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Faculty of Medicine and Life Sciences
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Master of Biomedical Sciences

Master's thesis

Unveiling The Therapeutic Potential of Glycine Receptor Alpha 2 in Psychosis

Chelsea Hayen

Thesis presented in fulfillment of the requirements for the degree of Master of Biomedical Sciences, specialization Molecular Mechanisms in Health and Disease

SUPERVISOR :

Prof. dr. Bert BRONE

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dr. Elisabeth PICCART

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Unveiling The Therapeutic Potential of Glycine Receptor Alpha 2 in Psychosis*

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ABSTRACT

The striatum is a key structure in the coordination of motor and motivated behavior and integrates glutamatergic input from the cortex and dopaminergic input from the midbrain. In psychosis, however, dopamine release to the striatum is increased. Current therapies achieve an anti-psychotic effect by blocking dopamine receptors, yet systemic administration also induces severe on-target side effects. Here, we investigated the therapeutic potential of the glycine receptor alpha 2, an inhibitory chloride channel, which is the only functionally expressed glycine receptor in the dorsal striatum. We hypothesized that overexpression of glycine receptor alpha 2 in the dorsal striatum in a mouse model of schizophrenia would shunt excessive locomotor activity in response to dopamine. While we successfully established the animal model, overexpression of glycine receptor alpha 2 did not significantly reduce locomotion activity compared to control when including both male and female mice. However, in male mice only, there was a positive trend. We next sought to determine expression levels of glycine receptor alpha 2 in dorsal striatal tissue of human schizophrenia patients and control and revealed similar expression levels. We conclude that more extensive research is required to determine the therapeutic potential of glycine receptor alpha 2.

INTRODUCTION

Psychosis is a hallmark feature of many neuropsychiatric, neurodevelopmental, and neurodegenerative disorders and is generally described as a "disconnection from reality" (1, 2). This condition encompasses a diverse range of psychological symptoms, including hallucinations, delusions, paranoia, disorganized thoughts, and negative symptoms. While approximately 1.5 to 3.5% of the population may meet the diagnostic criteria for a psychotic disorder, an even greater number will encounter at least one psychotic symptom over the course of their lives (1, 3, 4).

One well-known neuropsychiatric disorder associated with psychosis is schizophrenia, which effects around 1% of the global population (5). Onset of schizophrenia typically occurs during early adulthood in males, and late twenties in females (6). Psychosis (positive symptoms) is mainly regulated by the dopaminergic system in the striatum. In addition, schizophrenia is characterized by negative and cognitive symptoms, which is mainly regulated by the glutamatergic system in the prefrontal cortex (7-10). Negative symptoms encompass anhedonia, depression, self-neglect, and social withdrawal, while cognitive symptoms manifest as deficits in executive function, working memory, and attention (7, 8, 10-12).

The widespread occurrence of psychosis in various disorders and extensive research has led to many advances in understanding the pathological disease processes that drive psychosis. Nonetheless, the conversion of this understanding to successful innovative therapies for those experiencing psychosis have been limited.

The striatum is the primary input station in the basal ganglia and is responsible for converging glutamatergic input from the cortex and thalamus

and dopaminergic input from the midbrain (13-16). The striatum can be subdivided into two main regions: the dorsal striatum and the ventral striatum. The dorsal striatum, compromised of the caudate nucleus and the putamen, is primarily associated with motor control, and receives inputs related to voluntary movements and sensorimotor integration (17, 18). However, recent evidence suggests that the dorsal striatum is also involved in motivated and goal-directed behavior (18-20).

The principal cell type of the striatum are the striatal projection neurons (SPNs), which represent 95% of all neurons in the striatum (21). The SPN population can be categorized into two distinct cell types based on their axonal projection patterns and their differential expression of dopamine receptors (14, 15, 17, 22). Striatonigral SPNs express the D1 dopamine receptor (D1DR) and directly project to the output nuclei of the basal ganglia, namely the globus pallidus pars interna (GPi) and the substantia nigra pars reticulate (SNr), forming the "direct pathway." In contrast, striatopallidal SPNs express the D2 dopamine receptor (D2DR) and project indirectly to the SNr via globus pallidus pars externa (GPe) and subthalamic nucleus (STN). Hence, striatopallidal SPNs are referred to as "indirect pathway' neurons (15, 22-26).

SPNs receive excitatory glutamatergic inputs on their spines and modulatory dopaminergic inputs on their spine necks (15, 23, 27). Additionally, SPNs exhibit "up" and "down" states. During the "downstate" (rest state), SPNs are hyperpolarized (-80 mV). Strong glutamatergic inputs can drive these cells into an "upstate," which, when combined with spikes in dopamine release, enhances the generation of action potentials (20). Essentially, dopamine spikes amplify strong cortical inputs while suppressing weaker ones.

Abnormalities in DA function is key to psychosis development (28). Converging evidence indicates an increase in dopamine release within the striatum, accompanied by an upregulation of striatal dopamine receptors, leading to dopaminergic hyperactivity in the striatum (7, 28, 29).

Currently, the most common treatment for schizophrenia and psychosis involves the use of antipsychotic medications (11). These medications can be broadly categorized into two groups: typical and atypical antipsychotics (5, 30). Both types of antipsychotics primarily target D2 dopamine receptors in the brain, albeit in different ways. Typical antipsychotics primarily block the D2 receptors, thereby reducing dopamine activity in certain brain regions. On the other hand, atypical antipsychotics modulate dopamine transmission by interacting with both D2 and other neurotransmitter receptors, such as serotonin receptors (10, 13). However, despite the advantages offered by antipsychotics, both types of medications suffer from a significant limitation: their nonspecific targeting in the brain. Consequently, antipsychotic medications affect multiple brain regions beyond the intended target sites, resulting in severe side effects (5).

Collectively, there is a significant demand for innovative therapies that possess the capacity to attenuate the response of SPNs to dopaminergic hyperactivity, in the striatum specifically, to limit side effects.

Present research investigated the therapeutic potential of Glycine Receptor Alpha 2 (GlyRa2) to shunt excessive striatal activity in response to dopamine. Glycine receptors are members of the superfamily of pentameric ligand-gated ion channels, which include nicotinic acetylcholine receptors (nAChR), the serotonin 5-hydroxytryptamine type 3 receptor (5-HT3R), and the type-A y-aminobutyric acid receptor (GABAAR) (31, 32). GlyRs have four known α subunits (α1-α4) and a single β subunit, of which the composition differs between brain regions and developmental age (31-34). GlyRs assemble as homopentamers or heteropentamers, with each transmembrane subunit consisting of a large N-terminal extracellular domain (ECD), four transmembrane domains (M1-M4) connected by short intracellular and extracellular loops and a long intracellular loop connecting M3 to M4 (32, 33).

The Glycine Receptor Alpha 2 (GlyRa2) was traditionally thought to only be present during neurodevelopment and to gradually disappear after birth. However, Molechanova et al. demonstrated that functional GlyRa2 remains expressed in the adult striatum (35). When activated, activation of GlyRa2 induces a chloride current which drives the SPN's membrane potential toward the chloride equilibrium potential (-54 mV). When the SPN is at rest (downstate, -80 mV), GlyRa2 activation will depolarize the SPN. In contrast, when the SPN exceeds its upstate by strong depolarizing inputs, the membrane potential of the SPN will exceed the

equilibrium potential of chloride. Activation of GlyRa2 will result in inhibitory chloride currents and shunting inhibition of the depolarization (35). The GlyRa2 was investigated in a knockout (KO) mouse model to further prove these findings. In Devoght et al. (2023), DA terminals were optogenetically stimulated, and subsequent action potentials in upstate SPNs were recorded. They report that DRD1-expressing cells enhance cell excitability, which was even more pronounced in GlyRa2 KO animals. Furthermore, they showed that GlyRa2 KO enhanced locomotion in response to amphetamine, which increased phasic dopamine signaling (36).

Previously mentioned findings of GlyRa2 KO animals combined with the optimal location of GlyRa2 in the striatum suggest that GlyRa2 can become a novel treatment target for psychosis with limited side effects. **Therefore, we hypothesized that overexpression of GlyRa2 in the dorsal striatum will shunt excessive locomotor activity in response to dopamine, rescuing the psychosis-like behavior in an animal model of schizophrenia.**

EXPERIMENTAL PROCEDURES

Animals – All animal experiments were approved by and performed in accordance with the standards of the Animal Welfare Committee of BIOMED and Hasselt University and the EU directive 2010/63/EU on the protection of animals used for scientific purposes. Animals were maintained under a 12h/12h light/dark cycle with access to food and water ad libitum. During all experiments, wild-type and heterogenous D1Cre (B6.FVB(129S6)-Tg(Drd1a-cre)AGsc/KndIJ) littermates on a C57BL/6 background were used.

Subchronic PCP animal model for schizophrenia – C57BL/6 mice (6-8 weeks old) were administered either physiological saline or phencyclidine hydrochloride (10 mg/kg, i.p., Tocris Bioscience, Bristol, United Kingdom) for ten days (five consecutive days, followed by a rest period of two days, followed by another five consecutive days) at 1% body weight.

Subchronic Amphetamine animal model for schizophrenia – C57BL/6 mice (6-8 weeks old) were administered either physiological saline or amphetamine (Tocris Bioscience) injections in a repeated, intermittent manner over a period of three weeks. In week one, they were administered amphetamine on Monday-Wednesday-Friday with a dose of 1 mg/kg. In the second week they received 2 mg/kg amphetamine, followed by 3 mg/kg amphetamine in the third week.

Neonatal "Dual Hit" animal model for schizophrenia – Mouse pups were administered phencyclidine hydrochloride (10 mg/kg, s.c.) on post-natal day (PND) 7, 9, and 11. On PND 21, pups were weaned and placed in solitary housing. Control animals were administered saline and were group housed. Isolated PCP mice were held in the same housing room, to provide them with auditory, olfactory, and visual but no physical interaction.

Behavioral experiments – Behavioral experiments were conducted during the light phase of the light/dark cycle. All animals were handled daily for one week prior to the start of behavioral testing.

Open Field Locomotion Test – Locomotion activity was measured in a Plexiglas square open-field arena (49 x 49 cm). The baseline activity was recorded for a duration of 30 minutes. Subsequently, the mice were administered PCP (5 mg/kg: i.p.) or amphetamine (3 mg/kg, i.p.). Locomotion stimulated by either PCP or amphetamine was then measured for an additional 90 minutes. The distance traveled per 10-minute intervals, distance traveled per zone and total distance were tracked and analyzed with the EthoVisionXT video-imaging software (Noldus Information Technology BV, Wageningen, The Netherlands).

Forced Swim Task – Mice were placed in a glass cylinder containing water (10 cm deep) at room temperature (25°C) for a duration of five minutes. For analyses, the cylinder was divided into several zones (CM1-CM5). The first zone, CM1, represented a zone where the mouse's center point was located when it was actively swimming. As the mouse adopted a more passive floating posture, its center point shifted to lower zones. The center point of the mouse across the different zones was tracked using the EthoVisionXT video-imaging software (Noldus Information Technology BV).

Novel Object Recognition Test – Mice were first habituated to the arena (49 x 49 cm) for 10 minutes one day before the actual test. The following day consisted of two phases: a training phase and a test phase. During the training phase, the mice were allowed to freely explore one of the objects in the arena for a duration of 10 minutes.

After a gap of 4 hours, the mice were once again placed in the arena, which now contained the previously encountered familiar object as well as a newly introduced novel object. In this phase, each mouse received 10 minutes to explore both objects freely. The EthoVisionXT video-imaging software (Noldus Information Technology BV) was employed to track the time spent exploring the objects. To minimize any potential bias towards specific objects or locations, both the object and its position were randomized after each mouse. To assess the discrimination between the novel and familiar objects, a discrimination index was calculated using the following formula: (time spent on the novel object / total time spent exploring both objects) x 100%. Exploration of an object was defined as the mouse placing its nose within a 2 cm range of the zone where the object was located.

Y-maze – The Y-maze apparatus consisted of three arms, with each arm measuring 40 cm in length, 8 cm in width and 15 cm in height. The mice were placed in one of the arms and allowed to move freely through the maze for a period 5 minutes. The sequence of the arm entries was manually recorded. A triad is defined as the consecutive entry into all three arms. The percentage of spontaneous alternations is calculated by dividing the number of alternations with the number of possible triads x 100.

Genotyping – Mice were genotyped for D1Cre according to the instructions of the KAPA HotStart Mouse Genotyping Kit (KK7352, Sigma Aldrich, Massachusetts, United States). Primers used: 10 mM D1Cre104 forward (5'- TGG CGA TCC CTG AAC ATG TC -3') and 10 mM D1Cre204 reverse (5'- AGA CAG TGT GAA AGC AAG CG -3') oligonucleotide primers (Integrated DNA Technologies, Iowa, United States).

Stereotactic surgery – Synthetic Cre-inducible adeno-associated virus (AAV5-hSYN-DIO-GLRA2-IRES-eYFP: AAV5-GlyRa2) was acquired from Vector Biolabs (Pennsylvania, United States). Stereotactic surgery was performed on 6-week-old mice to deliver four 100 nl injections AAV5-GlyRa2 at four specific sites within each dorsal striatum hemisphere. The infusion coordinates, relative to bregma, were the following: anterior-posterior 0,7 mm and 1,1 mm, mediolateral 1,6 mm, and 2,0 mm, and dorsoventral 3,0 mm relative to the skull surface. Sham animals received identical infusions of a virus containing only eYFP. Surgeries were performed under general anesthesia using Isoflurane (Zoetis). Before starting, the mouse was administered an analgesic (Temgesic, 0.3 mg/ml, BE112515) and eyes were lubricated. Bilateral burr holes were drill in the skull at coordinate site. Infusion of AAV5 was performed with a Hamilton syringe (Neuros, 7000.5, 0.5 µL) over the course of 2 minutes, after which the syringe remained in place for 5 minutes to allow diffusion of the virus.

Human samples – Brains samples of the striatum of patients diagnosed with schizophrenia and non-diagnosed controls were obtained from The Netherlands Brain Bank (Amsterdam, The Netherlands). All donors had given informed consent for autopsy and the use of their tissue for research purposes. Ethical approval was given by the Ethical Committee of Hasselt University.

Laser Capture Microdissection – Single SPN cells were microdissected using a 40x ocular lens along with a 350 nm laser (Leica LMD 7000, Leica Microsystems, MultiModal Molecular Imaging Institute, Maastricht, The Netherlands) to precisely cut the tissue. The microdissected cells were collected into LoBind Eppendorf tubes (Eppendorf AG, Hamburg, Germany). For each tissue section, microdissection was performed in triplicate, resulting in the excision of 3 sets of 200 cells per tissue.

RT-qPCR – RNA extraction was conducted according to the instructions of the Acturus Pico Pure RNA Isolation Kit (12204-01, Applied Biosystems, Foster City, United States). The purity and quantity of the isolated RNA was evaluated using the NanoDrop 1000 Spectrophotometer (Thermo Fisher Scientific). Purified RNA was then converted to cDNA using the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems). For RT-qPCR, Fast SYBR Green Master Mix (Applied Biosystems) was used, containing 2,5 ng of cDNA template, along with 10 mM forward and 10 mM reverse oligonucleotide primers (SUPP table 1, Integrated DNA Technologies). The reaction volume was adjusted to 10 µl with RNase-free water. Primer efficiency was tested using a serial dilution, resulting in values ranging between 93% and 101%. The Ct (threshold cycle) values for each gene were normalized against stable housekeeping genes, which were determined using Normfinder software (MDL, Aarhus University Hospital, Denmark).

RESULTS

The "dual hit" PCP animal model is the most representative model for psychosis –Psychosis is one of the main symptoms that can occur in Schizophrenia. Therefore, we decided to use an animal model of Schizophrenia, as this model has been used to study psychosis for many years. We decided to pilot three possible animal models for schizophrenia: [1] subchronic PCP model, [2] subchronic amphetamine model and [3] neonatal "dual hit" PCP model. To follow up the overall health of our mice after drug administration, mice were weighed before each injection **(Fig 1)**. We observed a significant decrease in body weight in subchronic PCP-treated mice [treatment ($F_{1,42}=22.86$, $p<0.0001$); time ($F_{1.774,74.50}=4.75$, $p=0.014$); treatment x time ($F_{9,378}=5.76$, $p<0.0001$)] compared to saline-

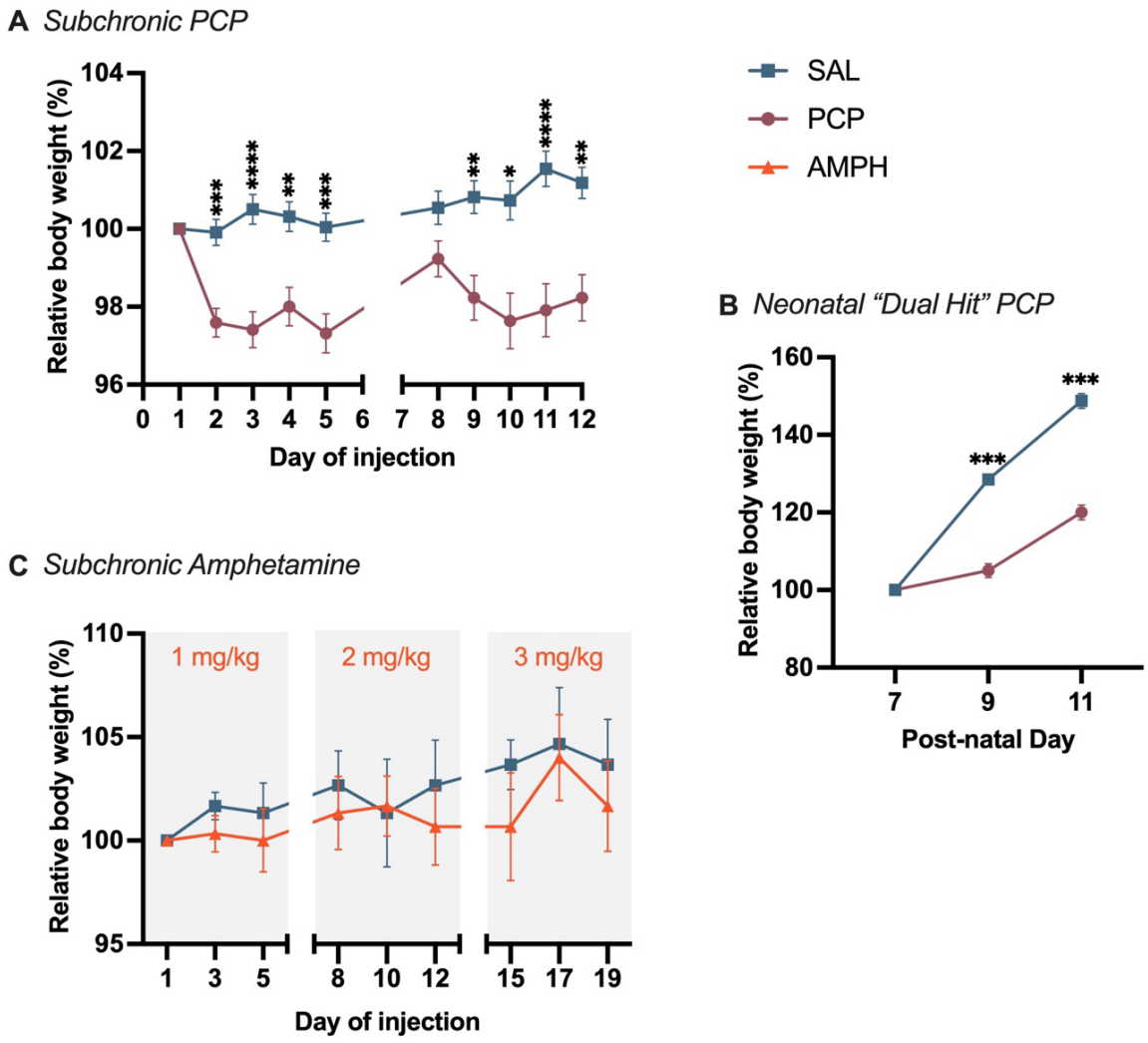


Fig. 1 – Subchronic administration of PCP significantly decreases body weight. (A) Subchronic PCP administration (n=22) significantly decreases body weight. Discontinuation of PCP administration during a two-day period, however, results in a near rescue of body weight loss. **(B)** Subchronic amphetamine administration (n=3) and increase in amphetamine dosage do not significantly decrease body weight. **(C)** Subchronic administration of PCP (n=4) in mice that are 7 to 11 days old significantly lowers the increase in body weight compared to saline-treated controls, which suggests a decline in overall development of PCP-treated mice. Data represented as mean ± SEM. Statistical analyses: two-way RM ANOVA with post hoc Sidak's multiple comparisons analyses. *p<0.05; **p<0.01; ***p<0.001; ****p<0.0001. PCP = phencyclidine; SAL = saline; AMPH = amphetamine.

treated controls, but this was not observed in the subchronic amphetamine-treated mice [treatment ($F_{1,4}$=0.36, p=0.580); time ($F_{2.528,10.11}$=2.70, p=0.108); treatment x time ($F_{8,32}$=0.46, p=0.875)]. In the neonatal "dual hit" PCP-treated mice, we observed a significantly lower increase in body weight in PCP-treated mice compared to saline-treated controls [treatment ($F_{1,6}$=130.7, p<0.0001); time ($F_{1.579,9.473}$=487.7, p<0.0001); treatment x time ($F_{2,12}$=96.71, p<0.0001)]. Taken together, we report that subchronic PCP administration, but not subchronic amphetamine administration, has a negative effect on the overall health in mice.

To investigate psychosis in the different mouse models, we performed the open field locomotion test. In rodents, dopaminergic hyperactivity is often reflected as increased locomotor activity. Acute amphetamine- or PCP-stimulation in an open field locomotion test is a standardized test that is often used to determine the efficacy of antipsychotic drugs (37).

Subchronic PCP-treated mice showed a significant decrease in locomotion activity over time after acute amphetamine administration (3 mg/kg, i.p.) (Fig 2A) [treatment ($F_{1,28}$=6.52, p=0.016); time ($F_{3.003,84.09}$=79.92, p<0.0001); treatment x time ($F_{14,392}$=2.67, p=0.0014)]. The significant reduction in locomotion activity is reflected in the total distance that they traveled after amphetamine administration (t=2.63, df=28, p=0.014) (Fig 2B). In contrast, we report no significant differences in locomotion activity (Fig 2C) [treatment ($F_{1,10}$=0.09, p=0.768); time ($F_{3.253,32.53}$=20.96, p<0.0001); treatment x time ($F_{14,140}$=0.93, p=0.530)] and in the total distance traveled (Fig 2D) (t=0.23, df=10, p=0.826) after acute PCP administration (5 mg/kg, i.p.) in PCP-treated mice compared to saline controls.

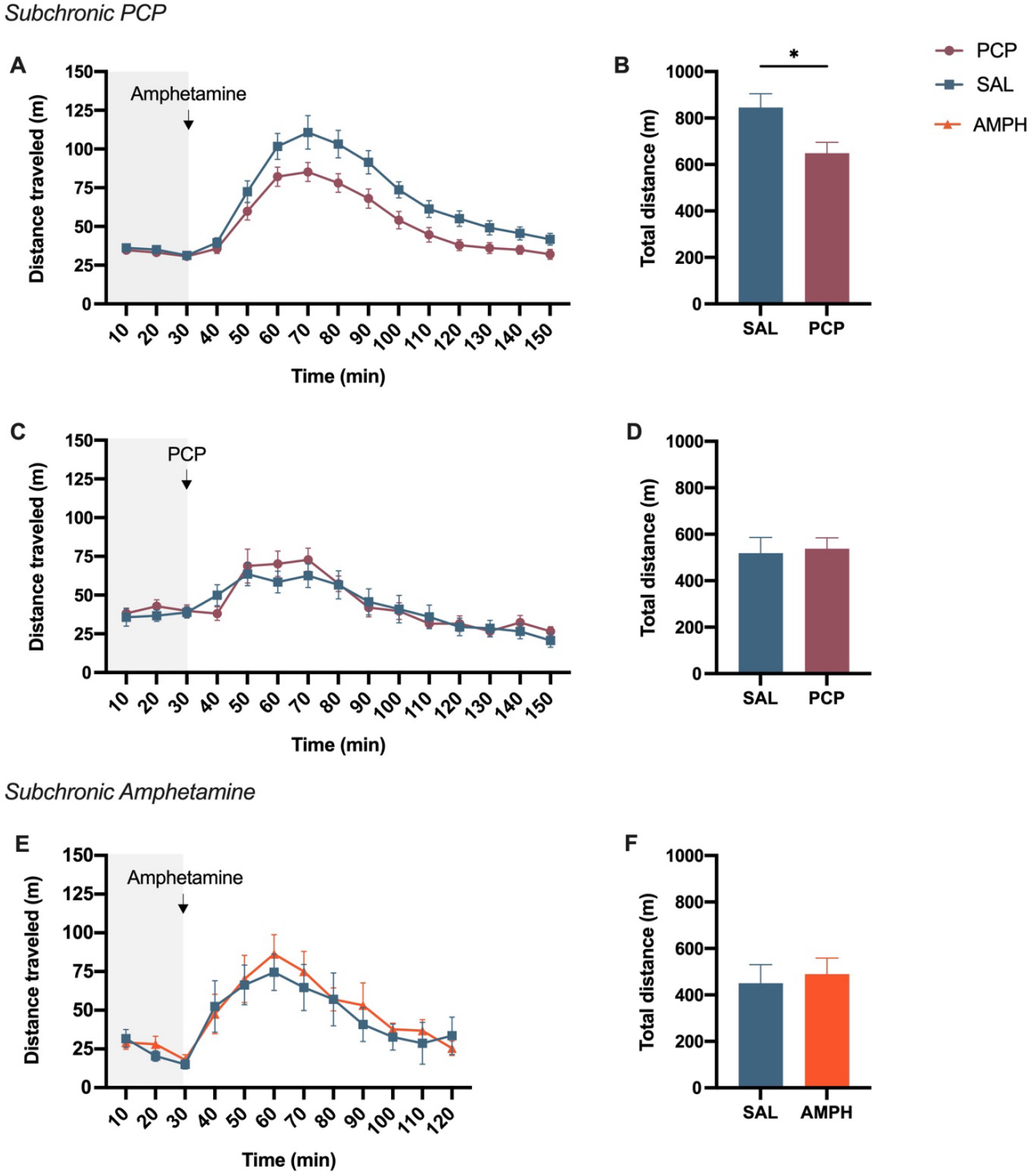
Subchronic amphetamine-treated mice showed no significant differences in locomotion activity (Fig 2E) [treatment ($F_{1,4}$=0.17, p=0.699); time ($F_{1.974,7.895}$=10.35, p=0.0006); treatment x time ($F_{11,44}$=0.28, p=0.699)] and total distance traveled (Fig 2F) (t=0.36, df=4, p=0.735) after acute amphetamine administration (3 mg/kg, i.p.) compared to saline-treated control mice.

In the neonatal "dual hit" PCP treated mice, we report no overall effect of treatment after acute PCP administration (5 mg/kg, i.p.) [main effect treatment ($F_{1,6}$=3.08, p=0.130)], however, there was a time-dependent increase in distance run in PCP-treated mice compared to saline-treated mice [treatment x time effect ($F_{11,66}$=2.55, p=0.0094)] (Fig. 2G). Following acute PCP administration, a few days later, the same mice were subjected to acute amphetamine (3 mg/kg) administration (Fig 2I). Here, we did not observe similar findings to acute PCP administration. Overall, there was no significant increase in locomotion activity in the PCP-treated mice after acute amphetamine administration [treatment ($F_{1,6}$=5.34, p=0.060); time ($F_{2.261,13.56}$=2.90, p=0.085); treatment x time ($F_{11,66}$=1.78, p=0.074)]. In contrast, afore mentioned results were not reflected in the total distance traveled after drug administration. A significant increase was observed after acute amphetamine administration (Fig 2H) (t=2.91, df=6, p=0.027), but not after acute PCP administration (Fig 2J) (t=1.94, df=6, p=0.100).

Although the focus of our hypothesis is on psychosis, we sought to utilize a complete representative model for schizophrenia, and thus investigated some of the afore mentioned models for both negative and cognitive symptoms.

To investigate negative symptoms that occur in schizophrenia, such as anhedonia, social withdrawal and depression, we performed the forced swim test (38). In this test, we measured the duration that the mouse was active swimming (mobility) and when it was passively floating (immobility). Active swimming behavior is displayed in the first few minutes of the test, as the mouse attempts to escape from the water. After a few minutes, the mouse will gradually transition to a passive floating posture, known as immobility. Immobility induced by forced swimming is thought to reflect a state of behavioral despair and is used to test the efficacy of antidepressant drugs (37, 39). Usually, data gathered from this test is represented as time spent immobile. However, even when the mouse adopts a passive floating posture, the mouse is still moving, making it difficult to accurately discern immobility from mobility. Therefore, we divided the cylinder in various zones, where the mice's center point is higher when the mouse is actively swimming compared to lower zones when the mouse is passively floating. Subchronic PCP (Fig. 3A) treated mice show no difference in immobility compared to control treated mice [treatment ($F_{4,150}$=1.016, p=0.401); zones ($F_{4,150}$=78.51, p<0.0001); treatment x zones ($F_{4,150}$=0.00058, p=0.981)]. Neonatal "dual hit"

PCP treated mice (Fig. 3B) show a decrease in active swimming behavior compared to the control mice [treatment ($F_{4,30}=1.94$, $p=0.129$); zones ($F_{4,30}=815.3$, $p<0.0001$); treatment x zones ($F_{4,30}=2.30$, $p=0.140$)]. Additionally, we performed the sucrose preference test in the subchronic PCP model. Anhedonia is a symptom of not only schizophrenia, but depression as well. Generally, depression entails that the ability to experience pleasure from activities or stimuli is diminished. The general hypothesis is that sucrose elicits a positive hedonic reaction. In case of anhedonia or depression, the mouse will not prefer sucrose water over normal water (37). During the experiment, we had to exclude two mice from the experiment due to excessive weight loss after the



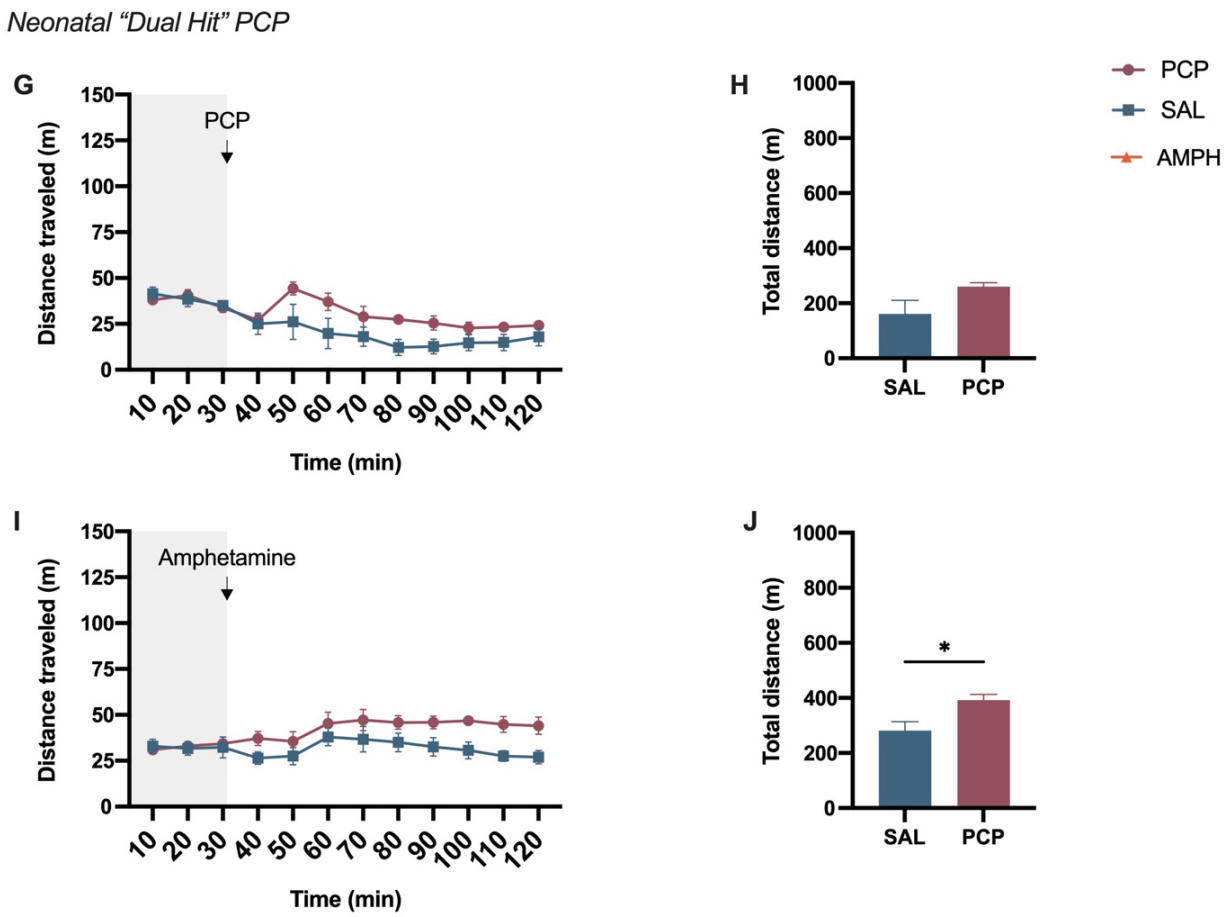


Fig. 2 – Neonatal "dual hit" PCP treated mice show significantly increased locomotion activity following acute amphetamine administration compared to control mice. (A-B) Locomotion activity and total distance travelled following acute amphetamine (3 mg/kg, i.p.) administration in subchronic PCP-treated mice (n=16) is significantly decreased compared to saline-treated controls (n=14), but not following **(C-D)** acute PCP (5 mg/kg, i.p.) administration (n=6). **(E-F)** Locomotion activity in subchronic amphetamine-treated mice is unaltered following acute amphetamine administration compared to saline-treated control mice (n=3). **(G-H)** In neonatal "dual-hit" treated mice no overall effect of treatment is observed following acute PCP administration (5 mg/kg, i.p.). There is, however, a time-dependent increase in distance run compared to saline-treated controls (n=4). **(I)** Acute amphetamine (3 mg/kg, i.p.) administration does not alter locomotion activity in a time-dependent manner in neonatal "dual hit" treated mice compared to controls (n=4). **(J)** In contrast, the total distance travelled in PCP-treated mice compared to saline-controls is significantly increased following acute amphetamine administration (3 mg/kg, i.p.). Data represented as mean ± SEM. *p<0.05. Statistical analyses: (distance traveled) two-way RM ANOVA with Sidak's post hoc multiple comparisons analyses and (total distance) unpaired t-test. *p<0.05.

24-hour habituation period. No significant difference in sucrose preference was observed in the subchronic PCP treated mice compared to control mice (p=0.341) (SUP Fig 1). These data indicate that these models are not able to mimic the negative symptoms that occur in schizophrenia.

To test for cognitive deficits in the subchronic PCP treated or neonatal "dual hit" PCP treated mice, we performed the novel object recognition test and y-maze. The Y-maze test was used to measure spatial working memory in the subchronic PCP mouse model (fig 4A) (39). We observed no differences in spatial alternations in the subchronic PCP treated mice compared to control mice (t=0.39, df=20.57, p=0.699). The novel object recognition test is widely used as way to test working memory in mice (39). We performed the novel object recognition test in both the subchronic PCP and

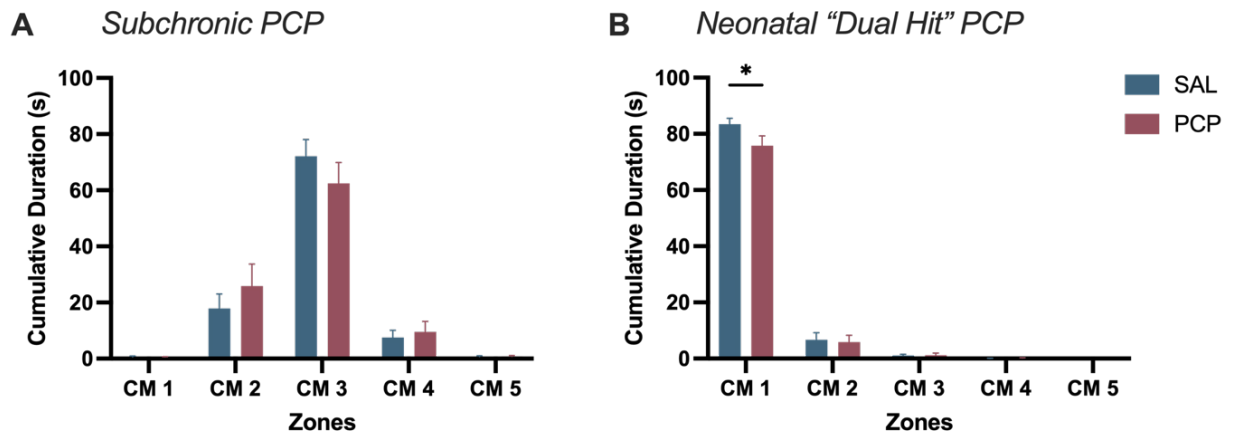


Fig. 3 – The neonatal "dual hit" PCP treated mice show increased behavioral despair compared to control mice. Forced swim task represented as the cumulative duration (s) in various zones of the cylinder to discriminate between active and passive swimming behavior. **(A)** The cumulative duration in the various zones is unaltered in subchronic PCP-treated mice (n=16) compared to saline-treated controls. **(B)** The cumulative duration of dual hit PCP-treated mice (n=4) in the first zone is significantly decreased compared to saline-treated mice, indicating increased passive floating behavior. Data represented as mean ± SEM. Statistical analyses: two-way RM ANOVA with post hoc Sidak's multiple comparisons analyses. *p<0.05.

neonatal "dual hit" model. However, due to object preference in the subchronic PCP treated mice, we were not able to analyze this data. By implementing the use of a different object and randomization of the object and object location, we were able to reduce object preference and potential bias. According to the data, there was no significant discrimination between the novel and familiar object in the neonatal "dual hit" PCP treated mice compared to control mice [treatment (F1,12=0.037,

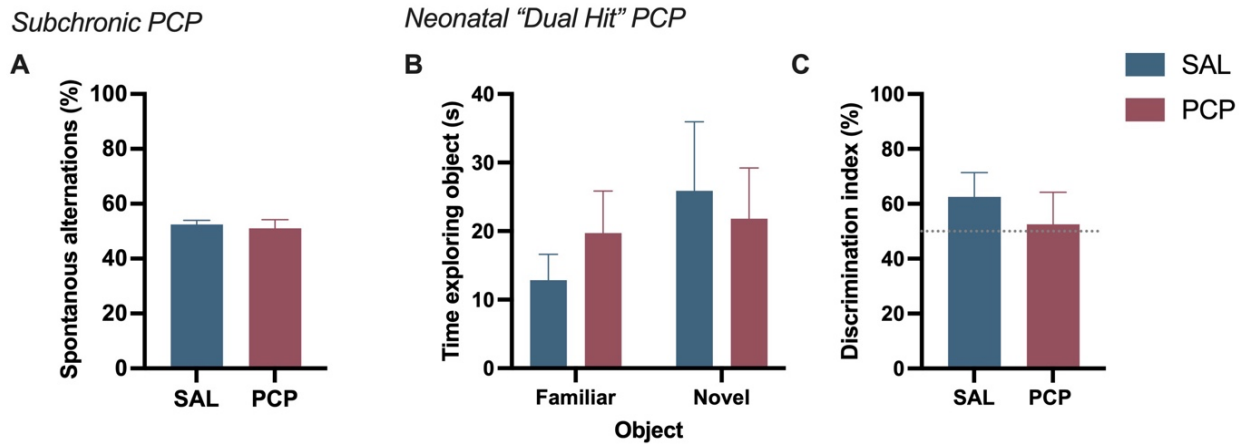


Fig. 4 – Subchronic PCP-treated mice and neonatal "dual hit" PCP-treated mice show no significant alterations in cognition. (A) Percentage of spontaneous alternations in the Y-maze test is unaltered in subchronically PCP-treated mice compared to saline controls, indicating unchanged spatial memory (n=6) (unpaired t-test with Welch's correction). **(B-C)** The novel object recognition test in neonatal "dual hit" PCP-treated mice show unaltered discrimination between novel and familiar objects compared to saline-treated mice (n=4), indicating unchanged working memory. **(B)** Represented as the time spent exploring both objects (ordinary two-way ANOVA with post hoc Sidak's multiple comparisons analyses) and **(C)** the percentage of time spent on the novel object in the 10-minute test phase (unpaired t-test). Data represented as mean ± SEM.

p=0.851); object (F1,12=1.10, p=0.314); treatment x object (F1,12=0.58, p=0.462)] (fig 4B-C). In summation, we report no deficits in cognition in both the subchronic PCP model and the neonatal "dual hit" PCP model.

In conclusion, this data indicates that the neonatal "dual hit" PCP model for schizophrenia is the most representative model to mimic psychosis in mice.

Overexpression of Glycine Receptor Alpha 2 does not rescue locomotion activity in a neonatal "dual hit" PCP animal model of schizophrenia – To determine the therapeutic potential of GlyRa2 in psychosis, we introduced overexpression of GlyRa2 in the neonatal "dual hit" PCP model of schizophrenia. As previously mentioned, mice were administered PCP (10 mg/kg, i.p.) on post-natal day 7, 9 and 11. As seen in figure 5A, PCP

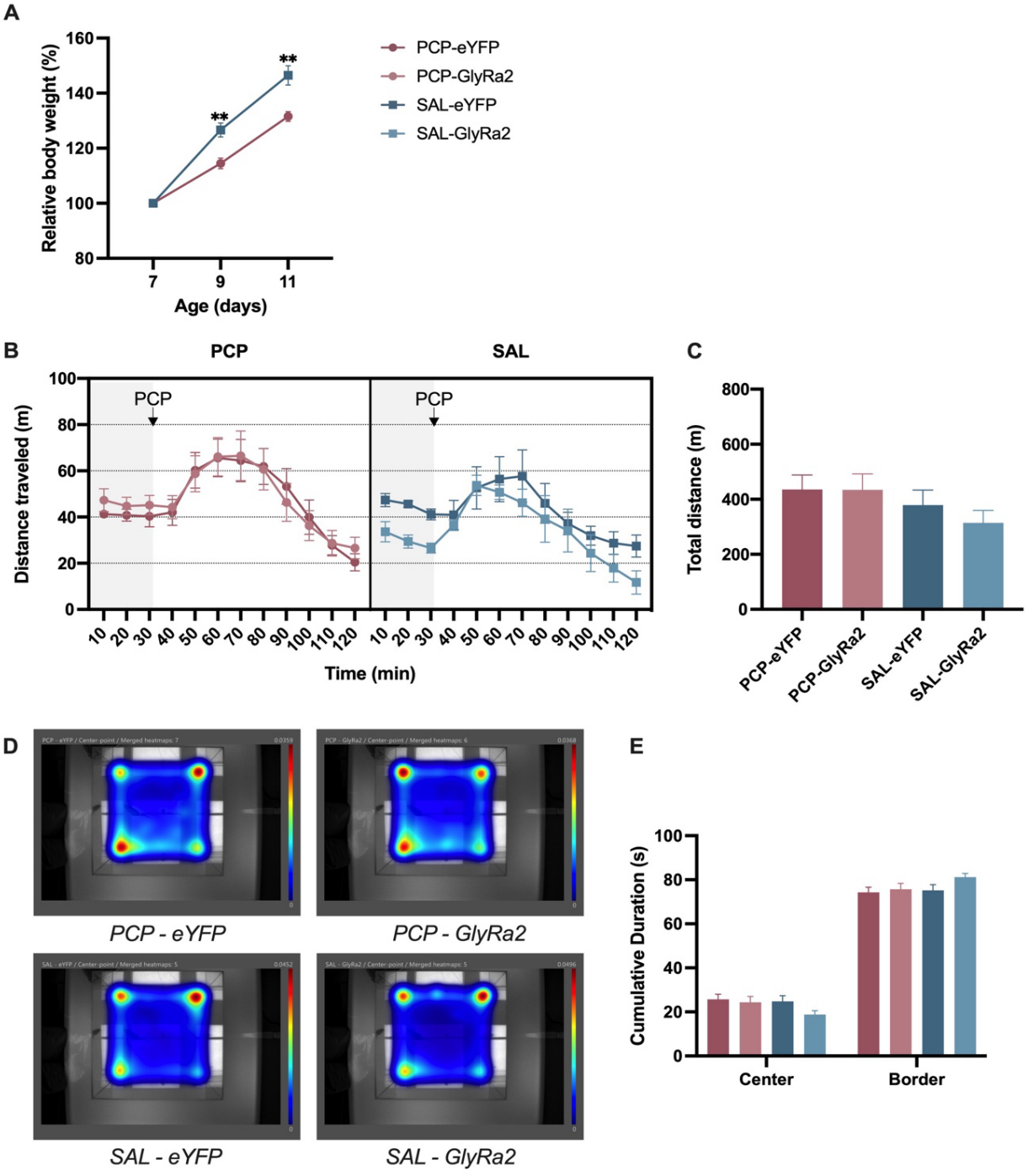


Fig. 5 – Overexpression of GlyRa2 reduces locomotion in control mice, but not in "dual hit" PCP-treated mice. (A) PCP-treated mice show a significant decrease in body weight gain, indicating impaired growth (two-way RM ANOVA with post hoc Sidak's multiple comparisons analyses). **(B-C)** Mice with GlyRa2 overexpression show a similar locomotion response to acute PCP administration (5 mg/kg, i.p.) compared to control mice, indicating that GlyRa2 overexpression is not able to significantly shunt dopaminergic hyperactivity in the striatum (three-way repeated measures ANOVA with Tukey's post hoc multiple comparisons analyses). **(D-E)** Time spent in the border zone is significantly higher compared to time spent in the center zone, indicating anxiety-like behavior. Overexpression of GlyRa2 does not alter anxiety-like behavior. **(D)** Representative images of merged heatmaps of the open field arena, depicting time spent in center and border zones of all conditions. **(E)** Quantified data of heatmaps, depicting relative time spent in center and border zones in the open field arena. (Ordinary two-way ANOVA with Sidak's post hoc multiple comparisons analyses). Conditions: PCP-eYFP (n=7); PCP-GlyRa2 (n=6); SAL-eYFP (n=5); SAL-GlyRa2 (n=5). Data represented as mean ± SEM. P-values: **p<0,01. GlyRa2 = glycine receptor alpha 2; eYFP = enhanced yellow fluorescent protein.

administration led to a significant reduction in body weight gain compared to the control mice [treatment (F1, 21=18.52, p=0.0003); time (F1.758,36.91=301.8, p<0.0001); treatment x time (F2,42=12.48, p<0.0001)]. At three weeks old, the PCP-treated mice were socially isolated. Following induction of the neonatal "dual hit" PCP model, mice received stereotactic injections of either AAV-GlyRa2-eYFP or AAV-eYFP (control) in the dorsal striatum at 6 weeks old. As these mice were Cre-inducible mice, this resulted in the overexpression of GlyRa2. To determine the effects of GlyRa2 overexpression on psychosis, we performed a PCP-stimulated open field locomotion test two weeks after stereotactic surgery. We report that overexpression of GlyRa2 does not significantly reduce locomotion activity following acute PCP (5 mg/kg; i.p.) administration (Fig. 5B) compared to controls [treatment (F1,19=3.175, p=0.0908); time (F3.029,57.54=29.86, p<0.0001); overexpression (F1,19=0.65, p=0.432); treatment x time (F11,209=1.18, p=0.432); time x overexpression (F11,209=0.16, p=0.999); treatment x overexpression (F1,19=1.06, p=0.317); treatment x time x overexpression (F11,209=0.98, p=0.4688)]. Additionally, we measured the time spent in the center zone versus the border zone of the arena (Fig. 5D-E). Mice that experience anxiety tend to spend more time in the border zone compared to the center zone of the arena. Mice of all conditions spent significantly more time in the border zone of the arena compared to the center zone of the arena [treatment (F3,38=0.0, p=0.999); zone (F1,38=961.0, p<0.0001); treatment x zone (F3,38=3.15, p=0.036)].

Important to mention, drugs of abuse often induce a greater response in male rodents compared to female rodents. We first observed this in the amphetamine-stimulated open field locomotion task in the subchronic PCP model for schizophrenia (SUP Fig 2A-B). The data indicates that there is a significant effect of gender [treatment (F1,26=5.87, p=0.023); gender (F1,26=5.20, p<0.031); treatment x gender effect (F1,26=0.41, p=0.525)]. Therefore, we decided to eliminate all female mice from our overexpression study, to investigate the effects of GlyRa2 overexpression in male dual hit mice (SUP Fig. 2C-D). We report a significant increase in locomotion in PCP-treated mice compared to saline-treated controls [treatment (F1,10=5.18, p=0.046)]. However, we did not observe a significant reduction in locomotion in GlyRa2 overexpression mice compared to control mice, or a significant interaction effect of both treatment and overexpression [time (F2.506,25.06=17.19, p<0.0001); overexpression (F1,10=2.48, p=0.146); treatment x time (F11,110=0.699, p=0.737); time x overexpression (F11,110=0.66, p=0.773); treatment x overexpression (F1,10=0.44, p=0.524); treatment x time x overexpression (F11,110=1.21, p=0.2885)].

Taken together, these data suggest that overexpression of GlyRa2 does not significantly reduce locomotion activity in response to acute PCP administration.

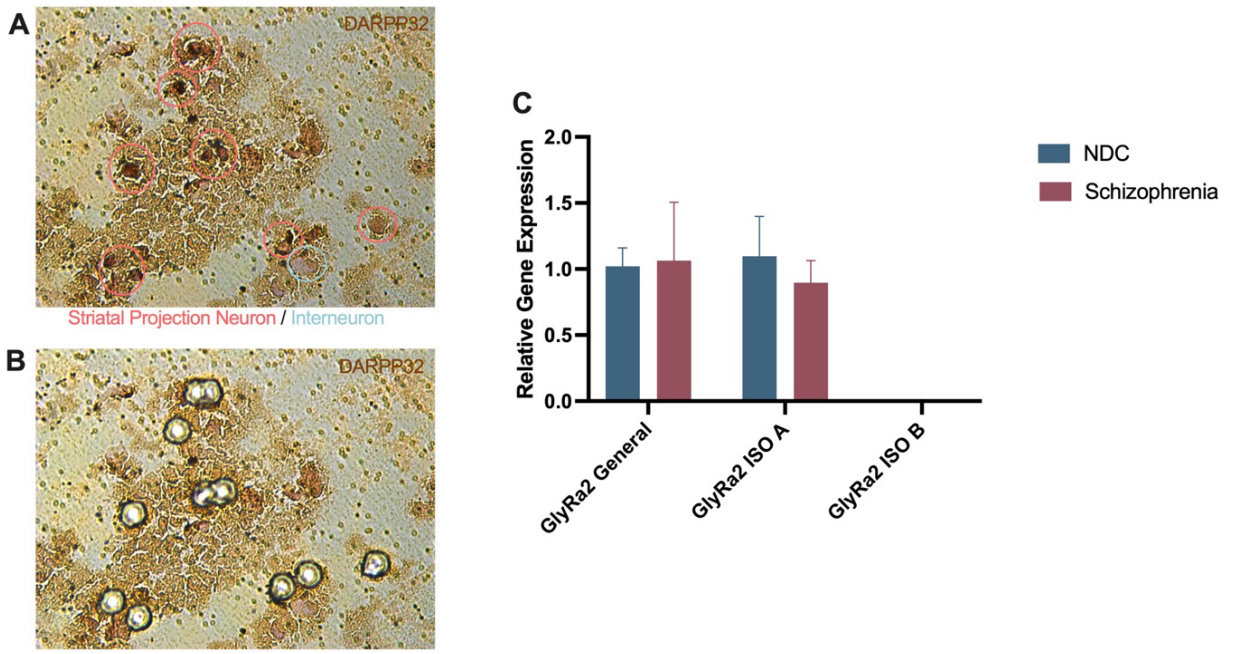


Fig. 6 – Isoform A of Glycine Receptor Alpha 2 is expressed in SPNs in the dorsal striatum of the human brain. (A-B) Representative images of DARRP-32 positive striatal projection neurons in the dorsal striatum **(A)** before and **(B)** after laser microdissection of striatal projection neurons. **(C)** GlyRa2 is expressed in SPNs in the dorsal striatum, more specifically in isoform A and not isoform B. Moreover, the relative expression GlyRa2 general, isoform A and isoform B is unaltered in patients diagnosed with schizophrenia compared to healthy controls. Data is comprised of 3 biological replicates per condition with n=200 cells and 2 technical replicates. Expression was normalized against the expression of stable housekeeping genes (GADPH and YWHAZ). Data represented as mean ± SEM. Statistical analyses: unpaired t-test.

GlyRa2 expression is not altered in striatal projection neurons in the dorsal striatum of patients diagnosed with schizophrenia – An important aspect of developing a target modulating therapy is to assess the functional expression of the target in the region of interest. To investigate if there are alterations in the relative gene expression of GlyRa2 in patients with schizophrenia compared to non-diagnosed patients (NDC), we performed a RT-qPCR. In this experiment, we collected SPNs originating from the dorsal striatum of the human brain via laser microdissection. GlyRa2 general was expressed in both NDC and in patients diagnosed with schizophrenia, with no significant alterations between relative gene expressions ($t=0.09$, $df=4$, $p=0.931$). Similarly, the GlyRa2 isoform A was expressed in both conditions, with no differences in relative gene expression ($t=0.58$, $df=4$, $p=0.591$). In contrast, the GlyRa2 isoform B gave "undetermined" as a result in the qPCR, indicating that there was no expression of GlyRa2 isoform B in both NDC and schizophrenic patients. Taken together, this data suggests that GlyRa2, and more specifically the isoform A of GlyRa2, is expressed in SPNs in the dorsal striatum of the human brain. Furthermore, no alterations in expression were found in relative gene expression between NDC and patients diagnosed with schizophrenia.

DISCUSSION

The present work investigated the potential of GlyRa2 overexpression in the dorsal striatum to shunt increased locomotion activity in response to dopamine in an animal model of schizophrenia.

The first objective to investigate our hypothesis consisted of validating an animal model for schizophrenia. We piloted three different models of schizophrenia: The subchronic PCP animal model, the subchronic amphetamine model and the neonatal "dual hit" model.

Subchronic administration of the N-methyl-D-aspartate (NDMA) receptor antagonist has been reported to not only introduce psychosis-like

symptoms in rodents, but negative and cognitive symptoms as well. Hence, the subchronic PCP model is represented as a more comprehensive model of schizophrenia (40, 41). Increased locomotion activity following acute-amphetamine administration has been reported in multiple studies (8, 41, 42). We, however, were not able to replicate these results in the amphetamine-stimulated open field task. We report a decrease in locomotor activity in subchronic PCP-treated mice compared to saline controls. Similar findings were reported where subchronic PCP administration blunted the stimulatory effect of amphetamine on locomotion (43, 44). In Tenn et al (2005), they suggest that PCP exposure may not lead to sensitization of the mesolimbic SA system in response to the stimulant effect of amphetamine. In this study, they performed an additional locomotion test, but with acute PCP administration instead of amphetamine. They observed that acute PCP administration significantly decreased locomotor activity compared to saline controls (43). Therefore, we decided to repeat the open field locomotion task in subchronic PCP treated mice with a PCP challenge. We report no significant increase in locomotor activity in PCP-treated mice compared to saline controls.

The subchronic amphetamine model of schizophrenia is based on the observations that amphetamine abuse can introduce psychosis in humans (45, 46). Amphetamine sensitization has been described to model schizophrenia-like symptoms. In previous studies, they report increased locomotion in amphetamine-treated mice compared to their respective controls (43, 45). In contrast, we do not report similar findings. The amphetamine-treated mice show an increase in locomotion after an amphetamine challenge, but this was not significant compared to saline controls. It is noteworthy that we only used three animals per condition. Therefore, it is feasible that by increasing the number of animals per condition, a significant effect could be observed.

The neonatal "dual hit" model was first described by Gaskin et al (2014) in rats (47). Briefly, mice were administered PCP at post-natal day 7,9 and 11, corresponding to the "first" hit. Following PCP administration, mice were weaned from their mothers at 21 days old and placed in solitary housing, resulting in the second hit. Many alterations in behavior with translational relevance to core symptoms observed in schizophrenia have been described due to social isolation of rodent pups after weaning. Examples are deficits in working memory (12) and hyperactive locomotion in a novel arena (48). In Gaskin et al, they report a significant time and time x PCP interaction effect, but no main housing or treatment effect (47). We did not investigate the effect of isolated housing versus group housing in our study, as we wanted to compare dual hit mice versus zero hit mice (group-housed saline treated mice). We reported similar findings, as we also observed a time and treatment x time effect, but not overall treatment effect. We were curious to investigate if acute amphetamine administration would induce similar results to the PCP challenge. We reported that that was not the case. However, we do need to note that these mice were not novel to the arena anymore, which could reduce anxiety-like behavior and thus overall locomotor activity.

Both the subchronic PCP and neonatal "dual hit" model have been reported to introduce negative symptoms in rodent (42, 47) To investigate the negative symptoms in both these models, we performed the forced swim task. We report that dual hit mice show increased immobility compared to their control counterparts. Mouri et al (2021) described that variation in mouse strains affects immobility responses in the forced swimming task, in subchronic PCP-induced specifically (49). They investigated immobility responses in mice with a C57BL/6J background compared to a C57BL/6N background. They described that immobility responses in C57BL/6J mice are less intense and durable compared to those in C57BL/6N (49). The mice we used in our study also come from a C57BL/6J background. We report similar findings in our subchronic PCP treated mice, as they show a slight decrease in immobility compared to their control counterparts. In conclusion, neonatal dual hit mice, but not subchronic PCP treated mice with a C57BL/6J background present with negative symptoms.

Taken together, we report that we were not able to replicate the behavior in subchronic PCP-treated and subchronic amphetamine-treated mice that was observed in previous studies. In contrast, we were able to replicate some of the behavioral findings in the neonatal dual hit model. As our focus was solely on psychosis and not

schizophrenia, we decided to use the neonatal dual hit model to investigate our hypothesis.

Our general objective was to investigate if overexpression of GlyRa2 can shunt excessive locomotor activity in response to dopamine. As there are currently no specific modulators of GlyRa2, we used stereotactic surgeries to introduce overexpression of GlyRa2 in the dorsal striatum by means of an adeno-associated viral vector. As previously mentioned, based on pilot studies performed in this project, we chose to utilize the neonatal dual-hit model to mimic psychotic behavior in mice. When the mice were six weeks old, we performed stereotactic surgeries to introduce the GlyRa2 overexpression. We then waited two weeks to test the mice in the PCP-stimulated open field task, to reach maximal expression in the striatum.

We report that there is no significant decrease in locomotion activity following overexpression of GlyRa2 in the neonatal "dual hit" PCP model of schizophrenia. Despite not observing a rescue of psychosis-like behavior in our model, it is conceivable that following certain adjustments to the experimental set-up, a significant effect can still be uncovered.

An important factor that influenced the significance of data is the high variability in data. Possibly, this is due to a small sample size, and consequently low statistical power (50). Ideally, sample size in behavioral tests need to be around 15 animals per group to achieve 80% power (51). Another possible factor might be that behavioral experiments in rodents are sensitive to subtle changes in the environment, which can alter outcome measures (39). There are many factors that can influence the readout of standard behavioral tests, such as housing, test location, handling as well as differences in genetic background and sex (52, 53). Sex is an important confounding factor to consider sex (52, 54). It has been well described that females are less sensitive to the effects of certain pharmacological compounds, which can be partly attributed to the protective effects of estrogen (11). Therefore, we decided to separate data obtained from female and male mice. No significant differences were observed, but differences were observed, nonetheless. Consequently, we suggest separating acquired female and male data in the future.

Future evaluation of injection sites will confirm expression pattern of GlyRa2 within the dorsal striatum. This is necessary to evaluate, as diffusion of GlyRa2 to other brain regions could introduce behavioral effects. In McCracken et al (55), they investigate expression of glycine receptors in forebrain structures and suggest roles for regulating neuronal excitability in the forebrain. Hence, overexpression of the inhibitory properties of GlyRa2 might result in decreased neuronal excitability. Therefore, it is crucial to validate the expression patterns of GlyRa2 in the dorsal striatum and so exclude expression of GlyRa2 in other regions of the brain.

An important aspect of developing a target modulating therapy is to assess the functional expression of the target in the region of interest. Therefore, we wanted to investigate the relative gene expression of GlyRa2 in schizophrenia patients compared to controls. Data was calculated using a method described by Vandesompele et al (2002) and Hellemans et al (2007) (56, 57). With this calculation, we were able to normalize the Ct values of the genes of interest against the Ct values of two stable housekeeping genes (GADPH and YWHAZ). Furthermore, in this calculation we also took primer efficiencies into account. GlyRa2 general and isoform A were both expressed in SPNs of the dorsal striatum. We report no differences between the relative expression levels of schizophrenic patients compared to controls.

CONCLUSION

In the search for a representative animal model of schizophrenia, we were able to validate the neonatal "dual hit" PCP model as a representative model for psychosis. We were not able to replicate the cognitive and negative symptoms that are present in schizophrenia. We report that overexpression of GlyRa2 did not significant decrease locomotor activity compared to control. We did observe a positive trend in male mice. Lastly, we determined the expression levels of GlyRa2 in dorsal striatal tissue of human schizophrenia patients and control and revealed similar expression levels. We conclude that more extensive research is required to determine the therapeutic potential of glycine receptor alpha 2

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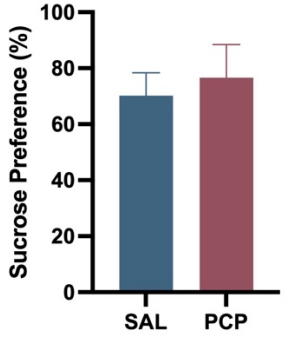
Author contributions – EP and BB conceived and designed the research. CH and EP performed experiments and data analysis. CH wrote the paper. All authors carefully edited the manuscript.

SUPPLEMENTARY MATERIALS AND METHODS

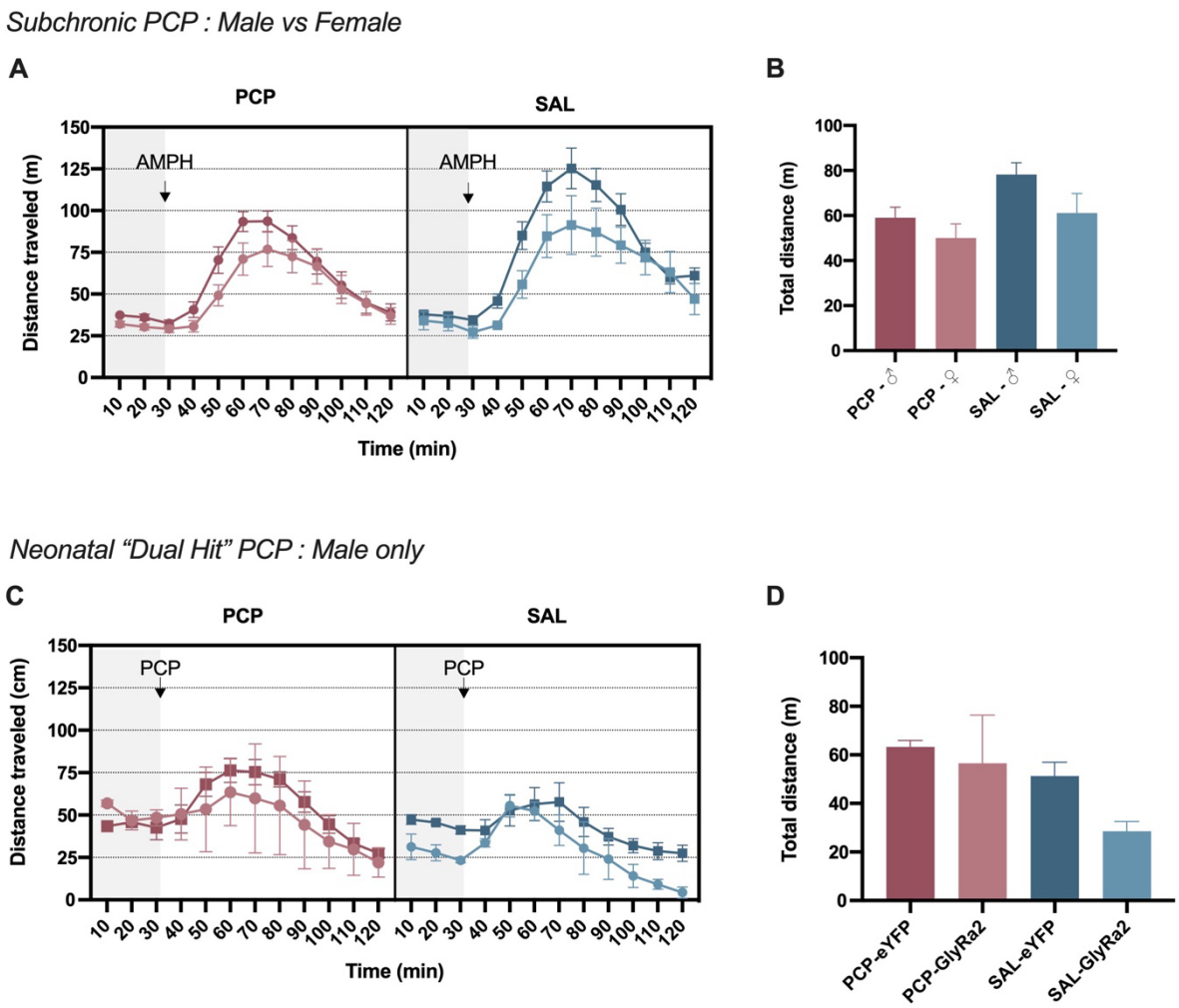
Sucrose Preference Test – The initial phase of the experiment involved the habituation phase (24 h), during which the animals were individually placed in cages containing two 15 ml tubes filled with either water or 10% sucrose solution. After the habituation phase, the mice underwent a 24-hour deprivation period during which they were water deprived. Following this deprivation phase, the mice were allowed to drink of both solutions' ad libitum for a duration of 8 hours. To determine the sucrose preference, the weight of sucrose water consumed was divided by the total weight of water and sucrose intake during the 8-hour period.

Immunohistology – Human brain sections (10 µm) were first placed in acetone at a temperature of -20°C for 10 minutes. Next, a few drops of HistoReveal was applied. They were then washed in PBS, followed by a 10 minute blocking step with protein block (X0909, Dako). Brain sections were incubated with the primary antibody (anti-DARPP-32, sc-271111, Santa Cruz H-3, 1:100) for 30 minutes. After dunking slices in PBS, secondary antibody (biotinylated anti-mouse, 1/400) was added for 15 minutes, and washed afterwards. Peroxidase block was added (1.5%) and left on for 5 minutes, washed with PBS. Brain slices were then incubated with streptavidin-HRP (P0397, Dako, 1:400) for 10 minutes and washed in TBS. For 1-8 minutes DAB was added (1 ml DAB buffer + 1 drop DAB chromogen), and checked for browning of the slice. Afterwards, slice was rinsed with distilled water and counter stained with hematoxyline for 5 minutes. Brain slices were washed and dehydrated: ethanol 70% > ethanol 95% > ethanol 100% > Xylene 1 > Xylene 2 (2 minutes).

SUPPLEMENTARY FIGURES



SUP Fig. 1 – Subchronic PCP treated mice show no difference in sucrose preference compared to control mice. (n=5). Data represented as mean ± SEM. Statistical analyses: Mann-Whitney U test.



SUP Fig. 2 – Male mice show increased locomotion after acute amphetamine administration compared to female mice. (A-B) Female mice show a reduced response to acute amphetamine (3 mg/kg, i.p.) administration compared to male mice in the subchronic PCP mouse model. Conditions: PCP-male (n=8); PCP-female (n=8); SAL-male (n=8); SAL-female (n=6). **(C-D)** GlyRa2 overexpression in male mice results in a reduced response to acute PCP (5 mg/kg, i.p.). Conditions: PCP-eYFP (n=4); PCP-GlyRa2 (n=2); SAL-eYFP (n=5); SAL-GlyRa2 (n=3). Data represented as mean ± SEM. Statistical analyses: three-way repeated measures ANOVA with Tukey's post hoc multiple comparisons analyses and (bar graphs) ordinary one-way ANOVA with Tukey's post hoc multiple comparisons analyses.