. In between the two cocaine phases a saline phase of 5 days was included.

The number of drug infusions and the total drug intake for each phase were investigated.

VI.2.3.2. Mechanistic IV self-administration Test with LSD cue (study ID number TOX10141)

The purpose of this study was to determine and assess the reinforcing properties of LSD in the self-administration paradigm. In addition different surgical procedures were investigated.

SPF Sprague-Dawley rats assigned to the study were provided with an indwelling jugular (5 rats) or femoral catheter (3 rats) and trained for LSD IV self-administration during 2 months. The test phase comprised 21 successive days during which the rats were presented LSD at a fixed ratio schedule of

reinforcement, but varying individually (FR3 - FR 5) with a maximum number of infusions of n=20 per daily session.

The number of drug infusions and the maximum daily intake were studied.

VI.2.3.3. IV self-administration Test of JNJ-X (a psychotrophic drug candidate) with cocaine as reference drug (study ID number TOX10046)

The purpose of this study was to determine and assess the reinforcing properties of JNJ-X in the self-administration paradigm. In addition, two different types of catheters were tested to evaluate their possible influence on this long-term IV administration.

JNJ-X, which interacts with the 5-HT_{2A} and the NMDA receptor and with the norepinephrine (NE) transporter, showed a partial stimulus generalization to cocaine (Chapter V). Therefore an IV SA study was initiated with cocaine (1 mg/kg/infusion) as the reference drug.

JNJ-X was used at one dose (2 mg/kg), prepared as an aseptic watery solution for self-administration at 0.2 ml/kg/infusion.

The pH, density and osmolality of the JNJ-X and of the cocaine solutions were measured and the concentration in and the stability of JNJ-X and of cocaine in the firstly prepared formulations were determined (quantitative determinations (HPLC). A new bottle of each formulation was used for each day of dosing.

Surgery (jugular procedure) was performed on 34 newly ordered SPF Sprague-Dawley (8 weeks old upon arrival), divided in 2 groups of which the first 17 were indwelled with a 13 cm 3Fr. CBAS® heparin coated catheter and the next 17 animals with a 13 cm 3FR polyurethane PU catheter. Both types of catheters were supplied by Instech. During the 10-day post-surgery recovery period and during the 5-week training period, patency testing was performed twice weekly. The positive testing, implying open and accessible internal catheters, was also a criterium for entering into the present study.

A fixed ratio (FR10) schedule of reinforcement was applied and the maximum number of infusions per daily session was set at n=20 for JNJ-X and at n=50 for cocaine.

The following self-administration parameters were studied: number of drug infusions, total drug intake, rate of responding and frequency of infusion rate during the 1-hour sessions. Additionally mortality and clinical observations, and the impact of the 2 different catheters on the outcome (based upon the survival rate) were considered as well. The toxicokinetic parameters of JNJ-X were also determined. For this purpose, a low and a high dose of JNJ-X were manually administered (IV) to 2 groups of rats (n=3/group) at 4 days after completion of the full IV SA study.

VI.2.3.4. IV self-administration Test of fentanyl with morphine as reference drug (study ID number TOX10211)

The purpose of this study was to determine and assess the reinforcing properties of fentanyl at a low (0.001 mg/kg/infusion) and a high dose (0.0025 mg/kg/infusion) in the self-administration paradigm. The volume of infusion was 0.1 ml/kg.

Morphine served as a positive control, chosen as comparator in view of the in-vitro receptor binding profile of fentanyl. It was dosed at 0.3 mg/kg/infusion. The volume of infusion was 0.2 ml/kg.

This study was also used to evaluate two surgical procedures (femoral versus jugular indwelling catheters) and three different types of catheters: 1) the 3FR CBAS® heparin coated catheter (Instech); 2) the 3FR Polyurethane (PU) catheter (Instech); and 3) the Prototype Hydro coated catheter (UNO). Surgery was performed on 46 naive SPF Sprague-Dawley rats as described in Table VI.1.

Fentanyl Low dose group (0.001 mg/kg/infusion) 23 animals	Fentanyl High dose group (0.0025 mg/kg/infusion) 23 animals
13 jugularly implanted animals:	13 jugularly implanted animals:
6 rats: CBAS® heparin coated catheter 4 rats: PU catheter 3 rats: hydro coated catheters	7 rats: CBAS® heparin coated catheter 3 rats: PU catheter 3 rats: hydro coated catheters
10 femorally implanted animals:	10 femorally implanted animals:
6 rats: CBAS® heparin coated catheter 4 rats: PU catheter	7 rats: CBAS® heparin coated catheter 3 rats: PU catheter

Table VI.1. Distribution of the rats into 2 fentanyl groups based upon the surgical procedure used and the type of indwelling catheter.

Twelve days post-surgery the training for morphine IV self-administration was started, which lasted for 6 weeks.

The final study comprised 5 phases (Fig. VI.3).

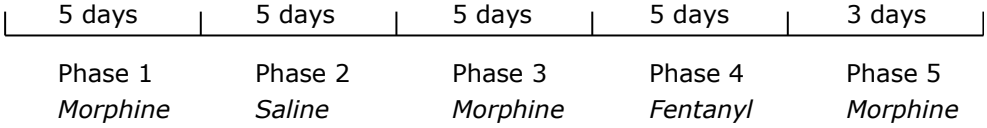


Fig. VI.3. Schedule of the final study with fentanyl.

From the start of the final study onwards, a fixed ratio schedule of reinforcement, varying individually (FR1 - FR 10) but kept as was on the last day of the training phase, was maintained and the maximum number of infusions possible per daily session was set at n=20. From the 4th day of the 4th phase onwards this number was increased to n=50 for both fentanyl and morphine self-administration.

The following self-administration parameters were studied: number of drug infusions, drug intake, and frequency of infusion rate. Additionally mortality and clinical observations were recorded. The 2 different surgery techniques (jugular versus femoral indwelling catheters) and the use of 3 types of catheters (CBAS® heparin coated, hydro coated and polyurethane catheters) were compared based upon the survival rate.

VI.2.3.5. Mechanistic IV self-administration Test to investigate cage enrichment, the maximum administrating volume in an IV SA paradigm and the use of a short-acting anaesthetic and its optimal dose for patency testing (study ID number TOX10287)

When conducting IV SA studies, the dose of a drug candidate (in mg/kg) to be self-administered is dependent on its solubility (mg/ml) and the limited volume of the infusions due to the timeframe of 2 to 5 seconds during which these have to be self-administered to obtain the effect of small and fast IV boli (see also VI.4.4.3.). The number of self-administered infusions under extinction conditions is determined at 20 or 50 infusions per session, depending on the toxicity profile of the drug candidate.

To date no data are available on the accepted maximum daily volume that rats can intravenously self-administer during long-term IV SA paradigms, without leading to any adverse effect on the rat's health or comfort. The consulted guidance "A Good practice Guide to the Administration of Substances and Removal of Blood, Including Routes and Volumes" (Diehl, 2001) states that usually a volume of 10% of the total blood volume (58 - 70 ml/kg b.w.) is taken into account as the maximum administered IV volume in a single 2h continuous infusion paradigm.

Based upon this guidance and the general outline of an IV SA study, a range of volumes was selected of which the highest volume was 3-fold the acceptable 10% of the total blood volume.

Rats (n=12) surgically prepared with an indwelling femoral or jugular catheter (CBAS® heparin coated or polyurethane catheter) (Table VI.2) were used. In addition, 3 naïve control rats were added to the study to serve as negative control.

Low dose group: 5 ml NaCl 0.9%/kg/day		
Rat ID	**Surgical procedure**	**Catheter type**
21	v. Jugularis	PU
22	v. Femoralis	PU
23	v. Jugularis	CBAS®
24	v. Femoralis	CBAS®
Mid dose group: 10 ml NaCl 0.9%/kg/day		
41	v. Jugularis	PU
42	v. Femoralis	PU
43	v. Jugularis	CBAS®
44	v. Femoralis	CBAS®
High dose group: 20 ml NaCl 0.9%/kg/day		
61	v. Jugularis	PU
62	v. Femoralis	PU
63	v. Jugularis	CBAS®
64	v. Femoralis	CBAS®

Table VI.2. Identification of the rats used in the study with the concomitant surgical procedures and catheter types. PU: polyurethane catheter; CBAS®: heparin coated catheter.

The volumes chosen in the present study (5, 10 or 20 ml NaCl 0.9%/kg body weight/day) accounted for approximately 7.8%, 15.7% and 31.5% of the total blood volume of a rat and were manually administered over 20 infusions during daily 1-hour sessions for 5 consecutive working days (Table VI.3), according to the IV SA paradigm.

Dosage groups (NC: negative control)	**NC**	**L**	**M**	**H**
- volume: ml/kg/infusion	0	0.25	0.5	1.0
- number of infusions/session	0	20	20	20
- maximum volume: ml/kg/20 infusions	0	5	10	20
- frequency of dosing	1-hour sessions/day (5 working days) with exception of NC animals)			

Table VI.3. Design of the administration of the chosen volumes of NaCl 0.9%. The aim was to reach a maximum volume administration of 7.8% (L), 15.7% (M) and 31.5% (H) of the total blood volume of a rat. L: Low dose; M: mid dose; H: high dose.

The variables studied included daily clinical observations (including mortality) and clinical pathology, comprising haematology, coagulation and clinical chemistry, determined 3 days before start of the study to obtain baseline values, and 24h and 7 days after the last daily dose administration.

In addition two types of cage enrichment were tested to enhance the animal welfare: Aspen wood block (Datesand, UK) and Sizzle nest (Bio Services, Datesand). During the first week a wood block and a portion sizzle nest were placed in the cages. During the second week sizzle nest only was provided at defined amounts of 10, 15, 20 and 25 g, whereas a wood block only was placed in the cages during the third week. The animals were observed for ease of comfort, behaviour related to the presence of the cage enrichment, and for possible signs of contamination that might lead to obstruction of the vascular access button at the back of the animals or to infection of the area around.

Finally the short-acting anaesthetic Propofol was tested at 2.5, 5 and 7.5 mg/kg/bolus in order to pick the optimal dose for performing the patency testing in the IV SA rat, based upon the duration of anaesthesia.

VI.2.3.6. Mechanistic IV self-administration Test to investigate the possible use of different lock solutions (study ID number TOX10412)

The purpose of this study was to investigate the possible use of a heparin lock solution in IV SA implanted rats. Two solutions (100 I.U. and 500 I.U. in a 30% dextrose watery solution) were administrated intravenously via the vascular access button at a fixed dose of 0.0549ml, which corresponded to the dead volume of the internal femoral catheter. Dosing was performed on Days 0, 3 and 6. Before each injection of the lock solutions it was tried to remove the previous dead volume. Patency testing was performed at the end of the study.

Overall remark: studies were performed sequentially as outlined in this section; all variables tested were hence implemented incrementally and as such retested in each following study. This added to the reproducibility of these variables on the outcome of the individual studies.

VI.3. Results

VI.3.1. Optimization of the methodology of the intravenous self-administration (IV SA) test for use in Drug Development

VI.3.1.1. Surgery and concomitant technical features

Based upon the survival rate and the cause of preterminal kill (post-mortem evaluation) in the various studies (see further: VI.3.2. Experiments), it was demonstrated that the femoral indwelling catheter procedure was superior to that of the v. Jugularis catheter implantation for use in long-term IV SA studies.

Of the three catheter types tested, the CBAS® heparin coated catheter and the polyurethane non-heparin coated catheter were considered superior to the hydro-coated catheter, as evidenced by the overall survival rate for the different catheters (Table VI.4).

	Overall survival rate			
	Jugular procedure		Femoral procedure	
Catheter type	X/N	%	X/N	%
Hepa	5/13	38%	10/13	77%
PU	5/7	71%	5/7	71%
Hydro	1/6	17%	/	/

Table VI.4. Survival rate (from surgery till finalization of study) based upon testing of the surgical procedure in combination with the three catheter types. Hepa: CBAS® heparin coated catheter; PU: non-heparin coated polyurethane catheter; Hydro: hydro coated catheter; X/N: incidence on total number of rats. Data from TOX10211 (see also VI.3.2.4.).

Combined testing of the three catheter types with the two surgical procedures showed that the indwelling PU non-heparin coated or the CBAS® heparin coated catheter, placed in the femoral vein was a superior method to improve the survival rate of animals in an IV SA paradigm. This was based upon the number of drop-outs after negative patency tests due to debris deposition at the catheter top, noticed during the macroscopic post-mortem evaluation.

VI.3.1.2. Development of a modified Vascular Access Button

The newly developed VAB has a small and light external top for tether connection, reduced in height from 2.7 cm to 1.2 cm, which was more comfortable and allowed free movements of the rats (Fig. VI.4).

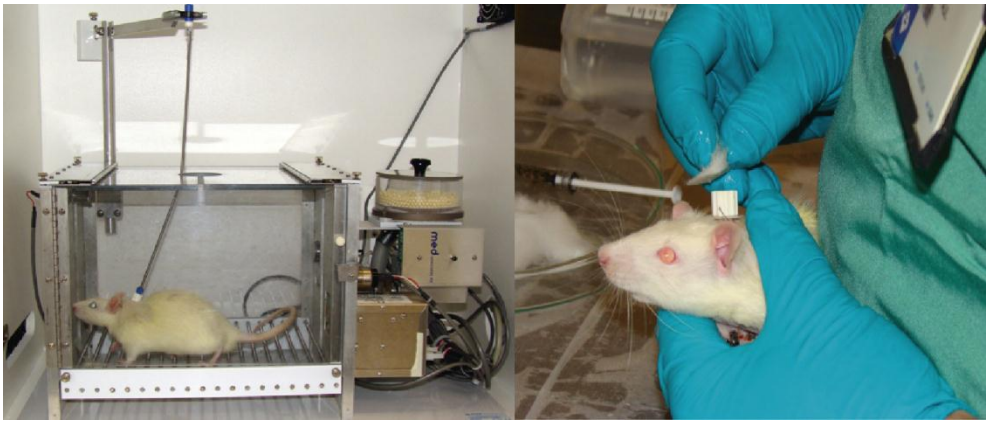


Fig. VI.4. Left: View of a rat connected to external tether via the newly developed VAB; Right: detailed view of the VAB.

It also allowed simple and aseptic access to the tether on a daily basis (Fig.VI.5a). The self-sealing internal septum membrane permits multiple punctures without a significant reduction in functionality (Fig. VI.5b). The gauze pad underneath (Fig. VI.5c) was modified in shape, size and material for adequate insertion subcutaneously. These modifications reduced the dermal and/or subcutaneous irritation that occurred with the commercial (plastic) forms. Lastly, the connection underneath between the VAB and the internal catheter was modified to avoid breaking loose (Fig. VI.5d).

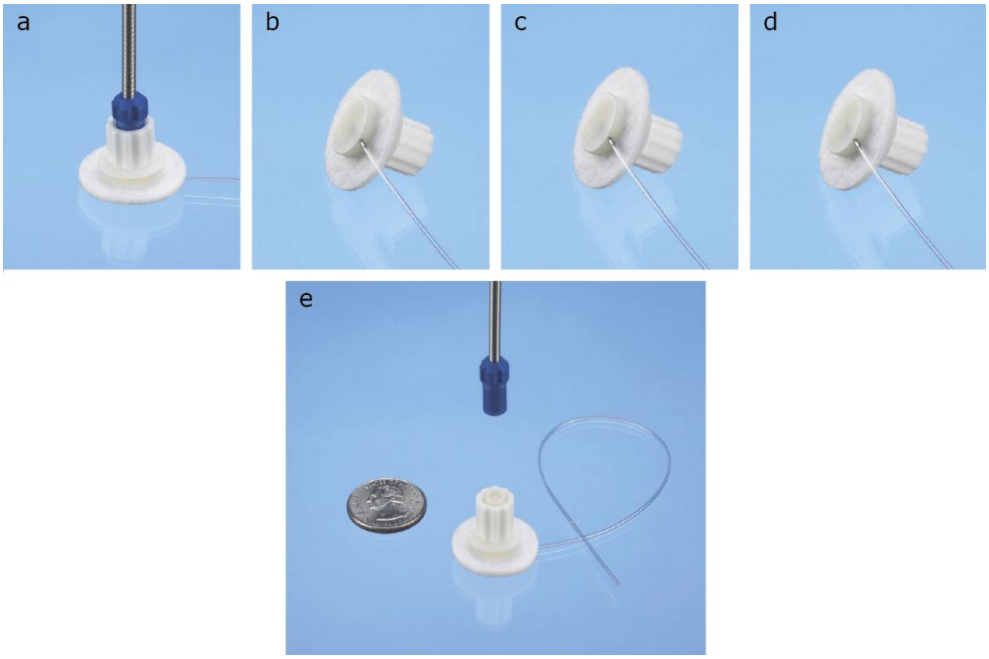


Fig. VI.5. The newly developed VAB: a: small and light external top; b: modified internal septum; c: new gauze pad; d: new connection; e: full picture.

VI.3.2. Experiments

VI.3.2.1. Mechanistic IV self-administration Test with two cocaine cues (study ID number TOX10139)

Sixteen out of the initially 19 males entered into the study, of which 13 rats (68%) finalized the three phases (cocaine–5 days / saline–5 days / cocaine–5 days).

Drop-outs were due to negative patency testing (5 rats) and one unscheduled death (resulting from a poor condition).

The total number of drug infusions during the cocaine phases in both groups was significantly higher ($p<0.01$-0.001) than that recorded during saline administration (Table VI.5).

The mean number of infusions per 1-hour session for each of the cocaine phases was higher in the 0.5 mg eq./kg dosed group than that recorded in the 1 mg eq./kg dosed group.

For the saline phase a similar number of infusions was obtained in the two groups.

Cocaine 0.5 mg/kg/infusion	Total number of infusions	N	Mean number of infusions/rat/day
Cocaine phase	5 days, 8 rats (40 individual sessions)	543	14***
Saline phase	5 days, 8 rats (36 individual sessions)	189	5
Cocaine phase	5 days, 7 rats (35 individual sessions)	370	11**
Cocaine 1 mg/kg/infusion	**Total number of infusions**	**N**	**Mean number of infusions/rat/day**
Cocaine phase	5 days, 8 rats (40 individual sessions)	331	8***
Saline phase	5 days, 8 rats (35 individual sessions)	177	5
Cocaine phase	5 days, 6 rats (30 individual sessions)	261	9***

Table VI.5. Total number and mean number of cocaine or saline infusions. The total number of infusions (N) was calculated based upon the total number of individual sessions per phase. Data were derived from all rats that entered into the study until termination of the individual IV SA due to negative patency testing. Significance computed by Mann Whitney test. ** $p<0.01$; ***$p<0.001$.

For the 0.5 mg/kg cocaine group, a mean daily intravenous intake of 6 mg/kg/day was calculated (based upon the number of infusions over the two 0.5 mg/kg cocaine phases). The maximum number of infusions obtained in this group was n=40, resulting in a maximum intake of 20 mg/kg.

For the 1 mg/kg cocaine group, a mean daily intravenous intake of 8 mg/kg/day was calculated (based upon the number of infusions over the two 1 mg/kg cocaine phases). The maximum number of infusions obtained in this group was n=15, resulting in a maximum intake of 15 mg/kg.

VI.3.2.2. Mechanistic IV self-administration Test with LDS cue (study ID number TOX10141)

Obstruction of the femoral catheter led to drop out of all these surgically prepared animals, thus prohibiting a comparison between the surgical procedures (jugular versus femoral procedure). Only the rats with indwelling jugular catheters started the study. Rats were trained for LSD IV SA during 2 months.

The final study lasted for 21 days and the daily number of drug infusions (at FR3) was low or even zero for most animals during the entire study (Table VI.6). One male (No. 12) experienced a somewhat higher response and was put on FR5 from Day 9 onwards. This resulted in a lower response rate.

The highest number of 7 daily infusions obtained in one daily session correlated with a maximum intake of 0.7 mg/kg LSD.

Day	No.11	No.12	No.13	No.14	FR and TO
	Number of responses per 1h-daily session				
0	4	3	4	2	FR1, TO5
1	4	5	4	3	FR1, TO5
2	4	5	2	1	FR1, TO5
3	2	6	2	0	FR1, TO5
4	1	7	2	0	FR1, TO5
7	2	5	1	1	FR1, TO5
8	1	3	1	0	FR3, TO20
9	0	7	1	0	FR3, TO20
10	0	2*	0	1	FR3, TO20; *: FR5
11	1	0*	0	1	FR3, TO20; *: FR5
14	1	0*	0	3	FR3, TO20; *: FR5
15	1	2*	1	3	FR3, TO20; *: FR5
16	1	0*	1	1	FR3, TO20; *: FR5
17	1	0*	1	1	FR3, TO20; *: FR5
18	1	4*	0	0	FR3, TO20; *: FR5
21	1	0*	1	2	FR3, TO20; *: FR5

Table VI.6. IV SA study with LSD (0.01 mg/kg/infusion). The number of responses per daily 1-hour session are shown for each individual rat. FR: fixed ratio of reinforcement; TO: time out or fixed interval (seconds) before a new infusion can be administered. Nos.11, 12, 13, 14: individual rat numbers. *: change from FR3 to FR5.

VI.3.2.3. IV self-administration Test of JNJ-X (a psychotrophic drug candidate) with cocaine as reference drug (study ID number TOX10046)

Twenty-four (24) out of 34 rats entered into the final study, of which 19 completed the 5 phases.

The mean number of infusions for each of the three cocaine phases (phase 1, 3 and 5) was approximately 8 and remained stable for all animals (n=19) throughout the study (Fig. VI.6). The mean daily intravenous intake was thus 8 mg/kg cocaine which was below its LD_{50} of 17.5 mg/kg.

When the possibility of self-administering saline (phase 2) was offered, an extinction burst (sharp increase in the number of infusions up to n=13) was noticed on the first day of this phase, followed by a gradual decrease in the number of responses to n=5.

The self-administration of JNJ-X (2 mg/kg/infusion) showed a gradual decrease in number of infusions to approximately n=4,5. No extinction burst was noticed during this phase.

Statistical analysis (Table VI.7) showed a significant decrease in the number of infusions during the saline phase and during the JNJ-X phase when compared to any of the cocaine phases (phase 1, 3 and 5). There was no evidence of a difference in the number of infusions in the saline versus JNJ-X phase.

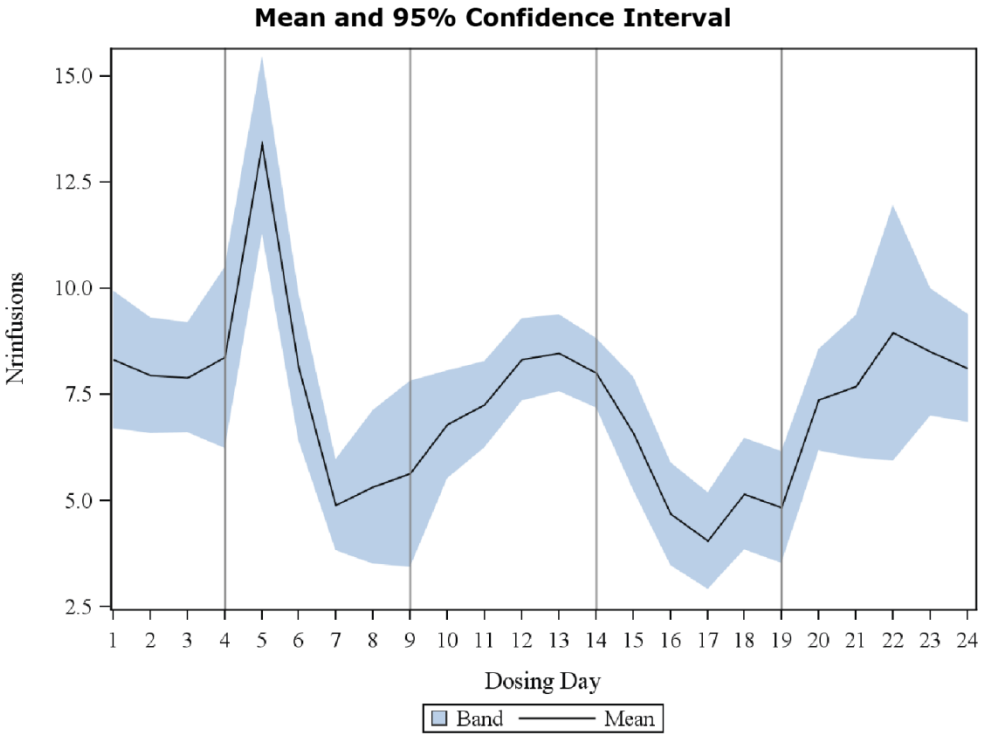


Fig. VI.6. Number of Infusions throughout the 5 phases of the study: observed average profile and its 95% pointwise confidence band. Phase 1 (4 days), Phase 3 (5 days) and Phase 5 (5 days): IV SA with cocaine; Phase 2 (5 days): IV SA with saline; Phase 4 (5 days): IV SA with JNJ-X

Phase	_Phase	Estimate	StdErr	DF	tValue	Probt
1. Training Drug I	2. Saline	2.7895	0.5155	259	5.41	<0001
1. Training Drug I	3. Training Drug II	-0.1930	0.5155	259	-0.37	0.7085
1. Training Drug I	4. Test Compound	3.3860	0.5155	259	6.57	<0001
1. Training Drug	5. Training Drug III	-0.3777	0.5241	259	-0.72	0.4718
2. Saline	3. Training Drug II	-2.9825	0.5155	259	-5.79	<0001
2. Saline	4. Test Compound	0.5965	0.5155	259	1.16	0.2483
2. Saline	5. Training Drug III	-3.1671	0.5241	259	-6.04	<0001
3. Training Drug II	4. Test Compound	3.5789	0.5155	259	6.94	<0001
3. Training Drug II	5. Training Drug III	-0.1847	0.5241	259	-0.35	0.7248
4. Test Compound	5. Training Drug III	-3.7636	0.5241	259	-7.18	<0001

Table VI.7. Number of Infusions: pairwise comparisons (repeated ANOVA) reveal a significant decrease of the number of infusions during the saline phase and during the JNJ-X phase when compared to the number of infusions during the cocaine phases. Training drug I, II, III: phases 1, 3 and 5 with IV SA cocaine (1 mg/kg/infusion); Test Compound: JNJ-X; Saline: saline phase.

The evaluation on the use of the catheter types to the outcome was made based upon the results of patency testing and on post-mortem examination of the animals. Of the initial 34 rats, 10 out of 17 rats indwelled with a CBAS® catheter and 9 out of 17 rats indwelled with a PU catheter completed the study.

The incidence and cause of preterminally killed or dead animals is enumerated in Table VI.8. There was no significant difference in survival rate related to the use of a specific catheter in this present study.

	Training phase		Final study	
Groups	**N**	**Cause of preterminal kill**	**N**	**Cause of preterminal kill**
Group A (CBAS)	6	1 dislocated catheter	1	1 difficult flushing
(17 rats)		2 difficult flushing		
		3 negative patency test		
Group B (PU)	4	1 death	4	2 deaths
(17 rats)		3 negative patency test		2 difficult flushing

Table VI.8. Macroscopic findings at necropsy of preterminally killed rats. N: number of rats.

With regard to clinical observations recorded during the study, it was noticed that intravenous self-administration of cocaine at 1 mg/kg/infusion led to head shaking and excitability. These observations were not present during the phases in which saline or JNJ-X was presented to the rats. Apart from difficult flushing or negative patency tests no other adverse clinical observations were noted during the ongoing study.

The minimum number of infusions with JNJ-X (n=1, corresponding to a minimal daily intake of 2 mg/kg) and the maximum number of infusions with JNJ-X (n=11, corresponding to a daily dose of 22 mg/kg) were taken into account for single dose IV administration of this drug candidate to evaluate its toxicokinetic parameters.

The minimum (2 mg/kg) and the maximum total drug intake (22 mg/kg), administered after completion of the study as a single intravenous bolus resulted in a C_{max} of 814 and 14150 ng/ml, respectively.

The mean C_0 and $AUC_{0-\infty}$ values were similar to somewhat higher in Group B (non-heparin coated catheter) compared to Group A (CBAS® heparin coated catheter) (Fig. VI.7).

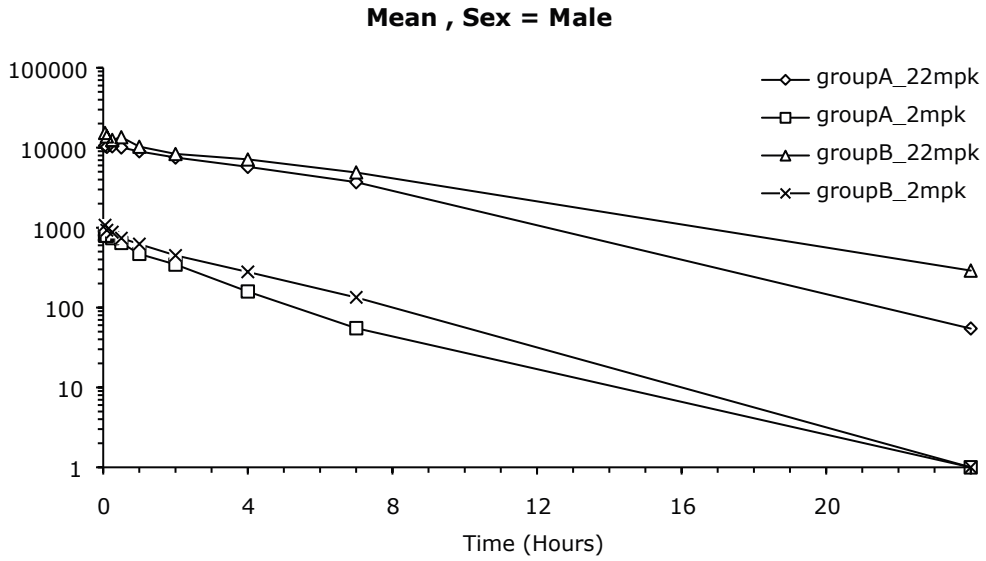


Fig. VI.7. Mean plasma concentrations versus time profiles after a single IV bolus of 2 and 22 mg/kg JNJ-X. Group A: CBAS® heparin coated catheter; Group B: non-heparin coated polyurethane catheter.

VI.3.2.4. IV self-administration Test of fentanyl with morphine as reference drug (study ID number TOX10211)

The overall outline of the executed study revealed that 34 out of the 46 ordered rats entered into the study. Twenty-six (26) out of these 34 animals or 76% finalized the study. Thus overall 56.5% of the 46 ordered animals went through the training and the 5 phases of the current study. The main cause of drop-out (20 rats or 43.5%) was a negative patency test (14 out of 20 rats). Other causes included a poor condition (2 rats) or a bitten VAB (2 rats). Two rats died during surgery.

Table VI.9 shows the drop-out rate (from surgery till finalization of study) based upon the surgical procedure in combination with the catheter type.

	Overall drop-out rate			
	Jugular procedure		Femoral procedure	
Catheter type	X/N	%	X/N	%
Hepa	8/13	62%	3/13	23%
PU	2/7	29%	2/7	29%
Hydro	5/6	83%	/	/

Table VI.9. Overall drop-out rate. The combination jugular procedure–use of Hydro coated catheters resulted in the highest number of drop-outs whereas the combination femoral procedure–heparin coated catheter resulted in the lowest number of drop-outs. Hepa: CBAS® heparin coated catheter; PU: non-heparin coated polyurethane catheter; Hydro: hydro coated catheter.

The FR used in the study was specified per animal and similar to the FR as set on the last day of training and ranged from FR1 to FR10 (Table VI.10).

Number of animals with their FR maintained during the study							
FR	Fentanyl L: low dose: 0.001 mg/kg/infusion			Fentanyl H: high dose:0.0025 mg/kg/infusion			
FR1 12 rats	3 Jug (Hepa)	1 Jug (PU)	1 Fem (Hepa)	1 Jug (Hepa)	1 Jug (PU)	3 Fem (Hepa)	2 Fem (PU)
FR3 9 rats	1 Jug (Hydro)	1 Fem (Hepa)	2 Fem (PU)	1 Jug (Hepa)	2 Jug (PU)	2 Fem (Hepa)	
FR5 4 rats	1 Jug (PU)	2 Fem (Hepa)	1 Fem (PU)	/			
FR10 1 rat	/			1 Fem (Hepa)			

Table VI.10. Number of animals with their individual FR, surgical procedure and catheter type used. Jug: jugular indwelling intravenous catheter; Fem: femoral indwelling intravenous catheter; Hepa: CBAS® heparin coated catheter; Hydro: hydro coated catheter; PU: non-heparin coated polyurethane catheter.

The mean number of infusions per 1-hour session during the three morphine phases (phase 1, 3 and 5) amounted to 11, 10 and 15, respectively and remained stable per individual animal throughout the separate phases (Table VI.11).

TOX10211		Phase 1: morphine (5 days)														
Low dose		Group	1	1	2	2	2	1	1	1	2	2	2	2	1	
Date	Day	Session	No. 2	No. 3	No.8	No.10	No.12	No.16	No.17	No.19	No.29	No.30	No.31	No.32	No.41	
		Box	box 1	box 2	box 1	box 2	box 3	box 3	box 4	box 5	box 5	box 6	box 7	box 8	box 8	Average per day
22-11-11	0	30	7	8	9	13	4	5	4	5	4	10	8	21	10	8
23-11-11	1	31	4	5	8	14	5	4	3	6	4	12	7	24	12	404 8
24-11-11	2	32	11	6	9	22	7	2	4	5	14	16	8	23	9	136 10
25-11-11	3	33	6	5	8	19	11	3	4	6	4	13	6	23	8	116 9
28-11-11	4	34	12	4	9	31	9	15	8	6	4	15	8	24	7	152 12
			40	28	43	99	36	29	23	28	30	66	37	115	46	**620** sum total
			8	6	9	20	7	6	5	6	6	13	7	23	9	**8** average per animal per session

TOX10211		Phase 1: morphine (5 days)														
High dose		Group	3	3	3	4	4	3	3	4	4	4	4	4	4	
Date	Day	Session	No. 4	No. 5	No.7	No.13	No.14	No.23	No.24	No.33	No.34	No.35	No.38	No.39	No.40	
		Box	box 1	box 2	box 3	box 1	box 2	box 4	box 5	box 3	box 4	box 5	box 6	box 7	box 8	Average per day
22-11-11	0	30	6	6	12	11	11	8	11	12	10	6	11	13	10	504 10
23-11-11	1	31	6	9	7	13	11	9	12	12	7	9	17	16	9	11
24-11-11	2	32	7	10	11	11	10	4	44	15	7	8	14	20	13	174 13
25-11-11	3	33	8	9	11	20	12	13	9	15	8	8	14	25	12	164 13
28-11-11	4	34	10	9	13	19	15	15	10	13	10	7	15	16	14	166 13
			37	43	54	74	59	49	86	67	42	38	71	90	58	**768** sum total
			7	9	11	15	12	10	17	13	8	8	14	18	12	**10** average per animal per session

Sum total	**1388 infusions**
Total mean	**11 infusions/ session**

TOX10211		Phase 3: morphine (5 days)														
Low dose		Group	1	1	2	2	2	1	1	1	2	2	2	2	1	
Date	Day	Session	No. 2	No. 3	No.8	No.10	No.12	No.16	No.17	No.19	No.29	No.30	No.31	No.32	No.41	
		Box	box 1	box 2	box 1	box 2	box 3	box 3	box 4	box 5	box 5	box 6	box 7	box 8	box 8	Average per day
6-12-11	10	40	4	4	6	23	6	5	11	3	2	13	25	7	8	369 9
7-12-11	11	41	7	5	6	23	7	1	10	4	1	10	4	23	9	8
8-12-11	12	42	12	3	6	18	13	6	7	7	0	1	8	20	9	110 8
9-12-11	13	43	12	5	8	20	7	7	10	3	1	13	6	21	10	123 9
12-12-11	14	44	11	4	8	31	9	10	10	5	9	13	2	20	4	136 10
			46	21	34	115	42	29	48	22	13	50	45	91	40	596 sum total
			9	4	7	23	8	6	10	4	3	10	9	18	8	9 average per animal per session

TOX10211		Phase 3: morphine (5 days)														
High dose		Group	3	3	3	4	4	3	3	4	4	4	4	4	4	
Date	Day	Session	No. 4	No. 5	No.7	No.13	No.14	No.23	No.24	No.33	No.34	No.35	No.38	No.39	No.40	
		Box	box 1	box 2	box 3	box 1	box 2	box 4	box 5	box 3	box 4	box 5	box 6	box 7	box 8	Average per day
6-12-11	10	40	0	6	14	16	11	14	5	18	2	2	8	25	10	462 10
7-12-11	11	41	3	9	11	12	12	16	10	18	6	4	3	21	9	10
8-12-11	12	42	9	8	5	18	11	11	9	16	10	7	1	21	10	136 10
9-12-11	13	43	7	8	12	14	8	15		13	10	6	12	25	13	143 12
12-12-11	14	44	10	8	15	23	11	14	13	19	9	7	15	24	15	183 14
			29	39	57	83	53	70	37	84	37	26	39	116	57	727 sum total
			6	8	11	17	11	14	9	17	7	5	8	23	11	11 average per animal per session

Sum total	**1323 infusions**
Total mean	**10 infusions/ session**

TOX10211		Phase 5: morphine (3 days)														
Low dose		Group	1	1	2	2	2	1	1	1	2	2	2	2	1	
Date	Day	Session	No. 2	No. 3	No.8	No.10	No.12	No.16	No.17	No.19	No.29	No.30	No.31	No.32	No.41	533
		Box	box 1	box 2	box 1	box 2	box 3	box 3	box 4	box 5	box 5	box 6	box 7	box 8	box 8	
20-12-11	20	50	6	3	12	21	13	19	11	10	13	16	13	37	9	183
21-12-11	21	51	10	4	11	21	11	17	15	11	12	18	9	37	13	189
22-12-11	22	52	10	0	9	17	9	14	12	8	13	17	9	34	9	161
			26	7	32	59	33	50	38	29	38	51	31	108	31	
			9	2	11	20	11	17	13	10	13	17	10	36	10	

Average per day
14
15
12
533 sum total
14 average per animal per session

TOX10211		Phase 5: morphine (3 days)														
High dose		Group	3	3	3	4	4	3	3	4	4	4	4	4	4	
Date	Day	Session	No. 4	No. 5	No.7	No.13	No.14	No.23	No.24	No.33	No.34	No.35	No.38	No.39	No.40	633
		Box	box 1	box 2	box 3	box 1	box 2	box 4	box 5	box 3	box 4	box 5	box 6	box 7	box 8	
20-12-11	20	50	10	15	21	37	18	14	14	15	20	13	10	24	21	232
21-12-11	21	51	11	12	17	34	17	11	8	15	17	10	9	32	18	211
22-12-11	22	52	10	12	18	26	14	4	10	18	17	13	10	19	19	190
			31	39	56	97	49	29	32	48	54	36	29	75	58	
			10	13	19	32	16	10	11	16	18	12	10	25	19	

Average per day
18
16
15
633 sum total
16 average per animal per session

Sum total	**1166 infusions**
Total mean	**15 infusions/ session**

Table VI.11. Number of infusions per animal and per day during the morphine phases 1, 3 and 5.

When the possibility of self-administering saline (phase 2) was offered, an extinction burst (sharp increase in the number of infusions with an average of 23 to 32 infusions per 1-hour session on the first day) was noticed during the first days (Table VI.12), followed by a gradual decrease in the number of responses during the following days.

TOX10211		Phase 2: saline (5 days)														
Low dose		Group	1	1	2	2	2	1	1	1	2	2	2	2	1	
Date	Day	Session	No. 2	No. 3	No.8	No.10	No.12	No.16	No.17	No.19	No.29	No.30	No.31	No.32	No.41	
		Box	box 1	box 2	box 1	box 2	box 3	box 3	box 4	box 5	box 5	box 6	box 7	box 8	box 8	
29-11-11	5	35	27	32	12	50	11	45	5	10	18	15	9	50	11	341
30-11-11	6	36	15	10	14	42	8	5	2	7	4	13	6	50	9	
1-12-11	7	37	13	10	8	26	4	12	1	9	0	4	11	22	3	123
2-12-11	8	38	10	2	7	35	11	7	0	9	3	5	2	25	7	123
5-12-11	9	39	5	3	8	22	7	6	3	2	2	11	7	16	3	95
			70	57	49	175	41	75	11	37	27	48	35	163	33	
			16	18	10	36	9	26	4	6	10	13	8	33	7	

Average per day		
8		
8		
10		
9		
12		
821	sum total	
15	average per animal per session	

High dose		Group	3	3	3	4	4	3	3	4	4	4	4	4	4	
Date	Day	Session	No. 4	No. 5	No.7	No.13	No.14	No.23	No.24	No.33	No.34	No.35	No.38	No.39	No.40	
		Box	box 1	box 2	box 3	box 1	box 2	box 4	box 5	box 3	box 4	box 5	box 6	box 7	box 8	
29-11-11	5	35	32	29	40	50	36	38	32	43	21	28	9	25	41	673
30-11-11	6	36	16	38	34	46	21	29	15	42	11	32	14	24	20	
1-12-11	7	37	9	20	16	50	6	24	12	40	8	28	3	31	12	259
2-12-11	8	38	7	25	13	34	9	14	4	46	6	9	2	25	16	210
5-12-11	9	39	2	24	12	34	13	15	2	43	4	7	8	19	21	204
			66	136	115	214	85	120	65	214	50	104	36	124	110	
			17	27	26	42	25	27	17	43	13	18	9	22	31	

Average per day		
32		
27		
21		
16		
15		
1329	sum total	
24	average per animal per session	

Sum total	**2150**
Total mean	**17 infusions/ session**

Table VI.12. IV SA data during the saline phase (phase 2). Number of infusions per animal and per day.

The IV self-administration of fentanyl showed an average in number of infusions of n=17 and n=13 for the 0.001 (low) and 0.0025 mg/kg/inf. dose (high), respectively (Table VI.13).

TOX10211		Phase 4: JNJ-35685-ADC (5 days)														
Low dose		Group	1	1	2	2	2	1	1	1	2	2	2	2	1	
Date	Day	Session	No. 2	No. 3	No.8	No.10	No.12	No.16	No.17	No.19	No.29	No.30	No.31	No.32	No.41	
		Box	box 1	box 2	box 1	box 2	box 3	box 3	box 4	box 5	box 5	box 6	box 7	box 8	box 8	
13-12-11	15	45	19	7	7	20	12	15	16	7	11	18	2	20	1	767
14-12-11	16	46	18	8	13	20	20	20	19	6	15	18	5	20	1	
15-12-11	17	47	20	8	11	20	20	20	20	8	18	20	13	20	0	198
16-12-11	18	48	17	7	16	32	21	28	20	13	21	33	18	42	12	280
19-12-11	19	49	17	8	19	33	19	31	20	13	19	33	15	47	15	289
			91	38	66	125	92	114	95	47	84	122	53	149	29	
			18	8	13	25	18	23	19	9	17	24	11	30	6	

Average per day		
12		
14		
15		
22		
22		
1105	sum total	
17	average per animal per session	

High dose		Group	3	3	3	4	4	3	3	4	4	4	4	4	4	
Date	Day	Session	No. 4	No. 5	No.7	No.13	No.14	No.23	No.24	No.33	No.34	No.35	No.38	No.39	No.40	
		Box	box 1	box 2	box 3	box 1	box 2	box 4	box 5	box 3	box 4	box 5	box 6	box 7	box 8	
13-12-11	15	45	9	7	10	13	2	8	10	12	15	9	9	12	9	556
14-12-11	16	46	9	10	14	18	3	12	13	14	20	2	2	20	14	
15-12-11	17	47	9	8	11	18	4	11	16	12	15	12	10	20	13	159
16-12-11	18	48	10	9	13	21	11	13	14	12	20	13	11	27	14	188
19-12-11	19	49	11	12	15	26	11	22	14	15	21	14	8	25	15	209
			48	46	63	96	31	66	67	65	91	50	40	104	65	
			10	9	13	19	6	13	13	13	18	10	8	21	13	

Average per day		
10		
12		
12		
14		
16		
832	sum total	
13	average per animal per session	

Sum total	**1937 infusions**
Total mean	**15 infusions/ session**

Table VI.13. IV SA data for fentanyl. Number of infusions per animal and per day during the fentanyl phase. Low dose: 0.001 mg/kg/infusion; High dose: 0.0025 mg/kg/infusion.

The overall result of IV self-administration during the 5 phases of the study is outlined in Fig. VI.8.

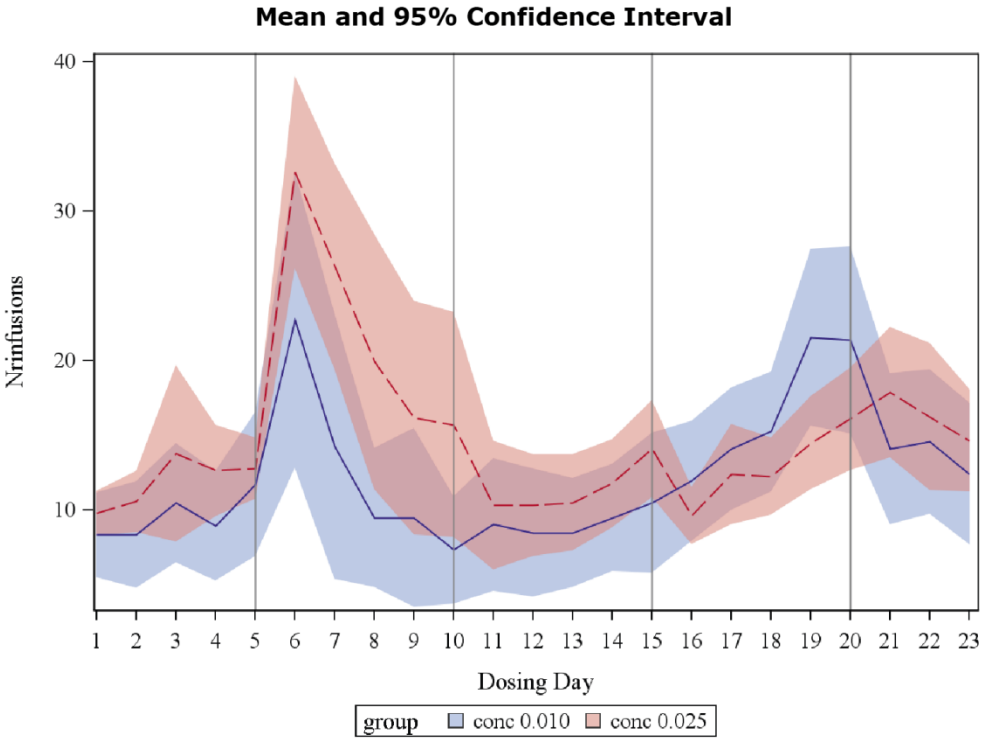


Fig. VI.8. Number of Infusions throughout the 5 phases of the study: observed average profiles per dose group and their 95% pointwise confidence bands. Conc 0.010: low dose group: 0.001 mg/kg/infusion; conc 0.025: high dose group: 0.0025 mg/kg/infusion.

Statistical analysis, calculated on the data of the 26 animals that finalized the 5 phases in total, showed a significant increase in the number of infusions during the fentanyl low dose phase when compared to any of the morphine phases (phase 1, 3 and 5) or to the saline phase (Table VI.14). In the fentanyl high dose group there was no significance for the increase in the number of infusions.

	Phase 1: MOR		Phase 2: Saline		Phase 3: MOR		Phase 4: Fenta Low		Phase 4: Fenta High		Phase 5: MOR	
	Total	Average	Total	Average	Total	Average	Total	Average	Total	Average	Total	Average
	26 rats / day		26 rats / day		26 rats / day		13 rats / day		13 rats / day		26 rats / day	
Day 1	235	9	719	28	248	10	155	12	125	10	415	16
Day 2	245	9	527	20	244	9	183	14	151	12	400	15
Day 3	310	12	382	15	246	9	198	15	159	12	351	14
Day 4	280	11	333	13	276	11	280	22	188	14	/	/
Day 5	318	12	299	12	319	12	289	22	209	16	/	/

Compare phases		Set	P-Value	Compare phases		Set	P-Value
Phase 2	Phase 1	1	0.26758	Phase 2	Phase 1	2	0.32788
Phase 3	Phase 1	1	0.44824	Phase 3	Phase 1	2	0.67676
Phase 4	Phase 1	1	0.00024	Phase 4	Phase 1	2	0.12012
Phase 5	Phase 1	1	0.01636	Phase 5	Phase 1	2	0.01367
Phase 3	Phase 2	1	0.47949	Phase 3	Phase 2	2	0.57446
Phase 4	Phase 2	1	0.00024	Phase 4	Phase 2	2	1.00000
Phase 5	Phase 2	1	0.00977	Phase 5	Phase 2	2	0.43604
Phase 4	Phase 3	1	0.00024	Phase 4	Phase 3	2	0.14795
Phase 5	Phase 3	1	0.00635	Phase 5	Phase 3	2	0.02344
Phase 5	Phase 4	1	0.00024	Phase 5	Phase 4	2	0.15039

Table VI.14. Total number and mean number of infusions per daily session during the various phases for the 26 animals that finalized the 5 phases, including statistics on the number of infusions. A significant increase in the number of infusions was obtained during the fentanyl low dose phase when compared to the number of infusions during the morphine phases. For the fentanyl high dose phase the increase was not significant. Statistics: pairwise comparisons using the Wilcoxon signed rank procedure based on the median of the last 3 observations per phase per animal. MOR: morphine; Fenta: fentanyl. Set 1: fentanyl 0.001 mg/kg/infusion; Set 2: fentanyl 0.0025 mg/kg/infusion.

The mean drug intake for the various phases was calculated based upon the individual and mean number of infusions. For morphine, a mean of 11 infusions per session per dosing day (calculated over the three phases) resulted in a mean daily intravenous intake of 3.3 mg/kg/day which remained below its LD_{50} of 223 - 265 mg/kg.

For fentanyl, a mean of 17 and 13 infusions per dosing day for the 0.001 and 0.0025 mg/kg/infusion dosed groups, respectively, resulted in a mean daily intake of 0.017 mg/kg for the low dosed group and of 0.0325 mg/kg for the high dosed group. The maximum number of infusions obtained in the low dosed group was n=47 (Table VI.13: male No. 32 of the low dosed group), resulting in a daily intake of 0.047 mg/kg. In the high dosed group one animal (Table VI.13: male No. 39) self-administered for 27 times as the highest number of total drug infusions per session, resulting in a daily intake of 0.1175 mg/kg.

The frequency of the infusion rate during the one-hour sessions per dosing day and per phase for all animals, expressed as cumulative rates was visualised with use of the QlikView tool (Fig.VI.9 and Fig. VI.10). In the present study the time-out period between 2 successive infusions was set at 5 seconds.

For the three morphine phases (1, 3 and 5) a similar pattern was present: high rates of cumulative infusions per minute were obtained, in particular during the first 5 minutes, thereafter gradually decreasing during the further course of the 1-hour sessions.

For the saline phase a high cumulative rate was recorded during the first 15 minutes of each 1-hour session. Thereafter the rate remained at a stable but lower rate of infusion.

When a low dose of fentanyl (0.001 mg/kg/inf.) was presented for self-administration a high rate of infusion was recorded during the first 40 minutes. During the last 20 minutes a slightly lower infusion rate was recorded. A high dose of 0.0025 mg/kg/inf. resulted in a self-administration pattern with a lower but stable cumulative ratio throughout the 1-hour session.

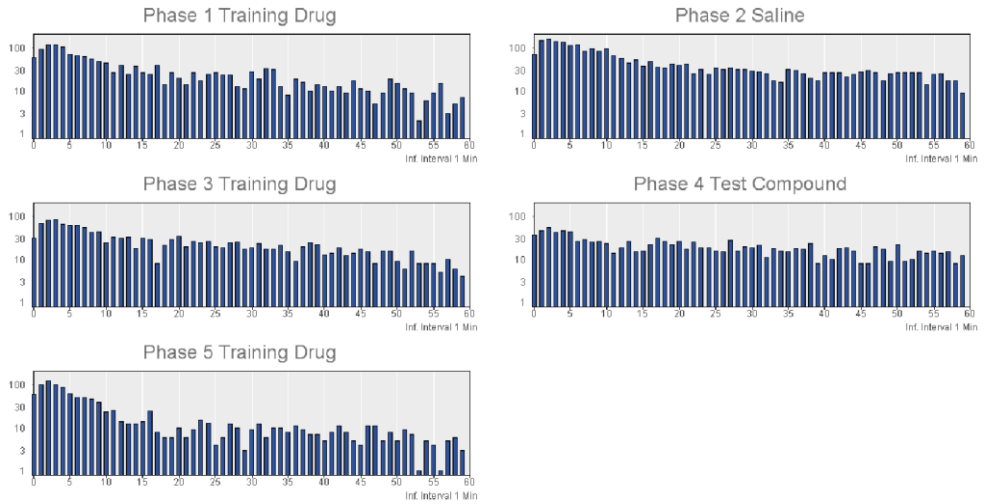


Fig. VI.9. Cumulative frequency rate of infusion rates during the 1-hour sessions per phase. Phase 4: fentanyl low dose (0.001 mg/kg/infusion).

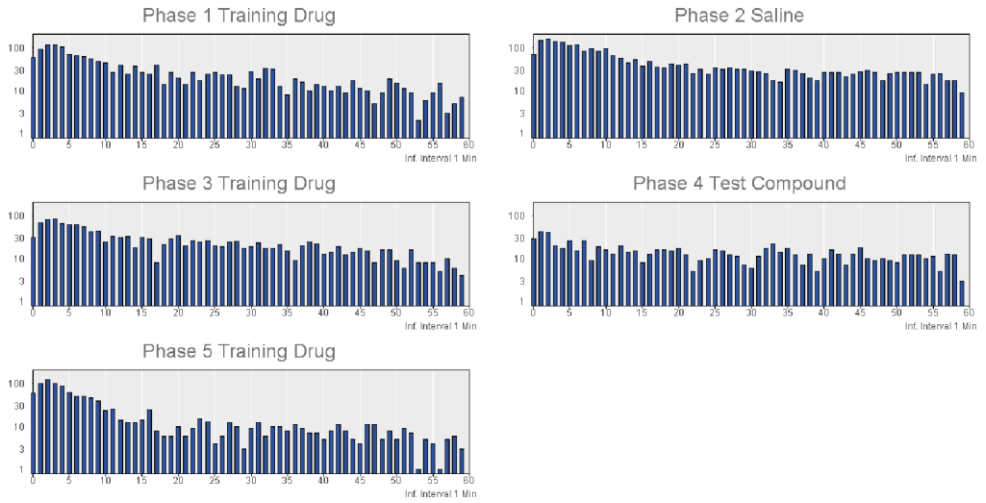


Fig. VI.10. Cumulative frequency rate of infusion rates during the 1-hour sessions per phase. Phase 4: fentanyl high dose (0.025 mg/kg/infusion).

Concerning the general clinical observations (Table VI.15), excitability was observed in a few animals during phase 1 (morphine) and phase 2 (saline). From phase 3 (morphine) onwards agitation was observed in several animals. Excitability was recorded in a few rats and 1 male showed compulsive behaviour twice during this phase. Many animals displayed automutilation (described as nibbling at the forepaws and abnormal licking).

During phase 4 where fentanyl was presented at a low and a high dose, agitation, excitability and automutilation remained present in the same extent. In the high dose group 2 animals experienced catalepsy. In addition, one male of the high dose fentanyl showed a decreased general activity, hypertonia and ataxia on the third day of dosing (number of self-administered infusions: 20). Ataxia was also noted on the fifth day of dosing (number of self-administered infusions: 25) in this rat.

During the fifth phase (morphine) agitation, excitability and automutilation were recorded in several animals. Catalepsy was observed in 10 animals. One male of the low dose fentanyl was in a poor condition, as evidenced by piloerection, chromodacryorrhea, dyspnea and a decreased general activity during the last two days of the study.

Apart from difficult flushing or negative patency tests and occasionally transient skin lesions around the vascular access button no other additional adverse clinical observations were noted during the ongoing study.

	Phase 1	Phase 2	Phase 3	Phase 4		Phase 5
				Low	High	
Excitability	4 rats	4 rats	2 rats	1 rat	3 rats	4 rats
Agitation			13 rats	8 rats	6 rats	16 rats
Compulsive behavior			1 rat			
Catalepsy					2 rats	10 rats
Automutilation (Evidence by nibbling)			13 rats	6 rats	7 rats	14 rats
Automutilation (Evidence by abnormal licking)			2 rats	1 rat	1 rat	5 rats

Table VI.15. Enumeration of the main general adverse clinical effects during the 5 phases of the study. Phases 1, 3, 5: morphine IV SA; phase 2: saline IV SA; phase 4: fentanyl IV SA: low: 0.001 mg/kg/infusion; high: 0.0025 mg/kg/infusion.

VI.3.2.5. Mechanistic IV self-administration Test to investigate cage enrichment, the maximum administrating volume in an IV SA paradigm and the use of a short-acting anaesthetic and its optimal dose for patency testing (study ID number TOX10287)

One rat of the high dosed group was preterminally killed after 2 weeks because of 2 successive negative patency tests. Post mortem evaluation showed a dislocation of the catheter top (at shoulder level). The top was encapsulated but leakage of the catheter (checked with Methylene Blue) was absent.

One rat of the high dosed group died towards the end of the study during the patency test.

The main general clinical observation noticed during the ongoing study was related to the difficult flushing of the catheters. The number of animals was too small to differentiate between the surgery technique or the catheter type.

Adverse clinical observations related to the various volumes of NaCl 0.9% administered intravenously were absent during the dosing period and during the 1-week recovery.

Clinical pathology revealed the following (Table VI.16): Haematological and coagulation pre-values indicated that the surgically prepared animals showed a higher value of fibrinogen (up to 21%), of reticulocytes (up to 50%) and of

neutrophils (up to 40%) when compared to the pre-values of naive control animals. There was an increase in the absolute and relative number of reticulocytes after 5 days of dosing (up to 44%) in the L, M and H dosed groups whereas in the naive control group the number of reticulocytes decreased. After a 1-week recovery period a slight increase was recorded in all groups.
No other relevant adverse effects were noticed when data were compared with the values of the naive control group for all time points and with the pre-values of the respective groups.

The haematological and coagulation parameters measured in the preterminally killed rat and in the dead rat were not adversely affected at any time point.

Pre-values of clinical chemistry indicated that the surgically prepared animals showed a lower value of cholesterol (up to 46%), of triglycerides (up to 52%) and of ALT (up to 25%). Urea nitrogen and creatinine pre-values were slightly increased (up to 15%) in these animals.

There were no adverse effects noticed on the various biochemical parameters after 5 days of dosing neither after a 1-week recovery period in the surgically prepared animals when compared with the values of the naive control group for these time points and with the pre-values of the respective groups. The male rat of the high dosed group which was preterminally killed on Day 15 showed a decrease in albumin (1.8 g/dl on Day 11 versus 3.4 g/dl on Day 5) and a slight increase in AST on Day 11. The biochemical parameters measured in the male that died were not adversely affected at any time point.

			NC	L	M	H
Fibrinogen	Prevalues	mg/dl	151 (3)	182 (6)	172 (6)	191 (6)
	24h after last daily admin	mg/dl	139 (5)	182 (4)	177 (6)	184 (6)
	After 1-week recovery	mg/dl	144 (5)	187 (3)	175 (6)	189 (6)
Reticulocytes	Prevalues	%	2.8 (0.2)	5.6 (0.2)	5.1 (0.7)	3.4 (1)
	24h after last daily admin	%	1.8 (0.2)	7.4 (1)	6.2 (0.6)	6 (1)
	After 1-week recovery	%	3 (0.2)	7.4 (0.6)	7.7 (0.3)	7.2 (0.8)
Reticulocytes	Prevalues	10^3/µl	236.6 (13.9)	413.2 (10.4)	380.5 (38.5)	283.9 (71.3)
	24h after last daily admin	10^3/µl	149.8 (11.2)	521.3 (58.5)	435.2 (36)	471.6 (60.7)
	After 1-week recovery	10^3/µl	248.5 (16.2)	533.8 (20.4)	544.5 (11.2)	549.5 (53.1)
Neutrophils	Prevalues	10^3/µl	0.82 (0.08)	1.25 (0.58)	1.35 (0.53)	1.18 (0.21)
	24h after last daily admin	10^3/µl	0.76 (0.07)	0.52 (0.16)	1.08 (0.46)	1.02 (0.25)
	After 1-week recovery	10^3/µl	0.84 (0.09)	0.7 (0.12)	1.48 (0.55)	1.06 (0.27)
Cholestorol	Prevalues	mg/dl	60 (5)	41 (5)	42 (6)	39 (5)
	24h after last daily admin	mg/dl	62 (8)	43 (4)	43 (7)	42 (3)
	After 1-week recovery	mg/dl	67 (8)	47 (3)	49 (8)	44 (4)
Triglycerides	Prevalues	mg/dl	71 (5)	44 (4)	39 (6)	37 (4)
	24h after last daily admin	mg/dl	98 (24)	74 (7)	52 (3)	50 (8)
	After 1-week recovery	mg/dl	124 (5)	83 (4)	79 (3)	88 (12)
AST	Prevalues	U/l	35 (2)	48 (11)	40 (4)	46 (4)
	24h after last daily admin	U/l	37 (4)	47 (12)	41 (5)	41 (3)
	After 1-week recovery	U/l	38 (2)	54 (15)	41 (6)	36 (10)
Urea Nitrogen	Prevalues	mg/dl	14.1 (/)	16.7 (1.4)	16.4 (0.5)	16.6 (0.3)
	24h after last daily admin	mg/dl	14.5 (0.6)	16.3 (0.5)	17.1 (0.4)	15.1 (0.6)
	After 1-week recovery	mg/dl	15.7 (15)	15.8 (0.4)	16.8 (0.02)	16.4 (0.5)
Creatinine	Prevalues	mg/dl	0.23 (0.01)	0.27 (0.01)	0.27 (0.02)	0.27 (0.02)
	24h after last daily admin	mg/dl	0.2 (0.01)	0.24 (0.01)	0.22 (0.01)	0.23 (0.01)
	After 1-week recovery	mg/dl	0.21 (0.02)	0.25 (0.01)	0.23 (0.01)	0.24 (0.01)

Table VI.16. Summary of the haematological, coagulation and biochemical parameters showing deviant values. Standard errors are given between brackets.

Cage enrichment which was investigated during three weeks revealed that wood blocks were hardly or not at all digested and that an amount of 10 g of sizzle nest per cage was sufficient to nidify. All amounts above these 10 g (15, 20 or 25 g) did not add value and prevented proper manipulation of the rats.

The duration of anaesthesia after the different doses of propofol revealed that a single IV dose of 5 mgkg/bolus was the optimal dose for patency testing in the SA paradigm, leading to 3-4 minutes of anaesthesia (Table VI.17).

Dose group	Propofol (mg/kg/bolus) Diprivan® (ml/kg/bolus)	Anaesthesia (minutes) Mean over three sessions
Low	2.5 mg/kg/bolus 0.25 ml/kg/bolus	Less than 1 minute
Mid	5 mg/kg/bolus 0.50 ml/kg/bolus	3 to 4 minutes
High	7.5 mg/kg/bolus 0.75 ml/kg/bolus	6 to 7 minutes

Table VI.17. Duration of anaesthesia related to the dose propofol administered intravenously.

VI.3.2.6. Mechanistic IV self-administration Test to investigate the possible use of different lock solutions (study ID number TOX10412)

Both lock solutions could be easily administered via the vascular access button. However pulling back the solutions out of the catheters via the access button after 3 or 4 days was not possible. Repeatedly filling of the catheters could be easily performed.

All animals but 2 tested positive for patency testing at the end of the study (Day 10). At necropsy, sheet forming was noticed in nine out of 12 rats but the catheters showed no obstruction (evidenced by easy flushing at necropsy). Other macroscopic findings included a swollen spleen noticed in all animals, depressed foci at the kidney surface (8 rats), white or red foci at the lungs (4 rats) and swollen bronchial lymph nodes (2 rats).

VI.4. Discussion

VI.4.1. Final outline of the three phases of an IV SA model (surgery, training and final study) with regard to methodology and technology

Six mechanistic studies were conducted in order to optimize the methodology and the technology of the intravenous self-administration (IV SA) model for use in Drug Development.

It was shown that both the surgical procedure used and the choice of the indwelling IV catheter were critical for the survival of the rats in this long-term IV SA model and hence of major significance to the success of IV SA studies. Indeed, the main cause of forced termination of the IV SA procedure in an animal was due to obstruction of the indwelling catheter by deposition of debris, mainly at the internal catheter top. Coating of the catheters aims to prevent the debris sticking in the catheter to allow fluent. However, of the three catheter types tested in various studies, both the CBAS® heparin coated catheter and the polyurethane non-heparin coated catheter were considered superior to the hydro-coated catheter.

Concerning the surgical procedures, it was demonstrated that the femoral indwelling catheter procedure was superior to that of the v. Jugularis catheter implantation. Obviously, the known lower turbulence in the femoral vein versus the higher turbulence in the jugular vein near the entrance of the right atrium (Lilbert, 2004) implied that deposition of platelets and/or debris at the femoral catheter top occurs to a smaller extent, thus resulting in less catheter obstruction and as such a lower drop-outs of animals in studies.

The collaboration with the external company Instech resulted in the development of a new prototype of a modified Vascular Access Button™, which was successfully implemented after several pilot and mechanistic studies and which is now on the market (Instech, 2014), enhancing the rat's comfort and making its use feasible in long-term studies as in the IV SA model. This new VAB also facilitates the biweekly (manual) injections for patency testing.

This collaboration effort was rewarded within the Department of Toxicology of Janssen R&D in 2011.

The training phase, during which the surgically prepared animals are habituated to self-administer a known psycho-active drug following a fixed ratio of reinforcement schedule (FR) took 4 to 12 weeks in general. The appropriate dose of the psycho-active reference drugs that were selected for use in an IV SA study as reinforcers, was based upon their pharmacological and toxicological profile, internal data and literature (Chapter III). A scientifically founded and reliable concentration was selected for the following reference drugs: cocaine (0.5 mg/kg; 6 mg/ml; 0.08 mg/ml and 1 mg/kg; 12 mg/ml; 0.08 ml/infusion, maximum of 50 infusions/session), morphine (0.3 mg/kg/infusion, 1.5 mg/ml; 0.2 ml/infusion, maximum of 50 infusions/session), LSD (0.1 mg/kg, 0.5 mg/ml, 0.2 ml/infusion, maximum of 20 infusions/session), d-amphetamine (0.06 mg/kg; 0.3 mg/ml; 0.2 ml/infusion; maximum of 20 infusions/session), ketamine (0.1, 0.3 and 0.6 mg/kg; 0.75, 2.3 or 4.6 mg/ml, respectively; 0.13 ml/infusion; maximum of 50 infusions/session).

The final ratio for response to an intravenous infusion was set standard at a fixed ratio of reinforcement of 10 (FR10: 10 times pressing the lever before an IV infusion was obtained) according to the regulatory requirements and for the reference drugs that allowed this. The reinforcing properties of cocaine (0.5 and 1 mg/kg) or d-amphetamine (0.06 mg/kg) could be easily demonstrated during training at FR10. However when morphine (0.3 mg/kg) was selected as training drug, the criterion of FR10 could not be obtained. Therefore the flexibility to maintain an individual FR lower than FR10 but with stable responses for each individual animal was made feasible. In these cases a rationale for a lower FR was written.

We opted for a final test design consisting of five successive phases. Each phase lasted for 5 working days during which animals were placed in the operant chambers and presented for IV self-administration over 1-hour daily sessions. During the first, third and fifth phase, all animals were presented the selected reference drug to which they were habituated for IV self-administration. The second phase allowed these animals to self-administer saline. During the fourth

phase, animals were divided into four groups for IV self-administration of the control/vehicle, a low, mid and high dose of the test compound.

The sequence of the first three phases evidenced the seeking responses to the reinforcing properties of the reference drug, interrupted by the decline in responses when saline was made accessible. The fifth phase was included to ensure the sustained seeking behaviour of the animals, in case of absence of reinforcing properties of the test compound at the selected doses. It also evidenced a control to the patency of the catheters during the fourth phase in case no responses were obtained during IV SA of the test compound.

The pattern of an extinction burst followed by a gradual decline in the number of responses, was observed when presenting saline (phase 2). This phenomenon takes place when rats are restrained from self-administration of a psycho-active drug which they were trained for through replacement by saline (extinction procedure). A temporary increase in the number of responses (= extinction burst) can then be noticed during the first days of saline self-administration because the initial reinforcing drug is no longer present. Thereafter a gradual decrease in the number of responses for saline will be seen.

It was assumed that in rats, adequately habituated to a reference drug in the IV SA paradigm, an extinction burst would be elicited. In full studies, where rats were presented saline in phase 2 (saline phase) but also for a control solution (NaCl 0.9%) during phase 4 (test compound phase), it was demonstrated that the extinction burst followed by further extinction appeared in a similar way (see Chapter VIII). Therefore it was concluded that the sequence of the saline phase as phase 2 in a full test design was scientifically valid and thus maintained as such.

The duration of each of the phases in this design was evaluated. It was concluded that once the animals were properly responding to the IV self-administration of the reference drug, separate phases of 5 dosing days were sufficient to reach stable and scientifically valid data. Stable data on the number of infusions were needed during the last 3 dosing days of the previous phase before entering the next phase. The study design aimed for 5 phases of 5 dosing

days each. However it remained feasible to extend each of the phases if needed to obtain stable data.

Lastly, the maximum number of infusions was limited at 20 or 50 infusions per daily 1-hour session, depending on the toxicity and concentration of the reference drug and of the test compound, to avoid IV overdose.

VI.4.2. Investigation of the variables to optimize the design of an intravenous self-administration model for use in Drug Development

Besides the assessment of the reinforcing properties of drug candidates and of known psycho-active drugs, variables possibly impacting the outcome of these IV SA studies were investigated to further optimize the IV SA model for use in Drug Development. The outcome hereof is described below.

VI.4.2.1. Maximum self-administered volume per daily session

For ethical purposes it was investigated whether the maximum volume of IV infusions that the animals could administer themselves in an IV SA paradigm was justified and of no concern for the general condition of the animals. Volumes up to 20 ml NaCl 0.9%/kg/day, accounting for up to 31.5% of the total blood volume of a rat and administered according to the IV SA paradigm (*i.e.* within one hour as 20 short (2 to 5 seconds) infusions during 5 consecutive days) were shown ethically acceptable.

The higher pre-values of fibrinogen (up to 21%), of reticulocytes (up to 50%) and of neutrophils (up to 40%) in surgically prepared rats was in line what can be expected in these chronically catheterised animals and not related to the volume self-administrated intravenously.

The lower value of cholesterol (up to 46%), of triglycerides (up to 52%) and of ALT (up to 25%) in these animals were indicative of the previous food restriction (20g/day) as the rats were re-used for this particular experiment. Finally the urea nitrogen and creatinine pre-values, which were slightly increased (up to 15%) were considered a result of permanent catheterisation which can induce kidney dysfunction and/or kidney inflammation (internal communication).

VI.4.2.2. Patency testing

To control for patency of the internal catheters during the training phase and the full study, rats were injected with a short-acting anesthetic. If anesthesia was induced within a few seconds, patency testing was considered positive. Initially a mixture of Ketalar®/Dormicum®/saline at a ratio of 3/1.5/5.5 was employed as a short-acting anaesthetic for IV patency testing. However, in view of the purpose of the study type it was considered less convenient to use because of the psycho-active components ketamine and midazolam present, which are scheduled as CIII and CIV compounds for drug abuse, respectively, in the U.S. and worldwide. Therefore another short-acting anaesthetic, propofol was selected for further investigation within the IV SA paradigm. Several doses were tested to select a proper dose to use for patency testing. It was demonstrated that 0.50 mg propofol/kg IV resulted in an adequate and short anaesthesia of 3 to 4 minutes, which was optimal for patency testing in the SA paradigm.

VI.4.2.3. Toxicokinetic parameters

Determination of the toxicokinetic (TK) parameters has always been a point of attention in the IV SA paradigm (Swedberg, 2013; Teuns, 2014). Within Drug Development the TK data of single IV infusions (unit dose) of a test compound are captured upfront in a separate dose range finding study, in order to select a proper dose range for the final IV SA study. As stated before, the C_{max} of the high dose (one infusion) needs to reach a 2x to 3x multiple of the human efficacious dose (expressed as C_{max}). Conditions for this requirement included that 1) data from pharmacology and toxicity studies allowed this high dose and 2) that the test compound was soluble at this high dose. For the low dose a C_{max} being a 0.1x multiple is considered acceptable as discussed in the FDA dialogues session of November 2010.

A kinetic phase was added to the final study after completion of all phases, as a standard to investigate the toxicokinetic parameters.

Within the IV SA study, we have opted to calculate the C_{max} of the actual (total) drug amount of a test compound that was self-administered within a 1-hour daily session (lowest and highest drug intake by an individual rat) to compare with the human efficacious dose (expressed as C_{max}). These data are of value for

the translational approach of an acutely (within 1 hour) self-administered maximum tolerated dose, and also in view of obtained dose-exposure-effect (see further: VI.4.2.4. The bell-shaped dose-response curve in IV SA).

To determine the correct dosages for manual IV administration at the TK phase, the minimum and maximum self-injected dose (mg/kg) in any daily test session were calculated within each dosage group, based upon the real number of infusions obtained during the 1-hour IV SA sessions in individual rats. From these calculations the minimum and the maximum IV dose (mg/kg) was used. In addition a mid dose is also selected.

An example of the method and rationale for dose selection for TK purposes is given below, derived from the IV SA study with methylphenidate (MPH) (see Chapter VIII).

Example of the method and dose selection for TK purposes: methylphenidate (MPH)

Within the low dose group (0.1 mg/kg/infusion MPH):

Minimum number of infusions/hour in any test session:

5 (rat No. 32 on 1st day of phase 4)

This rat self-injected MPH at minute 2, 24, 36, 27 and 59 of the 1-hour session. These infusion data were captured for each individual rat during each daily session and visualized with QlikView (Fig. VI.11).

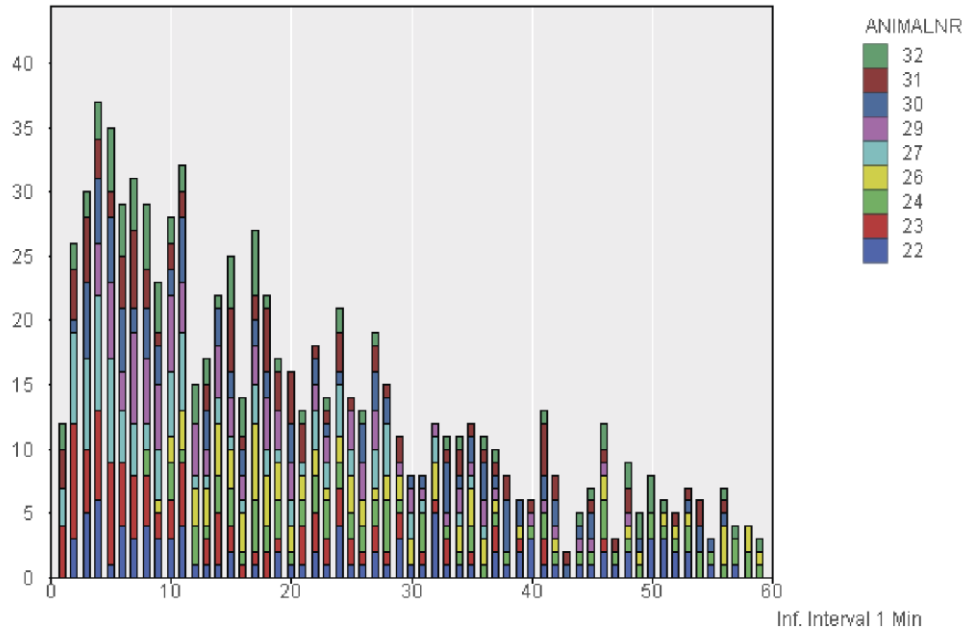


Fig. VI.11. Visualization of the cumulative data of the number of infusions per individual animal during one daily 1-hour session with 0.1 mg/kg/infusion MPH via QlikView.

Minimum dose administered in this low dose group:

0.1 mg/kg/inf x 5 inf = 0.5 mg/kg/hour

Maximum number of infusions/hour in any test session:

20 (5 out of 9 rats during various days of phase 4)

Maximum dose administered in this low dose group:

0.1 mg/kg/inf x 20 inf = 2 mg/kg/hour

Within the mid dosed group (0.5 mg/kg/infusion MPH):

Minimum number of infusions/hour in any test session:

0 (rat No. 42 on 3rd and 4th day of phase 4)

Minimum dose administered in this mid dose group:

0.5 mg eq./kg/inf x 0 inf = 0 mg/kg/hour

Maximum number of infusions/hour in any test session:

12 (rat No. 48 on 5th day of phase 4)

Maximum dose administered in this mid dose group:

0.5 mg eq./kg/inf x 12 inf = 6 mg/kg/hour

Within the high dosed group (1 mg/kg/infusion MPH):

Minimum number of infusions/hour in any test session:

2 (rat No. 64 on 1st day of phase 4)

Minimum dose administered in this high dose group:

1 mg eq./kg/inf x 2 inf = 2 mg/kg/hour

Maximum number of infusions/hour in any test session:

15 (rat No. 62 on 2nd day of phase 4)

Maximum dose administered in this high dose group:

1 mg eq./kg/inf x 15 inf = 15 mg/kg/hour

Dose range in self-administration paradigm over the groups

0	**0.5**	2	6	**15**	mg/kg/hour
M	L	L, H	M	H	dosed groups

Dosages for single dose IV administration for TK purposes (manual dosing):

Single dose at 0.5 mg/kg
Single dose at 5 mg/kg
Single dose at 15 mg/kg

The LD_{50} of MPH [48 to 50 mg/kg (Johnson Matthey, 2006; Separham, 2011] administered IV in the naive rat was also taken into account, and was in this example approximately 3 times the highest single dose to be administered intravenously.

During the TK phase, a single bolus of each of the selected doses used for TK purposes was slowly administered within 1 minute (3 rats per dose). The slow bolus administration was justified as follows: in our study set-up, IV SA infusions are administered within 2-5 seconds and with a TO of 20 seconds. Hence the maximum number of infusions per minute is 3, although not all animals showed

the same pattern for self-injecting MPH at a given dose during the 1-hour daily sessions. The maximum of 3 infusions per minute can be considered as a slow bolus.

Another example of the determination of the toxicokinetic parameters comprised TOX10046. The drug candidate JNJ-X, presented to male SPF Sprague-Dawley rats in a self-administration paradigm at a dose of 2 mg/kg/infusion with a FR10 showed no potential for abuse in the self-administration rat model using cocaine as concurrent positive control. The minimum number of infusions that was self-administered by the rat (n=1) and mimicked by a manually administered single intravenous dose of 2 mg/kg resulted in a C_0 of 875 ng/ml whereas the maximum number of infusions recorded during self-administration of JNJ-X (n=11, single dose of 22 mg/kg administered IV) showed a C_0 of 16931 ng/ml. These data correlated with 7.5 - 3.3 times (2 mg/kg/day) to 128.6 - 56.6 times the anticipated efficacious human dose level (110 - 250 ng/mL) and thus fell within the expected exposures needed (2 to 3-fold the human efficacious dose (C_{eff} Hu) determined by a C_{max} of 110 - 250 ng/ml).

Also in the study TOX10211 toxicokinetic parameters were included. For fentanyl, the maximum number of infusions obtained in the low (0.001 mg/kg/inf.) dosed group was n=47 (male No. 32), resulting in a maximum intake of 0.047 mg/kg. In the high dosed group one animal (No. 39) self-administered for 27 times as the highest number of total drug infusions per session, resulting in a maximum intake of 0.1175 mg/kg. An intravenous bolus of 2 µg/kg (0.002 mg/kg) in humans can lead to a serum concentration of 11 ng/mL, where in a large series of fatalities from fentanyl abuse, the mean blood concentration was 3 ng/mL (Lilleng, 2004). Referring to the linear dose-concentration curves of fentanyl (Choi, 2001) the dose of 0.047 mg/kg or the dose of 0.1175 mg/kg would result in serum concentrations of 258.5 or 646.25 ng/ml, respectively. Hence the total drug intake in rats of both groups reached sufficiently high concentrations of fentanyl when taken the C_{eff} in humans into account (0.24 to 0.91 ng/ml) (Lennernäs, 2005).

VI.4.2.4. Bell-shaped dose-response curve in IV SA

A bell-shaped curve is a typical and well-known phenomenon that occurs as a response to the different doses presented in an IV SA model for a variety of self-administered drugs (Van Ree, 1999; Piazza, 2000; Broadbear, 2004; Peana, 2010). The outer left limb demonstrates the inability to properly respond if the dose of the drug as presented by each single infusion is too low. When concentrations are increased, a higher number of responses will occur to ensure the reinforcing effects. The top of the curve represents the maximal reinforcement of an IV self-administered dose of a particular drug. The right limb shows the cumulative effect of the response rate when higher and increasing concentrations of a drug are presented, resulting in much lower IV SA responses during the 1-hour daily test sessions. This feature of inverse response to dose is a typical attempt to maintain a level of reinforcement not resulting in overdose or adverse effects.

Data from the executed IV SA studies with different drugs [cocaine (TOX10046), fentanyl (TOX10211), methylphenidate (TOX10335)] clearly confirmed the inverse dose effect, thus emphasizing the great importance of a correct choice of a dose/concentration range for the outcome of this type of studies.

VI.4.2.5. Heparin lock solutions

Heparin lock solutions are often being employed in human situations to prevent internal IV catheters from clotting and obstruction of the blood flow. Two lock solutions (100 I.U. and 500 I.U. of heparin/ml dissolved in a 30% dextrose solution) were investigated in the IV SA paradigm through repeated (every 3 days) intravenous administration via the vascular access button at the predefined dead volume for the implanted catheter (0.0549ml: dead volume of the internal femoral catheter). It was shown that the IV use of a 30% dextrose solution with either 100 or 500 I.U. heparine/ml every 3 or 4 days can be used to keep indwelling IV catheters open. This might be useful to tide over daily flushing during weekends.

The swollen spleen noticed in all animals, the depressed foci at the kidney surface (8 rats), the white or red foci at the lungs (4 rats) and the swollen bronchial lymph nodes (2 rats) were considered a consequence of the chronic IV

catheter implants and not related to the administration of the locker solutions (internal communication).

VI.4.2.6. Cage enrichment

In the IV SA studies, rats were housed individually to avoid damage to the external part of the catheter. Therefore cage enrichment is needed to improve the animal's welfare, thus fulfilling the requirements of animal care and welfare (AAALAC), but without affecting either study parameters. Obviously the classical polycarbonate tunnels could not be employed because of possible injuries at the VAB. Aspen wood blocks were nibbled at but not digested, thus not contributing to any additional food uptake in the feed restricted IV SA rats. The use of sizzle nest stimulated nidification, which is known to ameliorate the animal's comfort. In addition, use of sizzle nest was devoid of adverse effects on the external catheter (VAB) and surrounding tissue in terms of contamination or inflammation. In the final IV SA design the rats were thus provided with Aspen wood blocks in combination with 10 g of sizzle nest as cage enrichments.

VI.4.2.7. LSD in IV SA

LSD is a CI scheduled non-medical drug with high risk for physical and psychological abuse potential. However, from literature it is known that LSD is not generally self-administered by animals (Jerome, 2008; Bonson, 2012).

The data of TOX10141 confirmed that rats were irresponsive to LSD at a dose of 0.01 mg/kg/infusion. A possible explanation might be that LSD develops a sensory distortion (misinterpretation of real sensations) rather than a simple "feel good" effect in animals.

VI.4.3. Data management of an IV SA model for use in Drug Development and GLP compliance

VI.4.3.1. IT validation

The IV SA paradigm required an extensive investment of IT research to make the set-up and the concomitant procedures of IV SA infusions compliant with the GLP regulations. The IT package included the development, validation and implementation of a new, internally built IT system to perform the standard

study design and additional tools to facilitate the execution of the study according to the GLP requirements (state of the art: traceable and reproducible data). IT validation included an inventory of the business and compliance requirements, execution of the various test scripts to test system installation, security, compliance, functionality and performance, and a validation report.

The core of the IV SA system consisted of the commercially available MED-PC hardware and software. This system has been configured and customized in-house. These adaptations included, *amongst others*, the inactivation of 1 lever, the implementation of a standard time-out period of 20 seconds for retraction of the remaining lever during infusion phase, the activation of light stimulus (whenever the time-out of 20 seconds had passed the lights inside the chamber were (re)activated), the calibration of the hardware (commercial system from MED Associates Inc.), the development of some MED-PC procedures specific for self-administration studies, the introduction of a user interface in which various parameters can be captured, such as dose/concentration, number of infusions, session duration, infusion duration, rat body weights, FR, … (Fig. VI.12), and the incorporation of this whole modified package into a GLP environment through workarounds and vendor modifications.

The user interface (Fig. VI.12) was built in the shape of a template macro to ensure that correct data were obtained (calculation of infusion time based upon speed of the chosen pump, the dose concentration and dose volume of the drug and the individual body weight). In the background, a macro was generated with all the filled-in parameters collected from the user interface. This macro steered the SA session via the MED-PC software and gave each IV SA box the appropriate signal in order to conduct the study.

d-amphetamine: 0.1 mg eq./kg/infusion

Study Number*	TOX10335	**Responsible**	mkom2
Session Date (dd-mmm-yyyy)*	To be amended	**Protocol**	PROGRAM SA_Drug Self Infusion V1
Session Number*	1 - 3 - 5	**Template name**	DASAMacroSetup
Syringe Volume (ml)*	10	**Template version**	3.1
Motor type*	PHM-100-3.3		
Infusion speed (ml/s)	0.01837		
Catheter Type	Instech-VAB fem		
Dead Volume inf. Time (s)	2.98911		
Max Infusions	20		
Session Duration (min)*	60		

Fields marked with * are mandatory !!

Substance	Training Drug	Test Compound	Saline* (or any substance for which concentration = 0 and/or dose = 0)
Dose (mg/kg)	0.1		
Concentration (mg/ml)	0.5		
Dosage in Route (ml/Kg)	0.2	0	

Box	Animal Number	BodyWeight (g)	Fixed Ratio	Time Out (s)	Phase	Infusion Time (s)
1	1	250	10	20	1. Training Drug I	2.722323
2	2	300	10	20	1. Training Drug I	3.266788
3	3	350	10	20	1. Training Drug I	3.811252
4	4	400	10	20	1. Training Drug I	4.355717
5	5	450	10	20	1. Training Drug I	4.900181

Fig. VI.12. Example of a user interface (macro). The range of infusion time is between 2 to 5 seconds and is based upon the chosen pump, the dose concentration and dose volume of the drug and the body weight

Upon the end of the session, once the data were locally generated on the computer, they were transferred immediately to a secure location by means of an in-house developed and validated tool (SafeUploader), to avoid data tampering. Afterwards, the data were manually transferred to the database via another in-house developed and validated tool (DASAClient).

VI.4.3.2. QlikView

We selected a business intelligence tool (QlikView) with the ability to visualize the data of a particular IV SA study at, *amongst others*, an individual level or at a cumulative level per group, per session or per phase. Examples hereof are presented in Fig. VI.13, VI.14, VI.15 and VI.16.

This tool was also used to study the individual patterns of the infusions during the 1-hour daily sessions of individual rats or of dose groups.

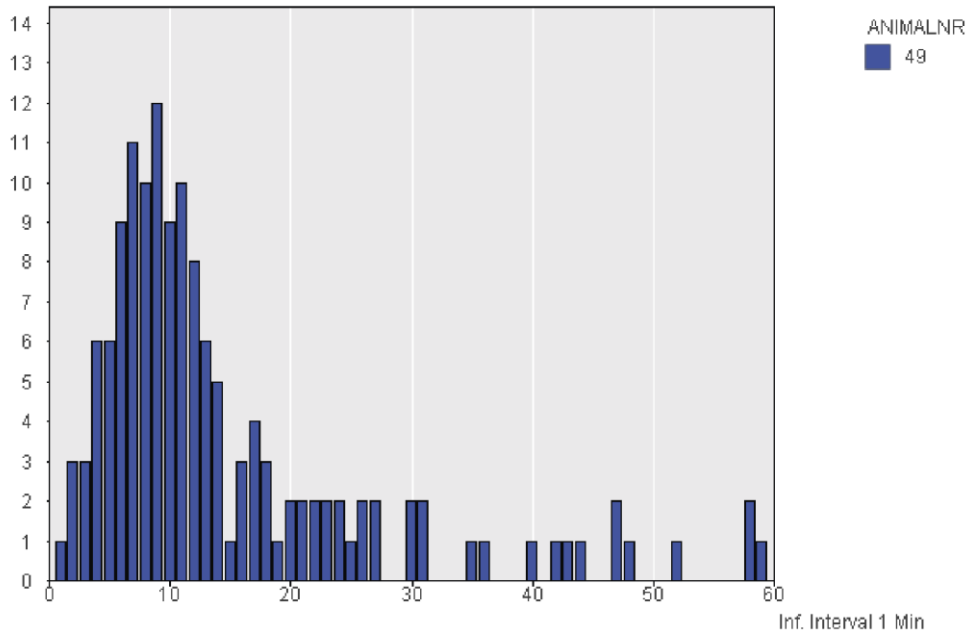


Fig. VI.13. Individual data of the infusion pattern during a daily 1-hour test session.

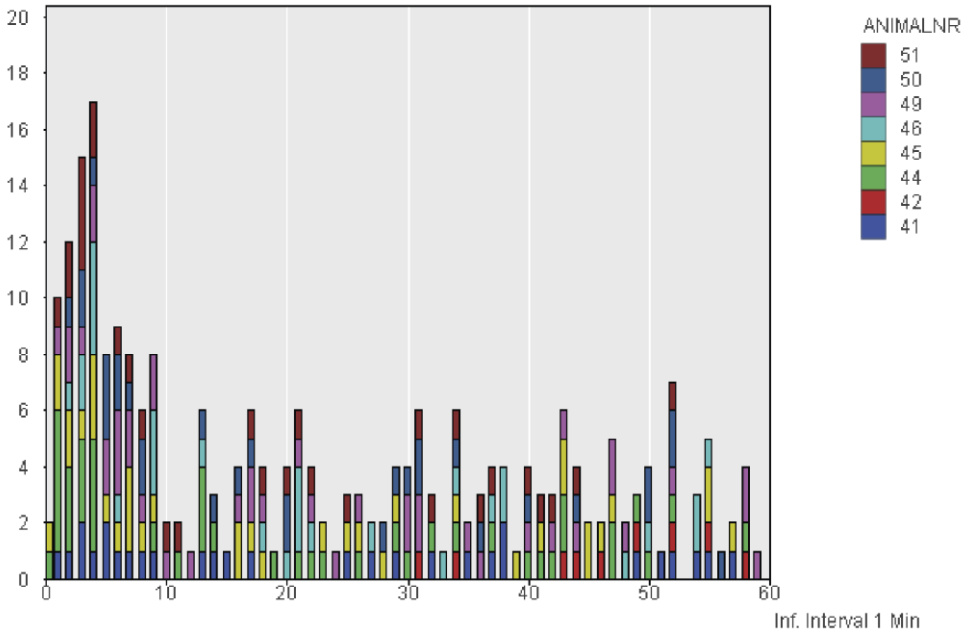


Fig. VI.14. Visualization of the cumulative data of the number of infusions per individual animal during one daily 1-hour session via QlikView.

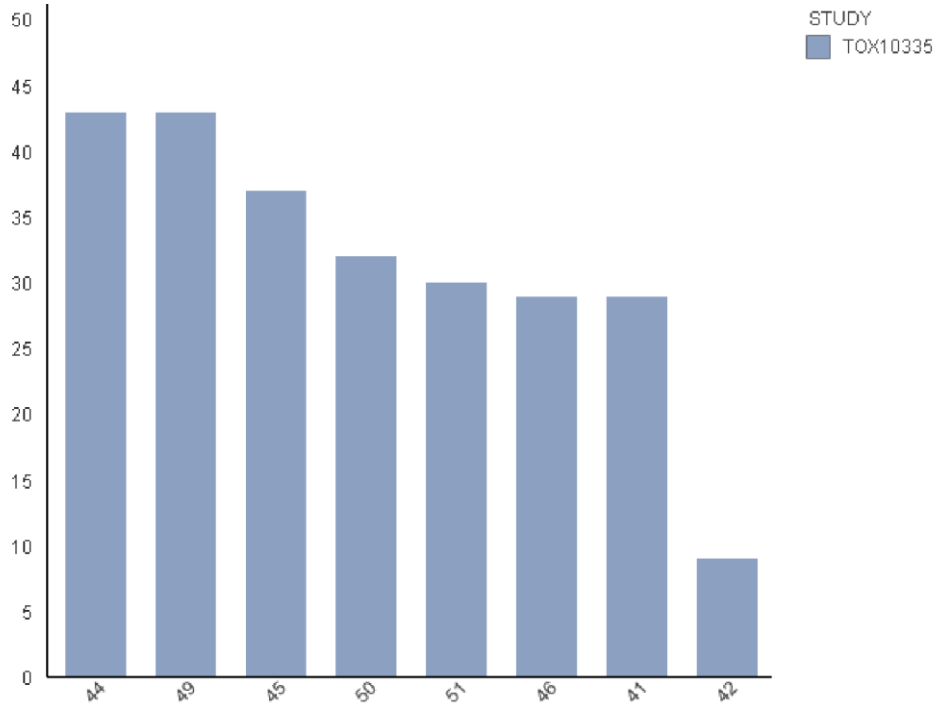


Fig. VI.15. Cumulative data of the number of infusions per animal during a 5-day test phase. Visualized via QlikView.

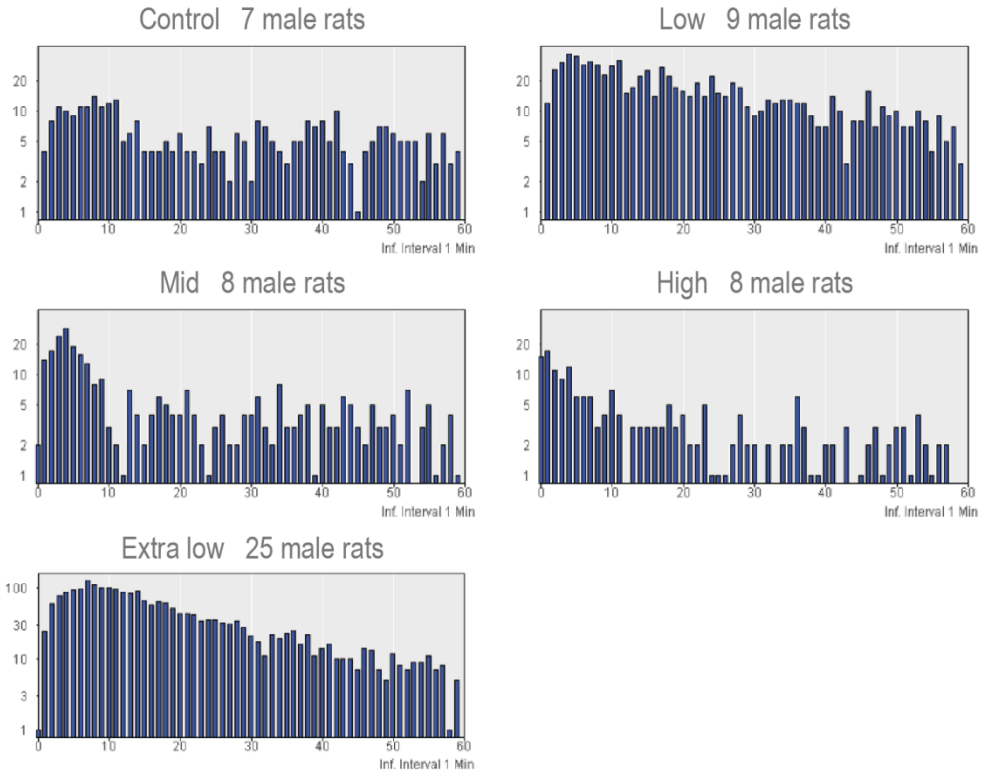


Fig. VI.16. Cumulative rates of the frequency of the infusion during the one-hour sessions per group over 5 dosing days. Visualized via QlikView.

VI.4.3.3. GLP compliance

The standard study design and tools needed to perform this type of study (the IT DASA system and the commercially available hard- and software inclusive) were made GLP-compliant. All systems were validated (IT validation, scientific validation) and all obtained data were retraceable and reproducible. The respective SOPs (for IT and for *in-vivo* procedures) were written and approved.

VI.4.4. Other considerations

VI.4.4.1. Priming with food or with the reference drug at initiation of the training phase

We investigated whether food reward would enhance the initiation of self-administering a known psycho-active drug. No beneficial effect was noticed. On the contrary, starting with food training resulted in a longer attempt to habituate

the animals to IV self-administration. This procedure was also not in favour because of the extra learning effort animals had to make in terms of conditioning and reinforcement. Therefore we did not select this food rewarding procedure as part of the training.

Priming by inserting the reference drug directly into the IV catheter when animals were placed in the operant chamber did not add to initiate lever press either. Therefore this procedure was not maintained.

VI.4.4.2. Inclusion of an additional extra dose in a full study

If, during the ongoing study, testing of an extra dose would be appropriate, a sixth phase can be added to the design. Such a design was executed in an IV SA study with methylphenidate (reference drug: d-amphetamine (AMP) 0.06 mg/kg/infusion) to study an additional lower dose (Fig. VI.17). This additional dose is presented after the fifth phase during which the reference drug was self-administered.

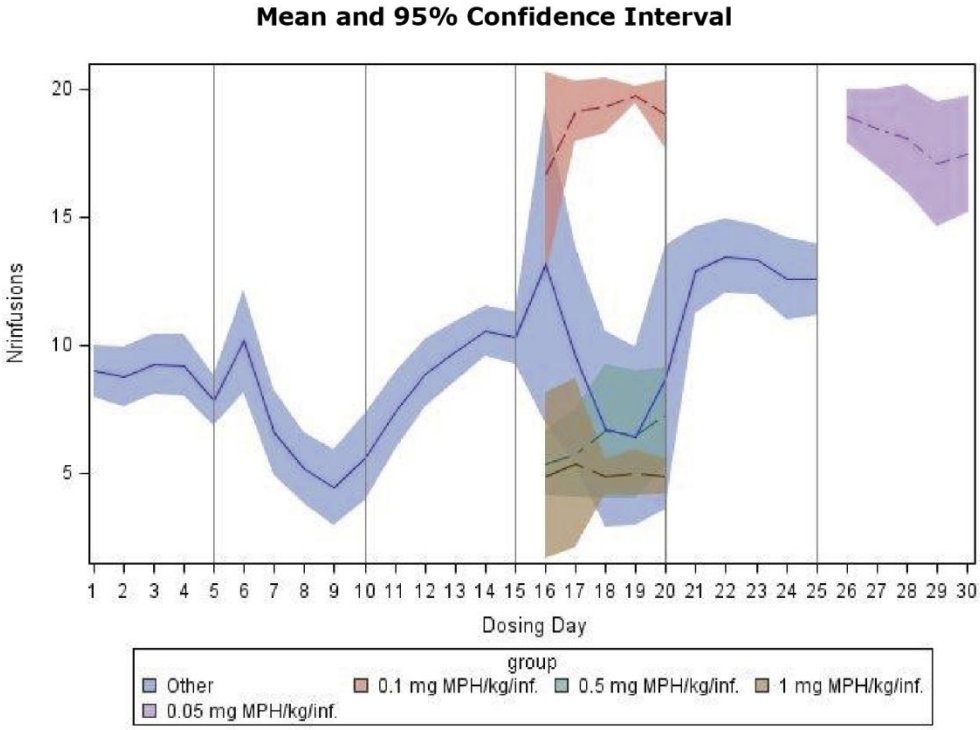


Fig. VI.17. Visualisation of the results of an IV SA study with methylphenidate (MPH). Number of infusions throughout the six phases presented as an observed average profile and its 95% pointwise confidence band. During the six phases of 5 days each, the following drugs were presented: d-amphetamine 0.06 mg/kg/inf. (32 animals): phases 1 (Day 1-5), 3 (Day 11-15), 5 (Day 21-25); and saline (NaCl 0.9%; 32 animals): phase 2 (Day 6-10), indicated as "Other". Phase 4 (Day 16-20): control solution (0 mg MPH/kg/inf.; 7 animals), Low: 0.1 mg MPH/kg/inf. (9 animals), Mid: 0.5 mg MPH/kg/inf. (8 animals); High: 1 mg MPH/kg/inf. (8 animals). Phase 6 (Day26-30): Extra low: 0.05 mg MPH/kg/inf. (25 animals).

VI.4.4.3. Investigation of different pumps to ensure correct infusion volumes

We investigated different pumps to correctly deliver the volume of the IV infusions as we sometimes faced difficulties regarding the concentration and the appropriate volume to be administered within the infusion time of 2-5 seconds.

The model PHM-100-3.3 (3.3 RPM) was used as the standard pump to initiate IV infusions. The correct volume to deliver is driven by the body weight and the

allowed time to infuse (2 to 5 seconds per infusion). Hence the intrinsic speed of the pump to deliver the requested volume per infusion is crucial (Fig. VI.18).

Infusion time (s) = (Dose(mg/kg) * BW (g))/(1000 * Concentration (mg/ml) * Infusion speed (ml/s))

BW (g)= (1000 * Infusion time (s) * Concentration (mg/ml) * Infusion speed (ml/s))/Dose(mg/kg)

Syringe volume (ml)	Infusion speed (ml/s)
1	0.00191670
2	0.0068
5	0.0117667
10	0.0183667

Dose (mg/kg)	60
Infusion time (s)	5

Concentration (mg/ml)	BW1 (g)	BW2 (g)	BW3 (g)	BW4 (g)
0.0005	0.0	0.0	0.0	0.0
0.0010	0.0	0.0	0.0	0.0
0.010	0.0	0.0	0.0	0.0
0.10	0.0	0.1	0.1	0.2
1.0	0.2	0.6	1.0	1.5
10.0	1.6	5.7	9.8	15.3
100.0	16.0	56.7	98.1	153.1
130.0	20.8	73.7	127.5	199.0
140.0	22.4	79.3	137.3	214.3
150.0	24.0	85.0	147.1	229.6
200.0	31.9	113.3	196.1	306.1
210.0	33.5	119.0	205.9	321.4
220.0	35.1	124.7	215.7	336.7
330.0	52.7	187.0	323.6	505.1
340.0	54.3	192.7	333.4	520.4
350.0	55.9	198.3	343.2	535.7
360.0	57.5	204.0	353.0	551.0
410.0	65.5	232.3	402.0	627.5
420.0	67.1	238.0	411.8	642.8
430.0	68.7	243.7	421.6	658.1
440.0	70.3	249.3	431.4	673.4
450.0	71.9	255.0	441.3	688.8
500.0	79.9	283.3	490.3	765.3

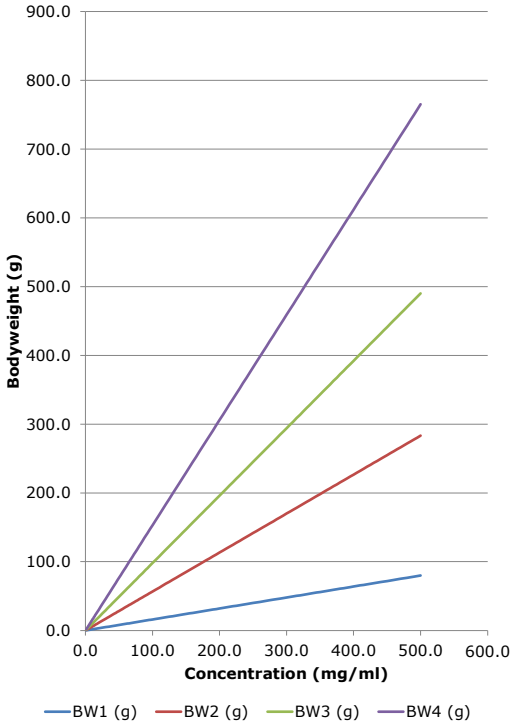


Fig. VI.18. Example of the possibilities to use the pump model PHM-100-3.3 in function of compound concentration (mg/ml) and body weight to administer an IV infusion during 5 seconds.

For some studies, the speed of these standard pumps was not high enough to inject the required volume per infusion in the limited imparted time (2 to 5

seconds at a maximum). Therefore, another pump model PHM-100-20 (20RPM), that should encounter this problem was selected and tested (Fig. VI.19). Today we have these 2 types of pumps available and validated.

Infusion time (s) = (Dose(mg/kg) * BW (g))/(1000 * Concentration (mg/ml) * Infusion speed (ml/s))

BW (g)= (1000 * Infusion time (s) * Concentration (mg/ml) * Infusion speed (ml/s))/(Dose(mg/kg))

Syringe volume (ml)	Infusion speed (ml/s)	Infusion speed (ml/min)
1	0.01146667	0.688
2	0.04076667	2.446
5	0.07060000	4.236
10	0.11020000	6.612
20	0.19648333	11.789

Dose (mg/kg)	30
Infusion time (s)	5

Concen-tration (mg/ml)	Dosage in Route (ml/kg)	BW1 (g)	BW2 (g)	BW3 (g)	BW4 (g)	BW5 (g)
1.0	30.000	1.9	6.8	11.8	18.4	32.7
2.0	15.000	3.8	13.6	23.5	36.7	65.5
3.0	10.000	5.7	20.4	35.3	55.1	98.2
4.0	7.500	7.6	27.2	47.1	73.5	131.0
5.0	6.000	9.6	34.0	58.8	91.8	163.7
6.0	5.000	11.5	40.8	70.6	110.2	196.5
7.0	4.286	13.4	47.6	82.4	128.6	229.2
8.0	3.750	15.3	54.4	94.1	146.9	262.0
9.0	3.333	17.2	61.2	105.9	165.3	294.7
10.0	3.000	19.1	67.9	117.7	183.7	327.5
11.0	2.727	21.0	74.7	129.4	202.0	360.2
12.0	2.500	22.9	81.5	141.2	220.4	393.0
13.0	2.308	24.8	88.3	153.0	238.8	425.7
14.0	2.143	26.8	95.1	164.7	257.1	458.5
15.0	2.000	28.7	101.9	176.5	275.5	491.2
20.0	1.500	38.2	135.9	235.3	367.3	654.9
25.0	1.200	47.8	169.9	294.2	459.2	818.7
30.0	1.000	57.3	203.8	353.0	551.0	982.4
35.0	0.857	66.9	237.8	411.8	642.8	1146.2
40.0	0.750	76.4	271.8	470.7	734.7	1309.9
45.0	0.667	86.0	305.8	529.5	826.5	1473.6
50.0	0.600	95.6	339.7	588.3	918.3	1637.4
55.0	0.545	105.1	373.7	647.2	1010.2	1801.1
60.0	0.500	114.7	407.7	706.0	1102.0	1964.8
65.0	0.462	124.2	441.6	764.8	1193.8	2128.6
70.0	0.429	133.8	475.6	823.7	1285.7	2292.3
75.0	0.400	143.3	509.6	882.5	1377.5	2456.0
80.0	0.375	152.9	543.6	941.3	1469.3	2619.8
85.0	0.353	162.4	577.5	1000.2	1561.2	2783.5
90.0	0.333	172.0	611.5	1059.0	1653.0	2947.3
95.0	0.316	181.6	645.5	1117.8	1744.8	3111.0
100.0	0.300	191.1	679.4	1176.7	1836.7	3274.7
110.0	0.273	210.2	747.4	1294.3	2020.3	3602.2
120.0	0.250	229.3	815.3	1412.0	2204.0	3929.7
130.0	0.231	248.4	883.3	1529.7	2387.7	4257.1
140.0	0.214	267.6	951.2	1647.3	2571.3	4584.6
150.0	0.200	286.7	1019.2	1765.0	2755.0	4912.1
160.0	0.188	305.8	1087.1	1882.7	2938.7	5239.6
170.0	0.176	324.9	1155.1	2000.3	3122.3	5567.0
180.0	0.167	344.0	1223.0	2118.0	3306.0	5894.5
190.0	0.158	363.1	1290.9	2235.7	3489.7	6222.0
200.0	0.150	382.2	1358.9	2353.3	3673.3	6549.4
210.0	0.143	401.3	1426.8	2471.0	3857.0	6876.9
220.0	0.136	420.4	1494.8	2588.7	4040.7	7204.4
230.0	0.130	439.6	1562.7	2706.3	4224.3	7531.9
240.0	0.125	458.7	1630.7	2824.0	4408.0	7859.3
250.0	0.120	477.8	1698.6	2941.7	4591.7	8186.8
260.0	0.115	496.9	1766.6	3059.3	4775.3	8514.3
270.0	0.111	516.0	1834.5	3177.0	4959.0	8841.8
280.0	0.107	535.1	1902.4	3294.7	5142.7	9169.2
290.0	0.103	554.2	1970.4	3412.3	5326.3	9496.7
300.0	0.100	573.3	2038.3	3530.0	5510.0	9824.2
310.0	0.097	592.4	2106.3	3647.7	5693.7	10151.6
320.0	0.094	611.6	2174.2	3765.3	5877.3	10479.1
330.0	0.091	630.7	2242.2	3883.0	6061.0	10806.6
340.0	0.088	649.8	2310.1	4000.7	6244.7	11134.1
350.0	0.086	668.9	2378.1	4118.3	6428.3	11461.5
360.0	0.083	688.0	2446.0	4236.0	6612.0	11789.0
370.0	0.081	707.1	2513.9	4353.7	6795.7	12116.5

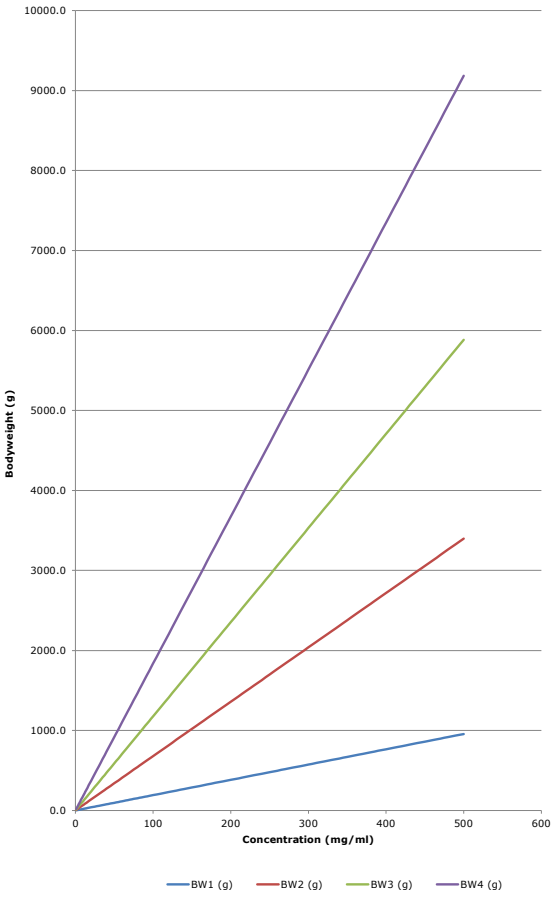


Fig. VI.19. Example of the possibilities to use the pump model PHM-100-20 in function of compound concentration (mg/ml) and body weight to administer IV infusions during 5 seconds.

VI.4.4.4. Statistics

Statistical analysis was worked out in detail. It was decided to analyze the data by means of group comparison plotted versus the reference drug (phases 1, 3, 5) and versus the saline (phase 2). Data were evaluated as follows:

- Individual profiles as well as observed average profiles (with 95% pointwise confidence bands) were plotted to explore the data.

- Summary statistics such as the number of animals (N) and the minimum, the maximum, the mean, 95% confidence limits of the mean, the median, 5% and 95% percentiles were calculated over all animals per session day.

- Only the last three days of observations within a phase were considered, hence taking into account only assumed stable responses.

- A repeated measures one-factor ANOVA with group (reference drug, saline, control, low, mid, high) as explanatory factor (Verbeke and Molenberghs 2001) was fitted to the data. Posthoc, all pairwise comparisons were evaluated using Tukey's method.

VI.4.4.5. Special features regarding husbandry

It was decided to feed the animals at a restricted regimen. This was a benefit to the health status of the animals and allowed to maintain the animals during these long-term studies in a good condition. The restricted feeding was set at 20 grams per day.

VI.4.4.6. Intracranial and oral self-administration

A literature search was performed on the possibility to use intracranial or oral self-administration (Goeders, 1987; McBride, 1999; Collins, 2012). However the variables involved for each specific route are large: the intracranial method is an invasive method, and the exact location of the probe is crucial but also limiting the outcome. This method is very interesting to study specific pathways but is not intended to be used as a standard procedure for predictivity of abuse potential in Drug Development.

The oral self-administration can eventually be used if the taste of the various psycho-active drugs and the test compounds can be neutralized, *for example* with use of sugar solutions.

To date the regulatory requirements support the use of the intravenous self-administration (IV SA) paradigm for investigation of drug abuse potential within drug development.

VI.5. Conclusions

The intravenous self-administration paradigm, using a fixed ratio schedule of reinforcement, is considered highly predictive for abuse liability testing of new CNS-active drug candidates within Drug Development and as such included in the preclinical Abuse Liability Assessment.

One of the critical issues regarding the methodology and technical features to execute these long-term intravenous studies comprised the proper choice of the surgical procedure and of the indwelling catheters. The femoral surgical procedure, combined with indwelling polyurethane non-heparin coated or the CBAS® heparin coated catheters, was considered the superior method to improve the survival rate of animals in an IV SA paradigm.

To enhance the animal's comfort a new vascular access button was developed in close collaboration with an external company (Instech), thus allowing IV SA testing without using harnesses. Moreover, the modifications of the newly implemented VAB ensured ease of freely moving when the rats were placed in their individual home cages, no irritation at the subcutaneous insertion site and a solid connection with the internal catheter. It also allowed easy and aseptic access to the tether and the possibility of multiple punctures during the studies.

The administration of drugs in an IV SA paradigm is not under control of the investigator but relies on the rat's behaviour to self-administer psycho-active drugs or test substances. Training the animals to self-administer psycho-active reference drugs precedes the actual test and can take as long as 3 months. Priming with food or with a manually administered intravenous injection did not add to shortening of this period and is not included in our final design.

As required by the drug-licensing authorities, a FR10 has to be obtained in the IV SA paradigm. However, some of the known psycho-active drugs cannot be used in the general FR10 schedule (*example*: morphine). Lower (individual) FRs may then be justified in a rationale.

The choice of a relevant reinforcer as the reference drug in an IV SA study is based upon the characteristics of the test substance, in view of its pharmacological or therapeutic class. Hallucinogenic drugs like LSD are not being self-administered in rats and testing drug candidates with these type of reference drugs will need to be executed with other preclinical models like, *for example*, the CPP (Bardo, 2000) or the DDL.

Other major issues to encounter in the IV SA paradigm are 1) the solubility of the test substance in order to enable intravenous self-administration, and 2) its concentration in the solution to fulfil the requirement of testing a multiple of the human efficacious dose. If feasible we propose a unit dose (1 infusion) with a concentration that enables plasma exposures relevant to the C_{max}Hu. Otherwise again a different model like the CPP needs to be taken into account.

The final standardized test consists of 5 phases during which the IV self-administration of the reference drug (phases 1, 3 and 5) is alternated with the IV SA of saline (phase 2) and the IV SA of 3 dosages of the test substance and its concomitant control/vehicle solution (phase 4). Each phase includes 5 days with daily 1-hour IV SA sessions. These 5 days have been shown to be sufficient in time to maintain stable responses, but can be prolonged if needed.

Testing a dose range of a substance with reinforcing properties will result in a typically bell-shaped dose-response curve and not in a linear dose-response as classically seen in toxicity studies. This indicates the importance of a well-considered dose selection for the outcome of this type of studies.

After finalization of these 5 phases, a toxicokinetic phase is added to determine the plasma concentration of a low, mid and high dose, administered manually as a single intravenous dose. The selection of these doses is based upon the drug substance intake calculated from the minimum (n=1) and maximum number of responses during phase 4 of the IV SA test. To date the C_{max} (C_0) and the time-exposure curves are determined for each TK dose and compared with the human efficacious dose (C_{eff} Hu, which is expressed as C_{max}).

During the investigation of this model, special attention was paid to fulfil the requirements of animal welfare guidances (European Convention, 1986, 2007; Belgian Law, 1991; AVMA, 2001). The effect of repeated volumes during these long-term studies, the proper cage enrichment and the fine-tuning of a reliable anesthetic usable in this type of studies added to the comfort of the rats and was accepted in the ethical protocol.

The standardized IV SA model, implemented as described was considered to be in full compliance with GLP guidelines (OECD, 1998) and in accordance with the requirements of the drug licensing authorities for abuse liability testing of new CNS-active molecular entities in Drug Development (EMA, 2006; ICH, 2009; FDA, 2010).

VII

Conditioned Place Preference test in rats: an alternative behavioural method to determine reinforcing properties of CNS-active compounds

VII.1. Introduction

The Conditioned Place Preference test (CPP) implies the investigation of the rewarding, and thus indirectly, of the reinforcing properties of a drug candidate through a learned association between environmental stimuli and the potential (subjective) drug effect experienced by the animal. (Bardo, 2000; Koob, 2000; Shippenberg, 2002; Ator, 2003; CPDD, 2006; Cunningham, 2006; Mueller, 2011; Huston, 2013).

The CPP is, in contrast to the IV SA model, a non-invasive and short-lasting method to measure the drug reward in naïve, untrained animals. This model also allows all routes of administration, which makes the clinical route (often oral administration) applicable for testing. As such solutions as well as suspensions can be administered and a dose range up to a (sub)toxic level can be reached, including dosages that are a multiple of the human efficacious dose, as required by the drug-licensing authorities (EMA, 2006; ICH, 2009; FDA, 2010).

The general outline of our CPP model was based upon literature (Tzschentke, 1998 and 2007; Bardo, 2000; Prus, 2009) and comprises three distinct phases: preconditioning (habituation and pretest), conditioning and post-conditioning (i.e. the test phase). A three-compartment test box was employed for testing (see Chapter III.3. Equipment). The habituation phase, part of the preconditioning, enables animals to become accustomed to the different environments of the test box, *i.e.* the white and black compartments, and in case of a three-compartment model, also the grey (neutral) compartment. During the 15-minute daily stay in the test box rats can explore all compartments. Time measures, recorded automatically during the habituation are not taken into account.

At pretest, animals can again freely move amongst the different compartments (15-minute stay in the test box), but the time spent in each compartment (including the grey -neutral- compartment) is recorded during this phase.

During the conditioning phase animals are treated daily, receiving the test compound or water/saline on alternate days. On those days that the test

compound is administered, animals are placed in the so-called drug-paired compartment for 45 minutes, whereas on the days that water/saline is administered, animals are placed in the so-called non-drug-paired compartment for 45 minutes. There are no time measures recorded during this phase.

At post-test, neither test compound nor water is administered and animals are given free choice to the three different compartments (15-minute stay in the test box). The time spent in each compartment (including the grey -neutral-compartment) is recorded. Classically, a conditioned place preference or positive reinforcing effect is obtained, if at post-test the animals spent more time in the drug-paired compartment than in the non-drug paired-compartment (Fig. VII.1).

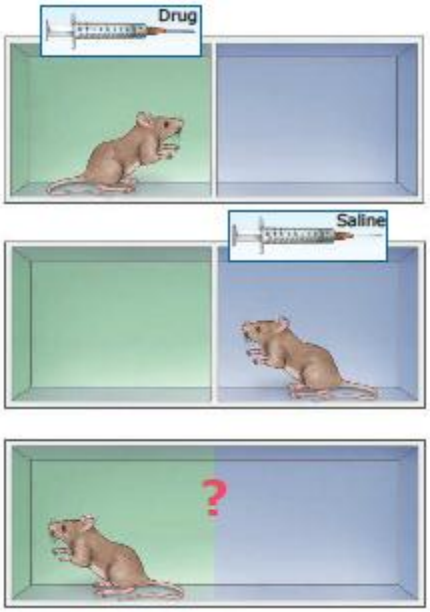


Fig. VII.1. General outline of the conditioning phase and the post-test: a test compound (drug) or water (saline) is administered on alternate days and paired with a specific environment during the conditioning phase. On the last day no drug- or saline treatment is performed and animals are allowed to freely move amongst the different compartments. Taken with permission from Camí, (2003).

Besides the CPP test, which is based on the rewarding properties of a drug, a conditioned place aversion (CPA) test is also often described in literature (Cunningham, 2006). In the latter, rats will spend less time in the drug-paired

compartment at postest. A conditioned place aversion is often related, but not limited to drugs that produce aversive effects. Examples hereof described in literature include naloxone (Cunningham, 2006) and lithium chloride (Prus, 2009).

The drug-licensing authorities consider the CPP as a valuable but less robust model to replace the IV SA in case of insolubility of the drug candidate (FDA, 2010). Therefore it was investigated whether modifications to the methodology could enhance the predictive value of this test. This research included six mechanistic studies (study identification (ID) numbers TOX10302, TOX10314, TOX10326, TOX10343, TOX10347, TOX10348) that were executed to investigate different variables possibly influencing the outcome and as such to develop a robust CPP design implementable within Drug Development. The order in which the experiments were conducted reflects the steps that were followed, starting from a basic design up to a fully outlined study.

Thereafter a validation study was performed to demonstrate the scientific validity and the face validity of the outcome, and to verify the GLP-compliance before the model was approved for use in Drug Development. The results of this study can be read in Chapter VIII.

After implementation, a conditioned place preference/aversion study was conducted with JNJ-Z as part of the Drug Development of this compound.

VII.2. Materials and Methods: optimization of the methodology of the conditioned place preference test for use in Drug Development

VII.2.1. Mechanistic Conditioned Place Preference Test with cocaine to evaluate variables possibly influencing post-test (study ID number TOX10302)

The purpose of this study was to test the general design as described in literature, to investigate the impact of different doses of the psycho-active drug cocaine and to compare the results of post-conditioning tests recorded at 24h versus those at 48h after the last cocaine pairing.

Cocaine was tested in 12 male Sprague-Dawley rats (n=6 per group) at 10 and 15 mg/kg, administrated subcutaneously at 5 ml/kg. A control group (NaCl 0.9% SC; n=6) was also added to the study.

The test was executed following a biased procedure. In a biased model, the place preference, determined by the time spent in the compartments and recorded at pretest, is taken into account to pair the drug treatment to the least preferred compartment (drug-pairing) and the saline treatment to the most preferred compartment during conditioning.

Sham treatment (SC) was performed during pre- and post-conditioning at 30 minutes before entrance into the CPP box, to mimic the dose administration as applied during the conditioning phase. The 30-minute time interval was chosen based upon the estimated maximal plasma exposure (C_{max}) of cocaine in the rat.

There were 8 conditioning days comprising 4 cocaine pairings at 10 (low dose group) and at 15 (high dose group) mg/kg cocaine SC, alternated with 4 NaCl 0.9% pairings (SC). In the control group rats were daily treated with NaCl 0.9% SC throughout the 8-day conditioning period.

The post-test was executed at 24 and 48 hours after the last cocaine pairing. The time spent in drug-paired and the non-drug-paired compartments was recorded for each rat.

The first conditioning day was defined as Day 0. After completion of the study, animals were discarded.

VII.2.2. Mechanistic Conditioned Place Preference Test to evaluate preconditioning (study ID number TOX10314)

This study was designed to investigate the evaluation of the individual preference of rats for either the white or black compartment during the habituation phase, and the possible influence of assigned boxes to the rats versus at random use of boxes during this phase.

12 naive non-treated female Sprague-Dawley rats (n=6 per group) were allowed to freely explore the 3 different compartments during 15 minutes per day on 5 successive days and observed for their spontaneous movement behavior in the test boxes.

In one group the animals were individually assigned to fixed test box numbers during the entire test period whereas in the second group the animals were placed individually at an at random basis in the various test boxes each day.

The time spent in the different compartments was recorded for each animal and the change of preference over the days was compared.

VII.2.3. Mechanistic Conditioned Place Preference Test with cocaine to evaluate conditioning factors (study ID number TOX10326)

The purpose was to study the possible gender difference, the impact of ranking based on initial preference (biased versus unbiased ranking) to further conditioning and the subsequent post-test, the impact of initial extreme preference at pretest (>75% for an environment) on conditioning and post-test data, and the relevance of post-test at 48 hours versus 24 hours after the last drug-pairing.

Cocaine was tested in 12 male (7-8 weeks of age) and 12 female (30-31 weeks of age) Sprague-Dawley rats at 10 mg/kg, administered subcutaneously (5 ml/kg).

The design included conditioning based upon both a biased [group H: n=(6 males+6 females)] and unbiased [group L: n=(6 males+6 females)] procedure. For the rats assigned to the unbiased model, the place preference recorded at pretest was not taken into account for pairing drug or saline with a specific compartment; in this design rats were assigned to a "predefined" drug-paired or non-drug-paired environment for conditioning using a body weight based stratified randomisation procedure.

Sham treatment (SC) was performed during pre- and post-conditioning at 30 minutes before entrance into the CPP box.

The conditioning phase comprised 8 conditioning days with 4 cocaine pairings. On alternate days saline (NaCl 0.9% SC) was administered.

The parameters recorded included the time spent in 3 different compartments (white, black and grey) at pretest, and at post-test at 24 and 48 hours after the last cocaine pairing.

A possible effect of the gender difference was evaluated at pre- and post-test.

VII.2.4. Mechanistic Conditioned Place Preference Test with cocaine to evaluate tactile stimuli (study ID number TOX10343)

This study was designed to investigate the impact of tactile stimuli on the results of the post-test by using different floor materials in the test boxes. The impact of extreme preference (>75% for an environment) was also determined.

Cocaine was tested in 24 male Sprague-Dawley rats (n=12 per group) at 10 mg/kg, administrated subcutaneously at 5 ml/kg. The control group received saline (NaCl 0.9% SC; n=12).

The unbiased procedure was employed (body weight based stratified randomisation) and 2 groups (L and M groups) were subjected to 8 conditioning days comprising 4 cocaine pairings (Table VII.1).

Dosage groups (Males)					
L		M		H	
21 - 32		41 - 52		61 - 72	
Solid floor		Classical floor		Classical floor	
Defined preference white	Defined preference black	Defined preference white	Defined preference black	Defined preference white	Defined preference black
21 - 26	27 - 32	41 - 46	47 - 52	61 - 66	67 - 72

Table VII.1. Randomization of the animals following an unbiased procedure. There are 12 rats per group, each divided in 2 subgroups (body weight based stratified randomisation).

One group (L group) was conditioned with cocaine pairings in boxes with solid floors in both compartments whereas in the second group (M group) conditioning with cocaine pairings was performed in boxes with classical floors (white compartment: grid floor; black compartment: bar floor). As technical issues prevented swapping the grid and bar floors from one compartment to another, the H group was redesigned as a control group (NaCl 0.9%) using the classical floors in the boxes.

Sham treatment (SC) was performed during pre- and post-conditioning at 30 minutes before entrance into the CPP box.

The time spent in 3 different compartments (black, white and grey) was recorded at pretest and at post-test (24 hours after the last cocaine pairing). The results were further evaluated with regard to the possible influence of the different tactile stimuli (floors) on the outcome of the study.

VII.2.5. Mechanistic Conditioned Place Preference Test with cocaine to evaluate the length of the conditioning phase (study ID number TOX10347)

The purpose of this study was to evaluate the length of the conditioning phase in view of the outcome.

Cocaine was tested in 24 male Sprague-Dawley rats (n=12 per group) at 10 mg/kg, administrated subcutaneously at 5 ml/kg. The concurrent control groups received saline (NaCl 0.9% SC; n=12 per group).

The unbiased procedure was employed (body weight based stratified randomisation) and 2 groups were subjected to 8 (4 cocaine pairings) or 16 (8 cocaine pairings) conditioning days, respectively (Table VII.2).

Dosage groups (Males)							
C1		H1		C2		H2	
1 - 12		61 - 72		101 - 112		161 - 172	
Conditioning: 8 sessions		Conditioning: 8 sessions		Conditioning: 16 sessions		Conditioning: 16 sessions	
Defined preference white	Defined preference black	Defined preference white	Defined preference black	Defined preference white	Defined preference black	Defined preference white	Defined preference black
1 - 6	7 - 12	61 - 66	67 - 72	101 - 106	107 - 112	161 - 166	167 - 172

Table VII.2. Example how rats are randomised via an unbiased procedure into the different dose groups. There are 12 rats per group, each divided in 2 subgroups (body weight based stratified randomisation).

Sham treatment (SC) was performed in all groups during pre- and post-conditioning at 120 minutes before entrance into the CPP box. The time interval was selected based upon the data of TOX10306 [Chapter V.3.4. Single dose toxicokinetic study of eleven psycho-active drugs in the rat (Study Identification number TOX10306)].

At pretest the time spent in 3 different compartments (black, white, grey) was recorded. The post-test was performed at 24 hours after the last cocaine pairing and the time spent in the 3 compartments was recorded. The results were further evaluated in view of the length of the conditioning phase.

VII.2.6. Mechanistic Conditioned Place Preference Test with cocaine to evaluate habituation (study ID number TOX10448)

This study was designed to evaluate the sequence of the habituation days (Days -5, -4 versus Days -3, -2) in view of the pretest data.

Cocaine was tested in 24 male Sprague-Dawley rats (n=12 per group) at 10 mg/kg, administrated subcutaneously at 5 ml/kg. The concurrent control groups received saline (NaCl 0.9% SC; n=12 per group).

The rats were divided into different groups (Table VII.3) following an unbiased procedure.

Dosage groups (Males)							
C1: NaCl 0.9%		H1		C2: NaCl 0.9%		H2	
1 - 12		61 - 72		101 - 112		161 - 172	
Habituation: Day -5, -4		Habituation: Day -5, -4		Habituation: Day -3, -2		Habituation: Day -3, -2	
Defined preference white	Defined preference black	Defined preference white	Defined preference black	Defined preference white	Defined preference black	Defined preference white	Defined preference black
1 - 6	7 - 12	61 - 66	67 - 72	101 - 106	107 - 112	161 - 166	167 - 172

Table VII.3. Overview of the dose groups according to the unbiased procedure for ranking. There are 12 rats per group, each divided in 2 subgroups (body weight based stratified randomisation).

The habituation phase included one 15-minute daily session on 2 successive days, either 3 and 4 days prior to pretest (defined as Day -1) or 1 and 2 days before. During these sessions the animals were allowed to freely move in the compartments, but the time spent in each compartment was not taken into account.

During pre- and post-test, sham treatment (SC) was performed at 120 minutes before entrance into the CPP box.

The cocaine groups were subjected to 8 conditioning days with 4 cocaine pairings. The control groups received NaCl 0.9%.

During pretest the time spent in 3 different compartments was recorded. The post-test was executed 24 hours after the last cocaine pairing and the time spent in the 3 compartments was recorded.

The pretest data were evaluated with regard to the former habituation patterns.

VII.2.7. Conditioned Place Preference/Aversion Test of JNJ-Z with fentanyl as positive control (study ID number TOX10829)

The purpose of this study was to evaluate the reinforcing or aversive properties of JNJ-Z through the Conditioned Place Preference/Aversion (CPP/CPA) paradigm. In addition, a combination with fentanyl administration (0.02 mg/kg SC) was added to the design, to investigate whether JNJ-Z could avert the reinforcing properties of fentanyl by dosing the test compound prior to each administration of fentanyl. It was also investigated whether the test compound

could interrupt the effects of fentanyl, by dosing the test compound after each administration of fentanyl. Fentanyl was also tested solely as the reference drug.

JNJ-Z, a CNS active novel drug candidate with the potential to treat opioid abuse, was administered subcutaneously at 10 mg/kg. Fentanyl was dosed at 0.02 mg/kg SC. The control solution was saline (NaCl 0.9% SC).

Forty male Sprague-Dawley rats (n=8 per group) were ordered for this study and were divided into 5 dose groups using a body weight stratified randomization procedure (unbiased procedure) (Table VII.4).

Dosage groups (Males)			
Vehicle	8 rats	Nos. 101 - 104	Black drug-paired compartment
		Nos. 107 - 110	White drug-paired compartment
JNJ-Z	8 rats	Nos. 201 - 204	Black drug-paired compartment
		Nos. 207 - 210	White drug-paired compartment
Fentanyl	8 rats	Nos. 301 - 304	Black drug-paired compartment
		Nos. 307 - 310	White drug-paired compartment
JNJ-Z + Fentanyl	8 rats	Nos. 401 - 404	Black drug-paired compartment
		Nos. 407 - 410	White drug-paired compartment
Fentanyl + JNJ-Z	8 rats	Nos. 501 - 504	Black drug-paired compartment
		Nos. 507 - 510	White drug-paired compartment

Table VII.4. Randomisation of the animals using an unbiased procedure (body weight based stratified randomisation).

The study comprised16 conditioning days with 8 drug pairings.
The detailed outline included the following:

1) Preconditioning: habituation period (Days -5, -4) and pre-test procedure (Day -1)

All groups received SC sham treatment during habituation period and on the pretest day to mimic dose administration (Table VII.5). The interval was chosen based upon the assumed T_{max} of JNJ-Z (15 minutes) and the T_{max} of fentanyl SC (30 minutes).

	-45 min	-30 min	-15 min	0h
V:	x	x	/	Start pretest
A:	/	/	x	Start pretest
PC:	/	x	/	Start pretest
A+PC:	x	x	/	Start pretest
PC+A:	/	x	x	Start pretest

Table VII.5. Outline of the sham treatment during preconditioning. Time points for sham treatment are based upon the time needed to reach C_{max} at the start of the pretest. V: vehicle; A: JNJ-Z; PC: positive control fentanyl; min: minutes; 0h: time point of starting the pretest (15 minutes in the test box).

The time spent in the three different compartments (black/white/grey) during the 15-minute sessions at pretest was recorded (individual animal data).

2) Conditioning phase

On the even study days (Days 0, 2, 4, 6, 8, 10, 12, 14) all groups were dosed with NaCl 0.9% SC (Table VII.6) and conditioned in the non-drug paired compartment during daily 45-minutes sessions.

	-45 min	-30 min	-15 min	0h: Start conditioning
V:	10 ml NaCl 0.9%/kg	1 ml NaCl 0.9%/kg	/	x
A:	/	/	10 ml NaCl 0.9%/kg	x
PC:	/	1 ml NaCl 0.9%/kg	/	x
A+PC:	10 ml NaCl 0.9%/kg	1 ml NaCl 0.9%/kg	/	x
PC+A:	/	1 ml NaCl 0.9%/kg	10 ml NaCl 0.9%/kg	x

Table VII.6. Dosing procedure for conditioning on even days: NaCl 0.9%: administered SC; Group ID: V: vehicle; A: JNJ-Z; PC: positive control fentanyl; min: minutes; 0h: time point of starting the conditioning session (45 minutes in the test box).

On the odd study days (Days 1, 3, 5, 7, 9, 11, 13, 15) rats were administered the appropriate SC dose of vehicle (V group), JNJ-Z (A group), fentanyl (PC group), JNJ-Z followed by fentanyl (A+PC group), fentanyl followed by JNJ-Z (PC+A group) (Table VII.7) and conditioned in the drug paired compartment during daily 45-minutes sessions.

	-45 min	-30 min	-15 min	0h: Start conditioning
V:	10 ml V /kg	1 ml NaCl 0.9%/kg	/	X
A:	/	/	10 ml A /kg	X
PC:	/	1 ml PC /kg	/	X
A+PC:	10 ml A /kg	1 ml PC /kg	/	X
PC+A:	/	1 ml PC /kg	10 ml A /kg	X

Table VII.7. Dosing procedure for conditioning on odd days; Group ID: V: vehicle; A: JNJ-Z; PC: positive control fentanyl; min: minutes; 0h: time point of starting the conditioning session (45 minutes in the test box).

3) Post-conditioning

On Day 16, at 24 hours after the last conditioning session with vehicle (V group), JNJ-Z (A group), fentanyl (PC group), JNJ-Z followed by fentanyl (A+PC group), fentanyl followed by JNJ-Z (PC+A group) (Day 15), the post test was executed. SC sham-treatment was performed as described for the pretest.

The CPP parameters included the time spent in the drug-paired and non-drug paired compartment and in the grey compartment at pretest and at post-test. In addition, clinical observations were recorded daily. Body weights were measured daily in order to calculate the dose volume. At the end of the study, all surviving animals were discarded.

Overall remark: studies were performed sequentially as outlined in this section; all variables tested were hence implemented incrementally and as such retested in each following study. This added to the reproducibility of these variables on the outcome of the individual studies.

VII.3. Results

VII.3.1. Mechanistic Conditioned Place Preference Test with cocaine to evaluate variables possibly influencing post-test (study ID number TOX10302)

The results at pretest indicated that 10 out of 18 animals preferred the black compartment and 8 out of 18 animals preferred the white compartment. Thus the animals were conditioned with cocaine administration paired with a 15-minute stay in the white (10 rats) or black compartment (8 rats), respectively (biased procedure).

A conditioned place preference was obtained in both groups (Table VII.8), though not more pronounced in the 15 mg/kg dosed group. Overall there was no major difference in the results when the post-test was executed at 24 or at 48h after the last cocaine administration.

Rat ID	Pretest (Day -1) time spent (seconds) in compartments		Last cocaine pairing in non-preferred compartment before posttest on Day 10	Posttest (Day 10) time spent (seconds) in compartments	
	Black compartment	White compartment		Black compartment	White compartment
C1	292.50	335.36	-0h	357.71	231.38
C2	335.53	246.28	-0h	341.39	241.30
C3	283.13	178.69	-0h	320.98	249.41
C4	367.28	355.30	-0h	406.27	127.92
C5	330.84	339.24	-0h	445.20	208.35
C6	370.61	306.15	-0h	299.14	390.05
L21	248.30	280.96	-24h	523.60	185.43
L22	355.49	298.45	-24h	242.33	333.38
L23	307.31	334.31	-24h	407.34	335.83
L24	294.07	425.32	-48h	623.55	113.91
L25	413.49	248.42	-48h	274.96	319.46
L26	280.73	196.28	-48h	327.43	245.81
H61	255.34	382.57	-24h	417.95	265.07
H62	406.07	248.43	-24h	295.66	406.88
H63	367.69	206.08	-24h	520.57	225.65
H64	299.78	328.01	-48h	403.66	200.14
H65	267.02	321.75	-48h	291.80	176.24
H66	430.50	268.38	-48h	348.76	389.40

Table VII.8. Individual data of the time spent (seconds) at pretest and at post-test (recorded at 24 or 48 hours after the last cocaine pairing). A biased procedure was used, thereby conditioning the animals in the non-preferred compartment that was individually defined at pretest. C: control; L: 10 mg/kg cocaine; H: 15 mg/kg cocaine.

VII.3.2. Mechanistic Conditioned Place Preference Test to evaluate preconditioning (study ID number TOX10314)

Overall, a slight physiological preference for the black compartment remained present throughout the present study when rats were allowed to freely move in the test boxes during 5 successive daily sessions of 15 minutes.

For those rats that were placed in the test boxes with fixed assignment to one particular box during the study (Fig. VII.2), the time spent in a particular compartment during the five testing days fluctuated mostly between 51 and 67%. One rat spent about 86 to 94% in a chosen daily compartment, but its preference for the white or black compartment was not fixed (W/B/B/W/B over the 5 days).

BOX	Animal		Preference Day 0	Preference Day 1	Preference Day 2	Preference Day 3	Preference Day 4
1	1	sec %	Black 349.44 51.91%	Black 361.35 55.84%	White 296.41 54.38%	White 469.15 61.23%	White 591.72 73.16%
2	2	sec %	Black 465.47 63.29%	Black 376.41 54.78%	Black 467.66 57.84%	Black 471.40 58.49%	Black 409.98 54.51%
3	3	sec %	White 375.10 56.90%	Black 297.64 51.87%	Black 205.76 52.63%	Black 259.60 66.70%	Black 267.42 53.41%
4	4	sec %	White 303.47 52.44%	Black 280.94 52.72%	White 347.26 52.10%	Black 330.50 67.13%	Black 321.07 51.00%
5	5	sec %	White 709.59 91.86%	Black 283.34 90.33%	Black 576.92 86.69%	White 750.32 91.76%	Black 511.22 94.38%
6	6	sec %	Black 369.47 54.45%	White 338.69 57.37%	Black 266.74 58.11%	Black 476.40 66.02%	Black 420.44 72.25%

Fig. VII.2. Habituation with fixed assignment to test boxes each day: Individual data of the time spent in the black or white compartment during 15 minutes per day (freely moving within the compartments).

When rats were placed at random in the test boxes each day (Fig. VII.3), 4 out of 6 animals (or 67%) spent the most time in the black compartment during the 5 consecutive days. Two rats showed a so-called extreme preference (>75% of the total time spent in a particular compartment) for the black compartment during some days.

Animal		Preference Day 0	Preference Day 1	Preference Day 2	Preference Day 3	Preference Day 4
11	sec %	Black 293.68 55.38%	Black 459.26 63.76%	White 345.79 52.17%	Black 391.48 58.28%	White 353.24 56.51%
	Box Nr	box 1	box 2	box 3	box 4	box 5
12	sec %	Black 320.18 54.37	Black 502.12 77.58%	Black 735.03 92.54%	Black 43.32 82.00%	Black 63.40 71.79%
	Box Nr	box 2	box 3	box 4	box 5	box 6
13	sec %	Black 414.35 57.14%	Black 469.96 68.09%	Black 343.53 56.27%	Black 370.25 68.19%	Black 401.73 63.37%
	Box Nr	box 3	box 4	box 5	box 6	box 1
14	sec %	Black 335.66 56.09%	Black 469.24 71.54%	Black 464.67 64.70%	Black 468.09 68.64%	Black 597.63 78.52%
	Box Nr	box 4	box 5	box 6	box 1	box 2
15	sec %	Black 284.77 53.92%	Black 379.93 56.30%	White 338.42 57.61%	Black 332.91 50.97%	White 425.49 67.13%
	Box Nr	box 5	box 6	box 1	box 2	box 3
16	sec %	Black 386.55 61.05%	Black 280.62 60.15%	Black 293.35 58.85%	Black 538.06 70.28%	Black 346.07 69.93%
	Box Nr	box 6	box 1	box 2	box 3	box 4

Fig. VII.3. Habituation with at random assignment to test boxes each day. Individual data of the time spent in the black or white compartment during 15 minutes per day (freely moving within the compartments).

VII.3.3. Mechanistic Conditioned Place Preference Test with cocaine to evaluate conditioning factors (study ID number TOX10326)

At pretest (Table VII.9), 2 males and 1 female of the high group (biased procedure) showed a preference for the white compartment; all other rats (9 out of 12 rats) of this group spent the most time in the black compartment. In the low group (unbiased procedure) 11 out of 12 spent the most time in the black compartment; for this low group these data were however not taken into account for pairing the drug to a non-preferred compartment during conditioning.

Two females of the low group (unbiased procedure) showed a so-called extreme place preference of 78.10 and 76.41% for the black compartment at pretest.

At post-test (Table VII.9), at 24 hours after the last drug-pairing, a drug-induced conditioned place preference for the drug-paired compartment was

recorded in 10 out of 12 rats (5 males and 5 females) conditioned following an unbiased procedure and in 10 out of 12 rats (6 males and 4 females) conditioned following a biased procedure.

Records after 48 hours revealed a decline in the response to 7 out of 12 rats for both groups.

		Pretest (Day -1)			Day 9			Day 10		
Preferred compartment at pretest (Day -1)	Rat ID	Time spent black comp (%)	Time spent white comp (%)	Drug-paired comp	Time spent black comp (sec)	Time spent white comp (sec)	X/N +CPP	Time spent black comp (sec)	Time spent white comp (sec)	X/N +CPP
Predef. white	L 21	63.01	36.99	Black	596.10	118.70	L: 5/6	256.36	137.11	L: 3/6
Predef. white	L 22	62.66	37.34	Black	506.77	151.32		503.29	215.89	
Predef. white	L 23	51.20	48.80	Black	386.94	276.46		489.32	219.79	
Predef. black	L 24	66.63	33.37	White	263.42	403.17		327.09	299.69	
Predef. black	L 25	60.04	39.96	White	235.91	249.19		295.78	200.05	
Predef. black	L 26	55.07	44.93	White	330.82	255.71		495.18	232.38	
Pref. black	H 61	55.83	44.17	White	300.04	317.72	H: 6/6	278.94	361.83	H: 5/6
Pref. black	H 62	60.75	39.25	White	248.88	288.03		317.74	330.15	
Pref. white	H 63	45.17	54.83	Black	581.08	164.58		709.07	101.09	
Pref. white	H 64	40.39	59.61	Black	317.90	305.51		400.18	336.72	
Pref. black	H 65	63.52	36.48	White	217.66	286.69		276.85	245.86	
Pref. black	H 66	63.23	36.77	White	324.52	334.58		330.11	350.60	
Predef. white	L 121	78.10	21.90	Black	752.89	77.80	L: 5/6	625.30	149.24	L: 4/6
Predef. white	L 122	69.83	30.17	Black	563.68	173.79		505.25	148.25	
Predef. white	L 123	61.76	38.24	Black	564.25	222.56		456.63	198.46	
Predef. black	L 124	57.62	42.38	White	318.35	352.63		453.54	318.07	
Predef. black	L 125	44.81	55.19	White	458.54	319.86		376.53	381.42	
Predef. black	L 126	76.41	23.59	White	266.08	429.97		429.26	266.01	
Pref. black	H 161	69.52	30.48	White	278.99	382.28	H: 4/6	463.53	238.68	H: 2/6
Pref. black	H 162	61.11	38.89	White	247,00	454.01		341.89	268.65	
Pref. black	H 163	60.36	39.64	White	208.96	477.26		221.74	500.23	
Pref. white	H 164	47.91	52.09	Black	267.94	228.48		317.51	273.08	
Pref. black	H 165	55.93	44.07	White	291.93	169.18		399.25	191.08	
Pref. black	H 166	61.94	38.06	White	546.67	200.99		414.51	257.37	

Table VII.9. Individual data of the time spent in the black or white compartment at pretest (in %) and at post-test (in seconds). L: unbiased procedure; H: biased procedure. Pref. black/white: preferred black or white compartment at pretest; predefined black/white: predefined non drug-paired compartment. L21-26 and H61-66: males; L121-126 and H161-166: females. Day 9: post-test data 24 hours after the last drug-pairing; Day 10: post-test data 48 hours after the last drug-pairing. X/N +CPP: incidence of conditioned place preference at post-test.

The 2 females that showed an extreme place preference for the black compartment at pretest developed a conditioned place preference for the drug-paired compartment after 24 hours; however, for the one female that was

conditioned with cocaine paired to the white compartment the preference reversed after 48 hours (i.e. conditioned place preference for the non drug-paired black compartment).

In the present study a clear gender difference was not picked; however, as the females were of older age (30-31 weeks at study start) compared to the age of the males (7-8 weeks at study start), this gender variable could not be investigated properly.

VII.3.4. Mechanistic Conditioned Place Preference Test with cocaine to evaluate tactile stimuli (study ID number TOX10343)

Subcutaneous administration of 10 mg/kg cocaine in the conditioned placed paradigm (4 cocaine pairings) led to a conditioned place preference for the compartment associated with the drug administration. In the M group where the classical floors were used, 10 out of 12 animals (83%) showed a conditioned place preference versus 9 out of 12 (75%) in the L group where the solid floors were used (Table VII.10). In the H control group to which a control solution (NaCl 0.9%) was administered and where the classical floors were used in the test boxes, 5 animals or 42% changed preference.

		Posttest(Day 8) time spent in compartments				
Animal ID	**Drug-paired compartment**	**Seconds in black compartment**	**% in black compartment**	**Seconds in white compartment**	**% in white compartment**	**Positive reinforcement**
L21	black	**297.56**	**65.73**	155.11	34.27	6/6
L22	black	**526.03**	**77.45**	153.12	22.55	
L23	black	**592.90**	**81.78**	132.08	18.22	
L24	black	**518.80**	**71.90**	202.80	28.10	
L25	black	**550.00**	**79.66**	140.47	20.34	
L26	black	**536.96**	**75.59**	173.44	24.41	
L27	white	250.15	49.24	**257.91**	**50.76**	3/6
L28	white	349.67	58.80	245.04	41.20	
L29	white	250.93	51.46	236.65	48.54	
L30	white	265.89	44.56	**330.84**	**55.44**	
L31	white	437.46	60.93	280.55	39.07	
L32	white	234.55	46.55	**269.34**	**53.45**	
M41	black	**578.42**	**86.18**	92.79	13.82	6/6
M42	black	**458.03**	**68.96**	206.12	31.04	
M43	black	**336.17**	**60.03**	223.87	39.97	
M44	black	**533.92**	**70.30**	225.57	29.70	
M45	black	**509.54**	**75.88**	161.98	24.12	
M46	black	**505.02**	**79.05**	133.88	20.95	
M47	white	424.34	65.85	220.03	34.15	4/6
M48	white	114.67	23.66	**369.99**	**76.34**	
M49	white	219.18	42.05	**302.11**	**57.95**	
M50	white	216.67	41.66	**303.37**	**58.34**	
M51	white	294.14	53.53	255.35	46.47	3/6
M52	white	263.52	41.34	**373.87**	**58.66**	
H61	black	**354.83**	**56.70**	270.99	43.30	2/6
H62	black	**345.40**	**58.76**	242.45	41.24	
H63	black	**412.84**	**58.90**	288.09	41.10	
H64	black	257.78	48.21	276.94	51.79	
H65	black	163.45	43.01	216.58	56.99	
H66	black	298.25	46.33	345.45	53.67	
H67	white	291.81	46.31	**338.37**	**53.69**	
H68	white	276.66	55.62	220.74	44.38	
H69	white	421.04	65.53	221.49	34.47	
H70	white	292.35	49.18	**302.07**	**50.82**	
H71	white	559.97	77.16	165.73	22.84	
H72	white	282.04	53.10	249.10	46.90	

Table VII.10. Time spent in the previously drug-paired compartments during post-test. L: solid floors in all compartments; M and H: classical floors in compartments. L, M: conditioning with cocaine (10 mg/kg); H: conditioning with NaCl 0.9% (control group)

In addition, in the 2 cocaine dosed groups (L and M groups) drug-pairing in the black compartment showed a higher conditioned place preference result than drug-pairing in the white compartment, as evidenced by significantly higher time spent in the black drug-paired compartment versus the time spent in the white drug-paired compartment (Fig. VII.4). This finding was not present in the H control group.

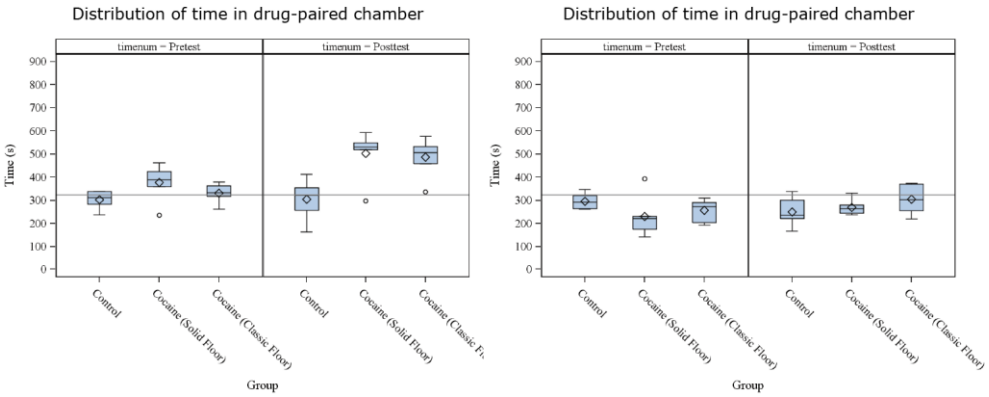


Fig. VII.4. Boxplot visualization of the distribution of the time spent in the black (left plot) versus white drug-paired compartment (right plot) using 4 cocaine-pairings. At posttest the time spent in the black drug-paired compartment was significantly higher than the time spent in the white drug-paired compartment. Data from TOX10343.

VII.3.5. Mechanistic Conditioned Place Preference Test with cocaine to evaluate the length of the conditioning phase (study ID number TOX10347)

Either 4 or 8 cocaine pairings (10 mg/kg cocaine) led to a conditioned place preference (11 out of 12 rats in both groups) for the compartment associated with the drug administration (Table VII.11). When the actual time spent in the different compartments was taken into account, the ratio of time spent in the drug-paired versus non drug-paired compartment was higher (though not statistically significant) in the H2 (8 cocaine pairings) group (ratio 2.14 versus ratio 1.71 for H1 (4 cocaine pairings) group). There was also a tendency of spending less time in the grey (neutral) compartment for the H2 versus H1 group.

Dose group	Number of rats	Number of rats with preference for drug-paired compartment[a]	Ratio black/white drug-paired compartment	Ratio total time spent in drug-paired versus non-drug-paired compartment[b]	Ratio total time spent in drug-paired compartment versus that of control[b]	Ratio total time spent in grey compartment versus that of control[b]	Ratio total time spent in non drug-paired compartment versus that of control[b]
C1 Control 0 mg/kg	12	5	5/0	0.87	1.00	1.00	1.00
H1 High 10 mg/kg	12	11	6/5	1.71 **	1.44 ***	0.90	0.73 ***
C2 control 0 mg/kg	12	3	3/0	0.89	1.00	1.00	1.00
H2 High 10 mg/kg	12	11	6/5	2.14 ***	1.59 ***	0.84	0.66 ***

Table VII.11. Overall results of study TOX10347. Study with 4 (H1) or 8 (H2) cocaine pairings demonstrated that the length of the conditioning period (number of drug-pairing) has an impact on the total time spent in the drug-paired compartments. [a]Significance for H1 (4 cocaine pairings) and H2 (8 cocaine pairings) computed versus Control C1 and C2, respectively by Fisher Exact probability test (two-tailed). [b]Significance for H1 and H2 groups computed versus C1 and C2 groups by Anova. ***: pvalue of comparison of H1 vs C1 and H2 vs C2 < 0.001; **: 0.001<pvalue of comparison of H1 vs C1 and H2 vs C2<0.01; *: 0.01<pvalue of comparison of H1 vs C1 and H2 vs C2<0.05.

The cutoff point of 324 seconds, a criterion for CPP expression as published by dela Cruz (2009) was set as a reference line in the box plot of the post-test data (Fig. VII.5). It was clearly demonstrated that the time spent in the drug-paired chambers (H1 and H2 groups) differed significantly from those of the control C1 and C2 groups, and exceeded the reference cutoff of 324 seconds. Again this was more pronounced in the H2 (8 drug-pairings) group.

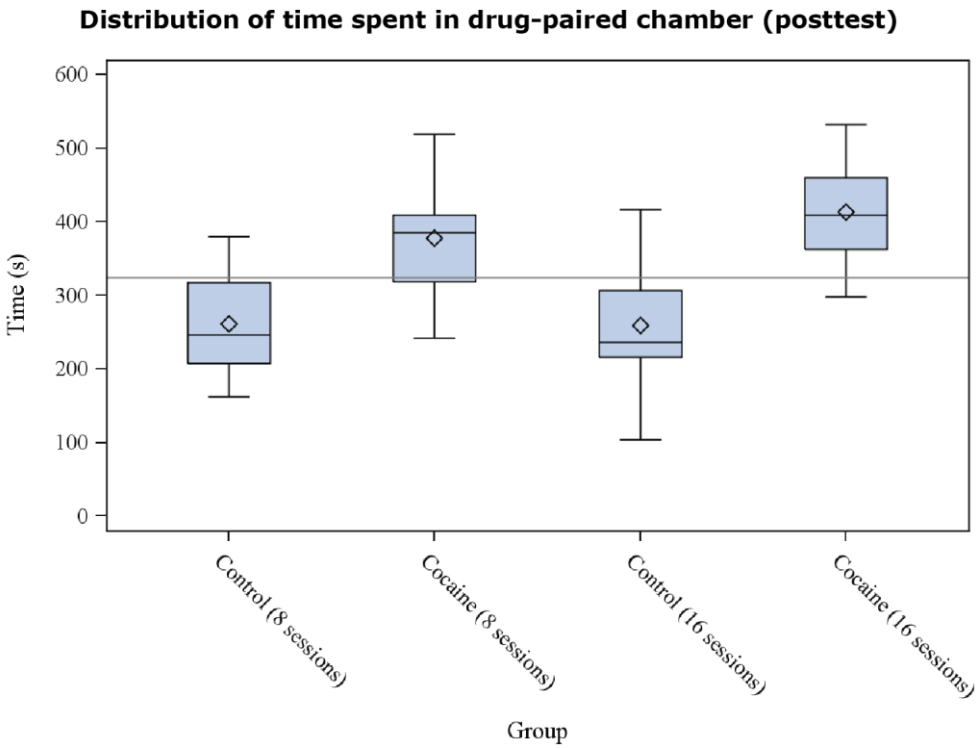


Fig. VII.5. Boxplot visualization of the distribution of the time spent in the drug-paired compartment at post-test. Drug pairings with cocaine clearly exceeded the reference cut off of 324 seconds.

VII.3.6. Mechanistic Conditioned Place Preference Test with cocaine to evaluate habituation (study ID number TOX10448)

When the total time spent in either compartment at pretest was taken into account (Table VII.12), a slightly better balanced distribution of the preference for the black, white or grey compartment was recorded when habituation was performed on Days -5 and -4 (groups C1, H1) versus habituation on days -3 and -2 (groups C2, H2). The preference for the black compartment at pretest was in all groups higher than that for the white compartment.

Groups	Time spent (seconds) Black comp.	Preference for black comp. (X/N)	Time spent (seconds) White comp.	Preference for white comp. (X/N)	Time spent (seconds) Grey comp.
C1	3984	6/12	3604	6/12	3212
H1	4132	8/12	3182	4/12	3487
Total time	8116		6786		6699
C2	4464	10/12	2967	2/12	3369
H2	4088	8/12	3114	4/12	3597
Total time	8552		6081		6966

Table VII.12. Pretest data: individual preference for the black or white compartment based upon the time spent in each compartment shows a preference for the black compartment in all groups.

At post-test a conditioned place preference for the drug paired compartment was obtained in both cocaine conditioned groups (8 out of 12 rats in H1 and 10 out of 12 rats in H2). The time spent in the drug-paired compartment was the higher in H1 accompanied with a lower time spent in the grey compartment (Table VII.13).

Post-test Total time spent (seconds)			
	drug paired compartment	grey compartment	non-drug paired compartment
C1	3424.5	4080.1	3295.4
H1	4413.0	2958.7	3428.1
C2	3060.3	3722.6	4016.8
H2	4193.1	3623.0	2983.9

Table VII.13. Post-test data: total time spent (per dose group) in the 3 compartments. C1, H1: habituation on Days -5 and -4; C2, H2: habituation on Days -3 and -2. In group H1 the total time spent in the drug-paired compartment was higher compared to group H2. In this group (H1) the time spent in the grey (neutral zone) was also lower.

VII.3.7. Conditioned Place Preference/Aversion Test of JNJ-Z with fentanyl as positive control (study ID number TOX10829)

Data from pretest (Day -1) revealed two rats with a >75% preference (76.1% and 88.4%) for the black compartment. This had no impact on the results at post-test.

Statistics showed no significance amongst the time spent in either of the compartments in any group at pretest, although a slight preference for the black (physiological) compartment was observed (Fig. VII.6).

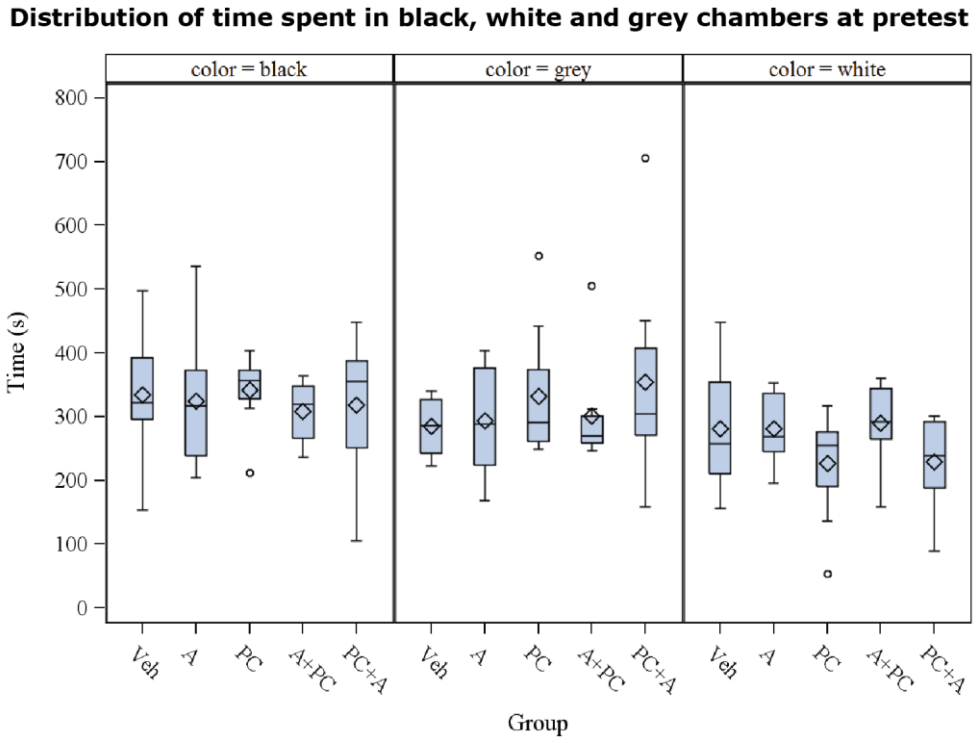


Fig. VII.6. Boxplot visualisation of the distribution of the time spent in the black, white and grey chambers at pretest.

The data at post-test revealed the following (Table VII.14):

Vehicle group: Four out of 8 vehicle animals spent the most time in the predefined drug-paired compartment when tested after 24 hours (ratio black/white: 2/2). The ratio of the time spent in predefined drug-paired compartment versus non-drug-paired compartment was 1.12, with a higher time spent in the drug-paired black compartment (1.15 versus 1.09). The total time spent in the three compartments at post-test was the highest in the grey (neutral) compartment (2552.8 seconds). Due to the high number of animals spending the most time in the predefined drug-paired compartment the vehicle used was further investigated. As neither NPH (N- Methylpyrrolidone) nor

captisol are known for their reinforcing properties, this result was considered not biologically relevant.

JNJ-Z group: Two out of 8 animals spent the most time in the predefined drug-paired compartment when tested after 24 hours (ratio black/white: 0/2). The ratio of the time spent in predefined drug-paired compartment versus non drug-paired compartment was 0.74. The ratio of the time spent in the non-drug-paired compartment thus amounted to 1.36; the ratio of the time spent in the non-drug-paired compartment versus the vehicle (V: ratio = 1.00) was 1.15. The total time spent in the grey compartment was higher (2836.2 sec) than the total time spent in the either the drug-paired or non drug-paired compartment.

PC group (positive control: fentanyl): Seven out of 8 rats spent the most time in the predefined drug-paired compartment when tested after 24 hours (ratio black/white: 4/3). The ratio of the time spent in predefined drug-paired compartment versus non drug-paired compartment was 1.53. The ratio of the time spent in the drug-paired compartment versus the vehicle (V: ratio = 1.00) was 1.11. The total time spent in the grey compartment was high (2677.8 sec) but lower than the total time spent in the drug-paired compartment.

A+PC group (JNJ-Z followed by fentanyl with time interval of 15 minutes (T_{max} of JNJ-Z): Six out of 8 rats spent the most time in the predefined drug-paired compartment when tested after 24 hours (ratio black/white: 4/2). The ratio of the time spent in predefined drug-paired compartment versus non drug-paired compartment was high (1.36) but the ratio of the time spent in the drug-paired compartment versus the vehicle (V: ratio = 1.00) was not significantly increased (1.03). The total time spent in the grey compartment (2806.3 sec) was within the same range as that of A group (JNJ-Z: 2836.2 sec), and higher than the total time spent in the either the drug-paired or non drug-paired compartment.

PC+A group (fentanyl followed by JNJ-Z with time interval of 30 minutes (T_{max} of fentanyl): Six out of 8 rats spent the most time in the predefined drug-paired compartment when tested after 24 hours (ratio black/white: 4/2). However, the ratio of the time spent in predefined drug-paired compartment versus non drug-paired compartment lowered to 1.04 compared to the previous group (A+PC).

The ratio of the time spent in the drug-paired compartment versus the vehicle (V: ratio = 1.00) was 0.94; the ratio of time spent in the non drug-paired compartment versus vehicle was 1.01. The total time spent in the grey compartment (2663.0 sec) was within the same range as that of the PC group (2677.8 sec), and higher than the total time spent in the either the drug-paired or non drug-paired compartment.

		I	II	III	IV	V	VI	VII	VIII	IX
Dose group	# rats	#		Ratio average time spent drug-paired (black or white) versus non-drug-paired compartment		Ratio average time spent in drug-paired compartment versus V	Ratio average time spent in grey compartment versus V	Ratio average time spent Non-drug-paired (white or black) versus drug-paired compartment		Ratio average time spent in non-drug-paired compartment versus V
V	8	4	2/2	B+W: 1.12	B: 1.15 W: 1.09	1.00	1.00	W+B: 0.89	W: 0.87 B: 0.92	1.00
A	8	2	0/2	B+W: 0.74	B: 0.67 W: 0.81	0.75	1.11	W+B: 1.36	W: 1.49 B: .1.23	1.15
PC	8	7	4/3	B+W: 1.53	B: 1.88 W: 1.24	1.11	1.05	W+B: 0.65	W: 0.53 B: 0.81	0.82
A+PC	8	6	4/2	B+W: 1.36	B: 1.78 W: 1.11	1.03	1.10	W+B: 0.74	W: 0.56 B: 0.90	0.85
PC+A	8	6	4/2	B+W: 1.04	B: 1.46 W: 0.71	0.94	1.04	W+B: 0.96	W: 0.69 B: 1.39	1.01

Table VII.14. Overall data of study TOX10829 at post-test. Column I: #: number of rats with preference for drug-paired compartment; Column II: rats with preference for drug-paired compartment: ratio black/white drug-paired compartment; B+W: ratio calculated from time spent in drug-paired black + drug-paired white compartment; W+B: ratio calculated from time spent in non drug-paired white + non drug-paired black compartment; B: black compartment; W: white compartment.

VII.4. Discussion

VII.4.1. Investigation of the variables to optimize the design of a conditioned place preference model for use in Drug Development: results of the six mechanistic studies

Several variables, linked to the three different phases of the CPP test were explored in order to enhance the predictive value of the classic model as described in literature, as such enabling to test possibly rewarding properties of CNS-active drug candidates with a new mechanism of action. These are discussed below, grouped for the three phases of the CPP.

VII.4.1.1. Variables linked to the preconditioning phase (habituation period and pretest)

Before the use of a biased or unbiased procedure was discussed, the physiological behavior of the rats during a repeated stay in the CPP apparatus was investigated (TOX10314), using the assignment to fixed individual test boxes or daily distribution at random to the test boxes as a variable. It was demonstrated that the rate of exploration was stimulated when rats were assigned to fixed individual test boxes, as evidenced by equally distributed individual preference for either the white or the black compartment, and which was situated mostly around 50%. On the contrary, when animals were daily placed at random in another test box, the time spent in the black compartment increased tremendously. This was regarded as an indication that repeated exposure to new situations drives the animals to their more physiological environment (i.e. a black compartment), prohibiting further exploration of the other environments. To avoid such behavioural changes, the regimen of fixed assigned boxes was maintained throughout an entire CPP study (habituation, pretest, conditioning and post-test).

In the mechanistic study TOX10448 using 4 cocaine-pairings per group, it was also demonstrated that a more balanced distribution of the individual preference at pretest was received if the habituation period was executed a few days before. This is in particular of importance when a biased procedure (see further) should be employed, but also add to consistent pretest values.

We have opted to perform the habituation period on Days -5 and -4 in our standard design.

For the assignment of animals to the drug-paired compartment during conditioning, a biased or an unbiased procedure can be employed. In a biased model, the individual place preference at pretest is taken into account to pair the drug treatment to the least preferred compartment and the water/saline treatment to the most preferred compartment. In the unbiased model, the individual place preference at pretest is recorded but not taken into account for pairing drug or water/saline to a specific compartment, and animals are assigned to a "predefined" drug-paired or non-drug-paired environment for conditioning using a body weight based stratified randomisation procedure prior to starting the habituation phase.

After investigating the value of a biased versus unbiased ranking procedure (TOX10326) on the outcome of a CPP test it was concluded that a biased procedure is not to be preferred over an unbiased procedure, because of 1) the subtle differences in the percentage of place preference to the different compartments at pretest when the prehabituation was performed on days -5 and -4; 2) the slightly more pronounced physiological preference to the black compartment; and 3) the non-significance of an extreme place preference at pretest on the final result at post-test.

In our standard design we have opted to implement the unbiased procedure and randomize the animals into the different groups on Day -6 using a body weight based stratified randomisation procedure.

An extreme place preference at pretest, comprising a time spent in a particular environment counting for >75% of the total time (15 minutes) was recorded in about 5% of the animals over the 6 studies. It was demonstrated that this "extreme" preference at pretest did not influence the post-conditioning results. Thus these animals do not need to be excluded from testing at post-test. However when the unbiased procedure, executed upfront, forced drug-pairing in this "extremely" preferred compartment, the post-test results could be more pronounced (as evidenced by the longer time spent in this compartment). This

was in particular seen with an individual extreme preference related to the black compartment. Therefore these animals are always discussed separately in the report.

VII.4.1.2. Variables linked to the conditioning phase

The impact of the visual stimuli (colour of and brightness in the different compartments) and subsequent drug-pairing on the results of the post-test was investigated through literature search and based upon internal expertise.

The rat retina consists mainly of rods (cells that perceive light and dark but no colour); only a limited (1%) number of cones (colour-sensitive cells) is present (Jacobs, 2001). The rat is also a dichromat (blue and green photoreceptor retinal cones but not red) thus having limited capacity to differentiate colours. As the rat is a nocturnal animal, this distinction between colours appears to be of minor importance and is as such considered a weak cue value for their vision. Moreover, the contrast of a dark (black) and light (white) environment seems of more importance to the albino rat, as the incoming light into the albino eye is not tampered. Therefore the choice of the CPP apparatus with black and white compartments was considered appropriate for use as a conditioning cue.

Using the unbiased procedure in our final design, we subdivided each dose group for equal drug-pairing in the white and black compartment, as such excluding any influence of the colour/brightness and the subjective effect of the drug.

Nevertheless, a slight physiological place preference to the black compartment remained present at post-test throughout the studies performed with different psycho-active drugs [cocaine (TOX10447 and TOX10448), d-amphetamine (TOX10342), methylphenidate (TOX10342), fentanyl (TOX10829)], as evidenced by the absolute time spent in the black drug-paired versus white drug-paired compartment in a conditioned place preference at post-test. This might remain a variable of importance when weak reinforcers or new drug candidates with an unknown mechanism of action need to be tested.

It was also demonstrated (TOX10343) that tactile stimuli (grid floors, bar floors) may enhance the conditioning but were not a major trigger to obtain a more robust conditioned place preference.

Evaluation of the length of conditioning phase (number of drug pairings) in view of the conditioned place preference (TOX10447) revealed that conditioning during 16 days (containing 8 drug-pairings on alternate conditioning days) resulted in a higher conditioned place preference in both the drug-paired black and the drug-paired white compartment with less time spent in the neutral grey compartment than conditioning during 8 days (containing 4 drug-pairings on alternate conditioning days). This confirmed the positive relationship of the number of drug-pairings and the solidity of the response (Cunningham, 2011).

Based upon these results we have chosen for a 16-day conditioning phase containing 8 alternate drug-pairings in the standard design, allowing to properly test new drug candidates with unknown or maybe weak reinforcing properties.

VII.4.1.3. Variables influencing the outcome at post-test

The impact of a dose range on the conditioned place preference was investigated for 2 psycho-active drugs (cocaine, TOX10302; methylphenidate, validation study).

For cocaine, the conditioned place preference was somewhat more pronounced in the 10 mg/kg group versus the 15 mg/kg group. Although the dose-effect curve of reinforcers generally follows a linear correlation/gradient in a CPP paradigm, it is known that psycho-active stimulants like cocaine can produce a more bell-shaped curve (Bardo, 1995, Mueller, 2011). This might point towards the aversion properties of cocaine at high doses when tested in a conditioned place model.

Methylphenidate, orally tested at a dose range comprising 2.5, 5 and 10 mg/kg, displayed a linear drug- and dose relationship when the absolute number of animals showing a drug induced place preference at post-test was taken into account (Teuns 2014), but was only biologically significant at 10 mg/kg. This was in line with data from literature (Mithani, 1986; Wooters, 2011), supporting

the hypothesis that the rewarding properties of methylphenidate are only demonstrated at high orally tested doses.

The results of the CPP at post-test recorded at 24 hours versus 48 hours after the last drug-pairing (TOX10326) indicated a more explicit drug-induced place preference after 24 hours. Hence we decided to perform post-testing at 24 hours after the last drug-pairing in the standard design. As a consequence the conditioning phase started with dosing saline/water in all groups on Day 0.

Using the 3-compartment CPP apparatus implicated both advantages and disadvantages. The so-called neutral (grey) compartment in between the black and white compartment allowed the rats to acclimatize for 5 minutes before exploration of the different compartments started (at pre- and post-conditioning). It also prevented placing the animals in a particular direction at start of the pre- and post-conditioning phases (example: with the nose towards the black or white compartment), what could influence the individual place preference.

Taking the time spent in this neutral (grey) compartment during post-conditioning into account, it was demonstrated that a significant decrease occurred when strong reinforcers were tested. An example hereof is given in Chapter VIII, where the validation study results of the CPP revealed that d-amphetamine and methylphenidate at 10 mg/kg but not at lower dosages clearly showed this feature.

The question was also raised whether the data of the neutral (grey) compartment in a conditioned place aversion model would be indicative of a shift towards the non-drug-paired compartment. Indeed, the results of the CPP/CPA study (TOX10829) illustrated that he time spent in the neutral (grey) compartment was somewhat higher (though not significant) in the JNJ-Z group and in the combination JNJ-Z+PC (fentanyl) group. However, currently no other data are available to demonstrate a firm relationship between the time spent in the grey compartment and place preference or aversion for a CNS-active compound when tested at different dosages. This topic should be of interest to

investigate whether it could serve as a possible criterion, thus refining the data interpretation and hence the biological relevance of the outcome of CPP studies.

With regard to the biological relevance of the outcome of CPP studies, not only the absolute numbers of animals showing a drug-induced place preference were taking into account. Also the ratios calculated for the time spent in the drug-paired versus non-drug-paired compartment, for time spent in the drug-paired compartment of the dose groups versus the control group, and for the time spent in the non-drug-paired compartment and in the grey (neutral) compartments of the dose groups versus the control group demonstrated a higher sensitivity to interpret the results, thus contributing to the evaluation of the biological relevance of the outcome of CPP studies. The latter was defined as follows:

- less that 25% (<25%) of rats with place preference or aversion, and ratio time spent in drug-paired versus non-drug paired compartment of < 25%: no alert for place preference or aversion;

- a percentage of 25 - 70% of rats with place preference or aversion, and ratio time spent in drug-paired versus non-drug paired compartment of 25% - 50% or vice versa: signal for place preference or aversion;

- more than 70% (>70%) of rats with place preference or aversion, and ratio time spent in drug-paired versus non-drug paired compartment of >50% or vice versa: full positively reinforcing properties (Horton, 2013) or full aversion.

Finally an extra parameter, defined as the cutoff point of 324 seconds for a drug-induced conditioned place preference, was included for the interpretation of the CPP data. This was based on a publication of dela Cruz (2009), in which it was stated that a time spent in the drug-paired compartment of 324 seconds, is a positive and relevant CPP signal, at least for testing cocaine. Our results from the various studies with cocaine (TOX10302, TOX10326,TOX10343, TOX10347, TOX10348 - statistical data available upon request) and with methylphenidate and d-amphetamine (validation study - statistical data available upon request),

indicate however that the cutoff of 324 seconds is strongly dependent on the selected dose, the duration of the conditioning phase (number of drug-pairings), the use of a biased versus unbiased design, the drug-pairing in the black versus white compartment (see Fig. VII.4), and the strength of the reinforcing properties of the investigated drugs (Fig. VII.7).

Therefore the cutoff line is considered not critical, but will be maintained as a possible criterion for further investigation.

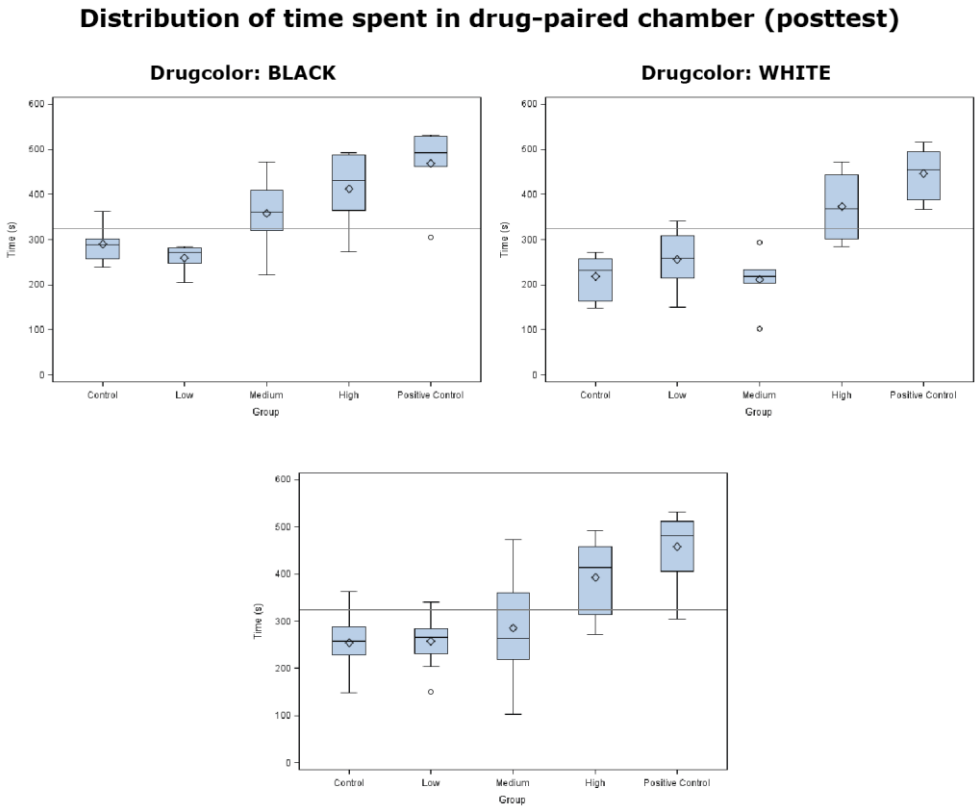


Fig. VII.7. Boxplot visualization of the distribution of the time spent in the drug-paired chamber at post-test: top left panel: drug-paired black compartment; top right panel: drug-paired white compartment; bottom panel: combined black+white drug-paired compartments. Data from the validation study with methylphenidate (2.5, 5 and 10 mg/kg) and d-amphetamine (0.8mg/kg). A clear drug-induced place preference was obtained at the high dose methylphenidate group (10 mg/kg) and in the d-amphetamine group (positive control).

A statistical analysis on the pre-and post-test data was implemented. Boxplots were used to visually represent the distribution of the data. Summary statistics such as the mean, median, standard error of the mean and 95% confidence limits of the mean were tabulated. ANOVA models were used for confirmatory analyses. Furthermore, all calculated ratios of the time spent in the different compartments (intra- and inter comparisons) were analyzed statistically. Finally the dependent variable, i.e. the excess amount of time spent in the drug-paired compartment at post-test as compared to pretest was statistically evaluated.

A possible gender difference in a CPP design was not picked up (TOX10326). A literature search revealed one study in which male rats were found less responsive to cocaine doses of 5 and 7.5 mg/kg compared to females (Schechter, 1992). In another study a sex-difference in the dose-response curve with morphine was noticed, explained by the higher susceptibility of females to the central action of morphine (Cicero, 2000). Since no further data of other drugs are available regarding the impact of gender on the responsiveness of rats to the CPP paradigm, and because historically the male rat is the gender of choice for behavioural studies at Janssen R&D, we have opted to use the male rat in the CPP test.

No strict research was executed towards the impact of age on the CPP model. Although data from literature were inconsistent regarding this variable, we endorse the hypothesis that age rather than gender may influence the outcome of a CPP (Bardo, 1995; Tzschentke, 2007). Based upon the available literature and with regard to the translational approach we have opted to use adult male Sprague-Dawley rats, aged 8 to 9 weeks at start of the conditioning phase.

VII.4.1.4. Final design of the CPP model for use in Drug Development

Before implementing the final design, some additional topics were investigated. Classically, animals do not receive any treatment during the pre- and post-conditioning. In our final design we have opted to perform sham treatment during the habituation phase, at pretest and at post-test to mimic dose administration during the conditioning phase. As such conditioning factors related to the dosing procedure were excluded, thus providing accurate

responses at post-test, purely correlated to the drug's effect and not affected by deviant behaviour (increased movements in all three compartments) due to procedural changes. The sham treatment followed the route of administration as executed with the test compound or with the reference drug (positive control drug) during conditioning, and was performed at $-T_{max}$ for both the test compound and the reference drug ($-T_{max}$ is defined as the time interval between dose administration and entrance into the test boxes, similar to the time needed to reach the highest plasma exposure after a dose administration).

In the final design, a toxicokinetic phase is added after completion of the CPP study. Toxicokinetic parameters are determined by administering an additional single low, mid and high dose of the drug candidate to a limited number of animals (n=3/dose group). Classically this phase is executed 24 hours after the post-test, but if the $T_{1/2}$ indicates sustained exposure, the TK phase can be postponed. The animals will then be kept in the study up to 7 to 10 days post-test, and the TK phase will be executed thereafter. Blood samples are taken on specified time points chosen in view of C_{max}, T_{max} and the $T_{1/2}$ of the test compound. This procedure is considered adequate to fulfil the requirements of the drug-licensing authorities, requesting the confirmation of testing a drug candidate at a dose range that includes a multiple of the human efficacious dose, expressed as C_{max} (EMA, 2006; ICH, 2009; FDA, 2010).

An example of a toxicokinetic phase is described in Chapter VIII for testing methylphenidate in the CPP paradigm.

Based upon all the above investigations, we developed the final design for use within Drug Development. The outline is visualized in Table VII.15.

Day -6:	Body weight base stratified randomization procedure
Days -5, -4:	Sham treatment at -T_{max}, using the intended route of administration of the test compound or the reference drug habituation at T_{max} for 15'
Day -1:	Sham treatment at -T_{max}, pretest at T_{max} for 15'
Days 0, 2, 4, 6, 8, 10, 12, 14:	Dosing: each group received water at -T_{max}, using the same route of administration as for dosing the test compound or the reference drug; conditioning at T_{max} for 45' in the non-drug paired compartment
Days 1, 3, 5, 7, 9, 11, 13, 15:	Dosing: test compound (C, L, M, H groups, respectively) or reference drug (positive control: PC group) at -T_{max}; conditioning at Tmax for 45' in the drug-paired compartment
Day 16:	Sham treatment at -T_{max}; using the intended route of administration of the test compound or the reference drug Post-test at T_{max} for 15'
Day 17, Day 24 or Day 27*:	Single dose administration of test compound (L, M, H) followed by blood sampling for toxicokinetic purposes.

*depending on the T1/2 of the drug candidate

Table VII.15. Outline of the final CPP design for use in Drug Development.

VII.4.1.5. A Conditioned Place Preference/Aversion test as employed in Drug Development, using JNJ-Z, a CNS-active novel drug candidate with the potential to treat opioid abuse

The final and approved CPP model was employed to test JNJ-Z for its possible drug-induced place preference or aversion. A 16-day conditioning period, comprising eight JNJ-Z drug-paired administrations alternated with eight NaCl 0.9% non-drug paired conditioning sessions showed a signal suggestive of drug-induced place aversion, as evidenced by the high ratio of time spent in the non-drug-paired versus drug-paired compartment.

Alternate dosing with fentanyl, a known drug of abuse with reinforcing properties, resulted in a high number of animals showing a drug-induced place preference and a high ratio of time spent in the drug-paired versus non drug-paired compartment, thus demonstrating a clearly drug-induced conditioned placed preference. These results were considered biologically relevant to the reinforcing properties of fentanyl.

The combination of JNJ-Z administered *prior* to each dose of fentanyl resulted in a high number of animals showing a drug-induced place preference and a high

ratio of time spent in the drug-paired versus non-drug-paired compartment. Although these numbers were slightly lower than those obtained with fentanyl only, they were indicative of a mild drug-induced conditioned placed preference and considered biologically relevant in view of the persistent reinforcing properties of fentanyl.

The combination of JNJ-Z administered *after* each dose of fentanyl (PC + JNJ-Z group) resulted in a high number of animals showing a drug-induced place preference but a low ratio of time spent in the drug-paired versus non-drug-paired compartment. This ratio was lower than that calculated for the vehicle group. Therefore the overall results were considered not indicative of a drug-induced place preference. Although not significant, the results of this group (PC + JNJ-Z) demonstrated a tendency to decreasing ratios which are indicative of place preference and increasing ratios which are considered indicative of place aversion. The decrease was in particular attributed to a shorter stay in the white drug-paired compartment and hence a longer stay in the black non drug-paired compartment. These results indicate that JNJ-Z treatment following administration of fentanyl might have induced some aversion, thereby influencing the strong reinforcing properties of fentanyl. It might be worthwhile to further investigate if testing a full dose range of JNJ-Z in combination with predosing of fentanyl can indeed confirm these data, hence giving rise to possible treatment methods for opioid abuse.

This test demonstrated the added value of the additional calculations of the ratios on the time spent in the drug-paired versus non-drug-paired compartments (*within* calculations and *between* calculations versus control) to detect subtle differences and to enhance the predictive power of the outcome.

VII.4.2. Data management of a conditioned place preference model for use in Drug Development and GLP compliance

The core of the CPP system consisted of the commercially available MED-PC hardware and software. This system had been configured and customized in-house. An appropriate software program (DA Services) was developed around it enabling to conduct the study according to the study design while following adherence to the GLP/Part 11 regulations (state of the art: traceable and

reproducible data). The whole CPP system has been implemented and validated in-house. This validation effort included, *among others,* an inventory of the business and compliance requirements, execution of the various test scripts to test system installation, security, compliance, functionality and performance, and of a validation report.

This newly built CPP system allowed automatic animal assignment to a test box and the subsequent drug-paired compartment at start of study, based on the study protocol. The CPP system also allowed online follow-up of the study (from rat assignment to boxes until study results) via a web-based interface.

With regard to the MED-PC Interface and combined with the data coming from the study protocol, the macros steering the MED-PC system are automatically generated for each session of the study phases, each session counting a maximum of 6 rats. On the study day, a collection of macros is made available locally on the computer in order to limit data tampering. Since there is still a small possibility of data tampering, the data integrity is checked after a study session by comparing the macro before and after the session (by means of a hash code). When the macro content has not been tampered with, the corresponding data files are moved to a secured location and the data is transferred to the database. From that moment on, the data results are available in the web-based interface for proper follow-up by the study team (Data management, laboratory team, Study Director, IT support, etc.).

The standard study design and tools needed to perform this type of study were made GLP-compliant. These tools included*, amongst others*, a check of 1) the test boxes for proper functioning of the photo-beamers; 2) automatic opening of the doors to the white and black compartment at pre- and post-test; 3) proper working of the lights in the test boxes; 4) the IT program, including the hard- and software. All systems were validated (IT validation, scientific validation) and all obtained data were retraceable and reproducible. The respective SOPs (for IT and for *in-vivo* procedures) were written and approved.

The definitive study design was validated through the drug-induced conditioned place preference test in the rat for testing methylphenidate, a known CII

scheduled drug, with d-amphetamine as reference drug. This can be read in Chapter VIII.

VII.4.3. Other considerations

In order to avoid behavioural changes of the animals related to other factors but conditioning with the test compound, it was decided to only partly replace bedding material of the home cages during the course of the study and as such allow a recognizable environment outside the test boxes. In addition, tunnels and food hoppers were not replaced but removed and re-used in the new, weekly changed clean home cages.

VII.5. Conclusions

The drug-induced conditioned place preference test in the rat (CPP) is employed to investigate the rewarding properties of a CNS-active drug candidate in development and as such part of the preclinical Abuse Liability Assessment. Although this paradigm is an indirect method for assessing reinforcement, it is considered a valuable method to serve as a substitute for the IV SA model, because insoluble drugs can be tested at a broad test range, utilizing the clinical route of administration.

The methodology of the classical CPP, as used in testing psycho-active drugs, was adopted in terms of technical features, to obtain scientifically grounded and reliable data. Several variables were investigated to improve the predictive validity of this test. The length of the conditioning phase, and subsequently the number of drug-pairings was, *amongst others,* considered a critical variable to test drug candidates with a new mechanism of action which may possess only weak reinforcing properties.

A major effort was also made on the methods to evaluate the post-test data and as such enhance the biological relevance of the outcome. Interpretation of the conditioned place preference data was not restricted to the absolute number of animals of the various groups showing a drug-induced place preference at post-test, but the calculated ratios of the average time spent in the drug-paired versus non drug paired compartment per dose group and the subsequent ratios of the average time spent in the drug-paired, non-drug-paired and grey (neutral) compartment, calculated per dose group versus control were also taken into consideration. This allowed fine-tuning of the interpretation, which is of added value in case of testing new CNS-active molecular entities.

Today our adopted CPP rat model is a GLP-compliant (OECD, 1998), valuable test model for use in a regulatory-driven preclinical environment, to test the rewarding properties of new, insoluble drug candidates, consistent with the requirements of the drug-licensing authorities (EMA, 2006; ICH, 2009; FDA, 2010).

VIII

Proof of Concept: predictivity of a translational approach for abuse liability testing using methylphenidate in four standardized preclinical study models

VIII.1. Introduction

The Chapters IV, V, VI and VII described how different variables, techniques and methodologies of 4 classical models, as employed in the fundamental research of abuse liability testing were investigated, adapted and optimized in order to obtain robust and scientifically well-founded test designs for the determination of the abuse potential of new CNS-active molecular entities within a Drug Development environment. This included also compliance with the requirements of the drug-licensing authorities (EMA, 2006; ICH, 2009; FDA, 2010), with the GLP regulations (OECD, 1998), and with the ethical considerations regarding animal welfare (European Convention, 1986, 2007; Belgian Law, 1991; AVMA, 2001).

Based upon the outcome of these investigations, a final design for each model was implemented and tested to demonstrate the scientific validity and the face validity of the results, and to verify full compliance before these models could be approved for use in a Drug Development environment. For this purpose, methylphenidate, a scheduled CII drug, was subjected to a non-precipitated withdrawal study, a drug discrimination learning study, an intravenous self-administration study and a conditioned place preference study. Methylphenidate is a psycho-stimulant, structurally related to amphetamine, and selected because of its known abuse potential, in particular in non-medical situations (Morton, 2000; Kollins, 2001; Freese, 2013). Based upon its abuse profile, the predictive validity and face validity of the four tests for determining the abuse potential of stimulants could be investigated.

These 4 studies were designed as GLP-like studies, including all GLP-requirements and regulations, with exception of the inspection visits from the QA-certified staff and the GLP statement in the final reports.

The non-precipitated withdrawal, the drug discrimination learning and the conditioned place preference tests involved oral administration of methylphenidate at doses of 2.5, 5 and 10 mg/kg. For the IV SA study, intravenous infusions at 0.05, 0.1, 0.5 and 1 mg/kg/infusion were presented to the rats. D-amphetamine was selected as the reference drug in all studies, at

0.8 mg/kg when dosed subcutaneously or at 0.06 mg/kg per IV infusion. The biological relevance of the outcome of all studies in view of the translational approach was discussed.

The full article is included in this Chapter.

VIII.2. Abuse Liability Assessment in Preclinical Drug Development: Predictivity of a Translational Approach for Abuse Liability Testing using Methylphenidate in Four Standardized Preclinical Study Models.

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Abstract

Objectives: Preclinical abuse liability assessment of novel clinical CNS-active candidates involves several tests, addressing different aspects characteristic for abuse potential, which are considered predictive for substance abuse of these candidates, thus ensuring an appropriate translational approach. To demonstrate how such a strategy could work, a known drug of abuse, methylphenidate was evaluated in a full rodent test battery, comprising four test models, and in accordance with the requirements of the FDA, ICH and EMA guidances.

Methods: Methylphenidate was tested orally at 2.5, 5 or 10 mg/kg for its physical dependence potential in a repeated dose non-precipitated withdrawal test, for its drug profiling in a drug discrimination learning procedure (single escalating doses), and for its reinforcing properties in a conditioned place preference test (alternate dosing days) and an intravenous self-administration procedure (0.05 to 1 mg/kg/IV infusion during 5 daily 1-hour test sessions). The stimulant d-amphetamine served as positive control and was administered subcutaneously at 0.8 mg/kg in the first three test models. In the intravenous self-administration procedure rats were habituated to intravenously self-administer d-amphetamine at 0.06 mg /kg/IV infusion prior to methylphenidate substitution.

Results and discussion: Cessation of subchronic dosing up to 10 mg/kg methylphenidate led to sustained or even exacerbated effects on locomotion and behavior, body temperature, body weight, food consumption, and alteration of the diurnal rhythm during withdrawal. Clear generalization to d-amphetamine was obtained in the drug discrimination test at 5 and 10 mg/kg. Distinct reinforcing properties were present in the conditioned place preference test at 10 mg/kg and in the intravenous self-administration study from 0.05 mg/kg/IV infusion onwards. The maximum plasma exposure after oral administration of methylphenidate over the dose ranges tested in the present rat studies covered at least 1.9-fold to 18.9-fold the recommended human therapeutic exposure of 10 ng/ml, a plasma level that is considered representative of the human efficacious methylphenidate dose. The ratio C_{max} Hu/rat calculated from the intravenous self-administration data ranged from 14.9 to 576.5. Consequently the regulatory requirements, stating that preclinical drug abuse liability studies should include high doses that produce plasma levels that are multiples of the therapeutic dose were fulfilled (FDA, EMA, ICH).

In conclusion, the presented preclinical models, implemented within a drug development environment, were considered highly predictive to assess the abuse potential of methylphenidate, and in accordance with the regulatory requirements of drug licensing authorities in terms of appropriate methods, dose selection and subsequent plasma exposure.

Key words: Preclinical drug development, abuse potential, predictivity, translational approach, methylphenidate, rat, withdrawal, drug discrimination, conditioned place preference, self-administration.

VIII.2.1. Introduction

The investigation of abuse potential of new CNS-active molecular entities has become a critical topic in drug development since the release of the EMA guideline (EMA, 2006), the ICH guideline (ICH, 2009), and recently the FDA draft guidance (FDA, 2010) and the subsequent FDA's decision tree (Bonson, 2011). In particular within the preclinical safety evaluation area the need of drug abuse liability testing has become a major issue as all compounds in

development exerting an activity in the brain (regardless of therapeutic area) are subject to these regulations.

In our lab four preclinical study models, investigating various aspects of abuse potential, i.e. physical dependence potential, discriminative properties and measures of direct and indirect reinforcing properties, were adapted from the known models in literature (Bardo, 2000; Koob, 2000; Shippenberg, 2002; Ator, 2003; CPDD, 2006; Cunningham, 2006), standardized, made compliant with the GLP regulations and implemented according to the regulatory requirements for testing CNS-active compounds in drug development. To date these adapted models are the basis of our preclinical abuse liability assessments and are evaluated in this article for their predictive validity using methylphenidate, a known drug of abuse. Methylphenidate (MPH), used to treat Attention Deficit - Hyperactivity Disorder (ADHD), has been scheduled for its abuse potential as CII in the US (FDA-DEA, 2012) and worldwide (Convention on Psychotropic Substances, 1971). It is structurally related to amphetamine and increases the extracellular dopamine in the striatum and the nucleus accumbens by inhibiting the dopamine transporter (Challman, 2000). Subsequently d-amphetamine-sulphate (AMP) was selected as a positive control in the present preclinical studies, based upon the comparable pharmacological action of both compounds. The d-form of amphetamine is a central nervous system stimulant and sympathomimetic with multiple mechanisms of action including adrenergic and dopaminergic uptake inhibition, release of monamines, and inhibition of monoamine oxidase (Shire, 2011). AMP has been prescribed in the treatment of narcolepsy and of attention deficit disorders and hyperactivity in children. It has been scheduled for its abuse potential as CII in the US (FDA-DEA, 2012) and internationally (Convention on Psychotropic Substances, 1971).

The preclinical abuse liability assessment as described here for MPH is a critical component in the development of CNS-active drugs supporting the safety evaluation with regard to abuse potential and possible scheduling. The translational approach of this example with MPH will be discussed based upon the outcome of the four presented standardized preclinical tests, conducted in male Sprague-Dawley rats, and in view of the known neuropharmacology and clinical findings of MPH. The question whether abuse liability studies performed

in ADHD diseased rats versus common Sprague-Dawley rats could add to a higher predictivity of the abuse potential of MPH will be discussed briefly. Finally the proper selection of an efficacious dose range, needed to comply with the regulatory requirements in terms of preclinically testing a several-fold of the human efficacious dose (Ceff Hu) and the biological relevance of the various test results within this dose range will be considered.

VIII.2.2. Materials and methods

VIII.2.2.1 Animals and husbandry

All tests were performed in an AAALAC-accredited (Association for Assessment and Accreditation of Laboratory Animal Care) testing facility. An ethical protocol was written and approved by the Ethics Committee for each of the four studies.

Housing and experiments were conducted in accordance with the European (European Convention, 1986, 2007) and Belgian guidelines (Belgian Law, 1991), and with the principles of euthanasia as stated in the Report of the American Veterinary Medical Association Panel (AVMA, 2001).

Young (6 to 9 weeks old upon arrival) Specific Pathogen Free (SPF) male Sprague-Dawley (Crl: CD®) rats were used in the four studies. All animals had an acclimatisation period of at least 5 days before being used in any experimental procedure.

Rats were housed separately in transparent polysulphone cages (floor area: 940 cm2) with a wire-mesh lid suspended in wheeled racks. Bedding material (Corn Cob size 12, Eurocob, France) and cage enrichment (wooden blocks, Sizzle nest and/or transparent polycarbonate tunnels) were provided. There was a 12/12 light/dark cycle and illumination did not exceed 700 lux. The test rooms were air-conditioned with their own supply of filtered fresh air. The standardized test conditions for temperature (20 - 23 °C), relative humidity (40 - 70%) and illumination (700 lux) were regularly controlled and recorded.

Rats were given free access to water. Food [R/M-H pelleted maintenance diet, Ssniff (Soest, Germany)] was provided ad libitum in the Withdrawal (WD) and the Conditioned Place Preference (CPP) tests but was restricted to 20 grams per

day in the Self-Administration test (IV SA) to maintain a stable body weight. In the Drug Discrimination Learning test (DDL) daily food supply was presented according to the following schedule: training days (Mondays to Thursdays): 9 g available in cage after training; test days (Fridays): 18 g available in cage after test sessions; weekends (no training or test sessions): 16 g on Saturdays; 13 g on Sundays. In this latter test food rewarding during training and during the test sessions was offered via sugar pellets (45 mg dustless precision pellets, Bioserv, US), (1 pellet per 10 lever presses).

Rats were checked at least once daily for general health, abnormal behaviour or unusual appearance, untoward clinical signs, toxic or pharmacological response, moribund state or mortality.

VIII.2.2.2. Equipment

A Plexx thermometer (Temperature reader (DAS 7007S) with implantable and programmed temperature transponders (IPTT-300) was used in the WD test.

For the DDL test operant conditioning chambers (Skinner boxes: modular test chamber ENV-007, Med Associates Inc; floor area: 750 cm2) with 2 response levers and equipped with a pellet dispenser (ENV-203-45, Med Associates Inc.) were used. The test chambers were placed in a steel soundproof box with light and ventilation.

The conditioned place preference apparatus (Med Associates Inc.) consisted of a test box divided into two separate compartments [21 x 21 x 68 cm (width x height x length)] interconnected by a short grey tunnel section (so-called neutral compartment). The compartments had different coloured sidewalls (black and white) with differently textured floors (smooth metal horizontal rods or wire mesh) in order to provide both tactile and visual environmental cues. The test box was housed in a sound-proof box to prevent any audible cues disrupting the conditioning.

For the IV SA test operant conditioning chambers (Skinner boxes: modular test chamber ENV-007, Med Associates Inc) with 2 respond levers (of which the left one was made inactive) were used. The chambers were put in a wooden box

with light and ventilation. An automated syringe pump system (Model PHM-100-3.3, MED-PC, USA) was configured to intravenously deliver the appropriate drug amounts via short infusions, with each infusion lasting between two and five seconds.

VIII.2.2.3. Drugs and dose rationale

VIII.2.2.3.1. Methylphenidate-HCl (MPH)

Methylphenidate is a piperidine derivative (methyl 2-phenyl-2-(piperidin-2-yl) acetate). The drug substance consists of a racemic mixture of two stereoisomers, (±)-threo-enantiomers with stereodescriptors R,R and S,S, respectively. The correct stereochemistry was established by utilizing the (±)-threo-ritalinic acid as the starting material (oral communication Noramco, Athens, GA, US). The oral LD50 of MPH in the rat is 350 mg/kg (Sigma-Aldrich, 2006) whereas the IV LD50 of MPH is 48 to 50 mg/kg (Johnson Matthey, 2006; Separham, 2011).

MPH is commercially available as a hydrochloride salt (Concerta®, Ritalin®) for treatment of ADHD. An overview of the maximum oral human dose of MPH and the subsequent maximum exposures (C_{max}) at peak time (T_{max}) is given in Table VIII.1 (Ritalin®, 2001; Concerta®, 2007), as well as intravenous dosages which are however rarely used in the clinic (Kerenyi, 1959; Janovski, 1978).

Drug	Route of administration	Dose MPH		C_{max}	T_{max}
Concerta®	OR	72 mg/day/70 kg	1.03 mg/kg	17.1 ng/ml (single dose)	6 h
				16.1 ng/ml (repeated dose)	6 h
Ritalin®	OR	21 mg/day/70 kg	0.30 mg/kg	7.8 ng/ml (adult)	2 h
		/	0.30 mg/kg	10.8 ng/ml (child)	2 h
MPH IV	IV	20–40 mg/70 kg (mean: 31.2 mg/70 kg)	0.28 to 0.57 mg/kg (mean: 0.45 mg/kg)	/	/

Table VIII.1. Overview of the oral (OR) human doses of MPH, commercially available as Concerta® (extended release) or Ritalin® and the corresponding maximum plasma exposures (C_{max}) at peak time (T_{max}). Intravenous (IV) human MPH doses are also listed.

In the present rat studies, oral dosages of 2.5, 5 and 10 mg/kg MPH were selected (WD, DDL, CPP) based upon literature data (Kollins, 2001; Botly, 2008; Wooters, 2011). Methylphenidate hydrochloride (CAS Nr. 298-59-9, Noramco)

was dissolved in NACl 0.9% (NaOH to pH = 7.4) at concentrations of 0.25, 0.5 and 1.0 mg/ml and the administered dose volume was 10 ml/kg.

In the IV SA study MPH was presented at 0.05, 0.1, 0.5 or 1 mg/kg/infusion (Kollins, 2001; Botly, 2008). Aseptic, aqueous solutions at concentrations of 0.25, 0.5, 2.5 and 5 mg/ml were prepared and the dose volume was 0.2 ml/kg/infusion.

VIII.2.2.3.2. d-Amphetamine sulphate (AMP)

The choice of d-amphetamine-sulphate as positive control in the present studies with MPH was based upon the comparable pharmacological action of both compounds.

Dextroamphetamine (d-Amphetamine, (2S)-1-phenylpropan-2-amine) is the dextrorotatory stereoisomer of amphetamine and is a slightly polar, weak base with lipophilic properties. The LD50 is 180 mg/kg when administered subcutaneously in the rat (Drug Information Portal, present; Warren, 1945). The LD50 of AMP administered IV to rats is 30 mg/kg (RTECS, 2013).

In the present rat studies (WD, DDL and CPP) a subcutaneous dose of 0.8 mg/kg AMP was selected based upon internal experimental and literature data (Spyraki, 1982; Levi, 2012). D-amphetamine sulphate (CAS Nr 51-63-8, Fagron, Belgium) was dissolved in NaCl 0.9% (NaOH to pH = 7.4; aseptic preparation of solution) at a concentration of 0.16 mg/ml and the administered dose volume was 5 ml/kg. In the IV SA study AMP was presented at 0.06 mg/kg/infusion (Carroll, 1997). Aseptic, aqueous solutions at a concentration of 0.3 mg/ml were prepared and the dose volume was 0.2 ml/kg/infusion.

VIII.2.2.3.3. Propofol

Propofol is a non-barbiturate sedative that was used to test the intravenous catheter patency testing. It is on the market as a sterile, non-pyrogenic emulsion containing 10 mg/ml of propofol suitable for intravenous administration (Diprivan®). It is not classified as a drug of abuse (FDA-DEA, 2013). Propofol was applied as a fast bolus at 5 mg/kg, corresponding to 0.5 ml/Diprivan®/kg/bolus. The chosen dose was based on literature data (Simons,

1991; Larsson, 1994; Naguib, 2003) and on a previously conducted internal experiment on proper dose selection of propofol (Teuns, 2013).

VIII.2.2.3.4. Heparin solution

An aseptically prepared 50 I.U. heparin/ml solution (NaCl 0.9%) was used for daily flushing (0.2 ml/rat) of the intravenous catheters of the IV SA rats.

VIII.2.2.3.5. NaCl 0.9%

Commercially available NaCl 0.9% (Kela Pharma, Belgium) was used whenever appropriate.

The stability of all the above formulations was checked at regular time-intervals to allow formulation preparations at appropriate points in time if applicable.

VIII.2.2.4. Methods

In all studies dosing (WD, DDL, CPP) or access to IV SA infusions (IV SA) commenced in the early morning (time range: WD and DDL: 7:30 - 9:00 AM; CPP: 7:00 AM - 1:00 PM; IVSA: 8:00 - 11:00 AM).

VIII.2.2.4.1. Withdrawal test

MPH was administered via oral gavage to naive rats (n=5 per dose group) at daily q.d. doses of 2.5, 5 or 10 mg/kg during 21 successive days, followed by a withdrawal phase of 7 days to assess possible signs of physical dependence. This procedure was also maintained for the control group (NaCl 0.9% OR) and the positive control AMP (0.8 mg/kg SC). Body temperature and behavioural observations were recorded in all rats on Days 1 (first day of study), 13, 20 and 21 of the repeated dose phase, at pre-dose (1 hour prior to daily dose administration), and at 1, 3 and 5 hours post-dose. Body weight and food intake were measured on these predefined days prior to daily dosing and at 5 hours post-dose. In addition rats were daily observed for general clinical observations at 1, 3 and 5 hours post-dose.

During the withdrawal period general and behavioural observations and body temperature were recorded on a daily basis at 0, 1, 3, 5 hours (0 hour = 24 hours after the last daily drug treatment) on Day 22 (first day of withdrawal), at

0, 3, 5 hours on Days 23 and 24 (0 hour = actual hour defined on Day 22); and at 0, 3 hours on Days 25 to 28. Body weight was measured daily at the defined 0, 3 and 5 hour time points. Food intake was recorded daily at the defined 0 hour time point. In addition, food intake was also measured at the 5 hour time point on Days 22, 23 and 24 of the withdrawal period to calculate the so-called daytime food consumption.

The toxicokinetic parameters were determined in additional satellite rats (see 2.6. Toxicokinetic parameters).

VIII.2.2.4.2. Drug discrimination learning test

Rats were trained to discriminate AMP (0.8 mg/kg, SC, pretreatment time: 15 minutes) from saline (5 ml/kg, SC, pretreatment time: 15 minutes) in a two lever food reinforced DDL procedure with a fixed ratio 10 (FR10) schedule of food reinforcement (FR10: 1 food pellet (reinforcement) after 10 correct responses). Daily AMP (D) or saline (S) injections were given according to 8-week alternating sequences, i.e., 1) D-S-S-D-S, S-D-D-S-S, S-D-S-D-D, D-S-D-S-D followed by 2) S-D-D-S-S, D-S-D-S-D, D-S-S-D-D, S-D-S-D-S. Rats with odd identification numbers started the training with the first sequence, whereas training of the even-numbered animals started according to the second sequence.

Acceptance criteria for each rat to participate in the actual study were a sustained FR10 and >100 responses on the drug lever (if AMP was injected) or the saline lever (if saline was administered) during at least 10 successive training sessions. The FRF value, which is a measure for the accuracy of the lever selection, and defined as the sum of the responses made on either lever before the first reinforcement occurs, had to remain below 15. The percentage of responses on the selected lever [i.e. the number of responses on the appropriate lever to the total number of responses on both levers during a 15-minute session] is indicative of a consistent responding and had to exceed 85%. Full training and testing procedures can be found in Meert, 1990.

During the present study, twelve trained rats were subjected weekly (each Friday: Study Days 1, 8, 15, 22, 29) to escalating doses of MPH, with a control

test (control solution) at the beginning and end of the series (0, 2.5, 5, 10 and 0 mg/kg), to test for stimulus generalization to AMP. MPH was administered (OR) 30 minutes prior to the start of the test sessions, whereas AMP or saline were injected SC at 15 minutes prior to starting the training sessions, to allow for the concentration achieved at maximum clinical effect of both active compounds (Concerta, 2007; Wooters, 2011; Teuns, 2012). Sham treatment was performed during the training sessions (OR, 30 minutes before start) and the test sessions (SC, 15 minutes before start) to avoid conditioning differences due to the routes of administration (OR for MPH versus SC for AMP or saline). All sessions lasted for 15 minutes and were conducted under non-extinction conditions (no limitation on the number of reinforcements). The following parameters were assessed: the selected lever, defined as the first lever (drug or saline lever) on which a rat received the first reinforcement (after 10 responses); the FRF value; the latency time till the first reinforcement occurred [the maximum latency time was 900 seconds (15 minutes), if a rat failed to perform the first FR10 during a session]; the total number of responses on both levers, and the percentage of responses on the selected lever. Outside the test sessions, the following variables were also recorded: the total number of responses on both levers, and the latency time in the last saline and in the last AMP training session during the 3 training days (i.e. Tuesday, Wednesday, Thursday) prior to any MPH substitution. During these training days, the criteria as prescribed for entering into the actual study (FR10, >100 correct responses, FRF<15 and minimum 85% response on selected lever) had to be fulfilled before MPH dose substitution on Fridays could be executed. Rats that could not be tested with a particular MPH dose that way, were allowed a retest at this MPH dose one week after the last escalation was performed; again the criteria had to be met during the 3 training days preceding this retest on Friday. This procedure was followed for 2 rats that did not meet the criteria on one training day prior to the second control (C2) substitution (1 rat) and the 10 mg/kg MPH substitution (1 rat). For ease of the reader the results of the retest on Day 36 were added to the regular substitution schedule (see Table VIII.4).

The biological significance of the outcome of a positive reinforcement, based upon the drug response after treatment with MPH at the various dosages, is considered as follows: <25% of rats with positive effect: alert; 25 - 70% of rats

with positive effect: signal; >70% of rats with positive effect: full generalization (Horton, 2013).

In addition toxicokinetic parameters were included (see 2.6. Toxicokinetic parameters).

VIII.2.2.4.3. Conditioned place preference test

The study comprised three distinct phases: preconditioning (2-day habituation period and pretest), conditioning and finally post-conditioning or posttest. One day before the habituation started, naïve rats were divided into five different dosage groups (n=12 per dose group) via an unbiased procedure, using a body weight based stratified randomisation procedure.

During the habituation days and during pretest, rats were allowed to freely move in the three compartments during 15 minutes, but only at pretest the time spent in each compartment was recorded.

Three groups were conditioned to MPH (OR), at doses of 2.5 (Low), 5 (Mid) or 10 mg/kg (High). A negative control group received NaCl 0.9% (OR) whereas the positive control rats were conditioned to AMP (0.8 mg/kg, SC). Conditioning in either the white or the black drug-paired compartments was evenly distributed in each group to exclude physiological preference for a distinctive environmental cue.

During the conditioning phase animal groups were treated daily, receiving MPH at the various doses (including the control solution) on odd days and water on even days. Dosing was performed at 30 minutes (for the 0, 2.5, 5 and 10 mg/kg MPH groups) before entrance into the compartments, to allow conditioning at T_{max}, corresponding to the highest MPH plasma exposure (C_{max}) after dosing (Concerta, 2007; Wooters, 2011). The same regimen was used for SC administration of AMP (odd days) and saline (even days) with SC dosing 15 minutes before entrance into the compartments, as for AMP the C_{max} is reached after 15 minutes (T_{max}) (Teuns, 2012). The daily conditioning sessions (16 days in total) lasted for 45 minutes.

At post-conditioning (posttest), rats were not treated and given free choice to all three compartments during 15 minutes. The time spent in each compartment (including the time spent in the neutral (grey) compartment) was recorded.

At pre- and post-conditioning, oral sham treatment was provided at 30 minutes (for the 0, 2.5, 5 and 10 mg/kg MPH dose groups) before entrance into the test box, in order to mimic the oral MPH dose administration during conditioning. In the positive control (AMP) group subcutaneous sham treatment was performed 15 minutes before the rats were placed in the test boxes.

Mortality, clinical observations and body weight were registered to follow up on the general condition of the animals. Toxicokinetic parameters were also evaluated (see 2.6. Toxicokinetic parameters).

VIII.2.2.4.4. Self-administration test

Forty-eight rats were surgically prepared with an indwelling 3FR CBAS® heparin coated catheter (Instech Solomon, Phymouth Meeting, PA, US) via the femoral vein. After a 10-day recovery period the training commenced for IV SA with AMP 0.06 mg/kg/infusion at a fixed ratio schedule of reinforcement of 1 (FR1) and with a time-out period of 5 seconds between successive infusions. No food reinforcement was given prior to start IV training. IV SA training sessions lasted for 1 hour. When stable responses were reached at 3 consecutive sessions, the FR was raised to FR3, FR5 and finally to FR10. The time-out period was set at 20 seconds after starting at FR3. To ensure the accessibility of the intravenous catheter, patency testing (propofol 5 mg/kg IV bolus) was performed twice weekly (during training and during the actual study).

Of the initial forty-eight rats, one rat died during surgery and fifteen rats were preterminally killed during training or during the actual study because of obstruction of the intravenous catheter or because of a poor general condition. The remaining thirty-two (32) rats trained to intravenously self-administer AMP at a dose of 0.06 mg/kg/infusion completed the actual study. The study consisted of 5 subsequent phases of 5 dosing days each. At all phases a fixed ratio schedule of reinforcement (FR10) was applied and the maximum number of infusions possible per daily 1-hour session was set at n = 20, with a time-out

period of 20 seconds between 2 successive infusions. During the first, third and fifth phase the 32 rats were presented AMP for IV self-administration whereas saline (NaCl 0.9%) was made available during the second phase. During the 4th phase MPH was offered for self-administration at IV doses of 0 (control: 7 rats), 0.1 (low dose: 9 rats), 0.5 (mid dose: 8 rats) or 1 (high dose: 8 rats) mg/kg/infusion. The extra low dose of 0.05 mg/kg/inf. was made available afterwards to 25 rats in a separate 6th phase. The following self-administration parameters were studied: number of drug infusions, mean and maximum drug intake and dose-response. The toxicokinetic parameters of MPH were also determined (see 2.6. Toxicokinetic parameters).

VIII.2.2.5. Statistics

All statistical tests in the four studies were performed at a two-sided significance level of 5 percent.

VIII.2.2.5.1. Withdrawal test

Statistics were performed on body temperature, body weight and food consumption. The significances for positive control (AMP), and for 2.5, 5 and 10 mg/kg MPH group(s) versus control were computed by Mann-Whitney-U (Siegel, 1988).

VIII.2.2.5.2. Drug discrimination learning test

A repeated ANOVA (Verbeke, 2000) was adopted on latency data and on the total number of responses to check for evidence of any difference among dose groups while accounting for the within-animal effects. A post-hoc Dunnett test (Dunnett, 1955) was performed to investigate all pairwise comparisons of each dose group (2.5, 5 and 10 mg/kg MPH) to the first dose administration of the control (referred to as C1) on Day 1.

VIII.2.2.5.3. Conditioned place preference test

The number of rats showing a drug-induced conditioned place preference were analysed using the Fisher Exact probability test (Siegel, 1988). ANOVA models (Neter, 1990) were fitted to the time spent in the drug-paired chamber at pretest as well as at posttest with group (AMP, 2.5, 5 and 10 mg/kg MPH dose groups) and colour (white and black) of the drug-paired chamber as explanatory

factors (Dela Cruz, 2009). The average time spent in the different compartments at posttest for the various groups was also analyzed using Anova.

VIII.2.2.5.4. Self-administration test

A repeated ANOVA (Verbeke, 2000) with group [AMP (positive control), saline, 0 (control), 0.05 (extra low), 0.1 (low), 0.5 (mid) and 1 (high) mg/kg/inf.] as explanatory factor was fitted to the data. Posthoc, pairwise comparisons versus control were evaluated using Tukey's method (Hochberg, 1987).

Statistical analyses were performed on the mean of the last 3 infusion data per animal per group per phase. The same animals were followed over phases 1 to 5. In phase 4 (MPH phase) the total number of 32 animals was divided amongst control (n=7), 0.1 mg MPH/kg/inf. (low dose: n=9), 0.5 mg MPH/kg/inf. (mid dose: n=8) and 1 mg MPH/kg/inf. (high dose: n=8,) dose groups. The 6th phase consisted of the remaining 25 animals that were all presented an extra low dose of 0.05 mg MPH/kg/inf. To account for the within-animal variability in comparison across different phases, a mixed effects model was fitted with a random animal effect (Verbeke, 2000).

VIII.2.2.6. Toxicokinetic parameters

The toxicokinetics (TK) of MPH were followed up in satellite animals (WD) or in the animals of the respective study after completion of the main experiment (CPP, DDL, IV SA).

A volume of 0.3 ml blood was taken from the tail vein for each sampling time point and put on K3EDTA. Bioanalytical analysis was performed on the plasma with a qualified research LC-MS/MS method and C_{max} and T_{max} were calculated.

VIII.2.2.6.1. Withdrawal test

Three satellite animals per dose group (2.5, 5 and 10 mg/kg MPH, OR) were used for blood sampling on Day 1 and Day 21 of the repeated dose phase (prior to daily dosing, 180 minutes-, 300 minutes- and 24 hour samples) to investigate the MPH exposure level after single versus repeated dose administration and the absence of drug at start of the withdrawal period.

VIII.2.2.6.2. Drug discrimination test

After the last substitution [Day 36: retest of 0 mg/kg (1 rat) and 10 mg/kg MPH (1 rat)], all rats were further trained according to the 8-week alternating sequence schedule during one week. On the next Friday, a single dose of either 2.5 (low dose) or 10 (high dose) mg/kg MPH (OR) was administered to 6 rats per dose, of which 50% received saline and 50% received AMP 24 hours before the respective MPH dose. Blood samples were taken 15, 30, 45 and 60 minutes after MPH administration.

VIII.2.2.6.3. Conditioned place preference test

One day after the posttest (or 48 hours after the last conditioning session with MPH), a single oral dose of 2.5, 5 or 10 mg/kg MPH was administered to the first 3 animals of each dose group. Blood sampling was performed after 30, 60, 150 and 300 minutes.

VIII.2.2.6.4. Self-administration test

A slow IV bolus injection (within one minute) of 0.5, 5 or 15 mg/kg MPH was administered manually to 6 animals per dose group. These dosages were calculated based upon the minimum and maximum number of infusions self-injected by the rats of the various dose groups during the study. Blood samples were taken at 3, 6, 20 and 60 minutes after this single dose administration.

VIII.2.3. Results

VIII.2.3.1. Withdrawal test

At 10 mg/kg MPH, body temperatures (Table VIII.2) were significantly higher compared with those of the control group, when recorded 1 and 3 hours after dosing on Days 13, 20 and 21. These values returned to the level of control values at 5 hours post-dose. Pre-dose measurements (-1h) revealed higher body temperatures, being significant on Day 20.

Similar significant findings were noted in the 0.8 mg/kg AMP dose group, but here the effects were present from the first treatment day onwards.

		MPH: C 0 mg/kg	AMP 0.8 mg/kg	MPH: h 10 mg/kg
d1	−1 h	36.5 (0.1)	36.8 (0.1)	36.7 (0.1)
	1 h	37.3 (0.3)	38.0* (0.1)	37.9 (0.2)
	3 h	36.4 (0.1)	37.2** (0.2)	36.9 (0.2)
	5 h	36.4 (0.1)	36.7 (0.1)	36.5 (0.1)
d13	−1 h	37.2 (0.1)	38.0** (0.1)	37.8 (0.2)
	1 h	37.6 (0.1)	38.2** (0.1)	38.3** (0.0)
	3 h	36.4 (0.2)	37.6* (0.2)	37.5* (0.2)
	5 h	36.4 (0.2)	36.7 (0.2)	36.4 (0.1)
d20	−1 h	36.8 (0.1)	37.9** (0.1)	37.9** (0.1)
	1 h	37.7 (0.2)	38.1 (0.1)	38.3* (0.1)
	3 h	36.4 (0.2)	37.7** (0.1)	37.1 (0.3)
	5 h	36.7 (0.2)	36.7 (0.3)	36.4 (0.2)
d21	−1 h	36.5 (0.2)	37.2** (0.1)	37.3 (0.2)
	1 h	37.1 (0.2)	38.1** (0.1)	38.2** (0.1)
	3 h	36.6 (0.2)	37.9** (0.1)	37.7** (0.2)
	5 h	36.3 (0.2)	36.8 (0.2)	36.3 (0.1)

		MPH: C 0 mg/kg	AMP 0.8 mg/kg	MPH: L 2.5 mg/kg	MPH: M 5 mg/kg	MPH: h 10 mg/kg
d22	0 h	36.5 (0.2)	37.5** (0.1)	37.3* (0.3)	37.1 (02)	37.4** (0.1)
	1 h	36.9 (0.3)	37.3 (0.1)	37.2 (0.3)	37.2 (0.1)	37.4 (0.1)
	3 h	36.6 (0.2)	36.9 (0.2)	36.7 (0.2)	36.8 (0.3)	37.0 (0.2)
	5 h	36.2 (0.2)	36.6* (0.1)	36.3 (0.1)	36.3 (02)	36.4 (0.1)
d23	0 h	36.2 (0.2)	37.2** (0.1)	37.0* (0.2)	36.6 (02)	37.4** (0.3)
	3 h	36.5 (0.3)	36.6 (0.1)	36.6 (0.3)	36.3 (02)	36.9 (0.2)
	5 h	36.2 (0.2)	36.7 (0.1)	36.4 (0.2)	36.3 (02)	36.5 (0.1)
d24	0 h	36.3 (0.2)	37.1* (0.3)	36.9 (0.3)	36.7 (02)	37.0* (0.3)
	3 h	36.5 (0.3)	37.5* (0.1)	37.1 (0.3)	36.4 (0.3)	36.5 (0.1)
	5 h	36.4 (0.2)	36.5 (0.1)	36.5 (0.1)	36.4 (0.1)	36.4 (0.1)
d25	0 h	36.8 (0.3)	37.3* (0.0)	36.9 (0.2)	36.7 (02)	36.7 (0.3)
	3 h	36.4 (0.3)	36.5 (0.1)	36.9 (0.3)	36.7 (02)	37.1 (0.3)
d26	0 h	37.3 (0.1)	37.8* (0.1)	37.2 (0.3)	37.0 (02)	36.9 (0.4)
	3 h	36.7 (0.3)	37.0 (0.2)	37.1 (0.2)	36.9 (02)	37.1 (0.3)
d27	0 h	36.7 (0.2)	37.6 (0.3)	36.8 (0.4)	37.3 (0.3)	37.4 (0.3)
	3 h	36.1 (0.2)	36.6 (0.1)	36.8* (0.1)	36.3 (02)	36.6 (0.2)
d28	0 h	36.2 (0.2)	37.1 (0.3)	36.8 (0.2)	36.8 (0.4)	36.7 (0.3)
	3 h	36.3 (0.2)	36.6 (0.2)	36.8 (0.3)	36.4 (0.3)	36.6 (0.2)

Table VIII.2. WD test: Mean body temperature (degree Celcius, SEM between brackets) during the repeated dose phase (RD: Day 1 - Day 21) and the withdrawal period (WD: day 22 - day 28). Time points -1 hour pre-dose, +1, +3 or +5 hours post-dose (RD) or versus 0 hours (WD). Significance for Positive Control (AMP), 2.5 (L: Low), 5 (M: Mid), 10 (H: High) mg/kg MPH group(s) computed versus Control by Mann-Whitney U (two-tailed): * $p < .05$ ** $p < .01$ *** $p < .001$.

On the first three days of the withdrawal period (Days 22 to 24) a significantly higher body temperature was recorded in the 10 mg/kg MPH group at the 0h

time point. This was also seen in the 2.5 mg/kg MPH (significant on Days 22 and 23) but less pronounced in the 5 mg/kg MPH group (no significance). In the AMP group a significant increase in body temperature at the 0h time point was present from Day 22 up to Day 26. In this group significantly higher values were also noted on Day 22 (5h) and Day 24 (3h).

In the MPH groups locomotor activity increased during the repeated dose phase after 1 and 3 hours post-dose, becoming more prominent as doses raised (Fig. VIII.1). While the study proceeded, increased locomotor activity also appeared prior to dosing. During the withdrawal phase the same pattern remained present.

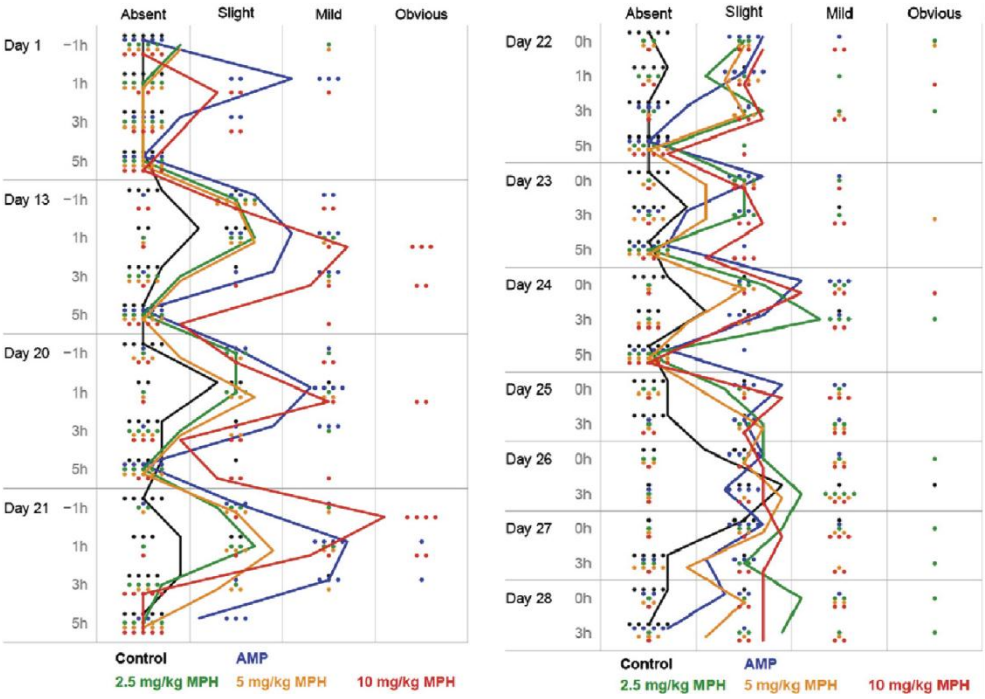

Fig. VIII.1. WD test: incidence of scores (absent, slight, mild, obvious) of locomotor activity per dose group during the repeated dose phase (RD: Day 1 - Day 21) and the withdrawal period (WD: day 22 - day 28). Time points -1 hour pre-dose, +1, +3 or +5 hours post-dose (RD) or versus 0 hours (WD). Dots represent individual data.

Increased locomotor activity, similar in time appearance and severity as in the MPH groups, was also recorded in response to AMP administration and during the subsequent withdrawal period.

Dosing up to 10 mg/kg MPH per day also led to a drug- and dose related incidence of sniffing, grooming, restlessness, hyperreactivity to touch, compulsive behaviour (5 and 10 mg/kg MPH only), excitation and an increased general activity, prior to and up to 3 hours after MPH administration. During the first 2 days after termination of drug treatment these observations (with exception of compulsive behaviour) remained present to the same extent or higher when animals were observed at the predefined time points (data not shown).

Overall body weight and food intake (Fig. VIII.2, Table VIII.3), recorded prior to and 24 hours after dosing on the predefined days of the repeated dose phase and daily (per 24 hours) during withdrawal, were not adversely affected in the various MPH and AMP groups. However, when daily 5 to 6-hour measurements were compared (so-called daytime measurements), body weight gain and food intake were higher (though not always significant) than those of the control group, where a body weight loss and a lower food consumption were measured during daytime (Table VIII.3).

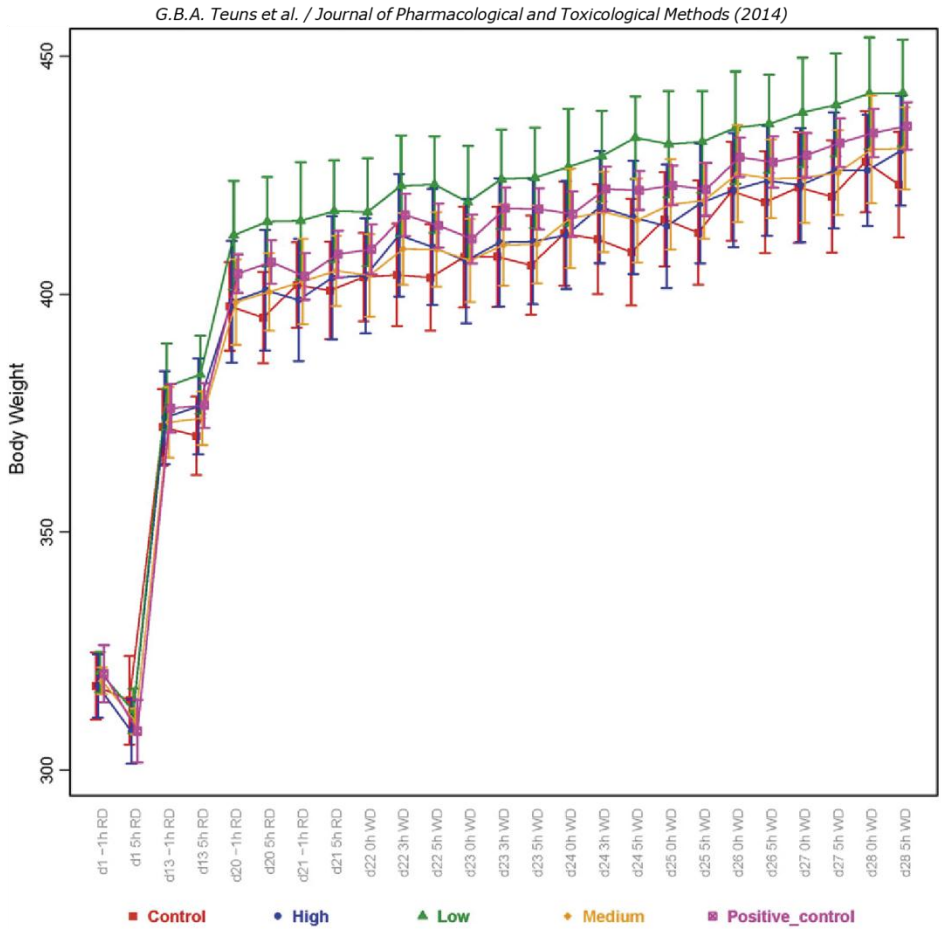

Fig. VIII.2. WD test: Mean body weight (± SEM) evolution during the repeated dose phase (RD: Day 1 - Day 21) and the withdrawal period (WD: day 22 - day 28). Time points -1 hour pre-dose, +1, +3 or +5 hours post-dose (RD) or versus 0 hours (WD). Control: 0 mg/kg MPH, Positive control: 0.8 mg/kg AMP, Low: 2.5 mg/kg MPH, Mid: 5 mg/kg MPH, High: 10 mg/kg MPH.

MPH	Control 0 mg/kg	Positive control 0.8 mg AMP/kg	Low 2.5 mg/kg	Mid 5 mg/kg	high 10 mg/kg
Mean body weight gain					
Repeated dose phase					
Day 1: +5 h vs. −1 h	−3 g	−12 g*	−8 g	−9 g	−10 g
Day 13: +5 h vs. −1 h	−2 g	0 g	+2 g	+1 g	+3 g
Day 20: +5 h vs. −1 h	−2 g	+2 g	+3 g	+2 g	+3 g
Day 21: +5 h vs. −1 h	−1 g	+5 g	+2 g	+2 g	+5 g
Day 20: over 24 h	+4 g	−1 g*	+3 g	+4 g	0 g*
Day 21: over 24 h	+2 g	+6 g	+2 g	+1 g	+5 g
Withdrawal phase					
Day 22: +5 h vs. 0 h	0 g	+5 g	+6 g	+5 g	+6 g
Day 23: +5 h vs. 0 h	−2 g	+6 g*	+5 g	+4 g*	+4 g
Day 24: +5 h vs. 0 h	−4 g	+5 g	+6 g	0 g	+4 g*
Day 25: +5 h vs. 0 h	−3 g	−1 g	+1 g	0 g	+5 g*
Day 26: +5 h vs. 0 h	−2 g	−1 g	+1 g	−1 g	+2 g
Day 27: +5 h vs. 0 h	−2 g	+3 g*	+2 g	+1 g	+3 g
Day 28: +5 h vs. 0 h	−5 g	+2 g*	0 g*	0 g	+4 g**
24 h weight gain: min/max ranges over 24 h from Day 22 to Day 27	+1 to 6 g	+1 to 6 g	+2 to 7 g	−1 to 9 g	+1 to 7 g
Food consumption					
Repeated dose phase					
Day 13:+5 h vs. 0 h	3 g	7 g	6 g	6 g	8 g*
Day 20: +5 h vs. 0 h	5 g	9 g*	8 g	7 g	8 g
Day 21: +5 h vs. 0 h	5 g	10 g	6 g	7 g	8 g
Day 13: over 24 h	26 g	30 g	29 g	28 g	29 g
Day 20: over 24 h	28 g	28 g	28 g	27 g	27 g
Day 21: over 24 h	29 g	32 g	28 g	27 g	30 g
Withdrawal phase					
Day 22:+5 h vs. 0 h	5 g	9 g	8 g	8 g	7 g
Day 23: +5 h vs. 0 h	5 g	11 g*	8 g	8 g	8 g
Day 24: +5 h vs. 0 h	4 g	9 g*	9 g	6 g	8 g*
24 h food consumption: min/max ranges over 24 h from Day 22 to Day 28	28 to 31 g	30 to 32 g	27 to 31 g	28 to 31 g	28 to 31 g

Table VIII.3. WD test: Mean body weight gain data and food consumption during the repeated dose phase and during the withdrawal period, calculated over daytime (+5 hours post dose versus -1 hour or versus 0 hour) and over 24 hours. Significance computed versus Control by Mann-Whitney U (two-tailed): * $p < .05$ ** $p < .01$ *** $p < .001$.

Mortality was absent during the ongoing study.

VIII.2.3.2. Drug discrimination test

Overall data are presented in Table VIII.4.

Day of study	Day 1	Day 8	Day 15	Day 22/36[a]	Day 29/36[b]
Dose groups: mg/kg MPH	C1: 0	L: 2.5	M: 5	H: 10	C2: 0
Selection drug (D)/saline (S) lever	0/12	3/12	10/12	11/12	0/12
% Response selected lever	100 (0.1)	95.24 (3.8)	98.57 (1.3)	99.88 (0.1)	100 (0.0)
FRF value	11 (0.4)	11 (0.4)	11 (0.3)	11 (0.6)	11 (0.3)
Latency (sec) at test session	30 (4.6)	55* (7)	55* (10.7)	83** (24.2)	34 (3.9)
Latency (sec) at last AMP session	53 (13.5)	58 (8)	46 (9)	71 (18.5)	51 (11.4)
Latency (sec) at last saline session	32 (141.1)	48 (5.1)	33 (7.5)	42 (9.8)	31 (4.3)
Drug lever responses	2 (1.1)	259 (146.2)	839*** (137.9)	842*** (117.3)	1 (0.4)
Saline lever responses	1596 (141.1)	1176 (203.9)	188*** (119.9)	87*** (86.2)	1629 (119.8)
Total number of drug + saline lever responses	1598 (141.4)	1435 (119.5)	1028*** (76.0)	928*** (90.0)	1631 (119.7)
Number of responses in the last AMP session	818 (89.4)	812 (70.8)	841 (63.9)	846 (81.2)	859 (74.9)
Mean no. of responses in the last AMP session in the entire study	835 (33)				
Number of responses in the last saline session	1514 (124.8)	1567 (134.6)	1580 (131.6)	1569 (100.2)	1573 (109.3)
Mean no. of responses in the last saline session in the entire study	1561 (52)				

% Response selected lever: Number of responses on the appropriate lever to the total number of responses on both levers in %. FRF value: sum of the responsesmade on either lever before the first reinforcement occurs; latency: time before the first reinforcement occurs; number of responses in the last saline or AMP (08 mg/kg SC) session: the last saline or AMP session on Tuesday,Wednesday or Thursday before each weekly substitution of MPH. Post-hoc Dunnett test: pairwise comparisons of each MPH dose group (2.5, 5 and 10 mg/kg) to the first dose administration of the control (C1) on day 1.
Remark: rat no. 7029 was excluded from confirmatory statistical analysis when tested at the high dose (did not meet the criterion of FRF value b15).
[a] One rat was substituted with 10 mg/kg MPH on day 36 because of failure to meet the acceptance criteria during a training day prior to substitution on day 22.
[b] One rat was substituted with control solution (C2) on day 36 because of failure to meet the acceptance criteria during a training day prior to substitution on day 29.

Table VIII.4. DDL test: Overall data after single escalating doses of MPH (OR). C1: 0 mg/kg MPH, L (Low): 2.5 mg/kg MPH, M (Mid): 5 mg/kg MPH, H (High): 10 mg/kg MPH; C2: 0 mg/kg MPH (SEM between brackets). [#]: one rat was substituted with 10 mg/kg MPH on Day 36 because of failure to meet the acceptance criteria during a training day prior to substitution on Day 22. [@]: one rat was substituted with control solution (C2) on Day 36 because of failure to meet the acceptance criteria during a training day prior to substitution on Day 29. % responses selected lever: number of responses on the appropriate lever to the total number of responses on both levers in %. FRF value: sum of the responses made on either lever before the first reinforcement occurs; Latency: time before the first reinforcement occurs; Number of responses in last saline or AMP (08 mg/kg SC) session: last saline or AMP session on Tuesday, Wednesday or Thursday before each weekly substitution of MPH. Post-hoc Dunnett test: pairwise comparisons of each MPH dose group (2.5, 5 and 10 mg/kg) to the first dose administration of the control (C1) on Day 1. Remark: Rat No. 7029 was excluded from confirmatory statistical analysis when tested at the high dose (did not meet the criterion of FRF value <15).

Doses of 2.5, 5 and 10 mg/kg MPH resulted in an amphetamine-like drug lever selection in 3, 10 and 11 out of 12 rats/dose group, respectively; the overall drug lever responses on the selected drug lever, expressed as a % of the total number of responses emitted on both levers during the test session were 25.0%, 83.3% and 91.7%, respectively. Of the twelve rats tested at 10 mg/kg MPH, one rat reached a FRF value of 17, indicating a failing lever selection at the beginning of the session (FRF value needs to be < 15). In all other cases, animals made direct lever selections with FRF values < 15.

The drug lever responses for MPH at 5 and 10 mg/kg dose were statistically significant from saline and comparable to those obtained during the last amphetamine training session preceding the drug testing (Fig. VIII.3). On average drug lever responses > 95% of total responding were reached.

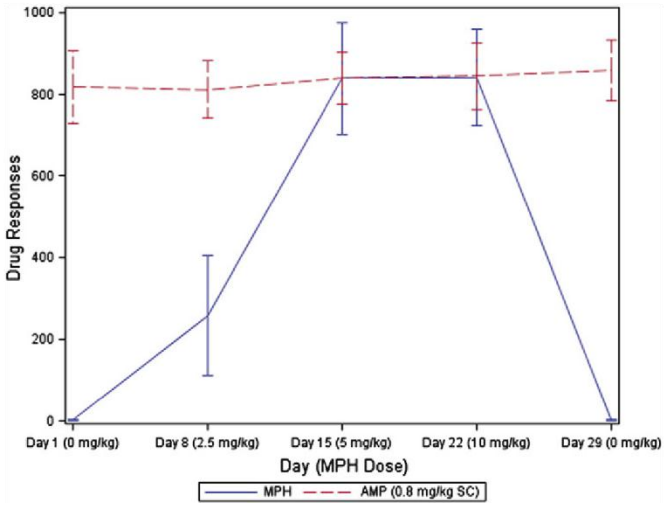

Fig. VIII.3. DDL test: Dose-response curve. MPH (OR): single escalating dose of 0, 2.5, 5 and 10 mg/kg. AMP (SC): 0.8 mg/kg: drug response to AMP at the last training sessions on Tuesdays, Wednesdays or Thursdays prior to weekly substitution of single MPH doses on Fridays (= Days 1, 8, 15, 22, 29).

The total number of responses on both levers decreased in a dose linear manner (significant from 5 mg/kg MPH onwards), indicative of rate reducing properties of higher doses of MPH.

The test-latency time before the first reinforcement occurred increased with dose and was significantly higher for all MPH doses compared with the control group C1 on Day 1, but in line with or slightly higher than the latency time recorded during the last AMP sessions in the various dose groups.

The mean number of responses for saline (n = 1561) during all last saline training sessions in the study was substantially higher than that for AMP (n = 835).

Rats remained in a good general condition during the study period and mortality was absent.

VIII.2.3.3. Conditioned place preference test

Four rats of the various dose groups experienced an individual preference of more than 75% for the black or white compartment during pretest. This was not taken into account for conditioning as an unbiased procedure (based upon body weight data) was used for ranking the animals into different dose groups and subsequent subgroups, with 50% of the rats assigned to conditioning in the white drug-paired compartment and 50% assigned to conditioning in the black drug-paired compartment.

Table VIII.5 shows the overall results of the present study.

Dose group MPH: OR AMP: SC	Number of rats	A	B	C	D	E	F
		Number of rats with preference for drugpaired compart-ment[a]	Ratio of black/white drug-paired compartment	Ratio of average time spent in drug-paired versus non-drug-paired compartments[b]	Ratio of average time spent in drugpaired compartment versus that of control[b]	Ratio of average time spent in gray compartment versus that of control[b]	Ratio of average time spent in non-drug-paired compartment versus that of control[b]
MPH control, 0 mg/kg	12	3	2/1	0.79	1.00	1.00	1.00
MPH low, 2.5 mg/kg	12	6 (0.4003)	2/4	0.99 (0.5233)	1.01 (0.8914)	1.18 (0.0415)	0.81 (0.0418)
MPH mid, 5 mg/kg	12	6 (0.4003)	5/1	1.05 (0.2499)	1.12 (0.2821)	1.06 (0.5003)	0.85 (0.0983)
MPH high, 10 mg/kg	12	9 (0.0391)	5/4	1.54 (0.0073)	1.55 (b0.0001)	0.77 (0.0102)	0.80 (0.0306)
AMP, 0.8 mg/kg	12	11 (0.0028)	5/6	2.16 (b0.0011)	1.80 (b0.0001)	0.71 (0.0011)	0.66 (0.0005)

[a] Significance for AMP, 2.5 (low), 5 (mid) and 10 (high) mg/kg MPH group(s) computed versus control by Fisher exact probability test (two-tailed).
[b] Significance for AMP, low, mid and higH MPH group(s) computed versus control by ANOVA (p-values between brackets).

Table VIII.5. CPP test: Overall results.

The number of MPH-conditioned rats with preference for the drug-paired compartment increased in a dose dependent way, being statistically significant at 10 mg/kg MPH (Table VIII.5 column A). At this dose a difference in the preference for the black or the white drug-paired compartment in the subgroups (column B) was no longer present (ratio 5/4 for black/white drug paired compartment) and the number of rats showing a drug-induced place preference amounted to that of AMP (9 out of 12 rats in the 10 mg/kg MPH dose group or 75% versus 11 out of rats or 92% in the AMP group). The average time spent in the drug-paired compartment (columns C and D) increased in a drug- and dose related pattern whereas the average time spent in the neutral (grey) compartment (column E) decreased in a drug- and dose related way, all being statistically significant at 10 mg/kg MPH. At this dose the time spent in the neutral (grey) compartment was as low as that of the AMP group (ratios: 0.77 and 0.71, respectively).

The time spent in the non-drug-paired compartment (column F) was lower in the MPH dosed groups compared with the control group but of equal order of magnitude for all three MPH dosed groups. In the AMP positive control group the time spent in the non-drug-paired compartment was significantly lower compared with the control group.

A confirmatory ANOVA analysis on the difference between posttest and pretest with respect to the time spent in the drug-paired compartment showed no evidence of an effect of the colour of the drug-paired compartment but again a significant difference ($p<0.0001$) between the different groups. Since a significant effect for group was picked up, the nature of that effect was further investigated by posthoc pairwise comparisons of the different groups. These results revealed that the dependent variable, i.e. the excess amount of time spent in the drug-paired compartment at posttest as compared to pretest) was larger in the AMP positive control group and in the 10 mg/kg MPH dose group than in the control, 2.5 and 5 mg/kg dose groups (P-values for the comparison of the PC versus C, L, M and H were <0.0001, <0.0001, 0.0010, and 0.2551, respectively; P-values for the comparison of the 10 mg/kg MPH versus C, L and M were 0.0002, 0.0007 and 0.0238, respectively).

Neither mortality nor adverse clinical symptoms occurred during the course of the study.

VIII.2.3.4. Self-administration test

Data are presented in Table VIII.6.

Phase	Dose (mg/kg/inf.)	Number of rats per phase	Mean number of infusions over the last three daily sessions per phase		
			Mean	SEM	p-Value
Phase 1: AMP	0.06	n = 32	9	0.51	0.0005
Phase 2: saline	0	n = 32	5	0.61	–
Phase 3: AMP	0.06	n = 32	10	0.44	<0.0001
Phase 4: MPH	0	n = 7	7	1.38	0.4634
Phase 4: MPH	0.05	n = 25	17	1.05	<0.0001
Phase 4: MPH	0.1	n = 9	19	0.40	<0.0001
Phase 4: MPH	0.5	n = 8	7	0.94	0.9749
Phase 4: MPH	1	n = 8	5	0.27	0.9985
Phase 5: AMP	0.06	n = 32	13	0.67	<0.0001

Table VIII.6. IV SA test: Number of infusions over the last three daily sessions per phase: summary statistics. Significance for AMP (phases 1, 3, 5), and MPH groups [phase 4: 0 (control: NaCl 0.9%), 0.05 (extra low), 0.1 (low), 0.5 (mid), 1 (high) mg/kg/inf.] computed versus saline (phase 2) by Anova. For ease of reading the extra low dose of 0.05 mg/kg MPH (presented during phase 6) is listed under phase 4.

The mean numbers of AMP infusions for the 32 rats during the last three days of each AMP phase were 9, 10 and 13, respectively (phase 1, 3 and 5 in the present study), all being statistically significant versus saline in phase 2.

The maximum AMP intake was calculated from the highest number of 20 infusions, obtained in three rats during the third reinstatement (phase 5), and amounted to 1.20 mg/kg AMP. This dose remained below the IV LD50 of 30 mg/kg AMP. The overall AMP mean daily intake (as IV self-administrations over 1 hour/day) was calculated based upon the mean number of infusions over the three AMP sessions for all animals and reached 0.6 mg/kg/day (mean of 10 infusions/daily sessions, data not shown).

When the possibility of self-administering saline (phase 2) was offered, a sharp increase or extinction burst was noticed in the number of infusions during the

session on the first day (average of 10 infusions per 1-hour session), followed by a gradual decrease in the number of responses during the subsequent days to a mean of 5 infusions over the last 3 days.

The self-administration of MPH showed an average of 19 infusions at the low dose (0.1 mg/kg/inf.), 7 infusions at the mid dose (0.5 mg/kg/inf.), and 5 infusions at the high dose (1 mg/kg/inf.) during the last three days of the 4th phase (Table VIII.6). When the extra low dose of 0.05 mg/kg/inf. was offered to the rats a mean of 17 infusions was obtained during the last three days. The average number of infusions in the extra low and low doses of MPH showed a statistically significant increase versus saline, comparable to that of AMP.

For the self-administration of the control solution (NaCl 0.9%) an extinction burst was noticed during the first 2 days of this phase (means of 13 and 10 infusions per daily 1-hour session, respectively), declining to a mean average of 7 infusions per session during the last 3 days of this phase.

The maximum daily intake in the extra low dosed group (0.05 mg/kg/infusion), calculated from the maximum of 20 infusions obtained in 20 out of 25 rats, corresponded to 1 mg/kg MPH. In the high dosed group (1 mg/kg/infusion) one rat self-administered 15 injections as the highest number of total drug infusions per session, correlating to a maximum daily intake of 15 mg/kg MPH. This latter dose was still below the IV LD50 of MPH (48 to 50 mg/kg) in the naive rat.

A mean daily MPH intake of 0.9, 1.9, 3 and 5 mg/kg was calculated for the extra low, low, mid and high dose, respectively, based upon the mean number of infusions calculated over the 5-day test sessions (data not shown).

Of the 48 rats initially ordered, 16 rats did not complete the actual study. One rat died during surgery and fifteen rats were preterminally killed during the training phase (6 rats) or during the actual study part (nine rats). Main cause of prescheduled death was obstruction of the catheter (11 rats) or a poor general condition (3 rats). One rat was killed because of hind leg paralysis.

Excitability was observed in some animals during the AMP and/or MPH phases. Transient skin lesions around the vascular access button were noticed on occasion. Tremors and convulsions were recorded after the manually injected MPH boli on the day of blood sampling for toxicokinetic purposes.

VIII.2.3.5. Toxicokinetic parameters

VIII.2.3.5.1. Withdrawal test

Plasma exposures measured at 180 and 300 minutes after a single or repeated dose of 2.5, 5 and 10 mg/kg MPH showed an increase with dose, being more than dose proportional from 5 to 10 mg/kg (Table VIII.7).

WD	OR route	2.5 mg MPH/kg	5 MPH/kg	10 MPH/kg	
		Mean plasma concentration (ng/ml)			
Day 1 180 min		0.819	1.726	7.473	
Day 21 180 min		0.447	1.075	5.876	
Day 1 300 min		0.389	0.716	1.985	
Day 21 300 min		0.137 (n = 1)	0.202 (n = 2)	0.800	
Day 1 24 h		BQL < (0.100)	BQL < (0.100)	BQL < (0.100)	
Day 21 24 h		BQL < (0.100)	BQL < (0.100)	BQL < (0.100)	
DDL	OR route	2.5 MPH/kg		10 MPH/kg	
		Mean plasma concentration (ng/ml)			
15 min		17.01^{S}	12.25^{A}	171.17^{S}	231.00^{A}
30 min		22.31^{S}	18.37^{A}	138.63^{S}	270.33^{A}
45 min		13.92^{S}	12.27^{A}	111.33^{S}	210.67^{A}
60 min		10.83^{S}	9.67^{A}	113.00^{S}	157.67^{A}
CPP	OR route	2.5 MPH/kg	5 MPH/kg.	10 MPH/kg	
		Mean plasma concentration (ng/ml)			
30 min		23.47	69.03	96.37	
60 min		13.52	62.00	66.27	
150 min		2.51	11.99	12.00	
300 min		BQL < (1.00)	1.51 (n = 1)	1.59 (n = 2)	
IV SA	IV route	0.5 mg MPH/kg	5 mg MPH/kg	15 MPH/kg	
		Mean plasma concentration (ng/ml)			
3 min		161.33	1216.97	6226.67	
6 min		134.00	1263.33	4210.00	
20 min		74.23	882.67	4506.67	
60 min		37.17	407.67	1556.67	

DDL: ^{S}: Samples taken fromratswhich were dosedwith saline at 24 hs before the MPH dose; ^{A}: samples taken fromrats which were dosedwith AMP at 24 h before the MPH dose. IV SA: 0.5 mg MPH/kg: corresponds with the dose calculated from theminimumnumber of infusions/h obtained in one rat of the 0.05 mg/kg/infusion lowdose group. IV SA: 15 mg/kg MPH/kg: corresponds with the dose calculated from the maximum number of infusions/h obtained in one rat of the 1 mg/kg/infusion high dose group.

Table VIII.7. Toxicokinetics: Overview of the mean plasma concentrations of the various dosages in the four studies per chosen time points. Min: minutes; h: hours; BQL: below quantification limit; N: number of samples. DDL: ^{S}: samples taken from rats which were dosed with saline at 24 hours before the MPH dose; ^{A}: samples taken from rats which were dosed with AMP at 24 hours before the MPH dose. IV SA: 0.5 mg MPH/kg: corresponds with the dose calculated from the minimum number of infusions/hour obtained in one rat of the 0.05 mg/kg/infusion low dose group. IV SA: 15 mg/kg MPH/kg: corresponds with the dose calculated from the maximum number of infusions/hour obtained in one rat of 1 mg/kg/infusion high dose group.

MPH plasma concentrations were lower at all dosages after repeated dose compared to single administration; in the low and mid dose some plasma concentrations were below the quantification limit (0.1 ng/ml) when measured at time point +300 minutes at the end of the repeated dose phase (Day 21).

Bioanalytical analysis of the 24h plasma samples after single or repeated dosing confirmed that no measurable concentrations remained present, thus implying absence of drug in plasma from the first day of withdrawal onwards.

VIII.2.3.5.2. Drug discrimination test

Bioanalytical results of blood samples after a single dose of 2.5 and 10 mg/kg MPH (OR) confirmed that the T_{max} was 30 minutes post-dose, corresponding to the time-interval between dose administration and entrance into the operant chambers (Table VIII.7). Peak plasma exposures represented by mean C_{max} were more than dose proportional between the chosen doses.

The plasma exposures after a single dose of 2.5 mg/kg MPH (OR) were slightly lower when AMP was administered 24 hours prior to this MPH dose compared to those levels in rats which had received saline 24 hours before (ratios of 0.72, 0.82, 0.88 and 0.89 at the 15, 30, 45 and 60 minutes sampling time points, respectively).

Administrating 10 mg/kg MPH 24 hours after the last AMP treatment led to plasma concentrations that were 1.34 to 1.95 times higher than those measured in rats treated with saline 24 hours prior to this single MPH dose. The difference in exposure was most pronounced at peak time (30 minutes).

The rat peak plasma exposures showed a ratio of 1.7 to 2 up to 13 to 25-fold the human efficacious exposure (Table VIII.1, data Ritalin®, children) for the 2.5 and 10 mg/kg, respectively.

VIII.2.3.5.3. Conditioned place preference test

The mean bioanalytical results of blood sampled at 30, 60, 150 and 300 minutes after single administration of 2.5, 5 and 10 mg/kg MPH (OR) confirmed that peak plasma exposures (C_{max}) were reached at 30 minutes (T_{max}) for all dosages

(Table VIII.7). There was a more than dose proportional increase in C_{max} between 2.5 and 5 mg/kg but peak plasma exposures were less than dose proportional between 5 and 10 mg/kg.

The C_{max} obtained after a single dose of 2.5 mg/kg MPH (23.47 ng/ml) was 2-fold the human plasma exposure (10.8 ng/ml, corresponding to the recommended human daily dose of 0.30 mg/kg, Table VIII.1, data Ritalin®, children) whereas the 5 and 10 mg/kg MPH dose reached a nearly 7- and 10-fold exposure of this human efficacious dose (C_{max}), respectively.

VIII.2.3.5.4. Self-administration test

Bioanalysis of the blood samples taken at 3, 6, 20 and 60 minutes after a single IV dose administration of 0.5, 5 and 15 mg/kg MPH revealed an increase with dose at all time-points which was less than dose proportional from 0.5 to 5 mg/kg and more than dose proportional from 5 to 15 mg/kg (Table VIII.7).

From the present data it was shown that the peak plasma exposure of the lowest dose as obtained in this study (0.5 mg/kg MPH: 161.33 ng/ml) was nearly 16-fold the human efficacious concentration (10.8 ng/ml; Table VIII.1, data Ritalin®, children). The highest dose self-administered in this study (15 mg/kg MPH, obtained in one rat of the high dose group at 1 mg/kg/infusion) resulted in a peak plasma level of 6226.67 ng/ml which was more than 600-fold the human efficacious exposure.

VIII.2.4. Discussion

VIII.2.4.1. Results of the four studies

Methylphenidate, a CII scheduled drug of abuse, was selected to evaluate the predictivity of the four described standardised preclinical tests as applied within drug development to investigate abuse potential of CNS-active compounds. The overall result of these studies conducted with MPH is shown in Table VIII.8.

Study type	Dose methylphenidate per group					Adm. route	Positive control	Dose	Adm. route
WD	0	2.5*	5*	10* mg/kg		OR	D-Amphetamine	0.8 mg/kg*	SC
CPP	0^{-}	2.5^{-}	5^{-}	10^{+++} mg/kg		OR	D-Amphetamine	0.8 mg/kg^{+++}	SC
DDL	0^{-}	2.5^{++}	5^{+++}	10^{+++} mg/kg		OR	D-Amphetamine	0.8 mg/kg^{+++}	SC
IV SA	0^{-}	0.05^{+++}	0.1^{+++}	$0.5^{+/-}$	$1.0^{+/-}$ mg/kg/inf.	IV	D-Amphetamine	0.06 mg/kg/ inf.$^{+++}$	IV

*: Exaggerated pharmacological effects, no withdrawal symptoms. CPP, DDL: $^{-}$: no effect; $^{+}$: <25% of ratswith positive and/or statistically significant effect: alert; $^{++}$: 25–70% of rats with positive and/or statistically significant effect: signal; and $^{+++}$: >70% of rats with positive and/or statistically significant effect: full confirmation of effect as biologically relevant. IV SA: $^{+/-}$: inverse response to dose; and $^{+++}$: positive and/or statistically significant effect: full confirmation of effect as biologically relevant.

Table VIII.8. Overview of the results on prediction of abuse potential in the four studies: WD: withdrawal; CPP: conditioned place preference; DDL: drug discrimination learning; IV SA: intravenous self-administration. Adm. Route: route of administration (OR: oral, SC: subcutaneous). Effects at dose versus biological relevance: WD: *: exaggerated pharmacological effects, not indicative of physical dependence potential. CPP, DDL: -: no effect; +: <25% of rats with positive and/or statistically significant effect: alert; ++: 25-70% of rats with positive and/or statistically significant effect: signal; +++: >70% of rats with positive and/or statistically significant effect: full confirmation of effect as biologically relevant. IV SA: +/-: inverse response to dose; +++: positive and/or statistically significant effect: full confirmation of effect as biologically relevant.

Little information on preclinical testing of withdrawal of MPH in rats was found in the literature.

The current withdrawal study showed that the pharmacotoxicological profile associated with stimulants acting through the dopamine pathway (increasing extracellular dopamine by inhibiting the dopamine transporter) was clearly present in all MPH groups during the repeated dose phase. Dosing MPH up to 10 mg/kg resulted in a drug- and dose related excitation and concomitant stereotypic behaviour, increased locomotor activity and hyperthermia (only at 10 mg/kg) up to 3 hours after daily dosing. These features are considered specific observations for stimulants (Kuczenski, 2006; Shire, 2011; Levi, 2012). Also the alteration of the diurnal rhythm, as evidenced by a higher body temperature prior to dosing, increased diurnal body weights and food intake, and enhanced daytime activity is associated with this type of compounds (Gaytan, 2000; Askenasy, 2006; Levi, 2012). As dopamine is known to mediate the reward value of food, the sustained increased dopaminergic activity after repeated administration of MPH can indeed lead to non-physiologic overfeed behavior (Hajnal, 2000; Baldo, 2007; Volkow, 2008; Vucetic, 2010; Volkow,

2011). These diurnal body weight and food intake increases (measured over 5 to 6 hours) did however not lead to an increase in either overall body weight gain or in total food intake.

When the kinetics were taken into account, the lower concentrations measured after 21 dosing days were inversely proportional to the clinical effects, which might be indicative of a developed sensitization to MPH.

However, classical signs indicative of physical dependence after abrupt cessation were not present and this was in line with the clinically known low physical dependence of MPH. On the contrary, the effects noticed during the repeated dose phase remained present to the same extent or greater during the withdrawal period. This phenomenon cannot be explained by the toxicokinetic data as MPH was no longer present after 24 hours post-dose and as accumulation did not appear. From literature it is known that the half-life for recovery of the dopamine transporter (DAT) takes more than 6 days (Fleckenstein, 1996); in addition it has been demonstrated that for cocaine, another known stimulant, abrupt cessation led to a further decrease of DAT during the 10 days of withdrawal (Pilotte, 1996; Fleckenstein, 1996). The sustained suppression of DAT and its slow recovery might also be present after abrupt cessation of MPH (Swanson, 2003), thus explaining the persistent or exacerbating activities noticed during the treatment-free withdrawal period in the present study. Other data, showing that dopamine neuronal impulse activity was increased after 3 days of withdrawal from MPH when previously administered to adolescent rats (Brandon, 2003), might also be supportive for this hypothesis.

Repeated administration of AMP (0.8 mg/kg, SC) and subsequent cessation resulted in similar findings.

In the drug discrimination test it was shown that MPH generalized to AMP in a drug- and dose related overall response, ranging from 25% at 2.5 mg/kg to nearly 100% at 5 and 10 mg/kg. These results demonstrate a positive signal for the discriminative stimulus properties of MPH at 2.5 mg/kg, and an absolute confirmation of these properties at 5 and 10 mg/kg MPH (Table VIII.8). The

number of discriminative responses of MPH at 5 and 10 mg/kg (839 and 842 responses, respectively) were similar to the number of drug responses in the AMP stimulus training sessions during the study (mean of 835 responses), and supported the biological relevance of the results at 5 and 10 mg/kg MPH.

The toxicokinetic data confirmed that the 30 minutes-interval between oral administration of MPH and entrance into the operant chambers was optimal to reach peak plasma levels of MPH in the rats during testing. Considering the plasma concentrations, a more than dose proportional increase in exposure was measured over the chosen dose range.

When toxicokinetic data were further verified in detail, plasma exposures after a single dose of 2.5 mg/kg MPH were slightly lower (0.72 to 0.89-fold at the various sampling time points) when rats were exposed to AMP 24 hours before dosing compared to the plasma levels of rats exposed to saline 24 hours prior to this MPH dose.

On the contrary, remarkably higher exposure levels (1.34- to 1.95-fold at the various sampling time points, most pronounced at the T_{max} of 30 minutes) were measured in those rats that had received AMP 24 hours prior to the single 10 mg/kg MPH dose versus those plasma levels measured in rats that had received saline 24 hours before. Although it was found in literature that chronic dosing of AMP increases its half-life, no drug-drug interactions of MPH with AMP have been reported (Concerta®, 2012; Shire, 2011). It might be useful to maintain the described design for toxicokinetic blood sampling, in order to obtain valuable kinetic data when compounds of a pharmacological class similar to their training drug are being tested in drug discrimination generalization studies. Another possibility is to terminate further exposure to the training drug or to saline after the last substitution has been executed, and to perform blood sampling for toxicokinetic purposes in the tested rats after a 1-week washout period.

Interpretation of the conditioned place preference data was not restricted to the absolute number of animals of the various groups showing a drug-induced place preference at posttest, but the calculated ratios of the average time spent in the drug-paired versus non drug-paired compartment per dose group and the

subsequent ratios of the average time spent in the drug-paired, non-drug-paired and grey (neutral) compartment, calculated per dose group versus control were also taken into consideration.

With regard to the most time spent in the drug-paired compartment at posttest, a place preference was recorded in 25, 50, 50 and 75% of the rats when conditioned at 0, 2.5, 5 and 10 mg/kg MPH, respectively. In the AMP group this number amounted to 92%. However, the calculated ratio of the average time spent in the drug-paired versus non drug-paired compartment per dose group and the ratio of the average time spent in the drug-paired compartment per dose group versus control demonstrated that a statistically significant increase was only obtained in the 10 mg/kg MPH group and in the AMP group. Besides, using a 3-compartment place preference apparatus implies that the time spent in the neutral (grey) compartment is also a parameter considered of significant relevance to the evaluation of results. In the present study, the time spent in the neutral compartment at posttest was significantly lower at 10 mg/kg MPH (versus control), and equal to that of the AMP group.

Based upon these parameters it was concluded that a firm conditioned place preference for the drug-paired compartment was only achieved at 10 mg/kg MPH, comparable with that of the AMP group (Table VIII.8), thus demonstrating a clear signal for the reinforcing properties of MPH at this dose. This was also evidenced by the excess amount of time spent in the drug-paired compartment at posttest compared to pretest, being significantly higher in the AMP group and in the10 mg/kg MPH group versus that recorded at 2.5 and 5 mg/kg MPH.

Employing the unbiased procedure with subdividing the different dose groups further into groups of equal numbers of animals, being conditioned in either the black or the white drug-paired compartment for 16 days, demonstrated that at 10 mg/kg MPH the individual preference for either the black or the white drug-paired compartment as well as the physiological preference for the black compartment disappeared, thus strengthening the face validity of the study (Cunningham, 2003).

Since the time spent in the non drug-paired compartment was low but equal at all MPH dosages tested in the present study, it would be of interest to investigate whether the data of the neutral (grey) compartment in a conditioned place aversion model will be indicative of a shift towards the non drug-paired compartment as well.

MPH presented to rats in a self-administration model showed explicit reinforcing properties using AMP as reference drug. As the responses at 0.5 (mid dose) and 1 mg/kg/infusion (high dose) were lower than those recorded at 0.05 (extra low dose) and 0.1 mg/kg/infusion (low dose) it can be concluded that the dose-response in this IV SA study was not linear but followed a bell-shaped dose-response curve with the peak of responses of the low and extra low dose situated on the left ascending site of this curve and those of the mid and high doses positioned at the right descending site (Botly, 2008). This curve is also suggestive of a cumulative effect on the response rate that is obtained when animals are self-injecting higher and increasing concentrations of MPH at doses from 0.5 mg/kg/inf. (mid dose) onwards, thus resulting in much lower IV SA responses during the 1-hour daily test sessions compared to the 0.05 (extra low) and 0.1 (low) mg/kg/inf. MPH dosages. This phenomenon of inverse response to dose, visualised by the bell shaped dose-response curve, has been described for a variety of self-administrated drugs (Van Ree, 1999; Piazza, 2000; Broadbear, 2004; Peana, 2010).

The lowest daily MPH intake, recorded in the 0.1 mg/kg/infusion low dose group was 0.5 mg/kg/hour (5 infusions), and corresponded with a C_{max} of 161.33 ng/ml, whereas the highest daily MPH intake was 15 mg MPH/kg/hour (obtained in the 1 mg/kg/infusion high dosed group and correlating with 20 infusions and a C_{max} of 6226.67 ng/ml). When comparing the toxicokinetic data of both the CPP and the IV SA study, apparently a mean C_{max} of approximately 100 ng/ml (96.37 ng/ml at 10 mg/kg MPH in the CPP and 161.33 ng/ml at the lowest daily MPH intake in the IV SA) was needed to clearly demonstrate the reinforcing properties of MPH in rats, independent of the different models or administration routes used. Nevertheless, some differences between the two models remain: the dose-effect response was linear in the CPP and bell-shaped in the IV SA. The exposure versus dose curve in the CPP was more than dose proportional from

2.5 to 5 mg/kg but less than dose proportional from 5 to 10 mg/kg MPH, whereas in the IV SA peak plasma exposures showed a more than dose proportional relationship over all dosages tested in the IV SA (Table VIII.7).

Some rodent models have been described in literature as being ADHD models (Russell, 2005; Grund, 2006; Wickens, 2011; Sagvolden, 2012). Preclinical studies performed with different strains of rats [the Spontaneously Hypersensitive Rat (SHR) rat as an ADHD model versus the physiologically normal Wistar rat] to investigate the reinforcing properties of MPH did however not show significant differences in sensitivity (dela Peña, 2012), nor were pharmacological differences in brain activity detected (Grund, 2006). The results of the 4 preclinical studies presented here demonstrated that the common Sprague-Dawley rat is a valid strain for abuse liability testing of MPH. In addition, clinical studies did not show aberrant clinical reactions after oral administration of stimulants to healthy subjects versus ADHD patients (Wickens, 2011). However, healthy subjects are likely to be more susceptible to the reinforcing properties of MPH when used in non-medical situations (dela Peña, 2012; HHS, 2006).

Taking this into account, preclinical data of abuse liability testing, obtained in the common Sprague-Dawley rat can be considered representative for the abuse potential that might occur in a normal, non-diseased human population, thus adding to the predictivity of the translational approach for testing abuse potential of CNS-active compounds as MPH.

VIII.2.4.2. Discussion on the predictivity of abuse liability testing during drug development using the preclinical standardized tests as described and compliance hereof with the regulatory requirements.

MPH is considered a first-line drug treatment for ADHD (Evans, 2004; NHS, 2009), although it has been scheduled as a CII compound for abuse potential (FDA-DEA, 2012, Convention on Psychotropic Substances, 1971). The drug is considered safe when administered at oral therapeutic doses in a medical treatment paradigm (Ritalin®, 2001), because the maximum efficacious plasma exposure (around 10 ng/ml) is reached slowly (within 2 hours) and thus not

primarily accountable for acute euphoria. However questions remain when MPH is being misused by healthy subjects, as the risk for abuse seems to be higher with nonmedical treatment practices (dela Peña, 2012) or when other routes than the oral one are being explored (Morton, 2000).

Regulatory guidance suggests that the dose range used to evaluate preclinical abuse liability testing of CNS-active compounds, should be based upon the ratio between the rat plasma values and the human efficacious dose, both being expressed as C_{max}. The high dose should represent a several-fold this human peak plasma exposure, if not limited by adverse effects or other safety concerns (EMA, 2006; ICH, 2009; FDA, 2010; Bonson, 2011). The recommended clinical dose of MPH ranges from 18 to 72 mg per day, equivalent to 0.25 and maximum 2 mg/kg/day (Concerta® prescription label, present). A therapeutic dose of 0.30 mg/kg in children, also tested in human healthy subjects corresponded to a C_{max} of 10.8 and 7.8 ng/ml, respectively (Table VIII.1; Wargin, 1983; Ritalin®, 2001; Shram, 2012). These dose concentrations were used to calculate the ratio of rat peak plasma exposure versus human plasma exposure (rat/Hu) and are presented in Table VIII.9.

Species	Healthy human subjects			ADHD subjects	SD rat		
	[a]Male, 18–45 years	[b]Male, 21–40 years	[b]Male, 21–40 years	[b]Children, 7–12 years	[c]Male, 8 weeks to 6 months		
Dose mg OR	54	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
Dose mg/kg OR	n.a.	0.15	0.3	0.3	2.5	5	10
C_{max}	14.3	3.5	7.8	10.8	20.34–23.47	69	204.48-96.37
Dose mg IV	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
Dose mg/kg IV	n.a.	n.a.	n.a.	n.a.	0.5	5	15
C_{max}	n.a.	n.a.	n.a.	n.a.	161.33	1263.33	6226.67

C_{max} ratio rat/hu				
2.5 mg/kg OR	1.4–1.6	5.8–6.7	2.60–3.00	1.88–2.17
5 mg/kg OR	4.8	19.71	8.84	6.38
10 mg/kg OR	6.7–14.3	27.53–58.42	12.35–26.21	8.92–18.93
0.5 mg/kg IV	11.28	46.09	20.68	14.93
5 mg/kg IV	88.34	360.95	161.96	116.97
15 mg/kg IV	435.43	1779.04	798.29	576.54

[a] Shram et al. (2012).
[b] Wargin et al. (1983).
[c] Oral animal data: from present CPP and DDL (see Table 7 for details); and IV animal data: from present IV SA (see Table 7 for details).

Table VIII.9. Calculation of C_{max} ratio rat/hu: based upon the human (hu) plasma concentrations and maximum plasma concentration ranges obtained in the present rat studies. [a](Shram, 2012); [b](Wargin, 1983) [c]Oral animal data: from present CPP and DDL (see Table VIII.7 for details); IV animal data: from present IV SA (see Table VIII.7 for details).

The results show that a low oral dose of 2.5 mg MPH/kg tested in the rat CPP and DDL versus a human therapeutic dose of 0.3 mg/kg gave a peak plasma exposure ratio of 1.9 to 2.2 in children and of 2.6 to 3.0 in adults. Increasing doses of orally administered MPH up to 10 mg/kg (high dose) in the rat showed peak plasma exposure ratios (rat/Hu) ranging from 8.92 to 18.9 in children and from 12.4 to 26.2 in adults. When the data of the IV SA test were taken into account, peak plasma exposure ratios of 14.9 up to 798.3 were obtained (children and adults). In terms of doses for the latter test, IV SA clearly showed reinforcing properties at a very low dose from 0.05 mg/kg/infusion onwards in rats, correlating with mean daily intakes of 0.90 mg/kg or 3 times the human therapeutic dose of 0.30 mg/kg.

Overall the concentration ranges achieved by the effective doses of MPH in the present studies were in accordance with the proposed relation to human therapeutic concentrations as suggested in regulatory guidance.

The difference of T_{max} in rodents versus human subjects after oral dosing of MPH, showing a much faster onset in C_{max} when tested in rodents [T_{max}: 30 minutes in rats versus 2-6 hours in humans (Ritalin®, 2001; Swanson, 2003)] might give rise to an overestimation of the risk factor to abuse MPH orally. It would be interesting to further investigate this finding using various scheduled marketed drugs to assess the impact on predictivity hereof.

The efficacious dose at which a clear reinforcement of MPH was obtained in rodents was considered approximately 100 ng/ml (C_{max}), which was 10x higher than the human efficacious dose of 10 ng/ml (CHu eff).

In conclusion, the known abuse potential of MPH, studied by means of tests investigating the direct (IV SA model) or indirect (CPP model) reinforcement was easily demonstrated via the oral (CPP) route as well as the IV route (SA) at a plasma exposure of approximately 100 ng/ml, corresponding to about 10-fold the human efficacious dose of 10.8 ng/ml. The dose-responses were linear in the CPP but showed a bell-shaped curve in the IV SA model. It can be assumed that the CPP, as executed through the described standardized model, is a valuable method to test reinforcing properties if the IV route, as in the IV SA, cannot be utilized due to insolubility of compounds at the required doses.

Drug profiling via the DDL indicated a similar and biologically relevant response for MPH at 5 and 10 mg/kg as for AMP (0.8 mg/kg), showing a linear dose relationship.

Physical dependence, defined as adaptation of the body to the drug and as such evidenced by tolerance during the repeated dose administration and/or withdrawal symptoms after cessation, and tested in the WD, was not present as such since the effects noticed during withdrawal after repeated dose administration up to 10 mg/kg MPH were considered exaggerated pharmacological effects due to the slow recovery of the dopamine transporter

(DAT), more than being classical withdrawal symptoms. The absence of plasma exposures after 24 hours post-dose and during withdrawal, demonstrated that the effects during the withdrawal period were not caused by effective presence of MPH.

The presented four preclinical tests, completed as described, have proven their value to confirm the abuse potential of MPH and showed a predictive validity to study abuse potential of CNS-stimulants in a drug development environment, and can therefore be used for preclinical abuse liability assessments.

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IX

Summary, General Discussion and Future Perspectives

Summary and general discussion

Drug-induced addiction is a complex, chronically relapsing disease, clinically characterized by compulsive behavior to seek and take a drug, loss of control to limit the drug intake, and a negative emotional state when access to the drug is prevented (Camí, 2003; Koob, 2011; NIDA, 2012). Form a biological point of view, it is considered to derive from neuroplastic changes in the brain, which can even be introduced by a single drug administration only, in vulnerable individuals (Koob, 2010; Furst, 2013). The initiation of the biological cascade following drug intake induces a dynamic modification of the neuronal pathways situated in the nucleus accumbens (N.Acc.), the ventral tegmental area (VTA), the amygdala and the prefrontal cortex (PFC) (Everitt, 2005). The subsequent behavioural dysregulation is characterized by three pathological stages, encompassing initially the impulsive action of occasional drug taking (thereby experiencing the positive effects of the drug), secondly the withdrawal or negative effect stage (during which the absence of the drug increases stress and anxiety effects), finally leading to the third stage of compulsive drug seeking and drug taking behaviour (where the negative reinforcement drives the motivated behaviour) (Koob,2010; Furst, 2013). The neuromolecular changes in these reward and stress systems will also contribute to further dependence and future relapse. In addition, the individual genetic print and socio-economic factors are also known to substantially drive the drug-induced addiction (Vassoler, 2014).

Drug-induced addiction is well-known for a variety of *nonmedical drugs* like opioids (*example:* heroin), stimulants (*example:* cocaine), hallucinogens (*example:* LSD), and cannabinoids (*example:* marihuana). Nicotine and alcohol are two non-scheduled but also heavily abused drugs. Regarding the legal/medical drugs, quite a number of *prescription drugs* on the market are known for their addictive component (NIDA, 2012) and as such restricted in their therapeutic use. Examples hereof include, *amongst others*, drugs for treatment of anxiety (*example:* benzodiazepines), ADHD (*example:* methylphenidate) or chronic pain (*example:* oxycodone). Patients taking these

drugs are carefully monitored during the treatment period and guidance on the proper use is provided by the healthcare professionals.

However, to date the *nonmedical use of legal prescription drugs* by healthy subjects has become a major issue. The inappropriate use of these prescription drugs, in particular of painkillers, sleep medication, opioid analgesics and benzodiazepines has led to a tremendous increase of ER visits and unintentional overdose deaths, as evidenced by the fact sheets regarding substance abuse (NIDA, 2011; CDC, 2014). Especially in young people the abuse of pharmaceutical products is increasing alarmingly, probably also driven by the perception that these legal drugs are safe to use.

This health concern has created an increased awareness of both Pharma Industry and governmental agencies worldwide towards more profound investigation of the abuse potential of new CNS-active drug candidates in Drug Development.

As such, the drug licensing authorities released distinct guidances on the investigation of abuse potential of NMEs during the past recent years [the EMA guideline (EMA, 2006); the ICH guideline (ICH, 2009), the FDA draft guidance (FDA, 2010) and the subsequent FDA's decision tree (Bonson, 2011)].

Driven by these guidances/guidelines, the Pharma Industry is coerced on taking increased responsibility to identify in more detail the possible abuse potential of new CNS-active compounds in development, primarily to protect the patient who will use this medication as prescribed, but also to prohibit, in every conceivable way, the possibility of abuse practices within the healthy population. This comprises preclinical investigations of the physical dependence potential and of the possible rewarding and reinforcing properties of new CNS-active molecular entities in Drug Development, but has also consequences at the clinical, regulatory, and final product form level.

However, the implementation of these stringent guidelines with regard to execution of the preclinical procedures listed herein, and the determination of the biological relevance of the results obtained raised many questions. These

were reflected in the active discussions between the Pharma Industry and FDA during the dialogue Session in 2010 and in the recent releases of multiple publications on abuse liability testing within Drug Development (Moser, 2011a and 2011b; Kuss, 2012; Horton, 2013; Marusich, 2013; Swedberg, 2013; Mead, 2014). While numerous literature is available on basic research using animal models, implementing these within a Drug Development environment was not straight forward. According to the guidances, the Abuse Liability Assessment must be based upon a two-tiered approach of investigating abuse potential of a drug candidate (EMA, 2006; FDA, 2010). The primary or first tier investigates whether a new molecular entity has CNS-activity based upon in-vitro and in-vivo pharmacological characteristics and on the toxicological profile. The second tier involves neurobehavioural studies during which possible physical dependence and rewarding and reinforcing properties are investigated. The second tier investigations have to comply with the requirements as defined by guidances of the drug licencing authorities (EMA, 2006; ICH, 2009; FDA, 2010), which include, *amongst others*, to provide evidence that testing was executed within a dose range including a multiple of the human efficacious dose (expressed as C_{max}). They also need to fulfill the GLP regulations (OECD, 1998). With respect to animal welfare, care must be taken not to interact with the animal's comfort and health status (AAALAC).

Four behavioural procedures have been defined in the guidances for preclinical investigation of the abuse potential of new CNS-active drug candidates in development, each focusing on different aspects of drug abuse: the non-precipitated withdrawal test (WD) is performed to investigate the physical dependence potential of a CNS-active compound. The evaluation of discriminative stimulus properties of drugs, including drug profiling and functional resemblance to known psycho-active reference drugs, is executed through the drug discrimination learning test (DDL). Rewarding and reinforcing properties are determined indirectly via the conditioned place preference test or directly via the intravenous self-administration model.

The focus and value of this Ph.D. thesis was to develop, adapt or modify these models to a Drug Development mode with its typical aspects and criteria regarding traceability and reproducibility, to allow execution according to the

specific requirements as stated in the guidances and to improve the predictive power of the outcomes, as compounds with a novel mechanism of action might display only weak reinforcing properties. The translational approach towards the predictability of this preclinical abuse liability assessment was investigated through the determination of the biological relevance of the outcome of the results.

Chapter IV of this Ph.D thesis dealt with a series of four experiments performed to optimize the methodology of the *non-precipitated withdrawal test* for use in Drug Development. Employing psycho-active drugs of abuse like opioids (morphine), sedatives (diazepam), stimulants (cocaine) and alcohol, the drug- and/or dose related effects of these compounds were determined during the repeated dose phase and compared to the withdrawal effects upon cessation. These drugs are proven valid for use as reference drugs in this model.

We demonstrated that a 21-day repeated dose phase was sufficiently long to obtain neuroadaptive responses form the body to the test substance before initiating a withdrawal phase. This latter phase was designed at 7 days but can be extended if needed, in particular if the test drug has specific pharmacodynamic or -kinetic properties like, *for example*, a long half-life ($T_{1/2}$).

We also demonstrated that inclusion of multiple daily time points, at which critical parameters like the observation of behavioural changes (scored for both incidence and severity), and changes in body temperature, body weight and food uptake were determined, largely contributed to the robustness of the final design. These careful examinations allowed to trace even very subtle changes possibly related to drug dependence.

Careful consideration of the selected dose range of a drug allowed to demonstrate plasma exposures, which were proven multiples of the human efficacious dose (expressed as C_{max}) at the highest dose tested.

Finally, the newly built IT system was proven a valid system to capture in-life data for further processing and reporting.

The standardized design was validated using the prescription drug methylphenidate, a scheduled CII drug of abuse, with d-amphetamine being the reference drug (**Chapter VIII**). With this test we proved that the non-precipitated withdrawal model, executed according to the final design, was scientifically and technically valid, demonstrating the face validity of the outcome.

Chapter V described the five experiments in which several variables were investigated to optimize the *drug discrimination learning model* (DDL) as a test compliant with the requirements of the drug licensing authorities and the GLP regulations and as such to be used within Drug Development. We demonstrated that the adapted stimulus generalization design enabled determination of the discriminative properties of drug candidates via substitution of one or multiple cues. The inclusion of toxicokinetic parameters followed a unique design to exclude possible drug-drug interactions of the test compound with the training drug, which might affect the plasma exposure levels needed to comply with the requirements of the drug licensing authorities (ratio C_{max} rat/hu). This was clearly demonstrated in the validation study reported in **Chapter VIII**. Explicit criteria were defined to allow for retesting of a certain dose for a given individual rat, based on scientific evidence. Sham treatment, mimicking the route of administration of the test compound and of the reference drug, prevented a conditioned response during the study which might otherwise interfere with the discriminative responses. The final design also allowed, next to the classical dose-exposure effects where rats are tested whenever the defined C_{max} is reached, to study dose-effect relationships within a given time interval. This might be of interest for testing complex drug candidates like prodrugs or allosteric modulators, where the maximal effect of the drug not always coincides with the maximal exposure. Finally we also demonstrated that, with regard to animal welfare, chronic treatment with either of the psycho-active drugs [cocaine, fentanyl, nicotine, ethanol, chlordiazepoxide, LSD, DOM, ketamine, a D1-agonist (SKF 82958), and d-amphetamine,], did not lead to histopathological changes of the brain. With regard to GLP-compliance, records of the animal's history of correctly discriminating for the reference drug(s) versus saline up to 11 days prior to start of study were included, to demonstrate the that animals accepted for entrance into the full study met the presupposed criteria.

In addition, the maximum plasma exposure (C_{max}) and the time interval needed to reach the peak plasma exposure (T_{max}) of the commonly used eleven psychoactive drugs were verified and considered appropriate, with exception of cocaine and DOM, where the classical time interval was underestimated. This will be adapted in future DDL studies, if cocaine or DOM will be used as reference drugs.

The validated IT system met the requirements of the GLP regulations on capturing and reporting of the recorded data.

The research on the *intravenous self-administration paradigm* (IV SA) is depicted in **Chapter VI**, in which the results of six experiments were described. Major accomplishments were reached with regard to technology, as evidenced by the development of a new Vascular Access Button (VAB) to improve the animal's welfare and comfort in this long-term type of studies. As a consequence, the collaboration for this effort with an external company (Instech) resulted in the successful launch of the VAB on the market. We demonstrated that the overall survival rate in the IV SA was further enhanced by implementation of the femoral surgical procedure, combined with either an indwelling CBAS® heparin coated or a non-heparin coated polyurethane catheter and daily flushing with heparin (10 I.U.). We also demonstrated that priming with food or with the reference drug at start of training did not add to initiate lever press. To enable IV SA of the required concentrations of the drug candidate we implemented two different pump models for correct volume administration of the infusion within the infusion time of 2-5 seconds.

As the laboratory animals, and not the researchers, have full control over the intravenous self-administrations of a drug, we demonstrated that maximum administered daily volumes, accounting for up to 30% of the total blood volume, did not negatively affect the animal's health in terms of general condition and clinical pathology when tested through manual IV administration according to the IVSA paradigm.

Patency testing with propofol, a non-scheduled anaesthetic was considered superior to the mixture of Ketalar®/Dormicum®/saline in terms of the scheduled

drugs used within. Indeed, the psycho-active components ketamine and midazolam are scheduled as CIII and CIV compounds for drug abuse, respectively, and as such less convenient for use within the IV SA paradigm. It was demonstrated that an intravenous bolus of 0.50 mg propofol/kg resulted in an adequate and short anaesthesia of 3 to 4 minutes, which was optimal for patency testing in the IV SA paradigm.

It was proven that the adapted model clearly demonstrated the reinforcing properties of stimulants (cocaine, methylphenidate, d-amphetamine) and opioids (morphine and fentanyl). The negative results regarding the investigation of the reinforcing properties of the hallucinogen LSD confirmed the data in literature, stating that LSD is not generally self-administered by animals (Jerome, 2008; Bonson, 2012). This known false negative result is however counterbalanced by full stimulus generalisation in drug discrimination learning studies with LSD (Meert, 1990; NIDA, 1991; internal experiments, 2011).

To comply with the regulatory guidances of the drug-licensing authorities, inclusion of toxicokinetic parameters is needed to prove that a multiple of the human efficacious dose (expressed as C_{max}) is tested in the IV SA model. The procedure for this investigation is still under quite some debate. We tackled this problem by calculating the actual individual minimal and maximal drug intake and manually administered these doses intravenously (as a single, slow IV bolus) after completion of the IV SA study to determine the maximum exposure (C_0) as time-exposure profiles. As such we were able to demonstrate that the correct dose ranges were tested in view of testing a multiple of the human efficacious dose (expressed as C_{max}). An example hereof is also reported in **Chapter VIII**.

Besides the GLP-compliance of the *in-vivo* study design, the newly developed IT system for driving the IV SA daily sessions (*amongst others* the automatically created files to ensure the correct daily administration of the training drugs and the test compound) and for capturing the data and further processing hereof, was validated for its traceability and reproducibility and was proven compliant with the GLP regulations.

The investigations to optimize the study model for *conditioned place preference testing* (CPP) were outlined in six mechanistic studies and described in **Chapter VII**. The methodology comprised an unbiased procedure as our data evidenced that, apart from a slight physiological preference for the black compartment, little variation in preferential conditions towards a coloured compartment was noticed at pretest. We demonstrated that the number of drug pairings was of major importance to obtain a robust signal, in particular because new CNS-active drug candidates may have only weak rewarding or reinforcing properties. A major effort was undertaken to improve the interpretation of the data. We demonstrated that the calculated *within* ratios of the time spent in the drug-paired versus non-drug-paired compartments per dose group and the *in-between* ratios of the time spent in the drug-paired and non drug-paired compartments versus those of control and/or reference dug, significantly contributed to the interpretation of the biological relevance of the outcome. Indeed, the absolute number of rats showing a conditioned place preference does not solely determine the positivity of the test. In addition, the time spent in the grey -neutral- compartment has to be taken into account as well. A full example is given in the validation study described in **Chapter VIII**.

We also demonstrated that, when using the albino Sprague-Dawley rats, the brightness of light rather than colour or tactile stimuli present in the CPP apparatus are of importance for conditioning.

As for the other test designs, a toxicokinetic part was built in to prove that a multiple of the human efficacious dose, expressed as C_{max} was tested.

Finally, the newly developed web-based IT system fulfilled all requirements regarding scientific power and GLP-compliance.

In addition to the standardized CPP model, we also performed a combined CPP/CPA study to test a drug candidate for possible drug-induced place preference or aversion. The results are also denoted in **Chapter VII**.

In **Chapter VIII**, methylphenidate (MPH), a CII scheduled drug was selected as a first example to investigate the *prediction of its known human abuse potential*

via the four nonclinical study models. The translational approach was based upon the biological relevance of the outcome of the studies. It was clearly demonstrated that for a correct determination of this biological relevance all data need to be taken into account. Indeed, the absence of the clinical signs indicative of physical dependence after abrupt cessation was in line with the clinically known low physical dependence of MPH. Also, the partial stimulus generalization at lower doses of MPH but the full generalization at higher dosages stressed the impact of a correct dose selection for a given administration route. Regarding the conditioned place preference of MPH, the additional *within* and *in-between* analyses on the time spent in the drug-paired, non drug-paired and neutral (grey) compartment enabled correct interpretation of a drug-induced conditioned place preference at the high dose only. MPH presented to rats in an intravenous self-administration model showed explicit reinforcing properties following the classical bell-shaped dose-response curve, again putting emphasis on the impact of a proper dose selection. It was concluded that the four developed tests as presented showed a high predictability of a translational approach for drug abuse liability testing of methylphenidate. In addition the plasma concentration ranges, achieved by the effective doses of MPH in the four present studies were in accordance with the proposed relation to human therapeutic concentrations as suggested in regulatory guidance (*i.e.* being a multiple of the human efficacious dose).

The use of diseased rats (*example:* the ADHD rat model) was also discussed but considered not to be encouraged when performing these regular, preclinical tests, in order to stay aligned with the toxicity studies and safety pharmacology studies which are conducted in common rat strains, but also because the data obtained in common rat strains can be considered representative to mimic the human situation of nonmedical use of drug products by healthy subjects, thus adding to the predictability of the translational approach for testing abuse potential of CNS-active compounds (Teuns, 2014).

Lastly the need to study abuse liability of biologics was considered. From most large molecules it is known that, apart from their longstanding half-lives ($T_{1/2}$ up to 3 weeks or more), they have a poor ability to cross the blood-brain-barrier (BBB) to interact with brain receptors (Vargas, 2012). As such the risk for

developing abuse potential is unlikely. However, the development of new large molecules, able to exert activities in the brain [*example*: monoclonal antibodies to treat Alzheimer's disease (Panza, 2011)] is ongoing. Therefore this type of molecules must be kept in scope whenever testing for abuse potential is considered.

In conclusion, the biological relevance of the positive outcome of the various tests executed in the common Sprague-Dawley rat has been thoroughly considered (**Chapter IV to VIII**), as this is the key to a profound translational approach towards human and hence to the predictability of the preclinical data. Combining the results of the various studies is necessary to make a correct interpretation of the possible abuse potential of an investigated drug.

As stated before, the *physical dependence potential* needs to be studied for each CNS-active compound in development; however, this feature is not necessarily associated with addiction. Examples hereof imply antidepressants and glucocorticoids, which are unscheduled drugs although known for their withdrawal effects upon abrupt cessation. The possible psychological effects, characterized by *rewarding and reinforcing properties* of a drug of abuse, appear to count for a much more substantial extent to the reliability of abuse potential. At this point however, the choice of a correct test dose range is of major importance, as evidenced by, *amongst others*, the classically bell-shaped dose-responses in IV SA models. The standardized methodology to determine the kinetics of the selected dosages in the various study models is also of significant importance to a proper evaluation of the abuse potential. Indeed, demonstration of the presence or absence of reinforcing properties needs to be established with the plasma levels that are multiples of the therapeutic human dose.

Genetic and environmental factors, which are known to play a significant role in the development of addiction, are not included in the classic preclinical investigation of abuse potential of CNS-active compounds, which is executed as a mandatory part within the process of the Drug Development.

This project also included an educational section on abuse potential at different levels (internally and externally) as part of the UH Doctoral School

requirements. The internal training to scientific employees assigned to this project comprised an insight view on the complexity on drug abuse to understand the aim of the studies. The training of the *in-vivo* part included understanding of the set-up, discussions on the different parameters, implementation of the criteria characteristic for each design and concomitant coaching of the individual responsibility associated with decision making during the study, training sessions on the outcome of the studies and the interpretation hereof, and coaching on literature search with regard to study design and dose selection of psycho-active drugs.

Five study directors were initially trained to set up withdrawal and drug discrimination studies (protocol writing and coaching on selection of psycho-active drugs), to follow-up and to report.

Regional and global development teams (Preclinical and Clinical Development Leaders, Regulatory Affairs members, …) were trained to understand drug-induced abuse potential and how this fitted into the development program of CNS-active compounds [from a preclinical, clinical, regulatory, formulation point of view and on the mitigation strategy (REMS)].

Internal presentations were given on a regular basis.

At the yearly Safety Pharmacology Society Congress, Continuing Education Courses on Drug Abuse Liability Testing were organized (SPS, 2010; SPS, 2011). These courses encompassed presentations discussing abuse liability testing, with focus on the preclinical tests, but also on the pharmacology, clinical topics and regulatory issues (Teuns, 2011).

Other external presentations on drug abuse were given upon invitation to different scientific (OECD GLP representatives, IWT representatives, CRO scientists, pharmacologist consortium…) or technically skilled groups (IT specialists, infusion specialists, …).

Final conclusions and future perspectives

To determine the abuse potential of new CNS-active molecular entities with a novel mechanism of action, considerable knowledge of the pharmacology, toxicity and kinetics is needed to enable the choice of the correct tests, the selection of an appropriate dose range of the test compound and the proper choice of the psycho-active reference compound(s) or a scheduled comparator. In addition, one must have a thorough expertise of the current overall Drug Development process and subsequent requirements of the drug licensing authorities.

The outcome of this Ph.D. project has led to the implementation of four scientifically validated neurobehavioural tests in rodents, complying with the requirements of the drug licensing authorities, the GLP-procedures applicable in preclinical research in Drug Development and with the principles of animal welfare. The tests were proven efficient and highly predictive for the translational approach of abuse liability testing of CNS-active compounds.

To date the investigation of abuse potential of prescription drugs has reached a considerable level of attention from both the Pharma industry and the regulatory authorities, as evidenced by the recent release of a draft guidance on abuse deterrent opioid formulations (FDA, 2013). The continuing efforts of the Pharma industry to reduce the nonmedical use of prescription drugs has led to innovative solutions such as the OROS technology (Concerta®, containing methylphenidate), the Tamper Resistant Formulation tablets of Tapentadol® TRF (a centrally acting analgesic), the inclusion of antagonists in the final product (Embeda®, containing morphine + naltrexone), the development of prodrugs (Vyvanse®, containing lisdexamfetamine, an inactive prodrug for d-amphetamine) and the application of an aversive stimulus added with the product [Acurox®, containing oxycodone and niacin (vitamin B3-nicotinic acid)].

The demanding requirements will result in an extensive overall Abuse Liability Assessment, consisting of preclinical investigations of the physical dependence and of the rewarding and reinforcing properties of new CNS-active molecular entities in Drug Development, but also including consequences at the clinical,

regulatory, and final product form level. Therefore it is essential for a pharmaceutical company to create a structured approach to encompass all these aspects and to reach out to other partners/consortia to further harmonize on the investigation on abuse potential (Fig. IX.1).

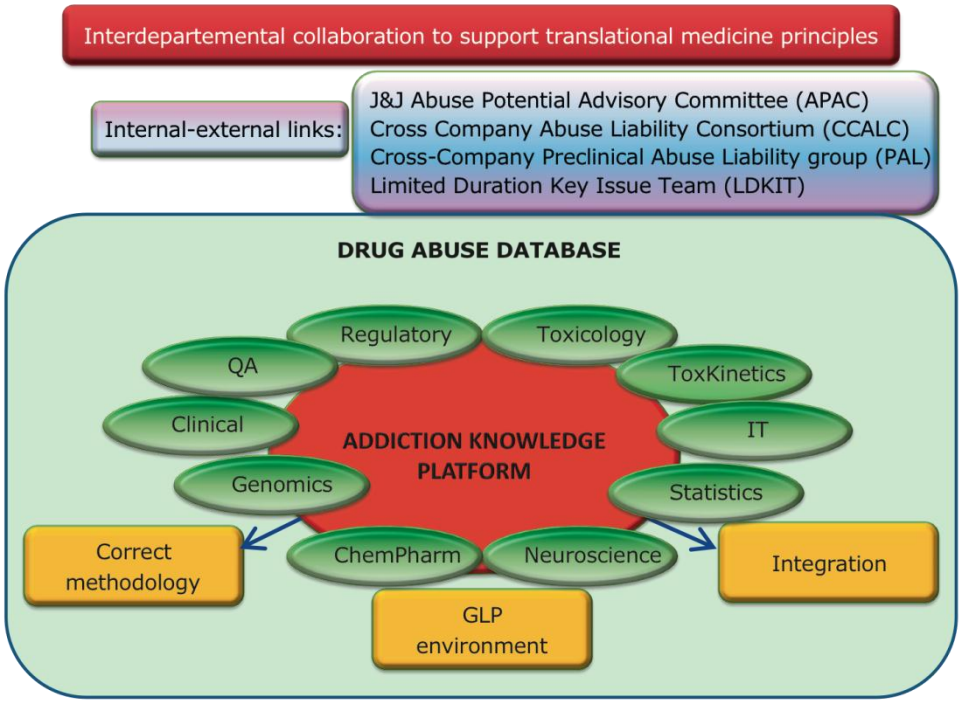

Fig IX.1. Working chart: integration of the addiction knowledge platform, to enhance the translational research of abuse liability testing.

Further perspectives also lie within the future Dialogue session with FDA and the Pharma Industry (2015) where, *amongst others*, the following topics should be further discussed:

- Filing of robust, reliable and thus highly predictive data from preclinical drug abuse liability testing might avoid human trials on drug abuse

- Reliable data of drug-induced abuse potential testing of new, marketed products, based on the innovative mechanisms of action (different receptor binding compared to the known psycho-active drugs of abuse), the delayed exposure-effect response, and the increasing knowledge of

the genetic impact on the development of addiction might give rise to different scheduling in future

- Impact of marketed drugs with a final product form that decreases misuse (ex. Oros technology)

- Drugs with novel mechanisms of action: choice of appropriate comparator(s) for investigation of drug abuse potential

- Discussion on the abuse liability program for large molecules/biologics

- Discussion on the abuse liability testing of "major metabolites"

- Risk/benefit: discussion on the necessity to investigate the abuse liability of, *for example,* drug candidates used in brain oncology.

In summary, the results of this thesis demonstrated that a robust, scientifically valid and overall compliant preclinical abuse liability testing battery was accomplished, allowing firm determination of the biological relevance of the data and hence a decent prediction of the abuse potential of new CNS-active molecular entities with a novel mechanism of action, which are currently under Drug Development. This translational approach involves a multidisciplinary knowledge and expertise at the level of, *amongst others*, pharmacology, toxicology, clinical development, regulatory demands, final product formulation and post-vigilance succession. The thorough assessment of the abuse potential properties and safety profile of novel drug candidates will unquestionably add to a decreased public health risk concerning misuse and/or addiction to prescription drugs.

X

Nederlandse samenvatting

Nederlandse samenvatting

De laatste decennia is er een enorme stijging waar te nemen in het misbruik van voorgeschreven medicijnen, niet in het minst in de leeftijdscategorie van 15-24 jarigen. Deze adolescenten menen ten onrechte dat dit type van medicijnen, zelfs wanneer gebruikt voor niet-medische redenen, veiliger is dan het gebruik van de klassieke illegale drugs, omdat voorgeschreven produkten immers grondig getest werden alvorens op de markt beschikbaar te komen.

Van overheidswege en vanuit de Pharma-industrie is er een grote en groeiende bezorgdheid dat dit misbruik kan leiden tot verslaving en zelfs tot sterfte door overdosis. Daarom worden medicijnen die nog in een ontwikkelingsfase zijn, en waarvan men weet dat ze bij inname door de bloed-hersenbarrière dringen en een aktiviteit in de hersenen uitoefenen, onderzocht op mogelijke fysische afhankelijkheid en psychische verslaving. Dit door de autoriteiten gedirigeerde onderzoek dient te gebeuren voor chemische molecules die specifiek worden ontwikkeld ter behandeling van een stoornis of aandoening in de hersenen (bv. voor depressie, voor ADHD of voor de ziekte van Alzheimer), maar ook voor molecules die voor een niet-neuronale aandoening worden ontwikkeld maar wel in de hersenen terechtkomen en daar een aktiviteit uitoefenen. Een gekend voorbeeld van deze laatste categorie zijn de centraal werkende medicijnen om gewichtsverlies te induceren.

Het onderzoek op verslaving wordt in eerste instantie uitgevoerd op proefdieren, bij voorkeur op ratten, zoals gespecificeerd in de richtlijnen van de autoriteiten. Deze richtlijnen geven ook aan dat verschillende aspecten, die kenmerkend zijn voor de ontwikkeling van verslaving, dienen te worden onderzocht.

In een eerste studie-opzet (de zogenaamde withdrawal test) gaat men dus na of een molecule fysische ontwenningsverschijnselen induceert bij het abrupt beëindigen van een langdurige dosering.

Een tweede onderzoek (drug discrimination learning test) omvat de vergelijking van de interoceptieve effecten van een molecule met deze van gekende psycho-aktieve stoffen zoals bv. cocaine, morfine, alcohol etc. Hiervoor worden ratten

getraind om een differentiatie te leren maken tussen het effect dat zij voelen wanneer een referentiestof (bv. cocaine) wordt toegediend en het effect dat zij voelen wanneer water wordt toegediend. Deze differentiatie wordt gevisualiseerd door het drukken op een pedaal geassocieerd met het toedienen van de referentiestof of op een pedaal geassocieerd met watertoediening. De training gebeurt op basis van verloning: de ratten krijgen een suikerpellet telkens wanneer zij een correcte keuze maken. De test zelf bestaat erin de test molecule toe te dienen, en dan na te gaan of het dier de effecten hiervan associeert met deze van de referentiestof of van water.

Een derde test (intravenous self-administration) bestaat uit het onderzoeken van bekrachtigingseffecten van een test molecule, die de relatie tussen het gedrag (het nemen van een drug) en de consequenties van dat gedrag (het drug effect) weergeven. Dit kan worden achterhaald door te testen hoeveel en hoe hard een rat wil werken om een intraveneus infuus (shot) te krijgen van een stof die hen een goed gevoel geeft (positieve bekrachtiging).

De vierde en laatste test (conditioned place preference) onderzoekt deze bekrachtiging indirect door een conditioneringspatroon op te bouwen bij het dier, bijvoorbeeld door een bepaalde omgeving te associëren met het (positieve) effect dat een drug teweegbrengt na toediening.

Deze vier testen werden reeds gebruikt in fundamentele research, maar dienden te worden aangepast of geoptimaliseerd om aangewend te kunnen worden binnen het kader van het wettelijk wetenschappelijk onderzoek van potentiele geneesmiddelen. In deze context moet immers voldaan worden aan de verplichtingen die van overheidswege worden opgelegd, en moeten ook de regels van de goede laboratoriumpraktijken worden gevolgd. Ook moet er zorg gedragen worden voor het welzijn en comfort van de dieren tijdens de testen.

In deze optiek werden verschillende aanpassingen doorgevoerd of nieuwe technieken ontwikkeld voor deze testen, zowel op wetenschappelijk als technologisch en IT-gerelateerd vlak. Er werd ook intens samengewerkt met externe partners (Instech), wat onder meer resulteerde in de ontwikkeling van een nieuwe technologie om intraveneuze catheters te verbinden met een

externe pomp die de rat voorziet van korte intraveneuze infusen (shots). Deze zogenaamde "Vascular Access Buttons" zijn nu beschikbaar op de markt en betekenen een grote meerwaarde voor het comfort van de rat in de intraveneuze zelf-administratie test.

In totaal werden 26 testen uitgevoerd waarin tevens verscheidene kritische variabelen werden uitgetest om hun effect op de eindresultaten te bestuderen. In het onderzoek werd aangetoond dat deze variabelen een significante impact hadden op de resultaten en werden als dusdanig ingebouwd in de studie-modellen. Voorbeelden hiervan zijn, *onder andere*, het vermijden van simulatie van stoftoediening (sham treatment) gedurende de periode waarin ontwenningsverschijnselen worden bestudeerd in een withdrawal test, de wijze waarop het toxicokinetisch onderzoek werd ingebouwd bij een drug discrimination learning test, de chirurgische procedure (intraveneuze femorale catheters i.p.v. jugulaire) in een intravenous self-administration test, en de lengte van de conditioneringsperiode (het aantal keren dat een rat in een specifieke omgeving wordt geplaatst na toediening van een aktieve stof) in een conditioned place preference test.

Het resultaat van dit aanzienlijk onderzoek heeft aldus geleid tot de implementering van vier gestandaardiseerde testen waarmee, binnen het geneesmiddelenonderzoek, molecules in ontwikkeling kunnen getest worden op mogelijk verslavingspotentieel. Deze testen voldoen aan alle vereisten met betrekking tot de wettelijke en kwaliteitsgerichte (GLP) verplichtingen en aan de vereisten aangaande dierenwelzijn binnen het geneesmiddelenonderzoek.

Samengevat kan men stellen dat de resultaten van de onderzoeken die beschreven werden in deze thesis hebben geleid tot de verwezelijking van een robuuste en sterk wetenschappelijk gebaseerde batterij van gevalideerde preklinische testen om het verslavingspotentieel te onderzoeken van nieuwe, CNS-actieve kandidaat geneesmiddelen met een innovatief werkingsmechanisme. Op basis van de resultaten verkrijgt men een accuraat beeld van de biologische relevantie, waardoor een hoge voorspelbaarheidsgraad wordt bekomen van mogelijke verslaving bij de mens.

Deze vertaalslag van proefdier naar de mens vereist wel een grondige kennis van de farmacologie en het toxicologisch profiel van de test molecule, alsook van de verdere klinische ontwikkeling, de regulatoire vereisten, de finale productvorm en de opvolging van het medicijn wanneer het op de markt komt. De grondige en diepgaande beoordeling van het mogelijke verslavingspotentieel en het veiligheidsprofiel van nieuwe kandidaat geneesmiddelen zal zeker bijdragen tot een verminderd risico voor de algemene gezondheid aangaande misbruik en/of verslaving van voorgeschreven medicijnen.

Curriculum Vitae

Greet Teuns (º1961) graduated as a Veterinarian (D.V.M.) at the University of Ghent, Belgium in 1985. She obtained a Master Degree in Zootechnical Sciences with distinction (Ghent, Belgium, 1987) and a Master Degree in Applied Toxicology with distinction (Guildford, UK, 2003), and is registered as a certified European toxicologist since 1998. She is a member of EuroTox, SPS, SFN, DIA, BST, EBPS and ECNP.

Greet started her career at Janssen Pharmaceutica N.V., Beerse, Belgium in 1985. She worked as a Study Director at the department of Toxicology, being involved in all areas of Toxicology (general and reproductive toxicology, safety pharmacology, environmental and safety toxicology, topical toxicology) for which she wrote over more than 1100 study reports. She played a major role as the toxicology lead of several Drug Development projects in Discovery and Early Development phases.

Greet headed the group of Study Directors in Safety Pharmacology & General Toxicology (Early and Full Development), and the group of Preclinical Formulation Unit and Analysis, thereby focusing on the safety evaluation of compounds in both early and in late development.

Currently, Greet is a Research Fellow/Scientific Director and since 2005 mainly working within the CNS area. In this role, she's the Team lead of the Drug Abuse Liability testing group at Janssen R&D, Preclinical Development & Safety, Beerse, Belgium. She is responsible for the Preclinical Abuse Liability Assessment of CNS-active compounds worldwide in late development, for which she designs the scientific program and supports the execution of the preclinical studies. She interprets the results and judges on the biological relevance hereof. Greet also supports promising compounds in Drug Discovery that might have added value to addiction treatment by executing modified designs of the regulatory abuse liability tests.

Another of Greet's roles is the investigation of the pharmacotoxicological pathways to explain off-target CNS and/or general toxicity of new compounds.

As such she is involved in most of the drug development projects in Early Development and in Drug Discovery. She is also the expert in preclinical Safety Pharmacology-CNS. Her broad expertise in toxicology adds great value to innovative projects like the colorectal cancer incubator project, the Transdermal Patch Community of Practice, and the internal joint collaboration on batch delivery.

Greet is actively involved in several internal and cross-Pharma working groups on drug abuse liability (CCALC, PAL, APAC), but also in cross-company workgroups to investigate, *amongst others*, preclinical markers for suicidal behaviour (IQ consortium working group), predictive markers and models for seizure sensitivity (Preclinical working group), and neurotoxicity markers (Hesi working group).

Greet is an active member of the abstract review committee (SPS) and an active reviewer of submitted articles in the Journal of Pharmacological and Toxicological Methods.

Greet has and still is coaching several junior scientists on general toxicology, CNS safety pharmacology, excipients-based safety evaluation, and on the process of Drug Development, and is an appreciated mentor within the company.

Greet lectures at various universities in Belgium and abroad, and organizes training sessions on toxicology, drug abuse liability and Drug Development for internal and external scientific and non-scientific groups. She is an active member of the Global Continuous Education Committee (SPS).

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"Life is an adventure of passion, risk, danger, laughter, beauty, love; a burning curiosity to go with the action to see what it is all about, to go search for a pattern of meaning, to burn one's bridges because you're never going to go back anyway, and to live to the end."

Saul D. Alinsky, Reveille for Radicals

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Oral presentations (invited speaker) (last 5 years)

Title: From Zebrafish to Non-Human Primates: Role of Animal Models in Drug Development.
Flemish Training Network Life Sciences (FTNLS) symposium: Drug Discovery anno 2014: from target identification to personalized medicine. 2014, Leuven, Belgium.

Title: To dose or not to dose: an insight view on toxicology.
SIG workshop (Special Interest Group of statistians in the pharmaceutical industry for Toxicology). 2013, University of Hasselt, Belgium.

Title: Drug abuse liability testing.
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Title: Drug abuse liability testing.
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Title: Safety Pharmacology Studies for Human Pharmaceuticals.
Modular Training Program in Applied Toxicology (M. Sc.). 2005, 2007, 2009, 2012, University of Guildford, Surrey, UK.

Title: Toxicology.

University of Antwerp and University of Hasselt, Belgium. Yearly from 2005 (Antwerp)/2009 (Hasselt) to present.

Title: Development and implementation of a new access button used in the rat self-administration design.
Infusion Technology Organisation. 2011, Barcelona, Spain.

Title: Drug Abuse Liability: an overview.
Safety Pharmacology Society (SPS) annual meeting. 2011, Innsbruck, Austria.

Title: Safety Pharmacology beyond the guidelines: CNS Issues in Drug Development.
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Poster presentations

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