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FACULTEIT GENEESKUNDE EN LEVENSWETENSCHAPPEN
master in de biomedische wetenschappen

Masterproef

Neuroregeneration in ethanol- and 6-hydroxydopamine-exposed planarians

Promotor :
Prof. dr. Karen SMEETS

Lore Colsoul

Scriptie ingediend tot het behalen van de graad van master in de biomedische wetenschappen

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List of abbreviations

6-OHDA	6-hydroxydopamine
AsA	Ascorbic Acid
BSA	Bovine Serum Albumin
BSO	Buthionine sulfoximine
CNS	Central nervous system
CREB	cAMP responsive element binding-protein
DAT	Dopamine membrane transporter
DMSO	Dimethyl sulfoxide
DPI	Diphenyleneiodonium chloride
EtOH	Ethanol
FASD	Fetal Alcohol Spectrum Disorder
H_2O_2	Hydrogen peroxide
MCI	Mild Cognitive Impairment
MetOH	Methanol
MOA	Monoamine oxidase
MRL	Murphy Roths Large
NAT	Noradrenaline membrane transporter
NDK	Nou-darake
NF-κB	Nuclear factor κB
PBS	Phosphate Buffered Saline
PBST	Phosphate Buffered Saline with Triton X-100
PD	Parkinson's Disease
PEG	Polyethylene glycol
pLMV	Planarian Locomotive Velocity Assay
qPCR	Quantitative polymerase chain reaction
ROS	Reactive Oxygen Species
RNAi	RNA interference
th	Tyrosine hydroxylase

Abstract

Background: To provide clues for the future treatment of neurodegenerative diseases, more insights in the specific pathways underlying neuroregeneration are essential. The planarian *Schmidtea mediterranea* is a small and inexpensive regenerative model organism that provides the opportunity to study neuroregeneration *in vivo*. It is therefore hypothesized that ethanol (EtOH)- and 6-hydroxydopamine (6-OHDA)-induced neurodegeneration in intact planarians is compensated by neuroregeneration and that the redox balance is involved in this process. Neurodegeneration caused by these neurotoxins was confirmed by phenotypical observation and visualization of the central nervous system (CNS). The role of the redox balance was investigated using ROS disturbers diphenyleneiodonium chloride (DPI) and buthionine sulfoximine (BSO).

Materials and methods: Planarians were exposed to the neurotoxins ethanol (0.5-2.5% dissolved in medium) and 6-OHDA (10-500 mg/ml dissolved in medium or ascorbic acid (0.3-0.6%)). Phenotypical alterations were monitored. Neuronal damage was determined via an anti-SYNORF immunostaining. The oxidative balance was disturbed using DPI (3 µM in 0.01% dimethylsulfoxide (DMSO)) and BSO (10 mM in 0.05% DMSO). The statistical significance of the results ($p<0.05$) was checked using one-way or multiple-factor ANOVA followed by the Tukey-Test.

Results: In planarians, EtOH mainly caused contracted immobility followed by sliding movements. When exposed to 2.5% EtOH during 24 hours, the planarians threw off their head. The appearance of blisters and lesions worsened when exposed to increasing 6-OHDA concentrations, although most animals displayed no phenotypical changes. All phenotypical effects were restored after seven days. A disturbed ROS balance during EtOH recovery had no effect on mobility and behavior.

Discussion and conclusion: It seems that prolonged exposure to EtOH causes a contracted and immobile phenotype followed by gut-sliding movements in intact planarians. The neuronal cause underlying these behavioral alterations remains unknown. Further research is needed to optimize and continue experimental exposures to both neurotoxic compounds and to identify how these compounds affect the CNS of planarians.

Samenvatting

Achtergrond: Meer inzichten in de onderliggende mechanismen van neuroregeneratie zijn noodzakelijk om aanwijzingen te verkrijgen voor de ontwikkeling van therapieën voor neurodegeneratieve ziektes. De platworm *Schmidtea mediterranea* is een klein en goedkoop modelorganisme dat ons de mogelijkheid geeft om neuroregeneratie *in vivo* te bestuderen. In deze studie werd onderzocht of ethanol (EtOH)- en 6-hydroxydopamine (6-OHDA)-geïnduceerde schade aan het zenuwstelsel gecompenseerd wordt door neuroregeneratie. Tevens werd de betrokkenheid van de oxidatieve balans tijdens dit proces bestudeerd. Neurodegeneratie, veroorzaakt door deze neurotoxines, werd onderzocht op basis van fenotypische veranderingen en visualisatie van het centrale zenuwstelsel. De rol van de redox balans werd geanalyseerd door gebruik te maken van diphenyleneiodonium chloride (DPI) en buthionine sulfoximine (BSO), allebei verstoorders van de redox balans.

Materiaal en methoden: De planaria werden blootgesteld aan EtOH (0.5%-2.5% opgelost in medium) en 6-OHDA (10-500 mg/ml opgelost in medium of ascorbaat zuur (0.3-0.6%)). Fenotypische veranderingen werden opgevolgd en neuronale schade werd onderzocht via een anti-SYNORF immunokleuring. De oxidatieve balans werd verstoord door gebruik te maken van DPI (3 µM in 0.01% dimethylsulfoxide (DMSO)) en BSO (10 mM in 0.05% DMSO). De statistische significantie van de resultaten ($p<0.05$) werd bepaald door éénweg of meervoudige variantieanalyse, gevolgd door de Tukey Test.

Resultaten: In platwormen veroorzaakt EtOH voornamelijk een opgetrokken en immobiel fenotype gevolgd door rupsachtige bewegingen. De wormen verloren hun hoofdje wanneer ze blootgesteld werden aan 2.5% EtOH gedurende 24 uur. Het verschijnen van blaren en letsels nam toe wanneer de dieren behandeld werden met stijgende concentraties van 6-OHDA. De meerderheid van 6-OHDA blootgestelde dieren vertoonde echter geen fenotypisch effect. Alle fenotypische veranderingen waren hersteld binnen zeven dagen. Een verstoorde redox balans gedurende herstel van EtOH blootstelling had geen effect op de mobiliteit en het gedrag van de dieren.

Discussie en conclusie: Langdurige blootstelling aan EtOH in planaria lijkt twee typische fenotypes te veroorzaken: een opgetrokken en immobiel fenotype gevolgd door rupsachtige bewegingen. De onderliggende oorzaak van deze gedragsveranderingen blijft echter onbekend. Verder onderzoek is nodig om experimentele blootstelling aan beide neurotoxische stoffen te optimaliseren en om te identificeren hoe deze stoffen een effect hebben op het centrale zenuwstelsel van planaria.

1. Introduction

1.1. Tissue regeneration

Tissue regeneration is a research topic of great interest since it holds the key to innovative and better therapies. Regeneration is the ability to restore damaged or lost structures, without the formation of scar tissue (1). Regenerative medicine provides the function to replace missing/injured tissue both structurally and functionally or to promote regeneration in the tissue itself (2). Neurodegenerative diseases, like Parkinson's disease (PD), are becoming increasingly common in human elderly populations. For these disorders, stem cell transplantation may provide a solution, since no effective treatment to reverse neuronal damage has been discovered to date (3). Improvement of human health and a better understanding of molecular pathways underlying this process, is being achieved in current research (4). However, more insights are needed in order to apply stem cells for therapeutic purposes. Therefore, it is important to invest in fundamental research concerning regeneration in various tissues and organisms. Only when the basic mechanisms of regeneration are known and understood, progress in the development of regenerative medicine can be achieved.

Over the years, continuous progress has been made in the development of stem cell-based treatments. These cells are already being applied in *in vitro* applications of stem cell therapy (5). However, those studies mainly focus on controlling mechanisms of differentiation, whereas much remains to be understood about the fundamental processes underlying regeneration. It is not yet clear where transplanted stem cells end up, how they integrate into existing tissue, which signals are needed to commit to a neuronal fate and how they can then restore normal functioning. Therefore, to gain more insights into these processes, stem cells must be examined in an *in vivo* context, preferably in systems with a high regenerative capacity (5).

1.1.1. Regeneration in various organisms

Many organisms are able to regenerate in the early stages of life. This ability is often lost upon metamorphosis or ageing. However, some organisms maintain the ability to regenerate all organs for life (1). Regeneration in adult humans is limited to only a few organs (e.g. human liver), in contrast with lower vertebrates (6).

A striking example of a mammal able to regenerate is the Murphy Rots Large (MRL) strain of mice. These animals are able to fully heal wounds in multiple tissues, without formation of scar tissue (7). Additionally, it has been proven that they can also regenerate damaged areas of the heart, thereby restoring myocardial function (8). In contrast to the limited amount of regeneration present in mammals, many lizards have intermediate regenerative capacities. They are able to drop (part of) their tail in order to escape a predator. In response they can grow a new one (9). Unfortunately, this new tail is not an exact copy of the original tail, it regrows into a cartilaginous tube where the spinal cord is replaced by an epithelial tube from which no nerves sprout (10). On the other hand, the zebrafish (*Danio rerio*) is a highly regenerative vertebrate which is able to recover amputated fins, lesions in the brain, heart and other tissues. They can regenerate neurons

within the retina, spinal cord and brain if residential glial cells are still present (11), but they cannot regenerate these structures *de novo*.

Hence, some organisms maintain the ability to regenerate for life, but the capacity to regenerate is particularly pronounced in the invertebrate planarian. *Schmidtea mediterranea* is a free living flatworm which is widely known for its regenerative potential due to the large population of stem cells in its body (12) (Figure 1). It also is one of the simplest metazoans in which regeneration is manifested (13). They can regenerate into complete individuals from parts as small as 1/279th of its original body size (5,14) (Figure 1). They can fully regenerate their central nervous systems (CNS) *de novo*, a unique ability which only a few species possess (15). This features makes them very interesting to study neuroregeneration.

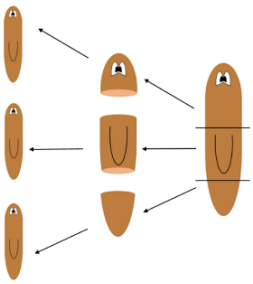


Figure 1. Regeneration capacity of the flatworm *Schmidtea mediterranea*. *Starting from miniscule body parts, they can regenerate into complete new individuals in only a matter of days.*

1.2. *Schmidtea mediterranea* as a model organism

1.2.1. Morphology of Schmidtea mediterranea

S. mediterranea planarians are organisms that are up to 20 mm in length and 3 mm in width. On the dorsal side they have a dark brown color, which is paler ventrally. They have a rounded head, containing two photoreceptors set in pigment-free patches (Figure 2A) (16). They have a well-defined bilateral symmetry, as well as anterior-posterior and dorsal-ventral axes (5).

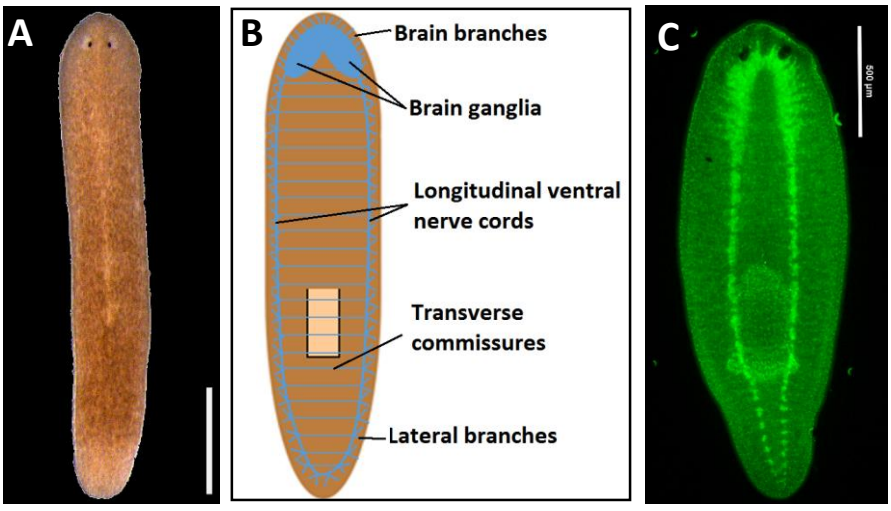


Figure 2. Morphology of the planarian *Schmidtea mediterranea*. *(A) Individual of the asexual strain of S. mediterranea. Dorsally, they have a dark brown color. The animals have a rounded head which contains two photoreceptors that are found in pigment-free patches. Scale bar: 1 mm. (B) Central nervous system of S. mediterranea, consisting of cephalic brain ganglia from which two longitudinal, ventral nerve cords sprout. The nerve cords are interconnected with transverse commissures. The photoreceptors are located dorsally to the brain. (C) Visualization of the central nervous system of the planarian species S. mediterranea by an anti-SYNORF immunostaining. Scale bar: 0.5 mm.*

Although planarians appear to be morphologically simple, they have a strikingly complex CNS, which they can regenerate completely. The CNS is divided into cephalic brain ganglia and two ventral, longitudinal nerve cords which are interconnected by transverse commissures (Figure 2B and C). The cephalic brain ganglia consist out of two lobes, which are connected by a single commissure. The CNS has distinct neuronal populations, based on neurotransmitter expression. The digestive system consists of a single pharynx in the center of the body and a gut with one anterior and two posterior branches. Various gut diverticula sprout from these branches (5).

1.2.2. Role of neoblasts during regeneration

The unique capacity of flatworms to regenerate missing body structures out of miniscule body parts (5,13) originates from a population of pluripotent stem cells called neoblasts (13,17). Neoblasts are the only mitotically active cells in planarians (14). Upon ageing or wounding, neoblasts migrate, proliferate and differentiate to replace lost cells (5,18). They account for 20-30% of all cells in planarians (5,19) and include both true pluripotent cells and already committed cells that retain neoblast-like features (5).

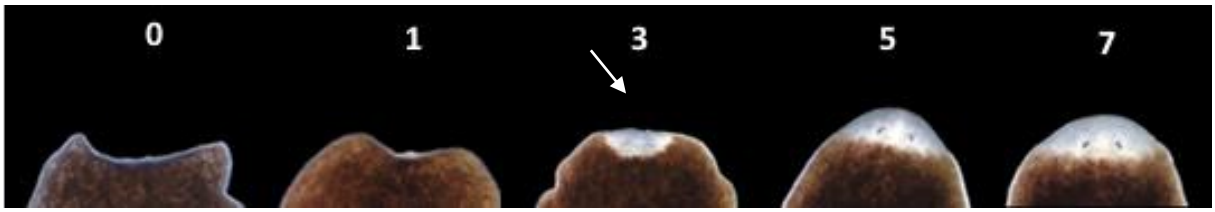


Figure 3. Regeneration process of the *Schmidtea mediterranea* head. *The blastema (visible at day 3, indicated with the arrow) is an unpigmented bud. One week after amputation, the anatomy of the animal is fully restored. Numbers refer to days post-amputation. Figure adapted from Newmark et al. (2002)* (17).

The process of regeneration is driven by injury, e.g. amputation (Figure 3). Rapidly, a protective mucosal coating followed by a thin layer of epithelium covers the wound. Afterwards neoblasts will migrate to the wounding site and will start to proliferate in order to produce a blastema. This is an unpigmented bud which is visible at day 3 post-amputation (Figure 3). During the following days, differentiated cells emerge from the blastema and the anatomy of the animal is recovered in approximately seven days. Subsequently, the behavioral characteristics are restored. The regeneration process is tightly regulated by either blocking the proliferation of neoblasts or by depletion of the neoblasts population (19).

1.2.3. Advantages in regenerative research

The use of *S. mediterranea* as a model organism holds many advantages. These include their small size and the fact that they are inexpensive and easy to breed (20). More than 100 years of scientific literature reporting experiments with planarians is available. These animals are easy to manipulate experimentally (13) and they have a pool of stem cells which is easy accessible (14). Therefore, by using planarians, it is possible to study stem cells *in vivo*. The planarian *S. mediterranea* is a stable diploid (2n = 8) with a genome size of about 4.8×10^8 base pairs,

which is nearly half that of other planarians (4). Its genome is fully sequenced (21), therefore the ability to perform molecular studies, like RNA interference (RNAi) (22) and *in situ* hybridization, is available (23).

S. mediterranea planarians have already been proven useful in other studies. For instance, they are used for investigating the effect of carcinogenic compounds (24). Since they are invertebrates, they are also used to reduce the use of laboratory animals (20). Planarians will never fully replace the use of higher organisms, but by performing pre-screenings, flatworms can limit their use.

Planarians are also relevant for vertebrate studies. In *S. mediterranea*, 85% of genes responsible for regeneration are conserved in other animals (25). Flatworms also use many of the neurotransmitters that are also present in mammals (26). Since there is a high degree of conservation, planarians are relevant to provide insights into the ability to activate recovery of mammalian neuronal networks in response to neurodegenerative diseases. Accordingly, they can improve our understanding of regulatory mechanisms that control neuroregeneration (27).

1.3. Neuroregeneration in planarians

Since the capacity to regenerate the CNS in mammals and lower organisms is limited, a lot of insights can be gained by studying animals that fully master this feature themselves, such as planarians. The rebuilding of their brain only lasts about four to seven days and is performed in three steps (Figure 4). When regeneration is induced by the amputation of the head, a blastema is formed. One day post amputation, a brain rudiment is present. This structure then grows and advances to a properly patterned brain. Finally, the planarian brain is functionally recovered when old and new nervous tissue connects (27).

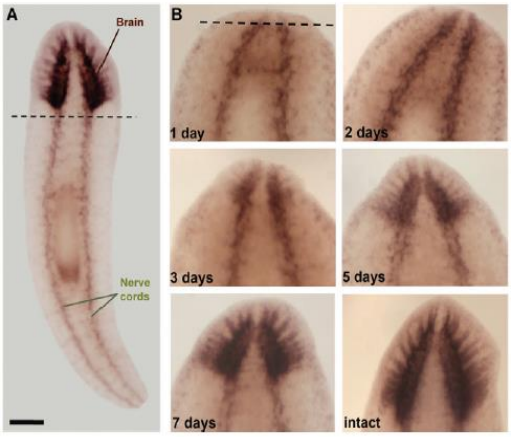


Figure 4. Planarian neuroregeneration. *The CNS of Dugesia japonica planarians was visualized by whole-mount in situ hybridization against a pan-neural marker (Cebrià et al. 2002) in (A) intact and (B) regenerating animals. During CNS regeneration, one day after amputation, a brain rudiment is formed. The brain is functionally recovered after seven days, when old and new neurons reconnect. Scale bar: 0.5 mm. Dashed line: place of decapitation. Figure from Gentile et al. (2011)* (27).

Previous research in *S. mediterranea* has demonstrated the importance of reactive oxygen species (ROS) during regeneration (28). ROS are reactive oxygen molecules that exert a dual function in physiological conditions. It can have detrimental effects when the redox balance is disturbed. This condition is associated with various pathologies such as neurodegeneration, cancer and cardiovascular diseases. On the other hand, ROS are essential for the regulation of diverse physiological processes, e.g. neurological functioning, wound healing, immunology and development (28).

The role of ROS during regeneration has also been investigated in other organisms. In the regenerative MRL mouse, it was hypothesized that ROS regulated p21- and/or p16-induced senescence, a potential mechanism to

increase the functioning of stem cells (30). An increase of H_2O_2 at the wound site following skin injury was observed in zebrafish larvae. This increase in ROS promotes the regeneration of the peripheral sensory axons (31).

In planarians, Pirotte *et al.* (2015) established that a ROS burst is initiated during wound healing in *S. mediterranea*. It was reported that this increase in ROS was essential for proper blastema and photoreceptor formation. The oxidative balance itself had no effect on wound closure, nor on the proliferation of stem cells. Otherwise, it was reported that ROS were essential to coordinate patterning and the localization, initiation and regulation of differentiation in planarians (28). When investigating the necessity of neuronal signaling during neuroregeneration, similar results were found. It was reported that neuronal degeneration prior to amputation resulted in regeneration defaults, such as decreased activity and C-shaped movements. It was hypothesized that a neuronal threshold was essential to initiate regeneration: a certain amount of neurons could be necessary to induce regeneration. This could imply that a correct balance of neuronal signaling is important for neuroregeneration. Neuronal signaling seemed to have no effect on proliferation, but it did influence differentiation, polarity and the ROS burst during amputation. Based on the similarities between ROS and neuronal signaling during regeneration, and the reduced ROS burst during neurodegeneration, it is likely that these two systems interact. On the other hand, both conditions caused different phenotypes: inhibition of ROS resulted in reduced blastema size and when too much neurons were damaged, regeneration was blocked. This implies that there is a possibility that both these processes can interact but can also work independently (29).

1.4. Neurodegeneration in toxicology

In order to induce neuroregeneration, degeneration of neuronal tissue must first be induced. This can be done using various neurotoxins. Accordingly, to create a Parkinsonian flatworm model, the animals can be exposed to 6-hydroxydopamine (6-OHDA) (32,33) while general neuronal damage can be induced by exposure to ethanol (EtOH) (29).

1.4.1. Ethanol

Alcohol (EtOH) is psychoactive and recreational drug used by humans. Although it is part of our current lifestyle, its effects are often underestimated. Heavy alcohol abuse can have acute and chronic adverse effects on the structure and function of the brain. This neurotoxin can interfere with the normal functioning of both mature, and more importantly, developing brains (34). Prenatal exposure to alcohol in humans leads to the impairment of the early CNS development. All diseases followed by prenatal alcohol exposure are gathered in one disorder, the Fetal Alcohol Spectrum Disorder (FASD). It is characterized by neurodevelopmental deficits, such as craniofacial dysmorphologies, growth deficits, deficiencies of the CNS and severe neurobehavioral impairments (35). In adults, alcohol is the cause of dementias ranging from Mild Cognitive Impairment (MCI) to severe dementias (36).

One mechanism by which alcohol causes neurodegeneration is by inducing pro-inflammatory enzymes, which in turn produce oxidative stress. Alcohol affects multiple pathways, including nuclear factor κB (NF-κB) and cAMP responsive element-binding protein (CREB) signaling. NF-κB is pro-inflammatory, whereas CREB is pro-survival. When alcohol is taken up, it causes upregulation of NF-κB. As a consequence, pro-inflammatory processes increase. On the other hand, CREB is downregulated, which causes a decrease in survival mechanisms. Consequently, protection from excitotoxicity and apoptosis is lowered and pro-inflammatory cascades in the brain are activated, which causes neurotoxicity by ways of atrophy, shrinkage, degeneration and inhibition of neurogenesis (37).

When exposed to low levels of alcohol, most humans experience disinhibition and euphoria. Increase in exposure results in incoordination and confusion (38). Changes in behavior in response to EtOH exposure have also been investigated in other organisms, including the fruit fly (*Drosophila melanogaster*), zebrafish (*D. rerio*) and planarians. In the fruit fly, a biphasic response is exhibited. It seems that low levels of alcohol stimulate locomotion, whereas high levels reduce it (38). These behavioral alterations are very similar to alcohol exposure in humans. In the zebrafish, among others, an increase in locomotion and aggression was observed when the animals voluntarily ingested EtOH (39). Another study performed by Shan *et al.* (2015) reported that zebrafish exposed to EtOH during gastrulation also showed behavioral alterations. They concluded that early EtOH exposure had detrimental effects on the CNS (40). In *Dugesia dorotocephala* planarians, in addition to C-shape movements (Figure S 1), motor dysfunctions and impaired negative phototaxis were also observed when exposed to 1% EtOH (41). In the planarian species *Schmidtea mediterranea,* decreased mobility and negative phototaxis were observed after 1% EtOH treatment (42). Pirotte *et al.* (2015) reported that 2% EtOH exposure for 24 hours caused degradation of the region in front of the photoreceptors in *Schmidtea mediterranea*. Behavioral alterations included decreased activity and C-shaped movements (29).

1.4.2. Six-hydroxydopamine

The neurotoxin 6-OHDA is a compound that specifically damages dopaminergic neurons, and is often used to create *in vivo* models of PD. PD is one of the most common neurodegenerative diseases and is characterized by loss of dopaminergic neurons in the *substantia nigra pars compacta* (43,44). The motor symptoms caused by this loss can, among others, be treated with dopamine agonists. Unfortunately, progression of the disease cannot be reversed (3). The etiology of PD is still unknown. It is thought that both genetic as environmental factors contribute to the development of this neurodegenerative disease (43). This hypothesis is stated by the fact that the pesticides paraquat and rotenone cause damage to dopaminergic neurons and subsequently induce similar symptoms as seen with human PD (45).

Since 6-OHDA is a hydroxylated analog of dopamine, it damages the CNS in a specific manner (Figure 5). The dopamine or noradrenaline membrane transporters (DAT and NAT respectively) take up 6-OHDA due to these structural similarities. Other frequently used compounds to create PD models include MPTP, and more recently

rotenone and paraquat (46). 6-OHDA does not cross the blood-brain barrier, which makes it a safer toxin to manipulate experimentally (47). An additional advantage is its presence in the urine of Parkinson patients (46).

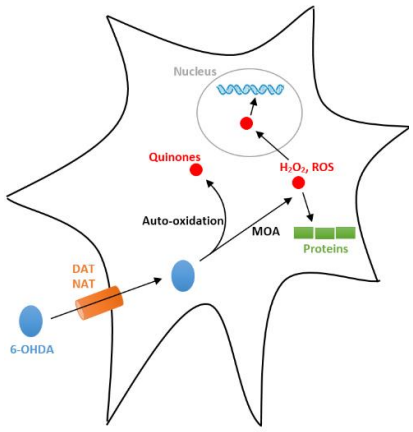


Figure 5. Underlying mechanism of 6-OHDA neurotoxicity. *The compound is intracellularly stored by the dopamine and noradrenaline membrane transporters (DAT and NAT respectively). There it excerts its effects by disturbing the oxidative balance via two mechanisms: deaminiation by monoamine oxidase (MOA) and auto-oxidation. The byproduct of the reaction facilitated by MOA is H_2O_2, a highly toxic oxygen radical. This molecule itself can give rise to other forms of reactive oxygen species (ROS). The compound can also undergo auto-oxidation and give rise to various toxic species such as H_2O_2, quinones and other radicals.*

The neurotoxic effects of 6-OHDA are mainly caused by two crucial events: (I) the intracellular storage of the compound into the catecholaminergic neurons via DAT and NAT and (II) the alteration of the redox balance. Hence, the neurotoxin exerts its effects by producing ROS through both enzymatic and non-enzymatic mechanisms, which in turn can be amplified by the endogenous elements iron and manganese. ROS is produced via two mechanisms: (I) deamination of 6-OHDA by monoamine oxidase (MOA) and (II) non-enzymatic auto-oxidation. MOA is an enzyme that has dopamine, and as a consequence 6-OHDA, as a substrate. This reaction generates H_2O_2, which is highly cytotoxic itself but also gives rise to other reactive intermediates. Under physiological conditions, 6-OHDA undergoes auto-oxidation. This results in the production of various toxic species such as H_2O_2, quinones and other oxygen radicals (43,47).

As a result of dopaminergic neuron loss, clinical symptoms in humans include dementia and motor deficiencies such as hypokinesia, tremor and rigidity (3,43). Behavioral alterations following 6-OHDA exposure have been described in various organisms, ranging from the invertebrate planarians to primates. All described behavioral alterations in these organisms are very similar to symptoms that are associated with human PD. This proves that the neurotoxin 6-OHDA is an ideal compound to model Parkinsonism for research purposes. The underlying mechanisms of neuronal damage, but also neuroregeneration processes and their potential implications for new therapies, can be elucidated by the use of this neurotoxin. A reduction of motor activity was described after systemic injection in Zebrafish by Anichtchik *et al.* (2004) (48). When the Grey Treefrog (*Hyla versicolor*) was exposed to 6-OHDA, both impairment of motor as cognitive functions was observed (49). In mice (*Mus musculus)* motor impairments and drug-induced rotational behavior were seen after infusion with the neurotoxin (50). This drug-induced rotational behavior was also observed in the common marmoset (*Callithrix jacchus*). Additionally, cognitive impairment was reported in this non-human primate (51,52). In planarians, Caronti et al. (1999) systemically administrated the neurotoxin in *Dugesia gonocephala s.l.* Their study was the first one to describe the neurotoxic effects of 6-OHDA in flatworms. After 24 hours of exposure, the animals displayed hypokinesia. This symptom worsened to complete immobility when exposed during seven days. These behavioral changes were caused by a significant reduction in catecholamine content in the neuropil regions after administration of the neurotoxin (30).

1.5. Hypothesis and objectives

Since the molecular mechanisms driving neuroregeneration are not understood yet, *in vivo* and *in vitro* models using neurotoxins are essential. These neurotoxins allow us to induce and study regeneration processes, in order to gain more insights into the fundamentals of neuroregeneration. Several neurotoxic compounds can be used to promote neuroregeneration following neuronal damage. Information about the specific function of involved pathways is necessary to improve research concerning regenerative medicine. This is done in order to reverse or overcome degenerative diseases. Planarians provide the opportunity to gain knowledge about regeneration in an inexpensive, easy manner. During this project, in order to elucidate the key mechanisms of neuroregeneration, planarians were exposed to EtOH and 6-OHDA to cause neurodegeneration, respectively in a general and neurotransmitter specific manner. Because similar experiments using these neurotoxins in planarians have already been carried out (29,32,33,42), EtOH and 6-OHDA are ideal compounds to induce neuronal damage to promote the regeneration process.

Since stem cells can differentiate into new neurons, they have the potential to recover the damaged CNS in planarians. Flatworms themselves hold the potential to be used as a disease model. It is therefore hypothesized that EtOH- and 6-OHDA-induced neurodegeneration in intact planarians induces neuroregeneration mechanisms for recovery. In order to confirm this hypothesis, neurodegeneration caused by these neurotoxins was established. Experimental conditions were determined by performing a morphological and cellular analysis. Concentrations and time points of exposure to the neurotoxic compounds were chosen by observing phenotypical changes, confirmed by visualization of the planarian CNS.

The second aim was to determine the ability of planarians to regenerate following damage to the CNS. Since it has already been confirmed that ROS are key players during neuroregeneration in amputated planarians (28), it was also hypothesized that redox balance was involved during neuroregeneration in intact worms. Therefore, the involvement of ROS during recovery was tested using the redox balance disturbers diphenyleneiodonium chloride (DPI) and buthionine sulfoximine (BSO), respectively a ROS inhibitor and ROS enhancer.

2. Materials and methods

2.1. Planarian cultivation

Asexual strains of *S. mediterranea* were cultivated in the dark at 20°C. They were maintained in planarian medium, containing millipore water and 1.6 mM NaCl, 1.0 mM $CaCl_2$, 1.0 $MgSO_4$, 0.1 mM $MgCl_2$, 0.1 mM KCl and 1.2 mM $NaHCO_3$. Once a week, the animals were fed with calf liver and the medium was refreshed. Prior to experiments, the worms were starved for one week to avoid non-specific binding results (53).

2.2. Experimental set-up

In order to damage the CNS of the animals, the neurotoxic compounds EtOH and 6-OHDA were used. Phenotypical alterations were monitored every day. The neuronal effects of EtOH exposure were assessed by staining the CNS using immunohistochemistry. The staining was performed using an anti-SYNORF antibody, a marker of synaptic vesicles. All assays were performed on intact animals. To assess regenerative capacities, the worms were maintained in culture medium during seven and 14 days after exposure.

2.2.1. Ethanol exposure

The planarians were exposed to various concentrations of EtOH (0.5-2.5%) (Merck Millipore; 100983) dissolved in medium. After 24 or 72 hours, exposure was ended and the worms were washed by rinsing them with culture medium to avoid prolonged EtOH exposure. Medium was used as control.

In order to disturb the oxidative balance during regeneration, the ROS inhibitors DPI (3 µM) (Sigma Aldrich, D2926) and the ROS enhancer BSO (10 mM) (Sigma Aldrich, B2515) were used. Both compounds were respectively dissolved in 0.01% and 0.05% dimethyl sulfoxide (DMSO) (Sigma Aldrich, 471267). DMSO itself was dissolved in medium. Control groups included medium, 0.01% DMSO and 0.05% DMSO (Figure 6).

Figure 6. Overview of experimental groups used during exposure to reactive oxygen species (ROS)-disturbers diphenyleneiodonium chloride (DPI) and buthionine sulfoximine (BSO). *DPI and BSO were respectively dissolved in 0.01% and 0.05% dimethyl sulfoxide (DMSO). Control groups included medium, 0.01% and 0.05% DMSO.*

2.2.2. Six-hydroxydopamine exposure

During optimization, the animals were continuously exposed to 6-OHDA (15-35 µg/ml) (Sigma Aldrich; H4381) and ascorbic acid (0.01-1%) (AsA; Sigma Aldrich; A0278) via their medium. The 6-OHDA solutions were refreshed every two days. Both compounds were dissolved in medium.

Subsequently, the planarians were exposed to various concentrations of 6-OHDA (10-500 mg/ml) dissolved in medium or ascorbic acid (0.3-0.6%) (to stabilize 6-OHDA) via injection. AsA was used as a vehicle control (32). Planarians were cold-anaesthetized on wet filter paper on ice. The animals were injected three times with 32.2 nl of a 6-OHDA solution during consecutive days. Injections were performed using a Drummond Scientific Nanoject injector.

2.3. Planarian Locomotive Velocity assay

Motility was assessed using the Planarian Locomotive Velocity (pLMV) assay designed by Lowe *et al* (2015) (42). The worms were placed in a petri dish on a grid of 0.5 cm and were given 30 seconds of acclimatization time. The pLMV was then quantified as the cumulative number of lines crossed or recrossed (#lines) by the planarian head during three minutes (42) (supplementary Figure S 2).

2.4. Fixation

The worms were fixed and mainly processed as described by Plusquin *et al*. (2012). The animals were placed in a 24-well plate (2 worms/well). For each compound, 1 ml was added to each well unless described otherwise. Incubation occurred in a shaking manner. First, the mucus of the planarians was removed with a fresh 2% HCl/PBS buffer during five minutes. During 2 hours at 4°C, the worms were placed in Carnoy's fixative (60% EtOH, 30% chloroform and 10% acetic acid) to prevent aggregation. Subsequently, the samples were rinsed with methanol (MetOH) for one hour at -20°C. In order to remove pigmentation, they were placed overnight under a cold lamp in 5% H_2O_2/MetOH.

2.5. Immunohistochemistry

Immunochemistry was performed based on the protocol published by Plusquin *et al.* (2012) (24). For each compound, 1 ml was added to each well unless described otherwise. Incubation occurred in a shaking manner. Following fixation, the worms were rehydrated through a graded series of MetOH/PBST washes (75%, 50% and 25% MetOH diluted in PBS containing 0.3% Triton X-100) for 10 minutes each. The samples were then washed two times 10 minutes with PBST. To block non-specific binding sites, the animals were placed in 300 µl 1% BSA/PBST during 2-3 hours. Afterwards, the worms were incubated overnight at 4°C with a primary antibody 3C11 (anti-SYNORF1, Developmental Studies Hybridoma Bank, dilution 1:50). In order to remove excess antibodies, the animals were washed seven times, 10 minutes each, with PBST. Subsequently, the samples were again transferred into 1% BSA/PBST for seven hours, followed by incubation with 50 µl secondary antibody (Alexafluor goat anti-mouse 488, dilution 1:400) in 1% BSA/PST at 4°C for 14-16 hours. On the last day, the samples underwent a series of PBST washes (six times 30 minutes) to remove excess antibodies. Lastly the animals were mounted in glycerol in order to be examined using a fluorescence microscopy with a Nikon Eclipse 80i (Nikon Instruments, Melville, USA).

2.6. Statistics

The statistical significance of the results ($p<0.05$) was checked using one-way or multiple-way ANOVA followed by the Tukey-Test. When necessary, the results were square root transformed in order to obtain normally divided data.

3. Results

3.1. Six-hydroxydopamine exposure

3.1.1. Phenotypical alterations

In order to induce Parkinsonism, the planarians were exposed to various concentrations of 6-OHDA (15, 25 and 35 µg/ml) via their medium. Not even after three days of exposure did these concentrations cause a phenotypical effect, although the solution was refreshed every two days. Following this, the animals were exposed to 6-OHDA dissolved in AsA via their medium to stabilize the neurotoxic compound (32). The controls were placed in AsA solutions. When the controls were exposed to 1%, 0.5% and 0.3% AsA, the animals almost instantaneously died. All concentrations between 0.01-0.3% AsA caused death of the planarians within seven days. Therefore, systemic exposure to 6-OHDA, dissolved in AsA, was not attempted.

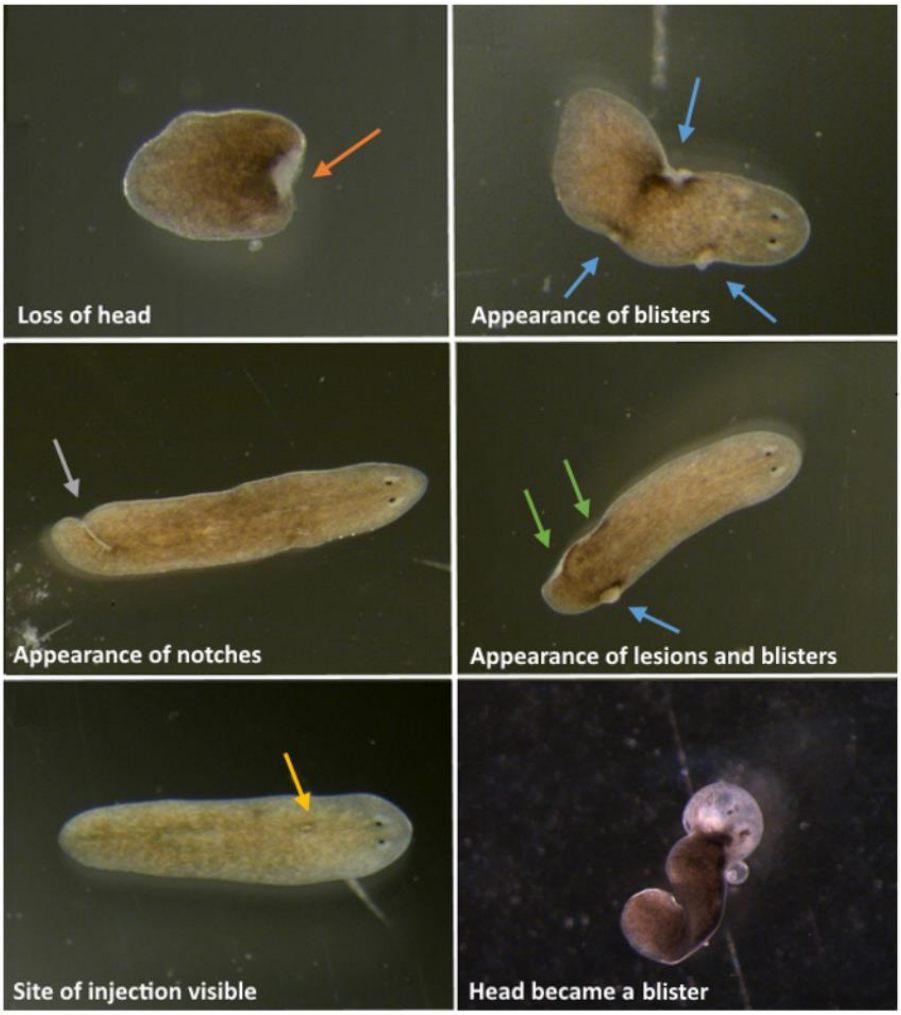
Because of these observations, the worms were injected with 100 nl 6-OHDA (15-500 µg/ml). Still no effects were observed using these concentrations. Therefore, the worms were injected with 6-OHDA dissolved in an AsA solution to stabilize 6-OHDA (32). The animals were injected multiple times in order to induce phenotypical changes. The alterations following injection were caused by various concentrations of 6-OHDA and AsA (supplementary Table S 1). Observed phenotypes included loss of the head part and the appearance of blisters, notches and lesions (Figure 7B). In some cases, the site of injection was still visible after 24 hours (Figure 7B). When higher concentrations of 6-OHDA (25-35 mg/ml) were used, the head of some animals seemed 'exploded'. Their heads became one big blister (Figure 7B).

Since the phenotype caused by 25 mg/ml 6-OHDA was too severe, two lower concentrations of 6-OHDA were chosen to induce (neuronal) damage: 15 and 20 mg/ml 6-OHDA, dissolved in 0.5% AsA. These solutions were injected for two consecutive day (Figure 7A). Six-OHDA exposure caused several phenotypical alterations 24 hours after the last injection (Figure 7B and C). When solely injected with AsA, the majority of animals displayed no effect. The remaining worms had a changed phenotype, including blisters, notches, the area of injection that was still visible and lesions. Some of these morphological changes occurred together. When injected two times with the lowest 6-OHDA concentration, a large proportion of the worms had either occurrence of blisters or no effect. Notches, lesions and loss of the head part was observed in a minority of cases. The appearance of blisters became more prominent when exposed to the highest concentration, 20 mg/ml 6-OHDA. This majority was followed by either no phenotypical effect, notches, loss of the head part and notches and blisters occurring together.

A


First injection
Second injection
48 hours after first injection
0 hours
24 hours
48 hours
... after first exposure

B


Loss of head
Appearance of blisters
Appearance of notches
Appearance of lesions and blisters
Site of injection visible
Head became a blister

C

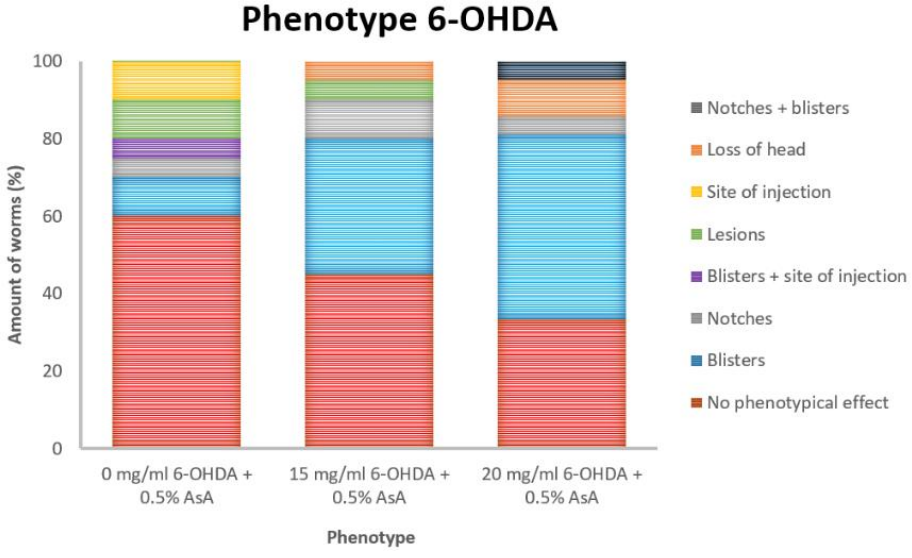

Phenotype 6-OHDA
Amount of worms (%)
100
80
60
40
20
0
0 mg/ml 6-OHDA + 0.5% AsA
15 mg/ml 6-OHDA + 0.5% AsA
20 mg/ml 6-OHDA + 0.5% AsA
Phenotype
Notches + blisters
Loss of head
Site of injection
Lesions
Blisters + site of injection
Notches
Blisters
No phenotypical effect

Figure 7. Phenotypical effects in planarians following 6-OHDA exposure, 48 hours after the first injection. *(A) Overview of observed phenotypes when injected with 15-20 mg/ml 6-OHDA, dissolved in 0.5% ascorbic acid (AsA), during two consecutive days. (B) Respectively, loss of the head part (orange arrow), appearance of blisters (blue arrow), notches (grey arrow) and lesions (green arrow), area of injection still visible (yellow arrow) and convergence of the head into a blister were seen. (C) AsA itself caused alterations in a minority of animals including appearance of blisters, notches and lesions. Additionally, the site of injection was still visible in 15% of the animals, of which 5% also had blisters. The effects of injection seemed to worsen when increasing concentrations of 6-OHDA were used. Besides displaying no phenotypical effect, exposure to 15 and 20 mg/ml 6-OHDA mainly caused the appearance of blisters. Animals were exposed to either 0.5% AsA (n=20), 15 mg/ml 6-OHDA dissolved in 0.5% AsA (n=20) and 20 mg/ml 6-OHDA dissolved in 0.5% AsA (n=21).*

3.2. Ethanol exposure during 24 hours

3.2.1. Phenotypical alterations

During a previous study, Pirotte *et al.* (2015) exposed the worms for 24 hours to a concentration of 2% EtOH in order to induce neuronal damage (29). Following this, two concentrations of EtOH were chosen for this project: 1.5% and 2.5% EtOH (Figure 8A) were used for the induction of neurodegeneration. When the flatworms were exposed this neurotoxin via their medium, the animals displayed various abnormal phenotypes.

When exposed to a low dose of EtOH (1.5%), the worms being contracted and immobile was the most common phenotype (Figure 8B and C). Another common phenotype was the movement of the animals in a gut-sliding manner. In order to move forward, the worm attached its head to the surface, stretched itself and then pulled itself forward (Figure 9). Other phenotypes included screw-like movement (curling up in the shape of a screw), C-shape of the planarian body, loss of the head part and death of the animal. A minority of the surviving worms showed regression of the photoreceptors: the pigment-free patches had disappeared. Two days following exposure, the animals that did not lose their heads, already seemed recovered since there was no noticeable difference between the exposed and control worms. The planarians that shed their heads recovered in a normal manner (Figure 3).

When using a high dose of EtOH (2.5%), the vast majority of worms displayed a C-shape (Figure 8B and D). Other phenotypes included being contracted and immobile, screw-like movement and death of the animal. In the surviving worms, the phenotype was often accompanied by disappearance of the head part. In this case, the worms followed a normal anatomical recovery pattern, as described before (Figure 3).

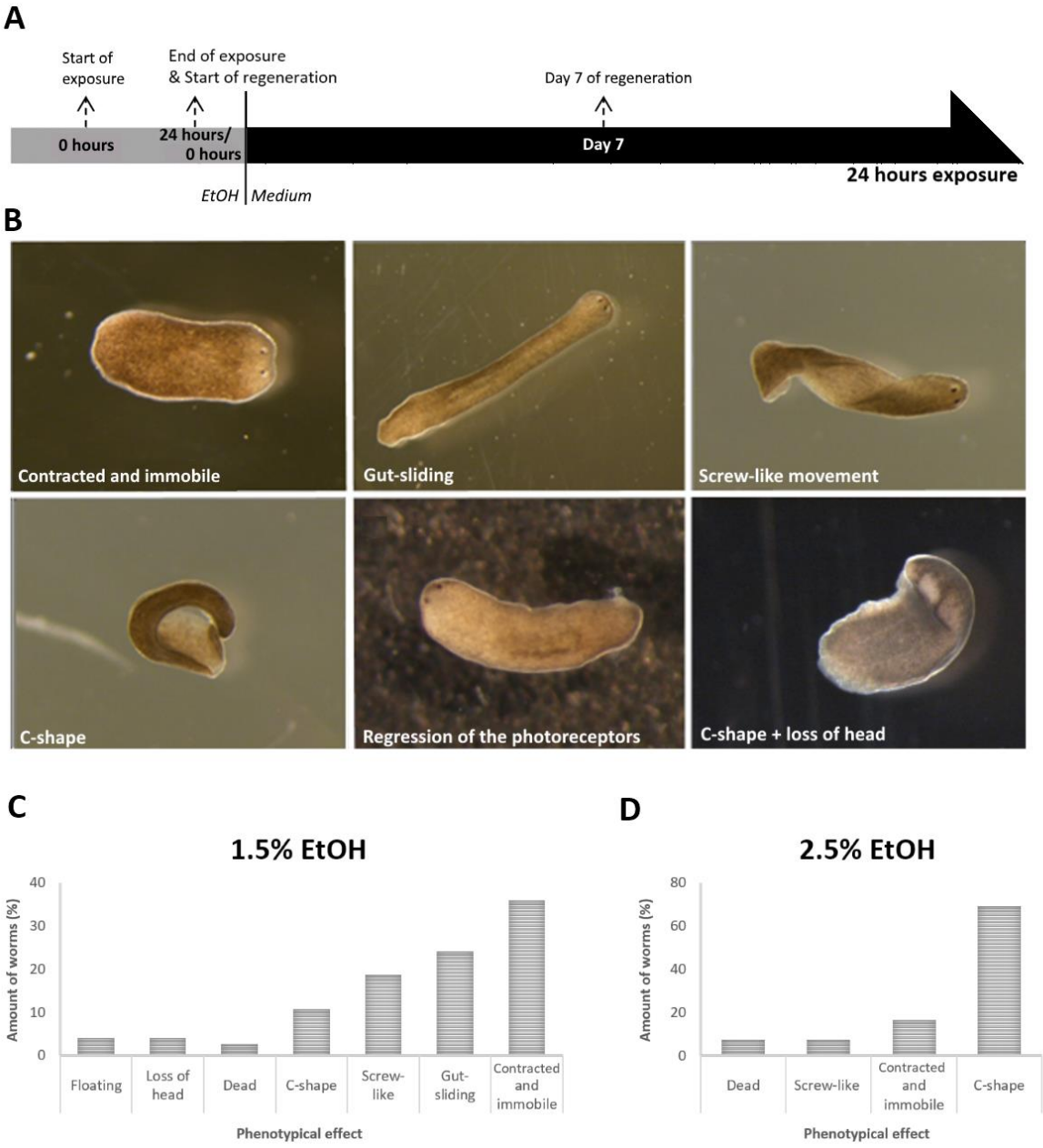


Figure 8. Phenotypical effects following EtOH exposure. *(A) Time frame of experimental conditions. Exposure to EtOH started at day zero. After 24 hours, the worms were placed in medium and regeneration was started. The animals were again analysed after seven days of regeneration. (B) Behavioral alterations followed by EtOH exposure included being contracted and immobile, gut-sliding, screw-like movement, C-shape, regression of the photoreceptors and C-shape accompanied by loss of head. (C) When exposed to 1.5% EtOH, the animals displayed various behavioral alterations. The two most common phenotypes were gut-sliding and being contracted and immobile. After two days of recovery in medium, there was no noticeable difference between the EtOH-exposed worms compared to the controls. (D) Exposure to 2.5% EtOH caused severe phenotypical changes. A C-shape was observed in the vast majority of animals. The head was lost in 51% of the surviving exposed worms (n=51). The worms followed a normal recovery pattern when placed in medium. Animals were exposed to 1.5% EtOH (n=75) and 2.5% EtOH (n=55).*

Figure 9. Gut-sliding performed by the planarian when exposed to EtOH. *In order to move forward, (A) the animal stretches itself (becoming thinner), (B) attaches its head to the surface and (C) pulls its body forward. Attached and stretched body parts are indicated with a line and arrow respectively.*

3.2.2. Anti-SYNORF immunostaining of the central nervous system

In order to confirm that the observed phenotypical effects after 24 hours of exposure (Figure 10A) were caused by the neurotoxin EtOH, an anti-SYNORF immunostaining was carried out. This anti-synapsin staining visualizes the CNS of planarians.

When exposed to 1.5% EtOH during 24 hours, the CNS of the animals did not appear to be damaged (Figure 10B and C). The cephalic ganglia remained intact. After seven days of regeneration, no remarkable differences were observed when staining the CNS (Figure 10B and D).

The loss of the head of the planarians, observed when exposed to 2.5% EtOH during 24 hours, was confirmed by an immunostaining of the CNS (Figure 10B). A gap was present between the two ventral nerve cords and the cephalic ganglia were lost. According to methods used by Hagstrom *et al*. (2015) (20), the brain size was quantified as the ratio of the brain width over the width of the head (supplementary Figure S 3). No significant reduction in the size of the brain was found between the control worms and worms exposed to 1.5% EtOH (Figure 10C). On the other hand, there was a significant difference between the control and 2.5% exposed planarians, since their heads were thrown off (Figure 10C). After seven days of regeneration, the cephalic ganglia had regrown to their former size (Figure 10D).

It should be noted that one general control was used during these experiments. Due to practical issues, the controls of the 24 hours experiments were not useable. Since previous experiments have shown that the CNS of intact worms does not differ greatly, the controls accompanying the regenerating worms were used for comparison of all conditions. Other limitations include the use of a small sample size and the presence of variation, since the experiments were carried out in living animals. In conclusion, these stainings give us a good indication, but in order to obtain correct results, the experiments have to be replicated.

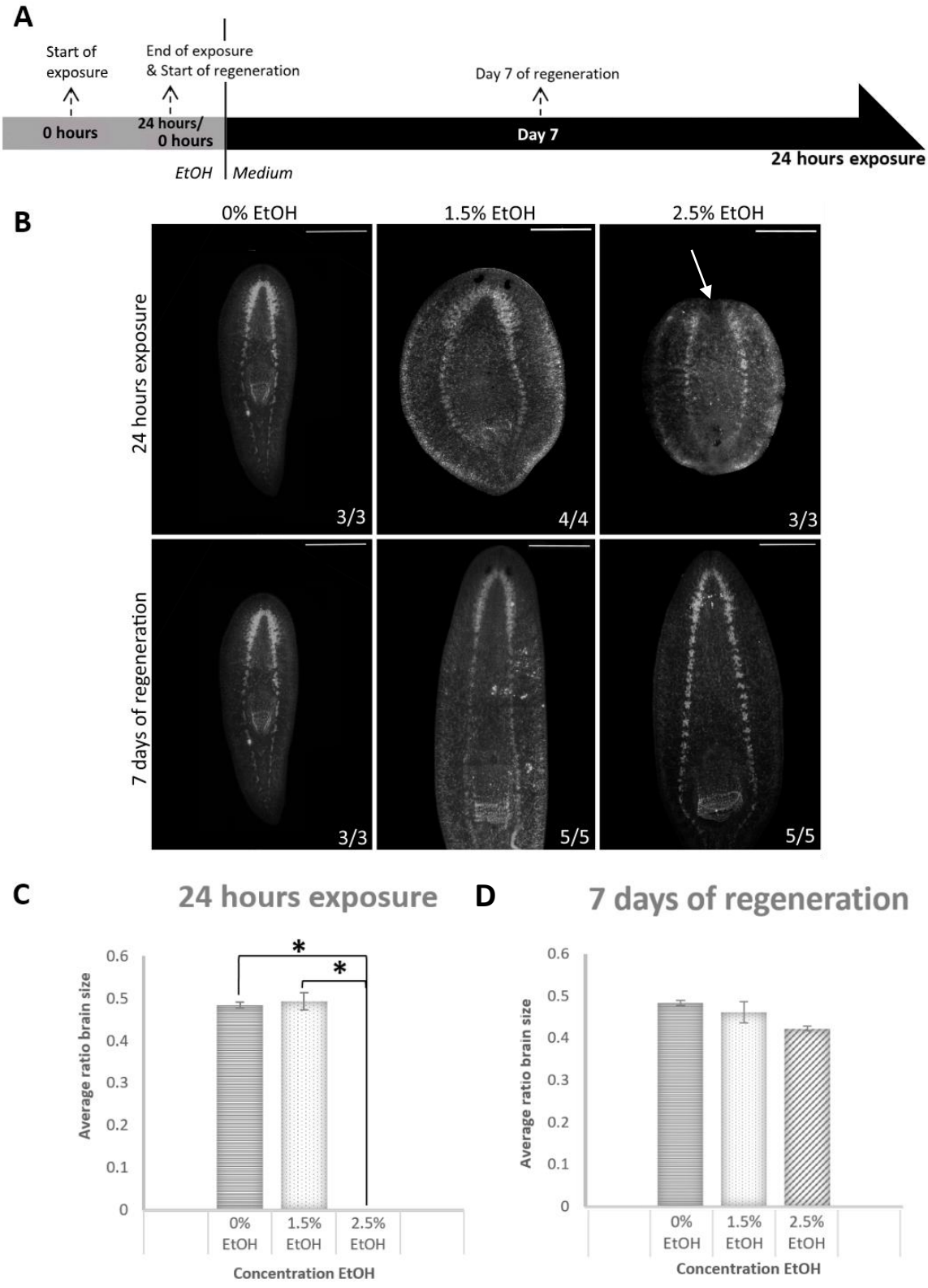


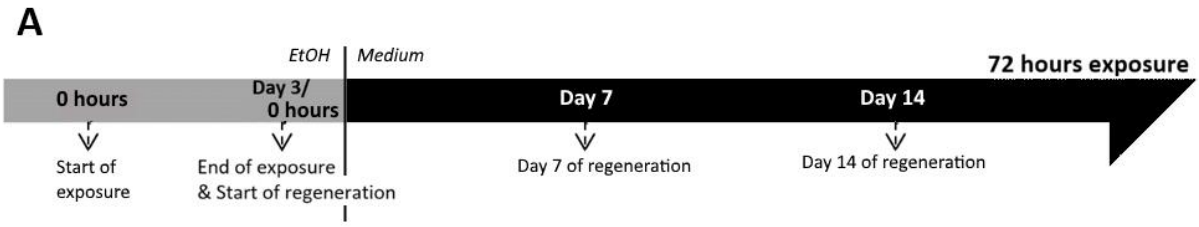
Figure 10. Morphology of the planarian CNS when exposed to EtOH during 24 hours. *(A) Time frame of experimental conditions. Exposure to EtOH (1.5-2.5%) started at day zero. After 24 hours, the animals were placed in medium and regeneration was initiated. The CNS of the animals was stained after seven days of regeneration time. (B) Anti-SYNORF immunostaining performed on EtOH exposed planarians. A concentration of 2.5% EtOH caused the animals to shed their heads, resulting in a gap between the two ventral nerve cords (as indicated with the arrow). Scale bar: 0.5 mm. (C) When*

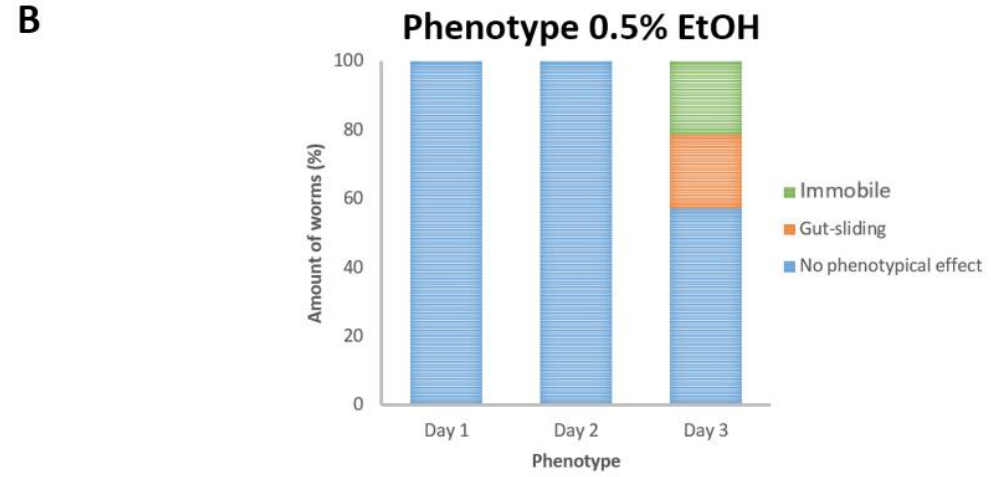
*exposed to 1.5% EtOH, no brain size differences with the control animals were observed. Since the animals exposed to the highest concentration (2.5% EtOH) lost their heads, their brain size was reduced to zero. The brain loss was significant compared to non-exposed or 1.5% EtOH-exposed animals. (D) After seven days of regeneration time, the 2.5% EtOH exposed worms had regrown their brain to a more or less normal size. No significant differences in brain size were found between the three conditions. Animals were exposed to medium (n=6), 1.5% EtOH (n=9) and 2.5% EtOH (n=8). Significant effects: * p < 0.1.*

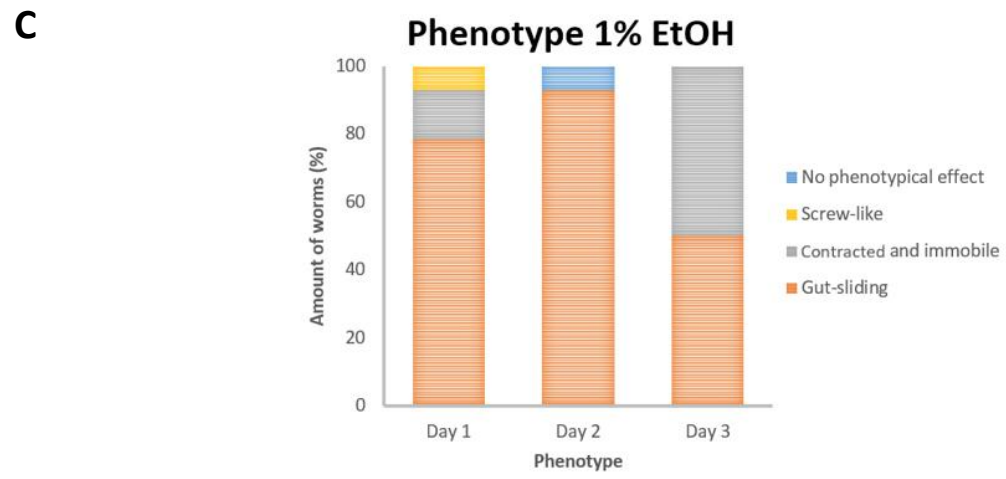
3.3. Ethanol exposure during three days

3.3.1. Phenotypical alterations

We were interested in the induction of CNS damage using EtOH, without regression of the head. Since 2.5% EtOH exposure induced head-loss, other concentrations and durations were chosen to test the impact on the CNS in intact worms. Because the effects of worms placed in 1.5% EtOH varied, this concentration was included in







D

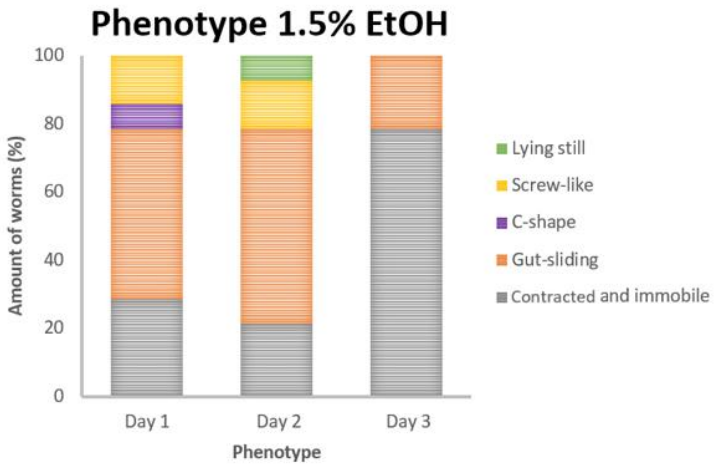


Figure 11. Phenotypical effects following three days of EtOH exposure. *(A) Time frame of experimental conditions. Exposure to EtOH started at day zero. After three days, the worms were placed in medium and regeneration was started. The animals were again analysed after seven and 14 days of regeneration. (B) When exposed to 0.5% EtOH (n=14) during two days, the animals did not show any phenotypical changes. At day three, a minority of worms showed gut-sliding and immobility. (C) At day one and two of 1% EtOH exposure (n=14), gut-sliding was the most observed phenotype. After three days of exposure, gut-sliding and being contracted and immobile were equally observed. (D) After three days, the highest concentration (1.5% EtOH, n=14) mainly caused gut-sliding and being contracted and immobility alterations. The other worms showed gut-sliding behavior.*

following experiments. When exposed to 0.5%, 1% and 1.5% for several days (Figure 11A), the animals displayed different behavioral changes. The solutions were refreshed every other day because EtOH is a volatile substance.

The lowest concentration (0.5% EtOH) only induced behavioral effects in a minority of worms after three days of exposure (Figure 11B). Observed phenotypes included gut-sliding and immobility. Not all worms seemed affected: about half of the animals behaved normally. When exposed to 1% and 1.5% EtOH, all worms displayed abnormal behavior after three days (Figure 11C and D). The two final phenotypes included gut-sliding and being contracted and immobile. In order to follow regeneration after exposure, a new experiment was carried out. We proceeded to work with 1.5% EtOH during three days of exposure, because no regression of the body and/or death of the worm was observed during this period using this concentration.

The same phenotypical alterations were seen as described before (Figure 11D and Figure 12B) when carying out new exposure to 1.5% EtOH during three days. The worms displayed gut-sliding movements and were contracted and immobile. When they were able to regenerate for seven and 14 days, all phenotypical changes were restored.

Subsequently, to determine if EtOH causes hypokinesia, a locomotive assay was carried to analyze motility. The movement of planarians was analyzed after three days of exposure and respectively seven and 14 days of recovery (Figure 12A). After three days of exposure, the worms were significantly less mobile compared to the control animals (Figure 12C). This effect was gone when the planarians were able to regenerate during seven

days. Compared to the controls, the EtOH-exposed worms were significantly faster. After 14 days of regeneration time, no significant differences were found between the two groups.

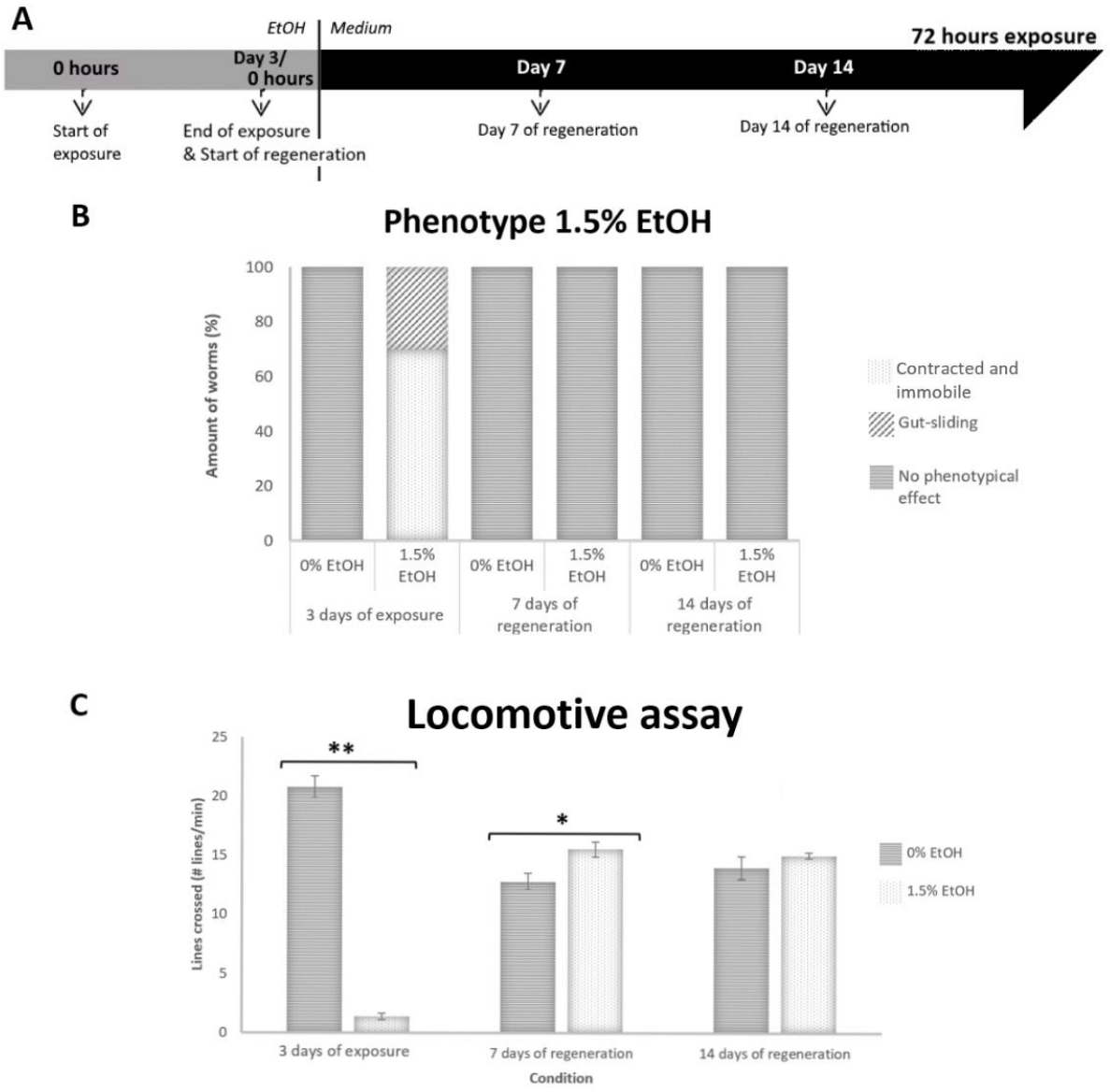


Figure 12. Planarian phenotype during regeneration after three days of exposure to 1.5% EtOH and seven and 14 days of regeneration time. *(A) Time frame of experimental conditions. Exposure to EtOH started at day zero. After three days, the worms were placed in medium and regeneration was initiated. The animals were again analysed after seven and 14 days of regeneration. (B) At day three of exposure, the typical gut-sliding and being contracted and immobile behavioral alterations were observed. When the worms were able to regenerate during seven and 14 days, the behavioral changes were restored. (C) After three days, the EtOH-exposed animals were significantly less mobile than the controls. When able to regenerate during seven days, the controls were slower than the worms that underwent EtOH exposure. After 14 days, no significant differences in mobility were observed. Animals were exposed to medium (n=10) and 1.5% EtOH (n=10). Significant effects: * $p < 0.05$, ** $p < 0.01$.*

3.3.2. Anti-SYNORF immunostaining of the central nervous system

In order to link the behavioral alterations to neuronal damage, an anti-SYNORF immunostaining was carried out on 1.5% EtOH exposed worms to visualize the CNS. The worms were stained after three days of exposure and seven and 14 days of regeneration time (Figure 13A). The cephalic brain ganglia of all worms from various

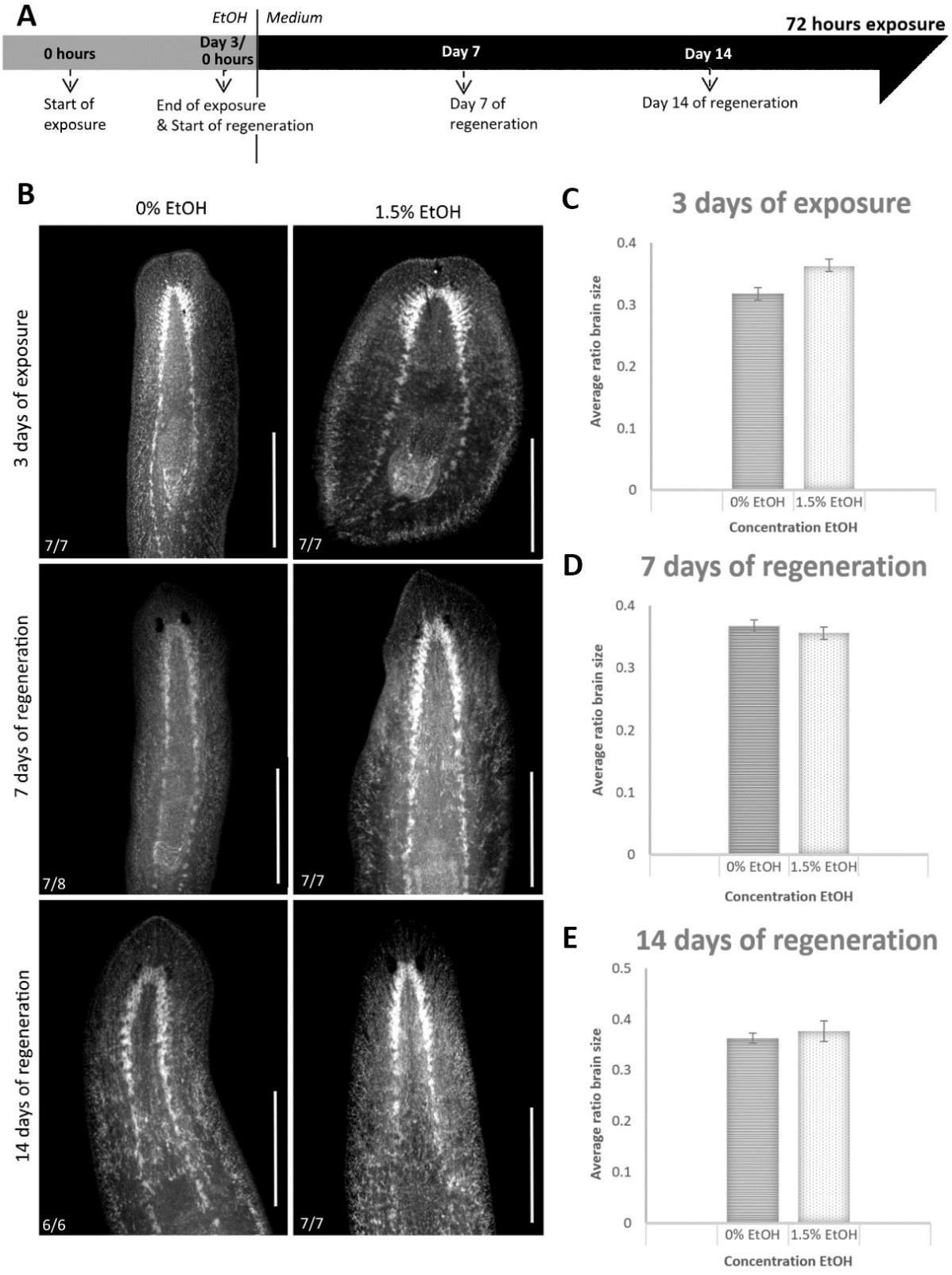


Figure 13. Morphology of the planarian CNS when exposed to EtOH during three days. *(A) Time frame of experimental conditions. Exposure to 1.5% EtOH started at day zero. After three days, the animals were placed in medium and regeneration was initiated. The CNS of the animals was stained after seven and 14 days of regeneration time. (B) Anti-SYNORF immunostaining performed on EtOH exposed planarians at different time points. (C, D and E) When the size of the planarian brain was quantified, no significant differences in brain size were found between the EtOH-exposed and non-exposed worms at different time points. Scale bar: 0.5 mm.*

conditions seemed similar (Figure 13B). The brain branches of the 1.5% EtOH during exposure were more prominent than the other groups. In contrast, when the brain size was quantified, no significant reductions were observed at all time points (Figure 13C, D and E). The brain size of the EtOH-exposed worms was similar to its control after three days of exposure and seven and 14 days of regeneration. Although brain loss was not demonstrated using this general CNS-staining, treatment with EtOH caused behavioral changes which probably originate from damage that is not visible using this technique.

3.3.3. Phenotypical alterations when the redox balance is disturbed

To determine the influence of the redox balance during potential neuroregeneration, planarians were first exposed to 1.5% EtOH during three days to induce neurodegeneration. Subsequently, the worms were treated with ROS disturbers DPI and BSO (dissolved in 0.01% and 0.05% DMSO respectively) during regeneration (Figure 14A). DPI causes a reduction in ROS production, whereas BSO induces an increase. Included control groups were placed in medium, 0.01% and 0.05% DMSO.

Three days of regeneration

When the non-exposed worms were placed in medium during three days, only BSO induced behavioral alterations (Figure 14B). The majority of animals appeared contracted and immobile. The remaining part displayed gut-sliding movements. In contrast, in the EtOH-exposed worms, changes were also seen when placed in the other compounds. Both concentrations of DMSO caused a contracted and immobile phenotype. About half of the DPI-exposed animals showed no alterations. The other half was also contracted and immobile. The majority of BSO-exposed worms displayed a C-shape, the remaining animals were again contracted and immobile. When the mobility was assessed using the locomotive assay, no significant differences were observed between the EtOH-exposed and non-exposed planarians (Figure 14C). Both groups had a significant reduction in mobility when placed in DMSO, DPI and BSO, compared to the control. Between 0.01% and 0.05% DMSO and between 0.01% DMSO and DPI on the other hand, no significant decrease in movement was observed. Interestingly, the BSO-exposed worms were significantly slower than the animals placed in 0.05% DMSO, the BSO solvent.

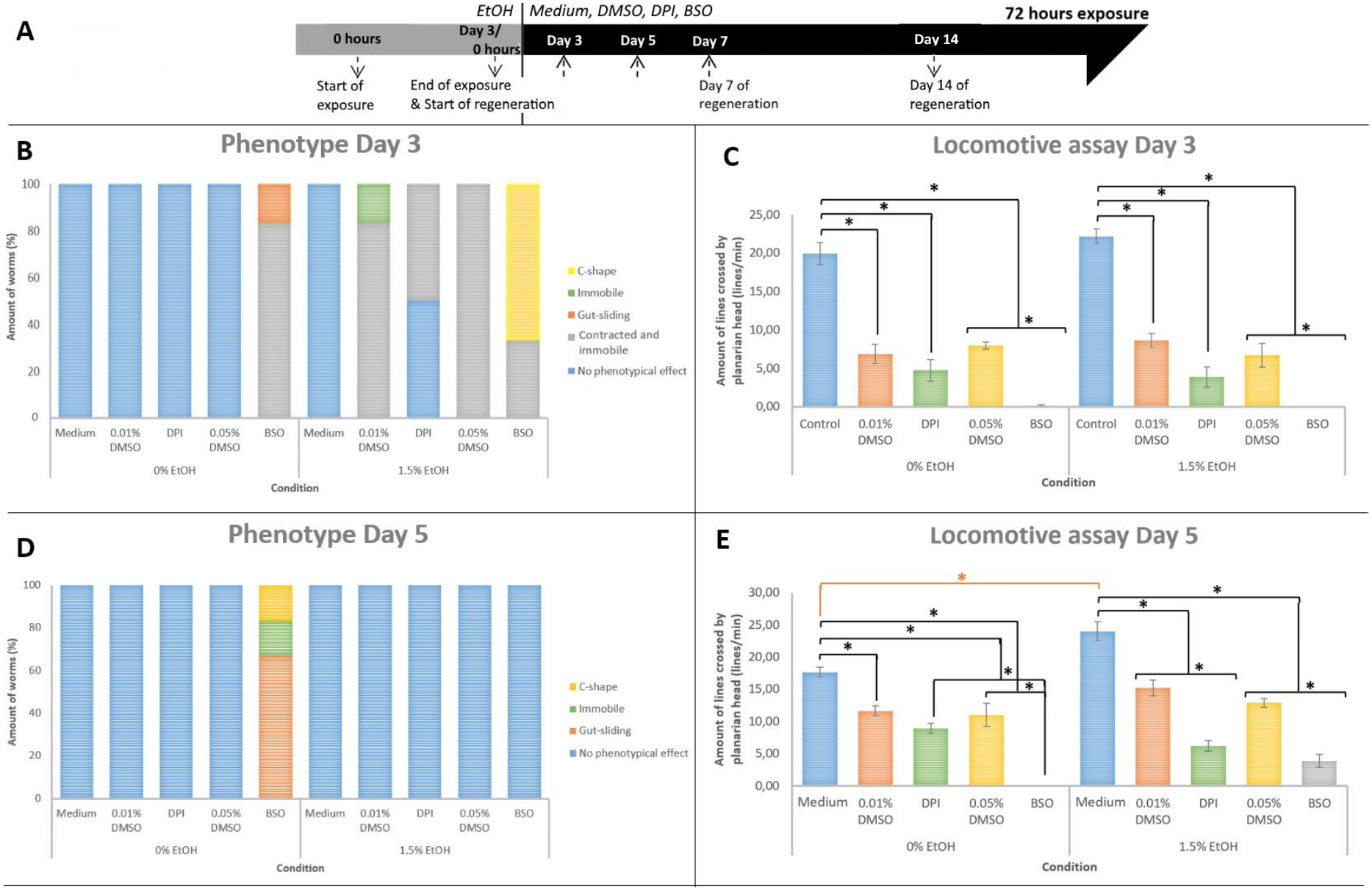
Five days of regeneration

At day five of regeneration, both the EtOH-exposed as non-exposed worms had no changes in their phenotype, except the non-exposed worms that were placed in BSO (Figure 14D). These animals showed gut-sliding, immobility and C-shape alterations. Differences in mobility between the EtOH-exposed and non-exposed animals

were observed in one condition: the worms placed in medium (Figure 14E). The EtOH-administrated worms were significantly faster after five days of recovery than their controls. As was the case after three days of regeneration, in both the 0% EtOH and 1.5% EtOH group, a significant decrease in movement was observed when placed in DMSO, DPI and BSO, compared to the control. The BSO worms in both groups were again significantly slower than the worms in the 0.05% DMSO solvent. Interestingly, in the non-exposed group, a significant change was found between DPI and BSO. In the EtOH-exposed condition, the animals exposed to DPI had reduced mobility compared to its solvent, 0.01% DMSO.

Seven days of regeneration

In both the 0% and 1.5% EtOH group, similar differences in planarian phenotypes were observed when able to regenerate during one week (Figure 14F). In the non-exposed worms, the majority of DPI-worms showed no effect. Additionally, the contracted and immobile phenotype was seen. All BSO-exposed worms showed alterations, ranging from gut-sliding to immobility and C-shape. The remaining animals were dead, due to crawling out of the solution. In the EtOH-exposed group, also the majority DPI-exposed planarians experienced no effect, whereas the other worms displayed gut-sliding behavior (Figure 14G). When placed in BSO, three phenotypes were observed: gut-sliding, C-shape and death of the animal. When the mobility of the planarians was analyzed, the exact same results were observed as seen after three days of regeneration. In both the 0% and 1.5% EtOH-exposed groups, the animals placed in medium were significantly faster than all other conditions and the BSO-exposed worms were slower than the once placed in the BSO-solvent, 0.05% DMSO. Between the EtOH-exposed and non-exposed planarians, no significant differences were found.


A
EtOH
Medium, DMSO, DPI, BSO
72 hours exposure
0 hours
Day 3/ 0 hours
Day 3
Day 5
Day 7
Day 14
Start of exposure
End of exposure & Start of regeneration
Day 7 of regeneration
Day 14 of regeneration
B
Phenotype Day 3
Amount of worms (%)
C-shape
Immobile
Gut-sliding
Contracted and immobile
No phenotypical effect
Medium
0.01% DMSO
DPI
0.05% DMSO
BSO
0% EtOH
1.5% EtOH
Condition
C
Locomotive assay Day 3
Amount of lines crossed by planarian head (lines/min)
Control
Condition
D
Phenotype Day 5
E
Locomotive assay Day 5

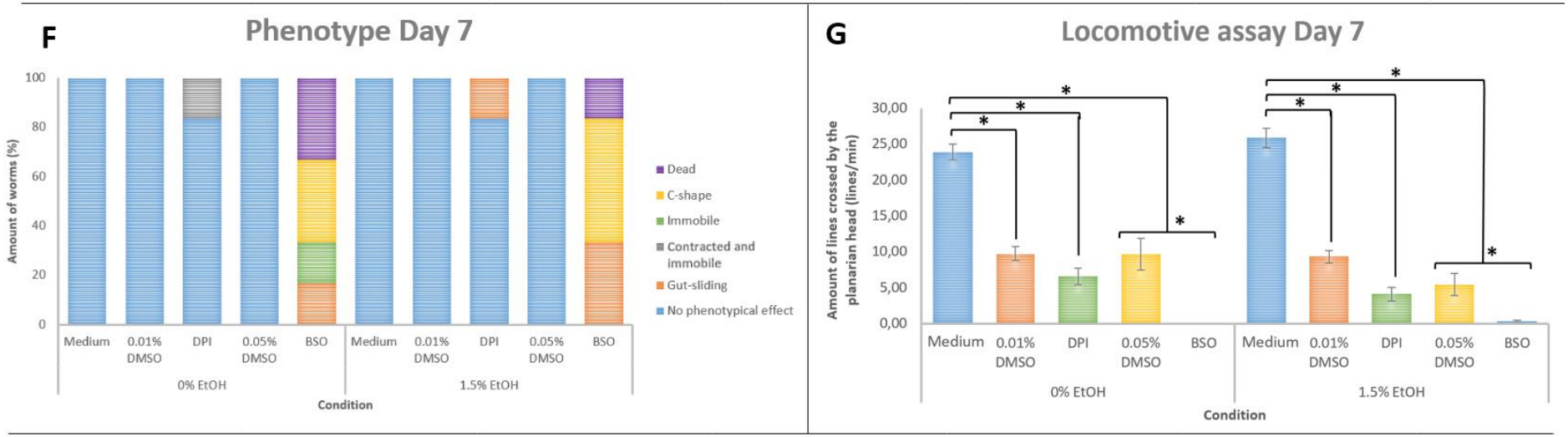


Figure 14. Phenotypical and locomotion analysis during regeneration of planarians exposed for three days to 1.5% EtOH. *(A) Time frame of experimental conditions. Exposure to EtOH started at day zero. After three days, the worms were placed in medium, 0.01% and 0.05% DMSO, DPI and BSO and regeneration was initiated (n=6/condition). The animals were then analysed after three, five and seven days of regeneration time in these various solutions. (B) After three days in medium in the 0% EtOH group, only the planarians placed in BSO showed behavioral alterations. The majority of BSO-worms additionally displayed a C-shape. (C) When the mobility of worms placed in these solvents was analysed after three days of regeneration time, no significant differences were found between the EtOH-exposed and non-exposed worms. Variations between the compounds was the same for both the 0% as the 1.5% EtOH-exposed planarians. (D) After five days of regeneration, both the EtOH-exposed and non-exposed worms placed in DMSO, DPI and BSO, displayed no phenotypical differences. This was with the exception of the 0% EtOH-exposed planarians placed in BSO. (E) When the locomotion of planarians able to regenerate during five days of regeneration was assessed, only one significant difference was found between the EtOH-exposed and non-exposed conditions. The animals placed in medium after exposure to 1.5% EtOH were significantly faster than their controls. (F) The phenotype of exposed planarians was analysed after seven days of regeneration for the last time. In both the EtOH-exposed and non-exposed animals, only the animals placed in DPI and BSO showed behavioral alterations. Similar results were found between the DPI-exposed worms in both the 0% and 1.5% EtOH groups. (G) When the mobility of the planarians was assessed after seven days, the exact same changes were observed as seen after three days of regeneration. Significant effects: * $p < 0.01$.*

4. Discussion

Although the ability to regenerate organs is lost upon ageing in most organisms, planarians maintain this feature for life. In addition, they are one of the few animals that can regenerate their CNS. Therefore, neurotoxins are used to induce planarian neurodegeneration, which allows us to study the following process of neuroregeneration. During this project, two neurotoxic compounds were used: 6-OHDA and EtOH. To create PD models, 6-OHDA is often used. This compound specifically damages the dopaminergic neurons, a characteristic of PD. In contrast, EtOH is a widely abused neurotoxin that induces general damage to the CNS. It is known that behavioral functioning in planarians is strongly associated with brain functioning. Since compounds acting on the CNS of planarians can induce behavioral responses, observation of these alterations can indicate potential damage to the brain (33).

4.1. Exposure to 6-OHDA induces phenotypical changes

Until now, only three studies have used the neurotoxin 6-OHDA to induce neurodegeneration in planarians. The first one was performed by Caronti *et al.* (1999) in the planarian *D. gonocephala s.l.* (33). They administered the compound via the planarian medium. The second study used *D. japonica* and was carried out by Nishimura *et al.* (2011) (32). By injecting 6-OHDA dissolved in a 1.0% AsA solution, damage to dopaminergic neurons was induced. Lastly, the third study carried out by Tsushima *et al.* (2012), also injected *D. japonica* planarians with 6-OHDA, dissolved in 0.3% ASA (54). Based on these studies, our planarians were first exposed to 15-35 µg/ml 6-OHDA (without AsA) via their medium. A possible explanation for the absence of an impact can be explained by the coloration of the solution after a few hours. When the concentration of 6-OHDA was higher, the red coloration of the solution became more intense. This might be caused by the fact that under physiological conditions this neurotoxin is rapidly oxidized by molecular oxygen, giving rise to various ROS. Subsequently, the compound quickly becomes ineffective because it is converted to its *p*-quinone form. Therefore, when AsA is added to the solution, redox-recycling is initiated and 6-OHDA is continuously regenerated from its *p*-quinone form, accompanied by the formation of ROS (55).

Subsequently, only after minimal two injections of 6-OHDA including AsA, phenotypical alterations were seen. Blisters were the most common observed alteration. It cannot be stated that the observed effects were solely caused by 6-OHDA, since the AsA control also displayed the same phenotypical differences. On the other hand, when increasing concentrations of the neurotoxin were used, the effects became more pronounced and increasingly common (e.g. appearance of more blisters in one worm). Additionally, the subset of animals having no effect became smaller. Thus, in order to induce potential neuronal damage, 6-OHDA has to be dissolved in AsA to maintain the redox-recycling of 6-OHDA.

Other studies in lower animals reported hypokinesia and reduction of motor activity in planarians and zebrafish respectively in response to 6-OHDA (33,48). In other organisms, both cognitive and motor dysfunctions were described (49–52). These behavioral alterations were not observed during this project (supplementary

Figure S 4), possibly due to differences in sensitivity. The death of the worms when treated with AsA alone might be explained by the fact that the compound is an acid, which might corrode the mucous layer surrounding the animal. This hypothesis is supported by the worms tolerating higher concentrations of injected AsA, in comparison with exposure via the medium.

Unfortunately, due to practical issues, it could not be confirmed whether 6-OHDA had an impact on the dopaminergic neurons via *in situ* hybridization. Additionally, a subset of worms displayed no phenotypical alterations when treated with 6-OHDA. This is why 6-OHDA was not taken along during the rest of the project.

4.2. Exposure to ethanol induces behavioral and phenotypical alterations

Various behavioral changes were seen when the planarians were exposed to a low dose of EtOH (1.5%), both during brief and prolonged exposure. Based on these findings, we can state that the phenotypes occurring most often were the worms being contracted and immobile followed by gut-sliding movements. It is known that planarian movement is mediated both by ciliary gliding, in which they produce a layer of mucus which propels them forward and muscularly movement, responsible for fine movements such as stretching. Hence, it is possible that EtOH affects the proper functioning of one or both of these mechanisms (56).

EtOH exposure followed by immobility of planarians has already been described before in a different context. Short EtOH (3%) treatment is a method developed by Stevenson *et al.* (2010) to immobilize worms for live imaging purposes (56). They hypothesized that planarian movement was primarily ceased because of inhibition of muscle-mediated movement on a neurological level. In mammals, a change in the firing activity of mouse brains, inhibition of synaptic currents and reduction of the excitability of nerves in the CNS have been described in response to EtOH (56). This is relevant because mammals and planarians share the same neurotransmitters (26). Since the worms were immobile but responsive to tactile stimuli by stretching, they thought that muscle-mediated movement is affected indirectly on a neurological basis. Additionally, they also observed ciliary loss and reduction of mucus production, probably contributing to planarian immobility following EtOH treatment (56).

Although EtOH clearly induces behavioral changes, both brief and prolonged exposure to a low dose of EtOH induced no differences in brain size when an anti-SYNORF immunostaining was carried out. Even though there was no significant difference between the EtOH and non-exposed planarians, the brain branches in the EtOH exposed worms seemed more pronounced. Many underlying reasons can be responsible for the absence of a significant decrease in the brain size of EtOH-exposed worms. As described earlier, EtOH causes a decrease in the production of mucus, reducing the protective layer (56). Therefore, the antibodies added during the immunostaining can possibly penetrate more easily into the body of the worm and bind to its target(s).

Based on these findings, it can be hypothesized that a low dose of EtOH treatment during three days is sufficient to cause neurological damage, since the majority of planarians in this project displayed contracted and immobile behavior. In the remaining animals, gut-sliding behavior was observed. Since these worms can still exhibit ‘fine

movements', such as gut-sliding, their CNS may not have been affected. This assumption is supported by an equal amount of 1% EtOH treated worms displaying gut-sliding movements and being contracted and immobile. When the concentration was elevated to 1.5%, gut-sliding was only observed in a minority of worms, which might imply that more animals were affected on a neurological basis. Additionally, the majority of worms exposed to both concentrations had reduced activity after three days, a phenotypic trait caused by EtOH and associated with neuronal damage, reported by other studies in planarians (29,42).

Although the most observed EtOH-induced phenotypes in intact planarians were gut-sliding and being contracted and immobile, these results have to be interpreted with caution. The phenotypical changes were behavioral, and not morphological, which implies that the observations are a snapshot of reality. A planarian that was immobile at the moment of observation, could have been displaying screw-like movement a few moments later. Additionally, it is an advantage that we can perform this study *in vivo*, but working with living animals also means that there is a lot of variability. On the other hand, a lot of worms (>50) were used during these experiments. Observed changes in behavior can thus give a good indication, but they do not tell the whole story.

The C-shape movements caused by high dosage (2.5%) exposure to EtOH during 24 hours, were also seen in other planarian studies (29,41). In addition, Pirotte *et al.* (2015) reported that 2% EtOH caused degradation of the region in front of the photoreceptors in *S. mediterranea*. We also observed that the worms shed their heads in response to a high dose EtOH. Hence, the brain size was reduced to zero when visualizing the CNS. This implies that the compound induces damage to the brain. Potentially, there is a narrow range between a small or no effect and an event that triggers the planarians to lose their head-part and regrow a new, functional brain. This narrow range of concentrations between low and high dosage of EtOH in planarians should be explored. Additionally, to determine whether prolonged exposure has an effect, the planarians can be exposed to 1.5% EtOH (or higher concentrations) for a continuous period of time. Loss of the head can be used as an endpoint during these observations. Another possibility is to follow the 2.5% EtOH-treated worms for a shorter time-range, to determine the duration of exposure necessary for the worms to experience damage, which causes them to shed their heads. Subsequently, a slightly shorter period of time can be used to observe to what extent EtOH causes neuronal damage.

Since the extent of neuronal damage caused by EtOH is not clear, it can be that hypothesized that EtOH damages neuronal populations which cannot be detected using a general anti-SYNORF immunostaining. Since the immunostaining is used to visualize the general CNS, damage to the individual neuronal populations is not visible. It has been reported that the neurotransmitters acetylcholine, dopamine and serotonin are the underlying mechanisms responsible for neuromuscular movements in planarians (57). As described earlier, the muscular-mediated movement is potentially affected by treatment with EtOH on a neurological level (56). Additionally, it has been described that EtOH can interfere with glutamatergic and dopaminergic pathways (29). Therefore, it is possible that EtOH affects general neuronal populations, which cannot be visualized using an anti-SYNORF immunostaining, resulting in the behavioral alterations. This hypothesis can be investigated by treating the planarians with EtOH followed by *in situ* hybridization to visualize these neuronal populations. Additionally,

neurotransmitter expression can be quantified using qPCR. With positive results, RNAi can possibly be used to knock down these genes and investigate whether this causes the same effects as EtOH exposure.

On the other hand, the similarities between the brain sizes observed during prolonged exposure to 1.5% EtOH can also be the effect of hormesis, a dose-response phenomenon. It is characterized by low-dose stimulation and high-dose inhibition. This model might explain why planarians exposed to 1.5% EtOH have similar brain sizes compared to non-exposed worms, while planarians exposed to 2.5% EtOH during 24 hours immediately shed their heads. It is possible that 1.5% EtOH is a sufficiently low dose to stimulate repair mechanisms which recover the EtOH-induced damage, but which also repair other injuries that were not recovered yet. This in contrast to the higher concentration, 2.5% EtOH, which might induce sufficient neuronal damage for the planarian to regrow its brain. It has already been reported in zebrafish and mammals that EtOH can have an hormesis effect (58,59). In addition to currently observed endpoints (behavior, locomotion and CNS-degradation based on an anti-SYNORF immunostaining), it can be investigated whether EtOH causes an increase in stem cell proliferation during low-dose exposure with an histone H3 immunostaining. Furthermore, by performing qPCR, markers of neuroregeneration in planarians (60) can be determined to check whether CNS repair mechanisms are activated during low-dose treatment.

4.3. Ethanol-induced behavioral and phenotypical alterations are restored within seven days

In order to follow regeneration after exposure to a low dose of EtOH during three days, the phenotype and mobility of the worms was assessed during recovery. After seven days of regeneration time, all behavioral changes were gone and no noticeable differences with non-exposed worms was observed. These findings are complementary with the results of the locomotive assay: the significantly reduced movements after three days of exposure to 1.5% EtOH were gone after seven days of recovery. Faster, but similar recovery was seen in the learning ability of fruit flies after exposure to EtOH (61).

When exposed to a high dose of EtOH, the planarians followed a normal recovery pattern: a head blastema was formed on the decapitated trunk. No significant differences in brain size were found between the EtOH-exposed groups and the control after seven days of regeneration. This might imply that the 2.5% EtOH-exposed worms have fully regrown their brains, although they appear to be smaller than the two other conditions. The apparently smaller brain size might be due to normal regeneration circumstances. In contrast, in planarians that were exposed to EtOH prior to amputation, one study reported failure of head regeneration in 30-40% of the animals (29). Another study in planarians reported that EtOH treatment prior to amputation caused a delay in the recovery of behavior, hence brain function reacquisition, within eight days (42). It seems that generally, the EtOH-induced behavioral alterations are reversible, although the recovery may be delayed.

4.4. Regeneration during oxidative balance disturbance

It has already been established that ROS are a necessity for (neuro)regeneration (28). In order to determine the potential role of ROS during this process, EtOH-exposed planarians were treated with DPI and BSO, both ROS disturbers. DPI is a nonspecific flavoprotein inhibitor which acts by interfering with different electron transporters. By using this compound during regeneration, a maximum reduction of ROS is achieved. On the other hand, BSO is an inhibitor of gamma-glutamylcystein synthetase which inhibits the synthesis of glutathione, an important anti-oxidant. Consequently, treatment with BSO during regeneration causes overproduction of ROS (28). Both compounds were dissolved in DMSO, a solvent used to enhance solubility (53). The behavioral alterations were checked during three, five and seven days of recovery, since it was established that behavioral changes following EtOH treatment were restored within seven days in medium.

Generally, the phenotypical analyses indicate that when the redox balance is disturbed in the beginning of recovery, different phenotypes are observed between the EtOH- and non-exposed groups, with exception of BSO. DMSO and DPI seem to have an effect on the EtOH exposed planarians which is not observed in the non-exposed worms. A possible explanation is that EtOH exposure induced damage which made it harder for the animals to cope with DMSO and DPI treatment during regeneration. This difference was gone after seven days of regeneration time: both groups experienced similar effects. These findings are in contrast with the results of the locomotion assay. Although minimal differences were observed between the 0% EtOH and 1.5% EtOH exposure groups, no clear alterations were seen at different time points. In conclusion, the effects of a disturbed redox balance were very similar between both groups. Whereas BSO clearly influenced the behavior and locomotion of the planarians, DPI treatment did not cause distinct alterations compared to its control (0.01% DMSO). Therefore, independent of potential neuronal damage, an increase in ROS production (BSO treatment) seems to have a greater effect on behavior and locomotion than a decrease (DPI treatment). Although multiple studies using DPI and BSO as ROS disturbers in various organisms have been published, often the impact of these compounds on the phenotype was not mentioned. Pirotte *et al.* (2015) reported phenotypical effects, but they were different from our observations. When exposed to DPI, they reported that the animals had regression of the head and when exposed to BSO, no phenotypical abnormalities were noticed. Long-term exposure (>7 days) to both ROS disturbers resulted in death of the animals (29), which was also seen in our animals. This comparison might also imply that, based on the small sample size, our findings are rather coincidental.

When DMSO was administered, locomotion was persistently affected, also in non-exposed animals. These effects have been described before (53). Additionally, the intrinsic toxicity of DMSO at different biological levels has been pointed out (53). Consequently, DMSO is not the ideal solvent to use for living animals, but its use is inevitable in toxicology because of its ability to solve hydropic compounds (53) (e.g. DPI and BSO). Another approach can be to use an alternative solvent, such as polyethylene glycol (PEG) 200 (62).

The results of behavioral analyses should always be interpreted with caution. The experiments were carried out on living animals, of which the behavior can differ from day to day for no reason. This already causes a lot of variability during the experiments. Additionally, the amount of planarians used was too small to draw conclusions

without repeating the observations. Another limitation is that the planarian locomotion was not assessed via an automated video tracking system and the assay was biased because the experiment was not blind. In order to confirm these results, the assays should be replicated or carried out in another manner.

5. Conclusion

With this study, we demonstrate that prolonged exposure to a low dose of EtOH causes a contracted and immobile phenotype followed by gut-sliding movements in intact planarians. These behavioral alterations were accompanied with decreased locomotion. When the animals were injected with 6-OHDA for two consecutive days, the most common phenotypical alteration was the development of blisters. *S. mediterranea* did not seem very sensitive to 6-OHDA treatment. All phenotypical changes were restored within seven days. No clear difference was observed in EtOH-treated animals when the redox balance was disturbed during recovery.

To increase the reliability of current findings, the experiments should be replicated since only a small amount of animals was used during each experiment. By performing an *in situ* hybridization, potential damage in response to EtOH treatment can be visualized in various neuronal populations. Furthermore, a lot of variation was inherently present since this study was carried out on living animals.

It is very likely that the reduction in activity and the behavioral alterations following EtOH exposure can be explained by neuronal damage in combination with loss of cilia and mucus. Since no neuronal damage was detected using an anti-SYNORF immunostaining, it is not clear where these effects arose from. When the planarians were exposed to a higher concentration of EtOH (2.5%), the animals threw off their head, the part of the body containing the cephalic ganglia. This could mean that EtOH induces damage to the brain and that the injured part is shed followed by the regrowth of a new, functional brain. Consequently, there seems to be a very narrow range of neuronal defects between the two EtOH concentrations that could be explored further.

It can be hypothesized that EtOH damages general neuron populations or that it induces an hormesis effect. Future research focusing on these questions could elucidate the effect of EtOH and 6-OHDA on planarians and this knowledge can then be used to study neuroregeneration processes.

Supplementary material

Figures

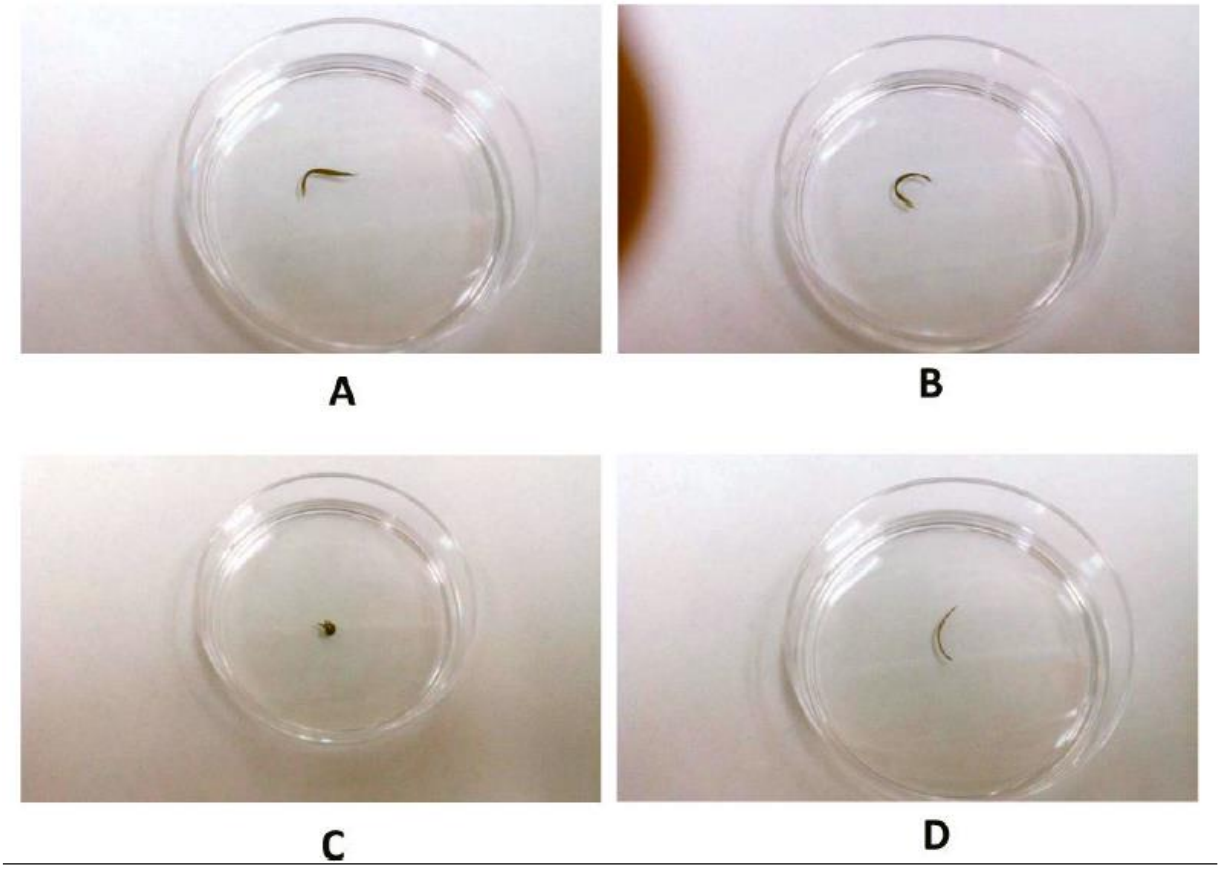


Figure S 1. Representation of a planarian displaying C-shape movements in response to 5 mM cocaine. *Tallarida et al. (2014) reported that concentration-related increases in C-shape movements were also observed when the animals were exposed to ethanol* (41).

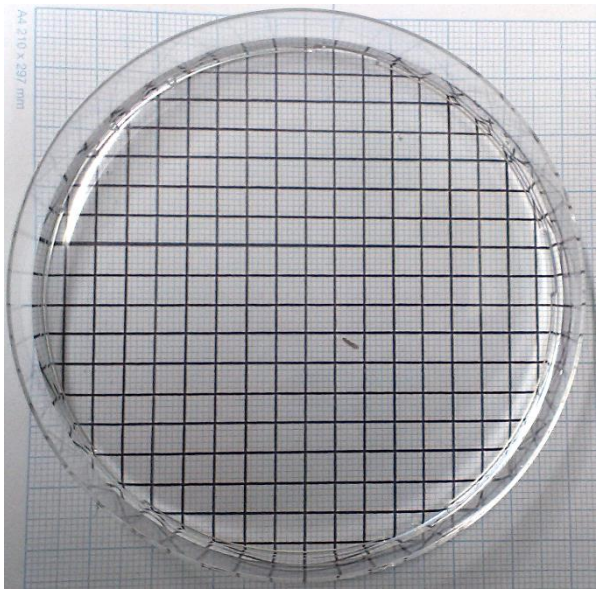


Figure S 2. Example of the locomotion assay. *Motility was assessed using the Planarian Locomotive Velocity (pLMV) assay designed by Lowe* et al *(2015)* (42)*. The worms were placed in a petri dish on a grid of 0.5 cm and were given 30 seconds of acclimatisation time. The pLMV was then quantified as the cumulative number of lines crossed or recrossed by the planarian head during three minutes* (42)*.*

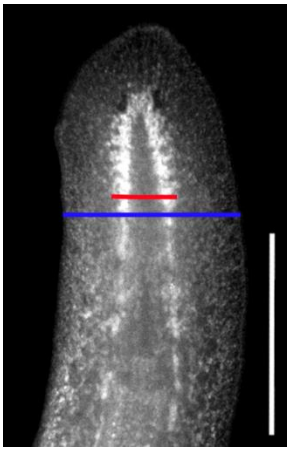


Figure S 3. Quantification of the planarian brain size. *According to methods used by Hagstrom* et al*. (2015)* (20)*, the brain size was quantified as the ratio of the brain width (red line) over the width of the head (blue line). Scale bar: 0.5 mm.*

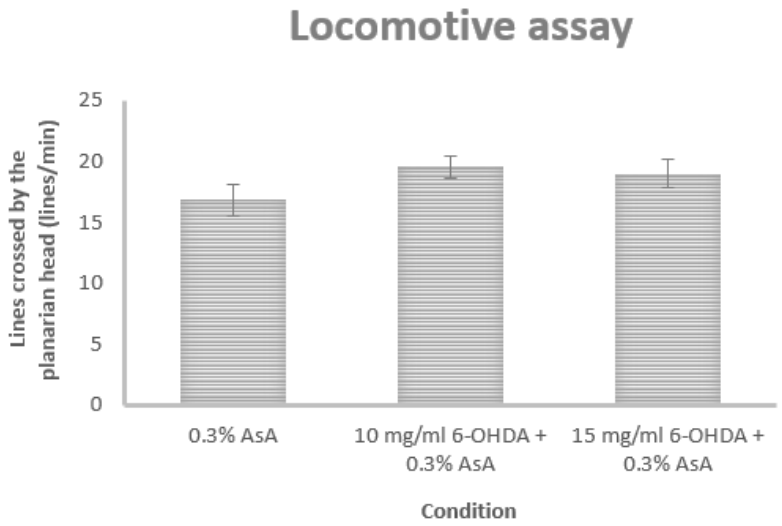


Figure S 4. Mobility of planarians injected for two consecutive days with 6-OHDA dissolved in ascorbic acid (AsA). *Animals were treated with 0.3% AsA, 10 mg/ml 6-OHDA and 15 mg/ml 6-OHDA (n=6/condition). No significant effects were obtained via one-way ANOVA analysis.*

Tables

Table S 1. Phenotypical effects during optimization of the administered 6-OHDA concentration. AsA = ascorbic acid.

Concentration 6-OHDA (mg/ml)	Concentration AsA (%)	Amount of injections	Effect
0	0.3	3	No phenotypical effect
		4	½ Appearance of blisters and notches, loss of head
			½ No phenotypical effect
0	0.6	2	Appearance of blisters
10	0.3	4	No phenotypical effect
10	0.6	2	Appearance of blisters and notches, loss of head
15	0.3	4	½ Appearance of blisters and notches
			½ No phenotypical effect
15	0.6	2	Appearance of blisters
20	0.3	2	No phenotypical effect
20	0.6	1	½ Appearance of blisters and notches
			½ No phenotypical effect
		2	Appearance of blisters and notches
25	0.6	2	Appearance of blisters and notches, loss of head, head became a blister
30	0.6	2	Minor lesions, head became a blister, loss of head
35	0.6	2	Lesions, head became a blister

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