HELLENIC REPUBLIC

National and Kapodistrian University of Athens

Department of Biology

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RESEARCH THESIS PROJECT

EFFECTS OF DOPAMINERGIC ANTAGONISTS ADMINISTRATION DURING THE EARLY POSTNATAL PERIOD ON RAT BEHAVIOR AND BRAIN NEUROCHEMISTRY

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2019

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Contents

Summary

Dopamine is a known neurotransmitter in the adult brain of mammals but is also present in areas of the developing brain, where it regulates neuronal proliferation, morphology and migration through its different receptors. However, there are still areas of the brain where we do not know how dopamine affects their development, like the prefrontal cortex (PFC). We used two models of reduced dopaminergic activity in the prefrontal cortex during the early life –a. Denial of Expected reward through maternal contact (DER) and b. intracerebral administration of the D1-antagonist SCH23390- and ran a series of behavioural and immunohistochemical assays to determine the function and structure of the PFC in adult animals. Behaviour wise, the two experimental groups, DER and SCH, displayed defects in reversal learning but none in the prepulse inhibition protocol compared to controls. Nevertheless, DER rats had reduced maximum response of their acoustic startle response and along with the SCH animals showed an increased startle latency. Moreover, both DER and SCH rats had reduced number of PFC neurons as well as reduced number of glutamatergic cells. Our work demonstrates a role for dopamine as a morphogen in the brain and more specifically in the PFC, where its reduced levels during development affect the behaviour and neurochemistry of the adult brain.

Keywords Dopamine, PFC, Neonatal experience, D1-like receptor antagonist, Prepulse inhibition

Introduction

Dopamine is a neurotransmitter that has distinct functions in the adult brain, such as regulation of motor functions and motivation. In the developing rat brain, dopamine appears to have a different role, not that of a neurotransmitter. Before the first synapses appear, dopamine is present in the neural tube of chickens, pointing to a potential role in the regulation of neural tube closure (1,2,3). Also, in rat, the mRNA of D1 and D2 receptors has been detected in various brain areas of the fetal brain as early as embryonic day 14 (E14) (4). Aside from being detected in the developing brain, dopamine and its receptors have been attributed roles regarding effects on neuronal morphology, proliferation and migration. *In vitro* studies have revealed that cortical neuron morphology is affected by the D1-like and D2-like receptors. More specifically, D1 receptor activation resulted in a reduction in neurite outgrowth of cultured progenitor cells (5). On the contrary, D2 receptor activation had the opposite effects, enhancing neurite outgrowth in cultured progenitor cells (5,6). In addition, *in utero* cocaine exposure of rabbits has been shown to affect dendritic development of pyramidal neurons in the medial prefrontal cortex and the anterior cingulate cortex, increasing the length of those dendrites (7). Cocaine is a drug known to affect the dopaminergic system as it blocks the dopamine transporter, effectively elevating the levels of dopamine in the extracellular space. In the previous study, they also showed that cocaine decouples the D1-like receptor from its Gs subunit stopping the signal transduction, while D2-like receptor coupling remained unchanged. It seems that D2-like receptor activation results in the elongation of neurites while D1-like receptor activation halts their growth.

Dopamine, also, seems to affect neuronal cell proliferation. Studies have shown that dopamine in the lateral ganglionic eminence and in the telenchephalon of CD1 mice affects the proliferation of neuronal progenitors in the ventricular and subventricular zone (8,9). The way that dopamine exerts its effects is dependent on the receptor that the progenitors express. When D2-like receptors were stimulated there were more cells entering the S phase of the cell cycle. In contrast, activation of D1-like receptor prevented the transition of cells from the G1 phase to the S phase in the cell cycle. The latter finding was also supported by an *in vitro* study of primary culture neurons of CD1 mice grown in the presence of EGF or FGF2 (10). Taking into account all of the above, Ohtani has proposed a model on how dopamine receptors are expressed in the ventricular and subventricular zones of telencephalic neuroepithelium and their expression ultimately affects the fate of the progenitor cells. (Figure 1)

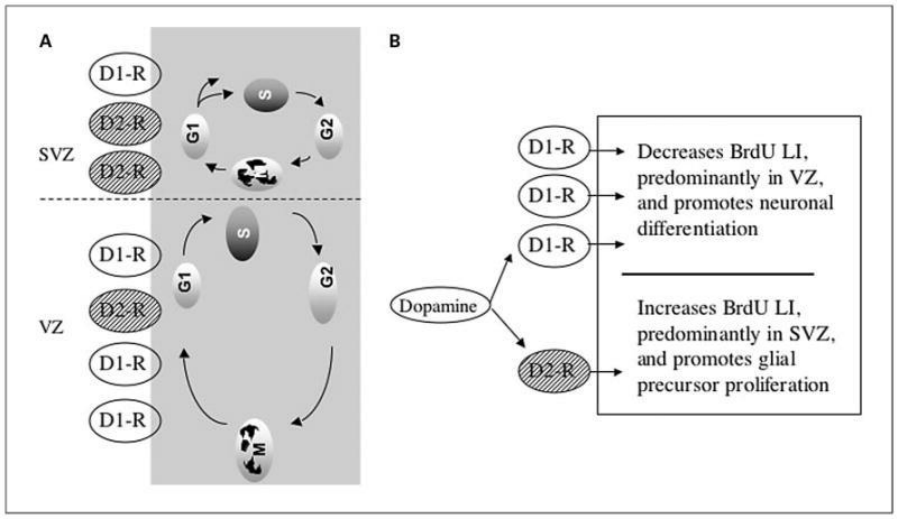

Figure 1: Scheme taken from Popolo 2004 that shows the how D1-like and D2-like receptors are distributed in the telencephalic neuroepithelium. Each group of receptors controls different aspects of development and proliferation of progenitor cells. The relative abundance of each receptor in a given brain area ultimately decides the fate of the cells born there.

In addition to the above, studies focusing on the D3 receptor have been carried out and have revealed that D3 receptor might play a role in the proliferation of neuronal progenitors. More precisely, *in vivo* (11) and *in vitro* studies (12) have shown that stimulation of the D3 receptor with specific agonists results in the proliferation of neural progenitor cells in the subventricular zone of postnatal and adult mice respectively.

Finally, dopamine seems to affect neurogenesis, not only in the developing brain, but also the developing spinal cord. This result comes from a study in Zebrafish embryos, where they found that the presence of dopamine is necessary for the generation of motor neurons (13). Interestingly, they found that dopamine is acting on spinal progenitor cells through the D4 receptor, which lowers the levels of cAMP and derepresses the Hedgehog pathway promoting the motor neuron fate of these progenitor cells.

The effects of dopamine on the neuronal migration during brain development are not well understood. Studies using models of cocaine exposure *in utero* are the only ones that have been used in an effort to stimulate conditions of dopamine imbalance during neuron proliferation and migration. In mice prenatal cocaine exposure of the pups leads to dramatic alterations of neocortical architecture (14). Lamination in the dorsolateral frontal cortex was largely disrupted and the vertical columns of post migratory neurons were poorly established. Also, in control animals, migrating neurons to the cortical layers show a predilection for layers IV-V, a phenomenon which is absent in treated animals where neurons are abnormally distributed throughout the six layers. Finally, the researchers focused on radial glial cells (RGCs), which are known for their role in guidance of newly born neurons migrating to the appropriate cortical layer. They found that the density of RGCs was severely reduced in experimental animals

compared to controls. All of the above led them to postulate a theory that the changes in cytoarchitecture they observed were a result of defects in cell generation and cell migration. Similar results were obtained from a study of *in utero* exposure to cocaine in rhesus monkeys (15), where abnormal cortical lamination was observed in the occipital lobe. This effect was more pronounced if the cocaine treatment happened at the approximate timeframe where cortical neurogenesis was happening (16). Finally, James E. Crandall using KO mice for D1 and D2 receptors and electroporation studies found that the activation of those two receptors can modulate the migration of GABA neurons (17). Specifically, activation of D1 receptor promoted migration of GABA neurons from the medial and caudal ganglionic eminences to the cerebral cortex in slices of mice embryo. On the contrary, D2 receptor activation had opposite effects where it decreased GABA neuron migration, decreasing migration of GABAergic neurons

Based on previous results obtained in an animal model of neonatal neglect developed in our lab [Denial of expected reward through contact with the mother, DER experience (18)], this adverse neonatal experience on PND10-13 resulted in reduced levels of dopamine in the neonatal PFC as well as its development with changes that can be seen in adult subjects (19). The aim of this study was to elucidate if there is a causal relationship between less dopamine in the PFC during development and the structure and function of the PFC in adult rats. To this end, we used an animal model of pharmacological dopamine depletion in the PFC of rat pups during the same developmental period as the DER experience takes place, namely from PND10 to PND13. In this model, we tried to mimic the conditions of the DER model with the use of the D1-like receptor antagonist SCH23390. Injections to the pups were made into the PFC area of the brain, in an attempt to lower the activity of the dopaminergic system in that area and observe how this functional depletion of dopamine during development affected the behaviour and neurochemistry of the adult rats. Using both of the above models, we ran a battery of behavioural and neurochemical tests and tested the hypothesis that SCH23390 administration can mimic the effects of DER-induced dopamine depletion.

Materials and methods

Animals

Wistar rats bred and grown in our colony were used. Animals lived under standard conditions (23 ± 1 °C, 12:12 h light/dark cycle) with food (Kounker-Keramari Bros. & Co., Athens, Greece) and water *ad libitum*. Prior to birth (postnatal day 0, PND0), each litter was randomly assigned to one of the following types: i. Exposed to the DER experience (DER group), ii. Controls (CTR), iii. Litters used for the intracerebral administration of the D1 antagonist SCH23390 (SCH-injected group) or vehicle-artificial CSF (aCSF group). Throughout the lactation period, fresh wood chip was added to the cage every 4–5 days, without disturbing the litter. On PND22, animals were weaned and housed in same sex, same group (DER, SCH, CTR, aCSF) of 2–4 animals per cage. Following weaning, cages were cleaned weekly by the experimenters with minimal disturbance of the animals.

Training in the T-maze

All animals of a litter were exposed to the DER experience (n=16) or were used as controls. Animals of the control group were left undisturbed with their mother throughout the lactation period. Training in the T-maze was performed as previously described (18) during PND10–13; briefly, a custom-made T-maze was used, with one arm leading through a small sliding mesh-wire door (9 cm x 11 cm) to the mother containing cage. For control purposes, at the end of the other arm of the T-maze a cage with a virgin female rat was present, albeit with no access from the T-maze. The door of the mother-containing cage remained always closed, blocking access to the mother. Each pup of the DER group was trained for ten trials, each trial of maximum 60 s duration, per training day. If a pup did not succeed to reach the entrance of the mother-containing cage before the end of the 60 s, it was gently guided to the entrance and had to remain in front of the closed door for 20 s, before the next trial started. At the end of the ten training trials the pups were returned to the home cage, containing the mother and the rest of the litter.

Intracerebral drug administration

Injections to the pups (n=22) were made on PND10-13. A local anesthetic containing lidokain 25 mg/g and pilocarpin 25 mg/g was applied on the skin over the area of the injections. Injections were performed, using 30G insulin needles, bilaterally into the IL/MO area of the PFC [according to the anatomical atlas of Ramachandra and Subramanian (2011)] with the use of a tailor-made pup head cast allowing the repetitive positioning of the needle and depth of injection into the same PFC area (verified in a pilot study). The volume of injection was 40μl per hemisphere and contained either artificial cerebrospinal fluid (aCSF, according to the Cold Spring Harbor Protocols) (n=8) or SCH-23390 (59.4nM) (n=14).

Behavioural testing

Learning and reversal learning behavioural task

Two-month old male rats (11 ctr/8 aCSF, 15 DER and 14 SCH23390-injected animals) were used that were food restricted 2 days prior to the task and throughout the protocol (50% of their normal daily food intake). The protocol extended in 5 consecutive days: 1 pre-training and 4 training days. Animals were trained in a custom made T-maze made of wood, colored black on the inside. The dimensions of the base of the maze were: 86.65 cm length, 23.6 cm width and 21.8 height. Each arm of the T-maze was: 13.6 cm length, 40.05 cm width and 21.8 cm height.

On the first day each animal was placed in the small arm of the T maze for 15 minutes. All over the floor of the maze, corn flakes were scattered in order for the animal to get accustomed to food seeking and eating inside the maze.

During days 2 to 5, no food was scattered on the floor. Instead, a white bowl of corn flakes was placed at the end of the right arm of the maze and a similar one but empty, at the end of the left arm. On each day, training consisted of 10 trials of 2 minutes max duration, for every subject. Additionally, before the 10 trials, there was one trial of "forced choice" (5 minutes max duration) where the only route available for the rat leads to the right arm where food was present. This helped the subject to remember that there was food on the right arm before the actual testing occurred. During the 10 testing trial, if the animal went to the right arm and poked its snout into the food-containing pot, its choice was considered correct and was left there to eat for 20 seconds before being moved back to the start of the maze. Should an animal stay at the short arm of the maze or in the intersection of the two long arms of the maze for more than 60 seconds, the trial was considered as a no-choice trials. No choice trials were counted as wrong choices during data analysis.

During the 5th day of the protocol and after a subject had completed the 10 training trials, reversal learning was tested. This happens with 10 more trials on the maze but on a different set up: The bowl with the food was located on the left arm and the empty one on the right. For those 10 trials, moving to the right arm was considered a wrong choice while moving to the left was considered a correct one.

Startle response

Startle chamber

Male rats (7 ctr/8 aCSF, 14 DER and 12 SCH23390-injected animals) 3 months of age that were previously used in the T-maze protocol were also used in this one. All acclimatization and testing occurred in a sound attenuating startle chamber (Panlab). Animals were restrained using a clear Plexiglas cylinder resting on a platform inside a ventilated and illuminated chamber. Sound and light were produced by a loudspeaker and a lamp respectively, inside the chamber. The whole-body startle

response of the rat caused vibrations of the Plexiglas cylinder, which were converted into signals by a piezoelectric accelerometer attached to the platform. The signals were analysed with the Packwin Software.

The protocol consisted of three days of acclimatization and one day of testing. The first day, animals were put in the Plexiglas cylinder in the sound attenuating chamber for five minutes in order to accommodate to the setting. The next day they were put in the chamber for ten minutes and on the third day, they were put for ten minutes in the morning (9-10am) and again for another ten minutes after two hours. Additionally, on the third day, we tested the startle response of the subjects in order to determine if a subject had a defect in its acoustic or motor system. Animals that performed poorly in this protocol were excluded from any further testing.

Acoustic startle response protocol

Throughout the acoustic startle response protocol, a background noise (70dB) and light were presented. After 30 seconds of acclimatization, 60 “Pulse” trials (40 ms) occurred. Six different sound intensities were used 70, 80, 90,100, 110 and 120dB with each one being presented for 10 trials in a pseudorandom order.

Pre-pulse inhibition protocol

The test session on the fourth day started with an acclimation period of five minutes where background noise (70dB) and light were presented the whole session.

After the acclimation period, there were two presentations of a startle inducing 120dB broadband noise pulse lasting 40ms (“Pulse”) to familiarize animals to testing. These trials were excluded from data analysis. Afterwards, rats were presented with “Pulse” trials (120dB, 40ms), “Pre-pulse” trials (85dB, 20ms), “No stimulation” trials in which no sounds occurred and “Pre-pulse+Pulse” trials, each on with a different inter-stimulus interval (ISI) of 40, 80 and 120ms, separating the prepulse and the pulse (onset to onset). At the end of the testing, two more presentations of “Pulse” occurred in order to compare them with the initial two “Pulse” and determine the magnitude of habituation animals exhibited during testing. 8 trials of each type were presented in a pseudorandom order and the inter-trial interval was fixed at 19 seconds.

For each session, the median of the 8 “Pulse” and the 8 “Pre-pulse+Pulse” trials were calculated. The percentage PPI was calculated as the [(median “Pulse” trials * median “Pre-pulse+Pulse” trials) / median “Pulse” trials] * 100.

Immunohistochemical staining

Some of the animals used in the behavioural tests were also used for the immunohistochemical experiments. Adult animals (3 ctr/4 aCSF, 6 DER and 8 SCH23390-injected animals) used for immunohistochemistry were deeply anesthetized with isofluorane, decapitated and their brains were isolated and flash-frozen in -40^{o}C isopentane. Sections of 20µm in thickness were cut on a freezing cryostat (Leica CM1900, Nussloch, Germany) at -15^{o}C, collected on silane-coated slides and stored at -80^{o}C.

Frozen brain sections were thawed, post-fixated with ice-cold 4% paraformaldehyde in 0.1 M phosphate buffer for 1 h at 4^{o}C. Following washing with phosphate-buffered saline (PBS) alone and PBS containing 0.4% Triton X-100 (SERVA, Germany), all slides were incubated in PBS containing 0.4% Triton X-100 and 10% normal donkey serum (NDS) (Dako, Denmark) for 1 h at room temperature. Afterwards, sections were incubated for 72 h at 4 °C with the primary antibody (1:200 dilution of mouse anti-NeuN (A60), Millipore, USA and 1:1000 dilution of goat anti-vGluT, AB1520, Millipore, USA) diluted in PBS containing 0.4% Triton X-100 and 10% NRS. Following incubation with the primary antibody, slides were washed in PBS and incubated for 2 h at room temperature with a biotinylated donkey anti-mouse secondary antibody (Millipore, USA) for NeuN and vGluT detection, at a concentration of 1:200 diluted in PBS containing 2% NDS. Following several rinses in PBS, slides were exposed to the ABC reagent (DakoCytomation, Denmark) for 60 min at room temperature. Slides were then washed in PBS and stained with 3,3′-diaminobenzidine (DAB) (1.7 mM, Sigma–Aldrich, USA) diluted in Tris–HCl buffer (10 mM, pH 7.6) and 0.03% H_2O_2 for 2–5 min at room temperature. Finally, they were washed, dehydrated, and coverslipped with DePex (SERVA, Germany) and analyzed microscopically under a brightfield microscope.

Cell counting

For each antigen and for each cortical area analyzed three-four systematic randomly selected sections were evaluated within the following anatomical borders, according to the anatomical atlas of Paxinos and Watson (2007): For medial orbital cortex (MO) from bregma 5.64 to 2.52; for anterior cingulate cortex (aCg1) from bregma 5.16 to 2.52. In each section immunostained cells were counted in two randomly selected, non-overlapping optical fields. For all immunolabelings, images were digitized using the Infinite Capture v.6.0 (Lumenera Co., Ottawa, Canada) software. Cell counting was performed for NeuN, in the MO and the aCg1 and for vGluT in MO. Cell quantification was performed “blindly” by two independent investigators. A “threshold” was set in the image analysis software (ImageJ v.1.46R, NIH, USA) based on the background, non-specific staining, and only cells above this limit were counted.

Statistical analyses

Behavioral and immunolabellling data did not differ between control animals and those neonatally injected intracerebrally with aCSF, thus the two groups have been collapsed into one group (ctr) for subsequent statistical analyses and data presentation.

Behavioural data were analyzed by a Generalized Linear Model which took into account the animal's litter, followed by a Bonferroni post-hoc pair-wise comparisons. Immunolabeling data were analyzed by one-way ANOVAs with the group of animals (DER, SCH, CTR) as the independent factor. The LSD for post hoc analysis was used when appropriate. The level of statistical significance was set at 0.05. All tests were performed with the SPSS software (Release 22, SPSS, USA).

Results

DER and SCH-injected rats exhibit defects in reversal learning in a T-maze setup.

We performed this behavioural assay to examine how well can rats reverse learn after being taught to find food in a specific arm in a T-maze setup. The correct choices made in the reversal part were counted and are shown in Figure 2. Rats in the DER group made the least amount of correct choices out of the three groups being significantly lower that both control and SCH-injected. SCH-injected subjects made more correct choices than DER but less than controls who had the biggest amount of correct choices (A generalized linear model was used for statistical analysis taking into account the litter animals belong to: $W_{2,42}$ = 57.228, $p < 0.001$; post-hoc test, ctr vs. DER $p < 0.001$, ctr vs. SCH $p < 0.001$, DER vs. SCH $p < 0.001$).

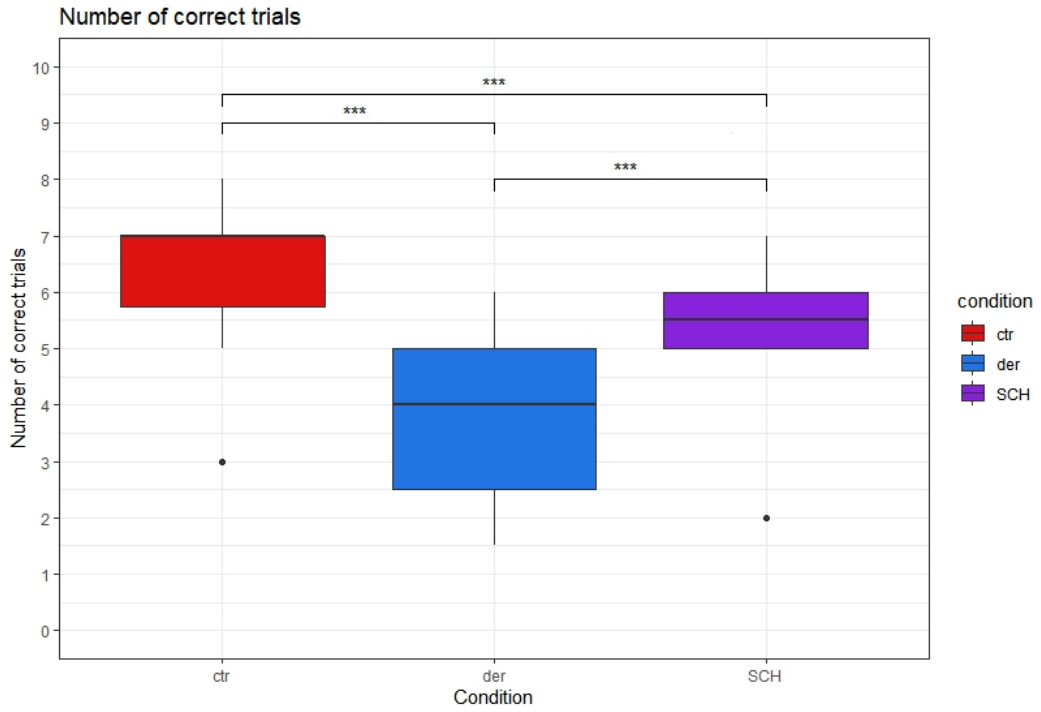

Figure 2. Number of correct choices made in the reversal part of the T-maze protocol. Subjects were trained to find food in the right arm of a T-maze for four days. In the fourth day, reversal occurred with food being placed on the left arm of the maze and correct choices were counted. DER and SCH-injected subjects made significantly fewer correct choices when compared to controls (***p < 0.001 ctr vs. DER, ***p < 0.001 ctr vs. SCH, ***p < 0.001 DER vs. SCH). Box plots, show median (horizontal lines inside boxes), quartiles (boxes), range (whiskers), and outliers (dots outside boxes).

As we shown in the figure above, DER subjects are most affected in their ability of reversal learning by making the least amount of correct choices when reversal happens. SCH-injected animals also made less correct choices than controls but more when compared to DER being the midpoint phenotype between control and DER. These data point to potential defects in reversal learning or set shifting for DER and SCH-injected rats, with the former displaying a much more severe phenotype than the latter.

Magnitude and latency of responses following a pulse differ in DER and SCH-injected rats compared to controls.

We performed a pre-pulse inhibition (PPI) protocol in order to examine the possible differences in sensory information processing by the prefrontal cortex of control, DER and SCH-injected rats. As described in materials and methods, the protocol mainly consisted of pulses alone and pulses that were preceded by a pre-pulse at three different intervals of 40, 80 and 120 ms. We did not observe differences between the three intervals either within a given group or between the three experimental groups (Figure 3).

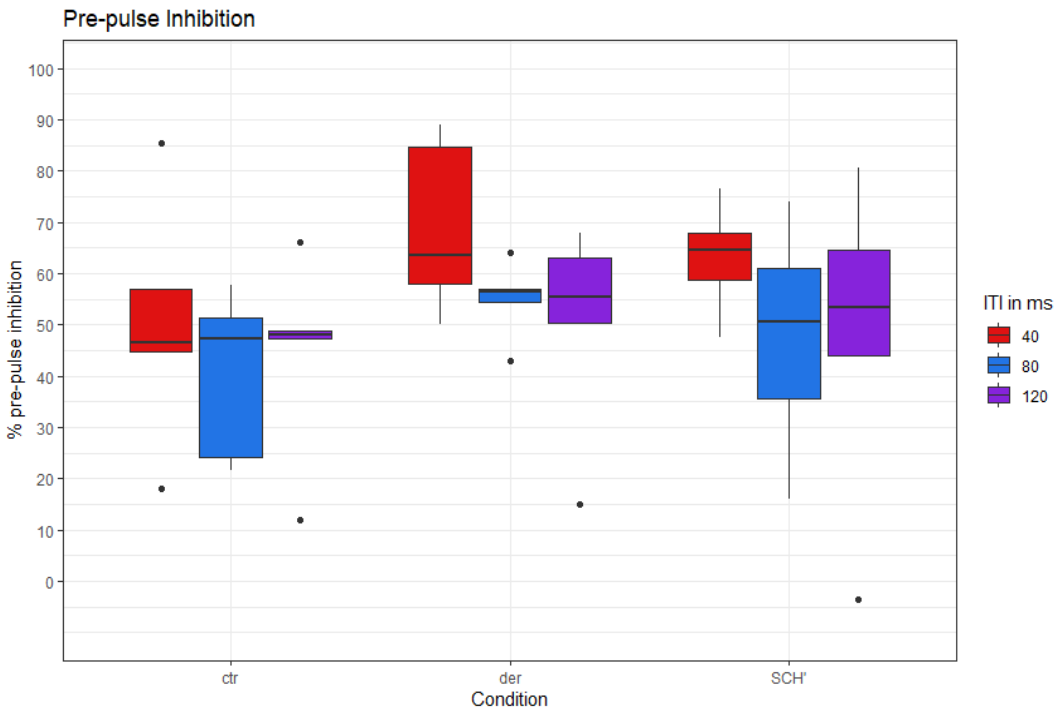

Figure 3. Percentage of pre-pulse inhibition (PPI) between control, DER and SCH-injected subjects. For each experimental group the PPI was measured for three interstimulus intervals between pre-pulse and pulse at 40, 80 and 120 ms. Box plots, show median (horizontal lines inside boxes), quartiles (boxes), range (whiskers), and outliers (dots outside boxes).

The above findings suggest that the sensory-motor gating in DER and SCH-injected subjects is similar to control ones. Also, inter-stimulus intervals of 40, 80 and 120 ms do not seem to alter the expression of PPI.

While no significant differences were observed regarding the magnitude of PPI between the 3 groups, there were statistically significant differences in the maximum response after the pulse-alone trials. More specifically, as seen in the Figure 4, DER subjects had a much weaker response when compared to the controls, which in turn had the most robust response out of all three groups. On the other hand, SCH rats seemed to respond in an intermediate way between controls and DER, without having any statistical differences with either group. ($W_{2,42}$ = 7.450, p = 0.02; post-hoc test, ctr vs. DER p = 0.007, ctr vs. SCH p = 0.122, DER vs. SCH p = 0.301)

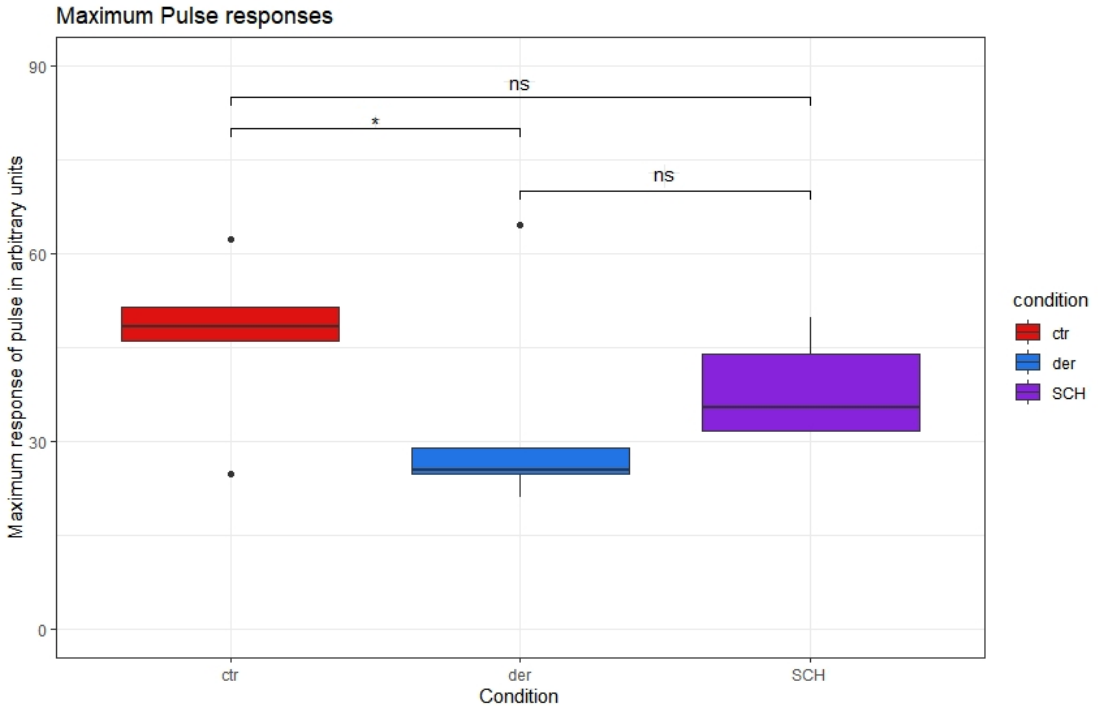

Figure 4. Maximum response in the pulse-alone trials of the PPI protocol as measured by the "Panlab" hardware in arbitrary units. The control group exhibits the highest responses out of the three groups, with DER subjects having the lowest ones and being statistically lower than controls. The SCH-injected group behaves somewhere in the middle of control and DER without having any significant differences from either (*p < 0.05 ctr vs. DER, p = 0.122 ctr vs. SCH, p = 0.301 DER vs. SCH). Box plots, show median (horizontal lines inside boxes), quartiles (boxes), range (whiskers), and outliers (dots outside boxes).

This result points towards a defective acoustic startle response in DER rats, that cannot be fully replicated by the SCH-injected group.

Along with the maximum responses in pulse-alone trials, we examined the latency of said responses measuring from the time the pulse was presented till the response of the subject exceeded the threshold of basal activity. Interestingly, we discovered that both DER and SCH-injected subjects displayed an increased latency in their responses to pulse alone trials when compared to the control group ($\underline{W_{2,42}}$ = 9.595, p = 0.008; post-hoc tests, ctr vs. DER p = 0.043, ctr vs. SCH p = 0.003, DER vs SCH p = 0.387). (Figure 5) No difference was observed between, DER and SCH-injected groups.

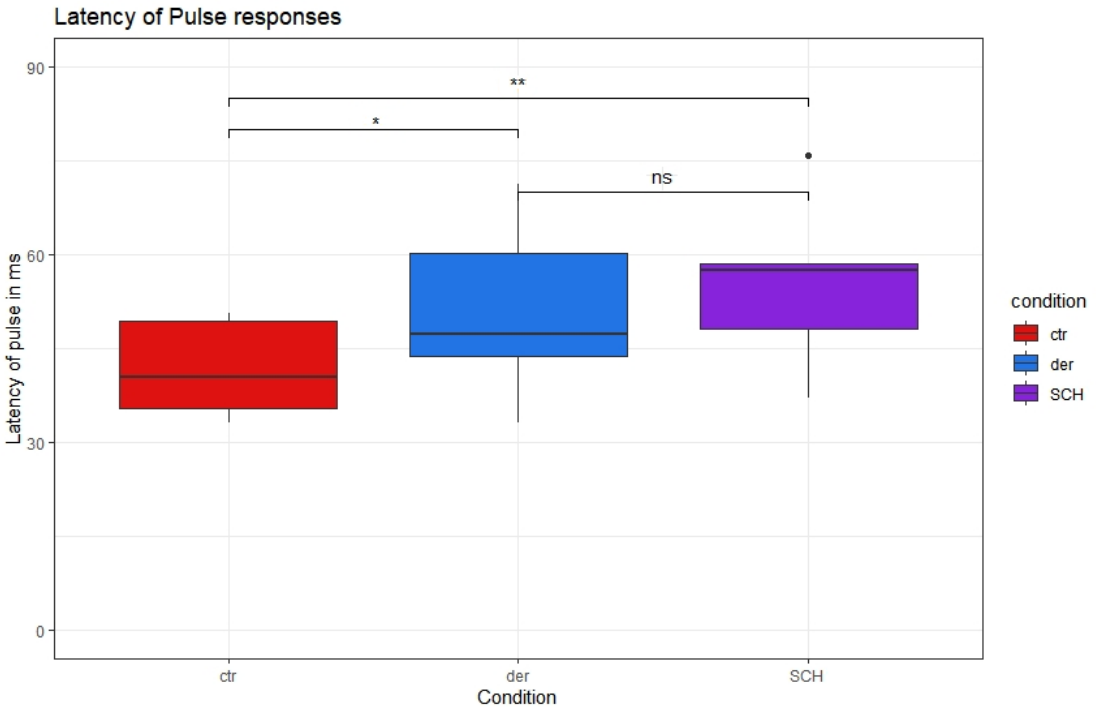

Figure 5. Startle latency in pulse alone trials of the PPI protocol. Startle latency is defined as the time period starting from the point a pulse-alone is heard till the response of the subject exceeds the threshold of basal activity. DER and SCH-injected subjects show an increased latency to respond to pulse-alone stimuli when compared to control subjects (*p < 0.05 ctr vs. DER, **p < 0.01 ctr vs SCH, p = 0.387 DER vs. SCH). Between DER and SCH-injected groups, no difference was detected regarding the latency to respond. Box plots, show median (horizontal lines inside boxes), quartiles (boxes), range (whiskers), and outliers (dots outside boxes).

The increased latency obsereved in both DER and SCH-injected rats bespeak of a potential defect in information processing in those groups compared to control subjects. Also, SCH administration during development seem to replicate the behaviour of DER in this aspect.

DER and SCH-injected subjects have fewer glutamatergic neurons in the medial orbital prefrontal cortex.

Since we observed alterations in the behaviour of DER and SCH-injected groups when compared to controls, we decided to investigate further by looking at the cytoarchitecture of the brain. Using a NeuN antibody, we stained the Prefrontal Cortex (PFC) for NeuN positive cells (Figure 6).

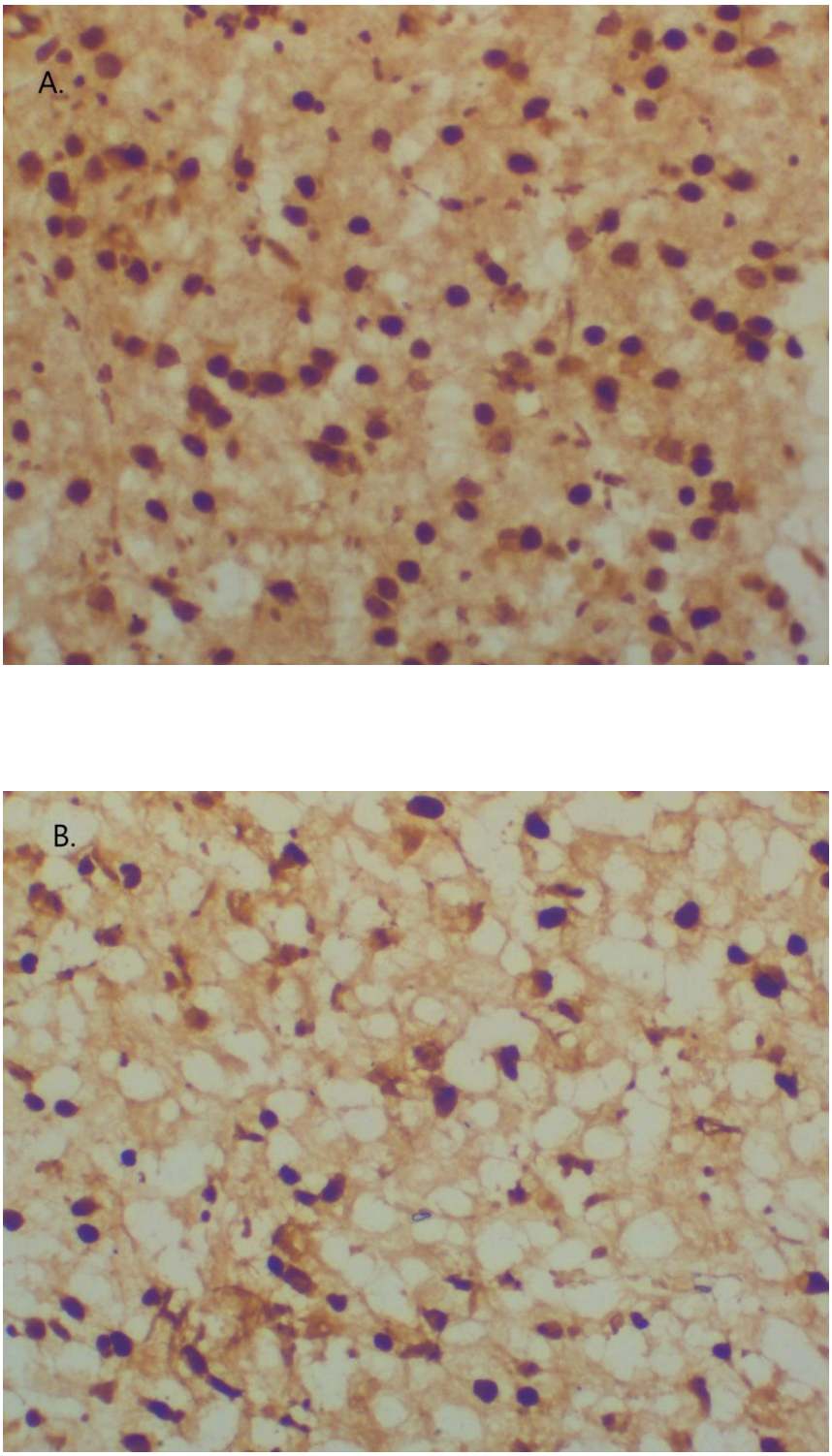

Figure 6. Immunohistochemical staining of cryotome brain sections with a NeuN antibody. The brain region examined was MO in the PFC in A. control and B. SCH-injected animals.

After staining with the NeuN antibody, we counted the stained cells in the MO area of control, DER and SCH subjects and found differences between the control and the experimental groups (Figure 7).

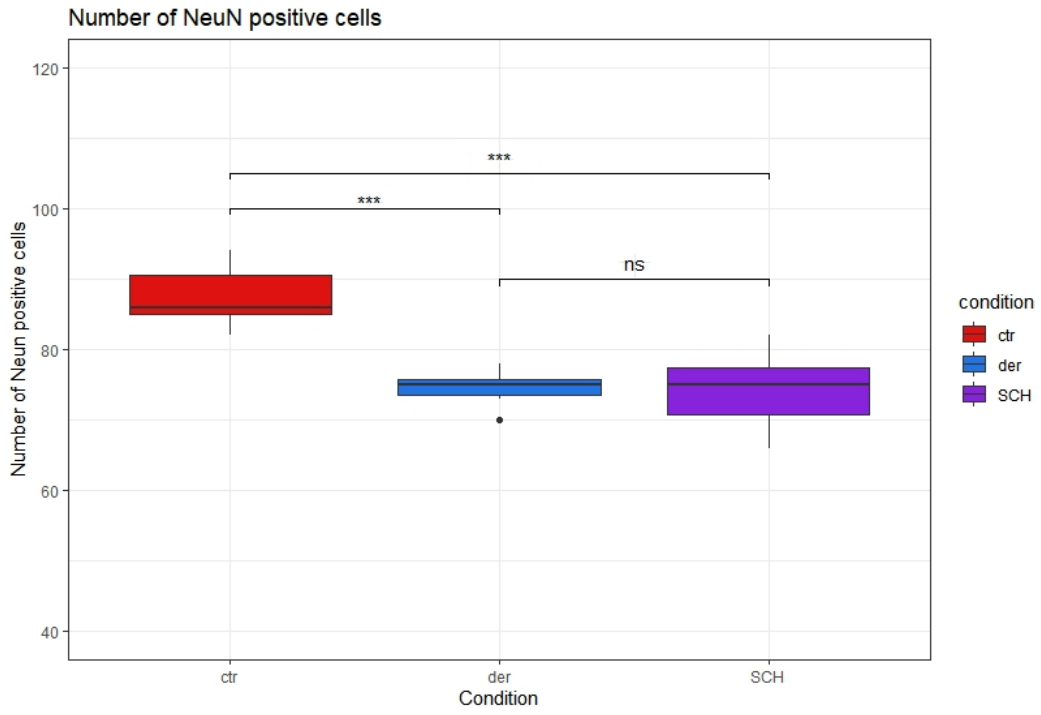

Figure 7. Number of NeuN positive cells counted in the MO area of the PFC. There is a statistical significant reduction in the number of the neurons in the MO area in the DER and SCH-injected subjects compared to the controls (***p < 0.001 ctr vs. DER, ***p < 0.001 ctr vs SCH, p = 0.879 DER vs. SCH). Box plots, show median (horizontal lines inside boxes), quartiles (boxes), range (whiskers), and outliers (dots outside boxes).

We observed a clear reduction of NeuN positive cells in the MO area of DER and SCH-injected groups when compared to the control, but no differences between them (one-way ANOVA: $F_{2,20}$ = 20,373, p < 0.001; post-hoc tests, ctr vs. DER p < 0.001, ctr vs. SCH p < 0.001, DER vs. SCH p = 0.879). Most likely, this reduction in cell number is not due to migration failure of the neurons destined to arrive in MO, as we examined the Cingulate Gyrus (CG) which is a nearby area of MO. We detected no change in the cell number of NeuN positive cells in the CG (Figure 8).

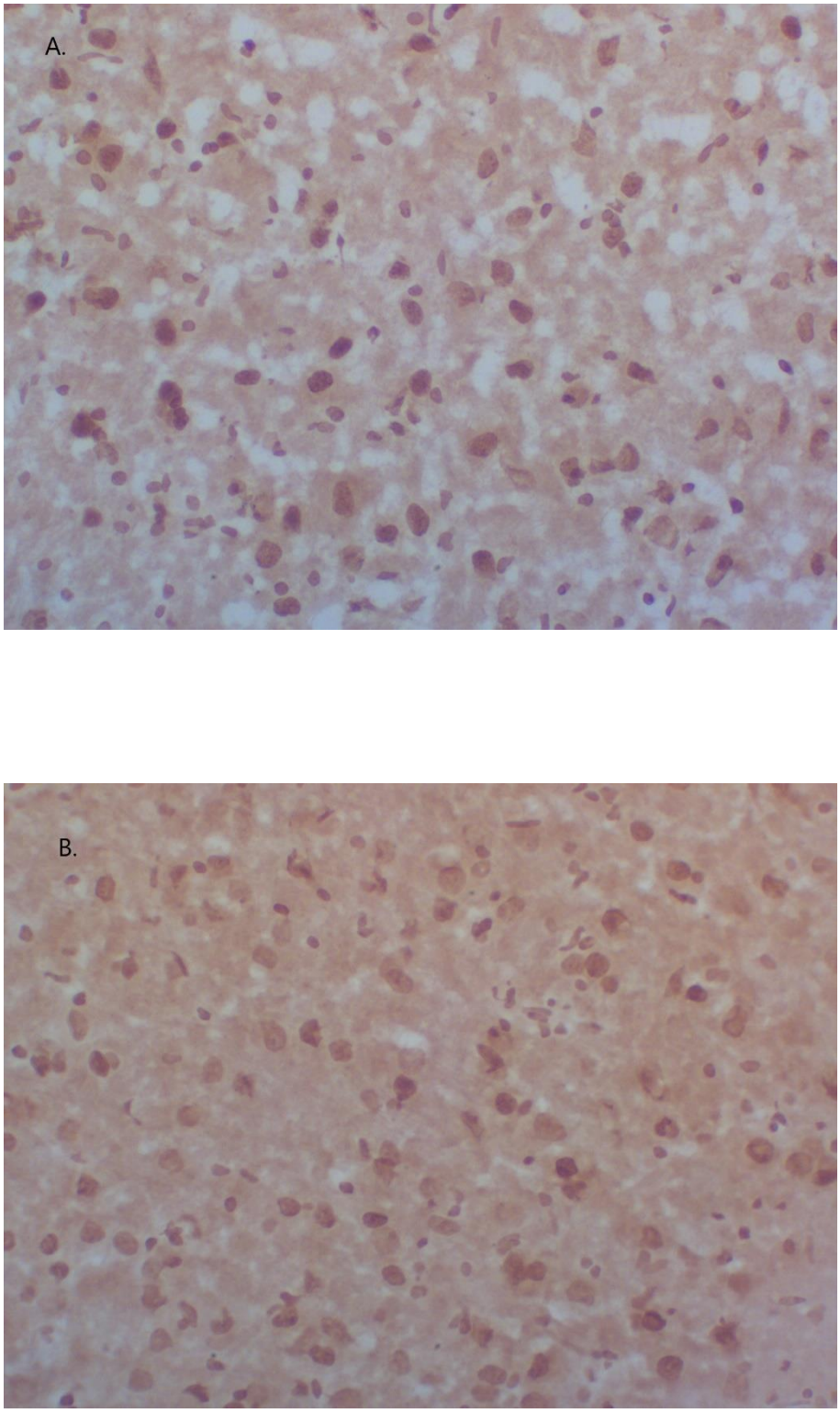

Figure 8. Immunohistochemical staining of brain sections with NeuN examining the Cingulate Gyrus (CG), an area above the MO in the PFC in A. control and B. SCH-injected animals.

Since we confirmed that there are alterations in the number of neurons in MO in our experimental groups, we further questioned which is the identity of those missing neurons. For that purpose, we stained brain sections with a vGlut antibody in order to find out if the missing neurons are of glutamatergic identity (Figure 9).

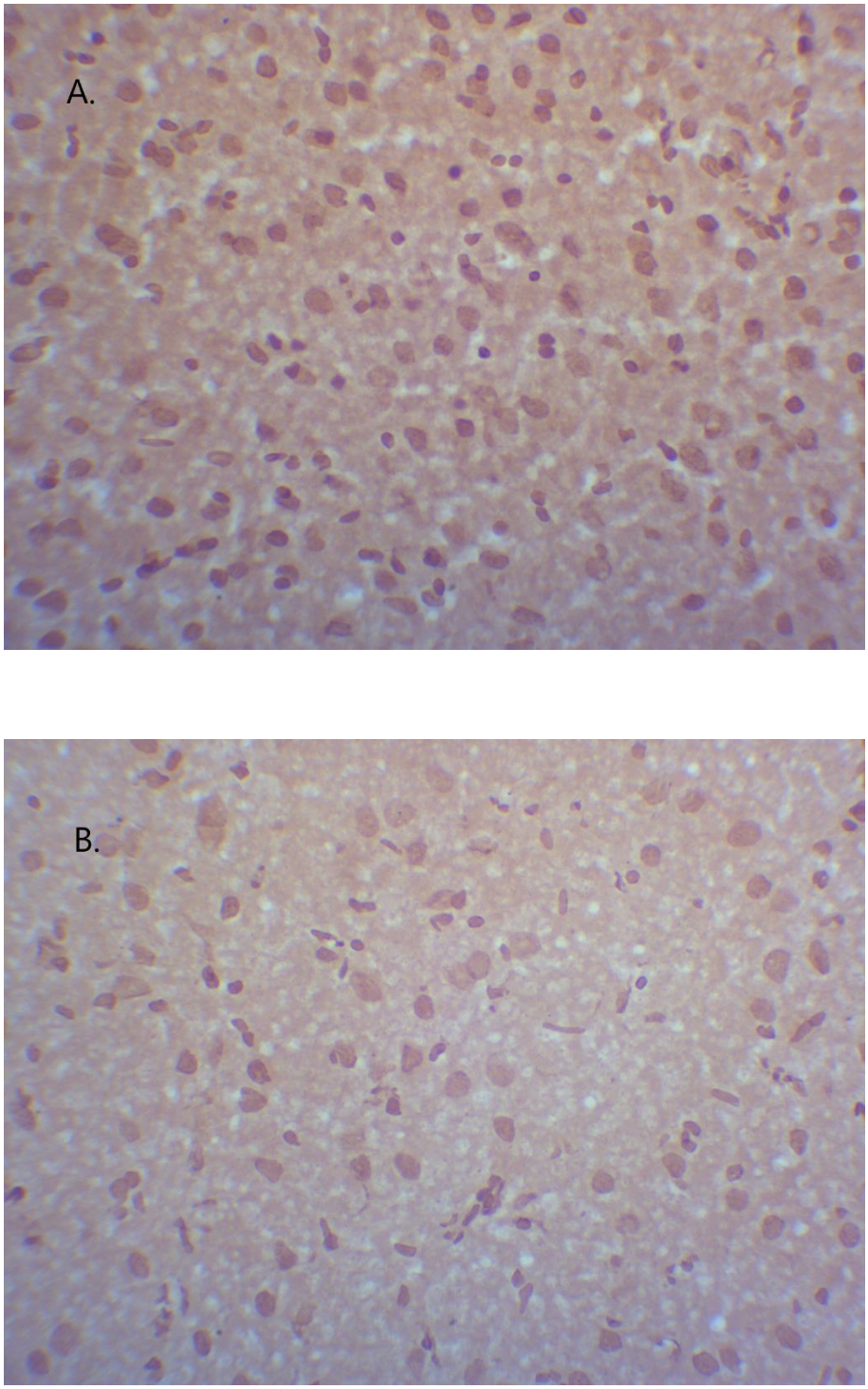

Figure 9. Immunochemical staining of the MO with a vGluT antibody of A. control animals and B. SCH-injected animals. There is a clear reduction in the number of glutamatergic neurons in the SCH-injected group when compared to the control group.

As is clearly shown in Figure 9, the SCH-injected subjects display a remarkable decline in vGlut positive neurons when compared to control subjects. This observation was further confirmed when we counted the glutamatergic cells in the MO/VO area of control, DER and SCH-injected subjects (Figure 10).

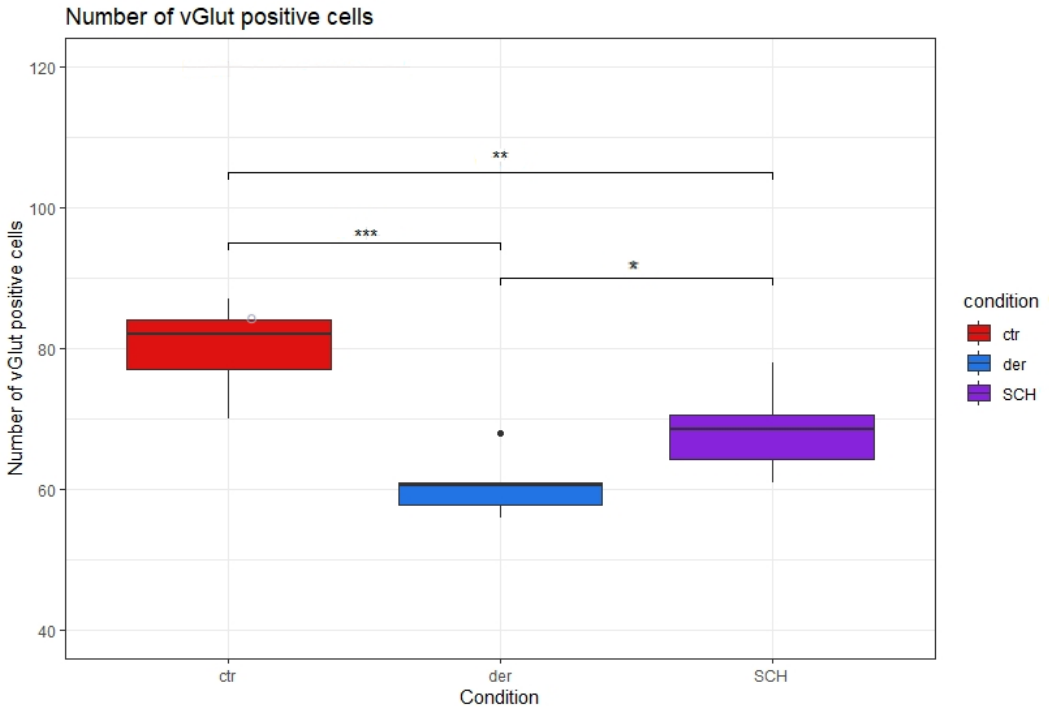

Figure 10. Number of vGlut positive cells in the MO/VO area of the PFC. DER and SCH-injected rats exhibit a significant reduction of glutamatergic neurons when compared to the control group. In addition, DER subjects seem to have even less glutamatergic neurons than SCH-injected (***$p < 0.001$ ctr vs. DER, **$p < 0.01$ ctr vs. SCH, *$p < 0.05$ DER vs. SCH). Box plots, show median (horizontal lines inside boxes), quartiles (boxes), range (whiskers), and outliers (dots outside boxes).

Aside from the decline in vGlut cells across the experimental groups, we also observed that the number differed between DER and SCH-injected subjects (one-way ANOVA: $F_{2,18} = 20{,}130$, $p < 0.001$; post-hoc tests, ctr vs. DER $p < 0.001$, ctr vs. SCH $p = 0.002$, DER vs. SCH $p = 0.028$). The DER group displayed a more severe phenotype with a bigger loss of glutamatergic cells when compared to the SCH-injected group.

Discussion

In the adult brain, dopamine is a neurotransmitter which has a well-studied role in various functions and is directly involved in the transmission in pathways such as the mesocortical pathway. However, the role of dopamine in the developing brain is unclear and very sparsely investigated. Functional synapses are not present in the developing brain, yet dopamine is present in specific areas. The above observations have led to postulations that dopamine plays a role in the developing brain different than the one in the adult brain. Findings show that dopamine is crucial during development at specific time points and brain areas, although the prefrontal cortex is one area that has not been studied.

Prompted by observations in an animal model of maternal neglect developed in our lab (the DER experience) linking dopamine depletion in PFC during development with aberrant PFC cytoarchitecture and PFC-dependent behaviours, we wanted to explore more the effect of dopamine during the prefrontal cortex development. Thus, we created another rat model of dopamine depletion in the prefrontal cortex during development at the same stages as DER experience takes place, using the dopamine receptor type 1 antagonist SCH23390 in order to see if we can phenocopy the DER model. Using the above models, we run a series of behavioural and immunohistochemical assays in order to determine the impact of dopamine ablation in specific areas of the prefrontal cortex.

First, we used a T-maze setup to test how well can adult rats learn and reverse learn the location of food in one of the two arms of the maze. After 4 days of learning how to find food we tested the ability of subjects for reverse learning based on the number of trials in the correct arm. The DER subjects were the ones that made the least amount of correct choices, significantly less than the control subjects. SCH-injected subjects performed better than DER, making more correct choices than them although they did less correct choices than controls. The literature for reversal learning is pretty rich and consistent in highlighting the PFC, its sub regions (20, 21) and dopamine (22) as crucial players in reversal learning and set shifting. However, the plethora of different sub regions of the PFC and set ups to investigate reversal learning, hamper our efforts to have a complete understanding of what regions and how they play a role in reversal learning. Nevertheless, our data indicate that a dopamine decline in the PFC during PND10-13, can impact the development of areas in the PFC which later in adult life are needed for reversal learning in a spatial discrimination task such as the T-maze set up we used. The more severe phenotype exhibited by the DER group, makes us hypothesize that other areas and neurotransmitters, that are affected during the DER experience, also play a role in reversal learning (23).

In addition, we performed a pre-pulse inhibition protocol with three different inter-stimulus intervals on control, DER and SCH-injected subjects where we showed that DER and SCH-injected groups show no difference from controls regarding the magnitude of pre-pulse inhibition either between groups or between the three inter-stimulus intervals. The lack of differences in PPI between groups can be attributed to the fact that the brain areas that are involved in mediation of PPI are located in the dorsal part of the PFC (24) which mature after the time point (25) we inject the pups with the D1 antagonist or have the pups go through the DER experience. As a result, we speculate that the dopamine decline during this time frame, affected the development of ventral areas of PFC while more dorsal one, which develop later, were unaffected. We also found that there were no differences in the PPI when the pre-pulse and the pulse were separated by 40, 80 or 120 ms in either control or experimental groups. The above is in accordance to studies that use variable ISIs and detect no differences in the PPI of control subjects in the range of 30-200 ms (26). Therefore, we are lead to believe that neither the DER nor the SCH-injected groups have their PPI affected at the ISIs we tested when compared to the control group.

Nevertheless, we did find that the maximum response to pulse alone in the control group was much bigger than the one in the DER group, while the SCH-injected group showed a midpoint response between the control and DER groups having no significant differences with either of them. The fact that only DER showed a reduced maximum response while the SCH-injected ones did not, points us to think that this phenotype is not a result of reduced dopamine in the prefrontal cortex during development. Data from previous studies in our lab, have shown that not only dopamine but also other neurotransmitters are affected during the DER experience (23, 27). Also, there are reports supporting that lesions in the Caudal Pontine Reticular Nucleus (PnC) reduce the maximum response of the Acoustic Startle Response (ASR) in rats (28). Due to the above, we hypothesize that the reduced ASR in DER subjects is due to perturbations in their PnC, possibly as a result of the DER experience. A hypothesis that remains to be explored in the future.

Along with the maximum responses in pulse alone trials, we also analyzed the time from the moment the pulse is presented till the subject's response exceeds a threshold of basal activity, called startle latency. Startle latency as a parameter of ASR has been studied extensively in human schizophrenia patients as a putative index of neuronal processing speed, with increased latencies being observed in schizophrenia patients when compared to controls (29,30), although other papers reported no difference in latency between schizophrenia patients and controls (31,32). Our data revealed that both our experimental groups, display a significant increase in the latency of their responses in pulses when compared to the control group. In contrast to the data for maximum response, we observe that DER and SCH-injected animals behave the same having greater latencies, so we speculate that the dopamine depletion in the PFC during development might play a role in increasing the latency of ASR. This is quite interesting, as reports studying pulse latency in rats are scarce and our data are among few, to our

knowledge, that link developmental dopamine insufficiency in the PFC with reduced neuronal processing speed in rats.

Since we detected differences in the behaviour of DER and SCH-injected, we wanted to investigate if the cytoarchitecture of the PFC was also affected. We used a NeuN antibody to stain for neurons in the PFC and found the SCH-injected subjects had fewer neurons in the MO area compared to control subjects. A similar finding with the above was published by our lab regarding the DER group were they also showed a reduced number of neuronal cells in the MO (19). These results lead us to postulate that the lack of dopamine in the developing PFC is responsible for the reduced number of neurons in the MO. These reduced numbers could be a result of increased apoptotic death or failure of newborn neurons to migrate properly to the MO. So, we counted the neurons in the CG, a PFC area dorsally to MO, and found no differences in the number of NeuN positive cells between controls and SCH-injected animals. This shows that neurons in SCH-injected animals most probably migrate properly but experience increased cell death which is a similar case with DER animals as we have seen in previous reports (33). In addition, we have mentioned before how crucial is dopamine in neurogenesis and how activation of D1-like and D2-like receptors have opposing effects on neuron proliferation. Since we used a D1-like receptor antagonist, we expect D1-like receptors to be occupied by SCH and all the available dopamine in the PFC is bound by D2-like receptors promoting neuronal proliferation. This aberrant proliferation could lead to increased neuronal death in the PFC, which we observe later in the adult animals. Ergo, our data strengthen our hypothesis that reduced dopaminergic activity in the PFC during development, leads to increased neuronal death in specific areas.

DER animals have also been shown to display fewer glutamatergic cells in the MO, indicating that this early experience could alter the excitatory neurotransmission (19). We performed a vGluT staining of the PFC in SCH-injected and control animals and we found that SCH-injected subjects display indeed fewer glutamatergic cells in the MO than controls but still more than the DER subjects. This is quite interesting as it shows that lack of dopamine in the PFC during development is necessary but not sufficient to cause the phenotype of altered excitatory neurotransmission that is observed in DER and other events during the DER experience seem to affect the number of glutamatergic cells in the MO.

In the current study, we developed a rat model of dopamine depletion during the postnatal period with the use of D1 antagonist. This model, along with the DER, one that has been developed previously in our lab where dopamine is depleted through denial of maternal contact, were used in behavioural and immunohistochemical assays to determine how detrimental is the lack of dopamine in the PFC during the perinatal period. Our data support that pharmacological depletion of dopamine affects certain aspects of behaviour controlled by areas of the PFC. At the cellular level, this is confirmed as the MO area displays fewer neurons than controls, which are of glutamatergic identity. Along with the above,

testing with the DER group revealed similarities with the SCH-injected but also stark differences, leading us to assume that the SCH model is not a complete phenocopy of the DER. This could be due to how the pharmacological depletion of dopamine was carried out or to the fact that the DER experience affects more neurotransmitters and brain areas other than dopamine and the PFC respectively. Nevertheless, the present work expands on how dopamine can affect the development of the brain in the perinatal period and how alterations of it during that period can last up to adulthood. More work needs to be done with dopamine depletion models in order to elucidate the precise areas of the brain that are affected and in which ways.

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