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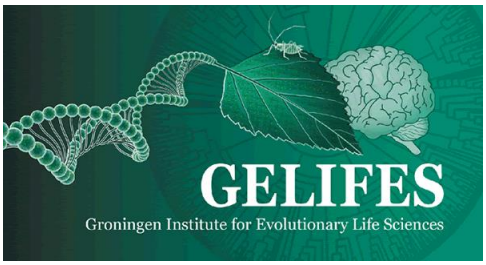

Influence of perinatal fluoxetine exposure on myelination during brain development

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Abstract

A high number of infants are exposed to antidepressant medication prior to birth and/or during lactation due to mental illness of the mother. The most used antidepressants are selective serotonin reuptake inhibitors (SSRIs), which can cross both the placenta and blood-brain barrier and are found in breast milk. Altered serotonin levels due to SSRI use during the developmental period can lead to disrupted brain development. Both human and animal studies have found that exposure to the SSRI fluoxetine during pregnancy and lactation impacts both behaviour and physiology in the offspring. Further, perinatal fluoxetine exposure can enhance myelin-related gene expression in the prefrontal cortex, and it can inhibit this gene expression in the basolateral amygdala at postnatal day 21 in male, but not female rats. This may suggest an acceleration of myelination and development. To determine whether myelination is accelerated due to perinatal fluoxetine exposure, gene expression of four myelin-related genes - myelin-associated glycoprotein (*Mag*), myelin basic protein (*Mbp*), Claudin-11 (*Cldn11*), and 2',3'-cyclic-nucleotide 3'phosphodiesterase (*Cnp*) - in the basolateral amygdala and prefrontal cortex at postnatal day 1, 7, and 14 were measured. Female Wistar rats received a daily dose of either fluoxetine (FLX; 10 mg/kg) or vehicle (autoclaved water) orally from gestational day 1 until weaning. Male and female offspring brains were collected at postnatal day 1 (n=24), 7 (n=33), and 14 (n=33) for molecular analysis. We hypothesized that myelin-related gene expression is increased in the FLX-exposed animals compared to control animals early in development. We also expect the effect to be stronger in males compared to females. Results show that the gene expression for *Mbp* and *Cnp* show no significant differences in myelin-related gene expression between the fluoxetine and control group. In addition, no sex differences are observed. Although our data is not normalized yet, these suggest that fluoxetine does not accelerate myelination from PND1 to PND14 in the BLA and PFC.

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Introduction

About 3.8% of the population worldwide suffers from depression, with the highest prevalence in individuals aged 18-25 (WHO, 2021). The prevalence of depression and/or mood disorders during pregnancy is 9-16% (WHO, 2021), and around 5% of pregnant women use selective serotonin reuptake inhibitors (SSRIs) during pregnancy to treat the depression (Andrade et al., 2008; Cooper et al., 2007). As a result, a high number of infants are exposed to anti-depressant medication prior to birth and/or during lactation. SSRIs block the serotonin transporter (5-HTT or SERT), resulting in a reduced reuptake of serotonin by the presynaptic neuron and a high amount of serotonin in the synaptic cleft. Fluoxetine is an SSRI and one of the most widely prescribed antidepressant drug (Andrade et al., 2008; Cooper et al., 2007). Both fluoxetine and its metabolite norfluoxetine can cross the placenta (Heikkinen, 2003; Kim et al., 2006; Rampono et al., 2009) and is found in breast milk in both humans and rodents (Heikkinen, 2003; Kristensen et al., 1999; Lester et al., 1993). During development, the expression of the serotonin transporter is widespread in the brain (Gaspar et al., 2003; Homberg et al., 2010). SSRIs can pass through the blood brain barrier (Feng et al., 2008), and therefore SSRIs can block the serotonin transporter in many brain regions and lead to high levels of serotonin throughout development. Serotonin acts as a neurotransmitter and is involved in fundamental brain functions, such as regulation of aggression, appetite, mood, sleep and wake rhythms, learning and memory, and reward (Brummelte et al., 2017; Muller & Jacobs, 2009). However, during development serotonin also acts as a neurotrophic factor and playing a role in the following neurodevelopmental processes: growth cone elongation, control of cell proliferation, cell differentiation, dendritic pruning, cell migration, cell division, cell death, synaptogenesis, and myelination (Gaspar et al., 2003; Teissier et al., 2017). Altered levels of serotonin during the developmental period can therefore lead to disrupted brain development and long-lasting effect on brain function.

Influence of fluoxetine on offspring

Both in humans and in rodents, the effect of fluoxetine exposure during development on the offspring has been studied. The most important findings of the clinical studies in humans are summarized in Table 1a. A meta-analysis performed by Halvorsen, and colleagues (2019) shows that in *utero* exposure to SSRIs in humans is significantly associated with the development of multiple mental disorders later in life. However, solely the effects of fluoxetine on the offspring cannot be measured in humans, because of the underlying depression of the mother. In addition, the depression of the mother, next to the fluoxetine exposure, can also influence the development of the offspring.

In animal models, it is possible to study the effects of fluoxetine during pregnancy without having the underlying depression. The most important findings in animal studies are summarized in Table 1b. Further, a meta-analysis performed by Ramsteijn and colleagues (2020) found that exposure to SSRIs during pregnancy leads to a reduction in activity and exploration behaviour, less efficient sensory processing, and a more passive coping style. Overall, perinatal SSRI exposure during the critical periods of development can lead to altered neuronal signalling, neuronal plasticity, gene expression, and protein metabolism (Brummelte et al., 2017). Changes in these important processes during development can lead to functional disturbance.

Table 1: Effects of fluoxetine during pregnancy on the offspring in humans (a) and rodents (b). References: (1) Review by Olivier et al., 2011; (2) Review by Kiryanova et al., 2013. Internalizing behaviours include sadness, shyness, withdrawal, and inhibition, all behaviours often associated with a variety of psychological disorders (Hansen & Jordan, 2020). Poorer psychomotor development leads to worse emotional, cognitive, motor, and social capacities (Cioni & Sgandurra, 2013). Serotonin withdrawal symptoms include dizziness, anxiety, gastrointestinal upset, sleep problems and hyperarousal, irritability, lowered mood, and headache (Coupland et al., 1996). Serotonergic symptoms are tremor, rigidity, irritability, shivering, eating and sleeping difficulties (Laine et al., 2003).

(a) Effects of fluoxetine during pregnancy on offspring in humans
↑ Preterm birth[1,2]
↓ Birth weight[1,2]
↑ Risk for pulmonary hypertension and other cardiovascular abnormalities[1,2]
Higher scores for serotonergic symptoms[1]
Serotonin withdrawal symptoms[1]
Altered HPA stress response[1]
↑ Internalizing behaviours[1]
Blunted somatosensory responses[1]
Poorer psychomotor development[1]

(b) Effects of fluoxetine during pregnancy on offspring in rodents
↓ Birth weight[1,2]
Shortened gestational length[1,2]
↑ Neonatal mortality[1,2]
↓ Litter size[2]
↑ Risk for cardiovascular malformations[1,2]
Serotonin withdrawal symptoms[1]
↑ Immobility time in forced swim test (more behavioural despair)[1,2]
↓ Exploratory behaviour[1,2]
↑ Anxiety-related phenotypes[1,2]
↑ Locomotor activity[1]
↓ Sexual behaviour[1]
↓ Aggression[1]
↑ REM sleep[1]
↓ Active sleep[1]
Anhedonia[1]
Blunted somatosensory responses[1]
↑ Sensitivity to rewarding effects of cocaine[1]
Improved learning and memory[1,2]
↑ Passive avoidance[1]
↓ Impulsivity[1]

Influence of perinatal fluoxetine on myelination

Myelination of axons is a process whereby brain oligodendrocytes produce layers of myelin which wrap around the axon (Doretto et al., 2011). Myelination plays a critical role in brain development and functioning, including fast nerve conduction, reduction of energy consumption, influence on neuronal circuit formation, and metabolic support to neurons (Review by Stadelmann et al., 2019). In humans, myelination starts at birth and spreads throughout the brain in four different fashions: central to peripheral, caudal to rostral, dorsal to ventral, and sensory to motor (Barkovich et al., 1988; Review by Lebel & Deoni, 2018; Review by Zhang & Gaillard, 2009). In humans, myelination is most rapid in the first two years and ends around early adulthood (Review by Lebel & Deoni, 2018). In rodents, myelination also starts at birth and continues to spread throughout the brain from caudal to rostral (Downes & Mullins, 2014). At postnatal day 21, the limbic system is the last region that needs to become myelinated and myelination is finished at postnatal day 70 (Downes & Mullins, 2014). Previous research has found that next to behavioural changes, perinatal fluoxetine can increase myelin-related gene expression in prefrontal cortex and inhibit this in the basolateral amygdala at postnatal day 21 in male rats (Ramsteijn et al., 2022). In female rats, myelin-related gene expression showed to be weakly and non-significantly downregulated in the basolateral amygdala (Ramsteijn et al., 2022). In addition, perinatal exposure to citalopram, an SSRI, leads to abnormal cortical network function and raphe circuitry and showed to have a more severe affect on male than female rats

(Simpson et al., 2011). Further, the results of this study show that perinatal exposure to citalopram may have an affect on the maturation of oligodendrocytes in the corpus callosum, leading to abnormal myelin formation. It is possible that perinatal SSRI exposure affects males and females differently due to sex-specific development of the serotonergic system (Brummelte et al., 2017). Further, changes in gene expression due to SSRIs differ per brain region and developmental time (Glover et al., 2015). A differential expression analysis between maternal adversity and perinatal fluoxetine exposure showed that maternal adversity and perinatal fluoxetine exposure interact to affect the expression of myelin-related genes, such as myelin-associated glycoprotein (*Mag*), myelin basic protein (*Mbp*), Claudin-11 (*Cldn11*), and 2',3'-cyclic-nucleotide 3'phosphodiesterase (*Cnp*) (Ramsteijn et al., 2022). The myelin-associated glycoprotein (*Mag*) gene encodes for the MAG protein that attaches the myelin sheath to the axon (Review by Stadelmann et al., 2019). The myelin basic protein (*Mbp)* gene encodes for the MBP protein, which binds the membrane layers around the axon (Review by Stadelmann et al., 2019). The Claudin-11 (*Cldn11)* gene encodes for the CLDN11 protein, which composes the tight junctions that are connecting the outer layers and therefore this protein has a barrier function (Review by Stadelmann et al., 2019). The 2',3'-cyclic-nucleotide 3'phosphodiesterase (*Cnp)* gene is responsible for the synthesis of the CNP protein which in turn creates cytoplasmic channels in the myelin sheath (Review by Stadelmann et al., 2019).

Normally, at PND21 myelin gene expression is at its highest point and the limbic system, including hippocampus and amygdala, is starting to become myelinated. Myelination spreads throughout the brain from caudal to rostral, with the prefrontal cortex being one of the last regions to become myelinated (Downes & Mullins, 2014). In offspring exposed to perinatal fluoxetine, a reduced myelin gene expression in the basolateral amygdala and an increased gene expression in the prefrontal cortex leads to the hypothesis that myelination is almost finished in the basolateral amygdala and is already starting in the prefrontal cortex at PND21. This suggests that myelination takes place in an accelerated fashion (*Figure 1*).

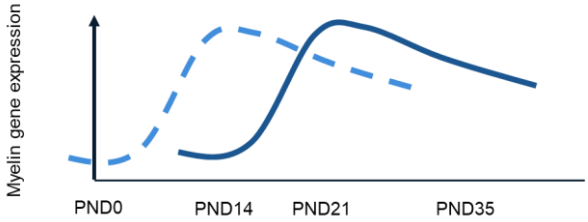

Figure 1: A scheme on the rate of myelin-related gene expression at several postnatal day timepoints. The solid line represents the rate of myelin gene expression under normal conditions. The dashed line represents the accelerated rate of myelination in offspring that are exposed to perinatal fluoxetine. At PND1, myelin-gene expression in BLA and PFC will be the same in the experimental and control group. At PND7, myelin-gene expression in BLA will start to higher in the experimental group, while myelin-gene expression in the PFC will be the same in the experimental and control group. At PND14, myelin-gene expression will be higher in the BLA and PFC in the experimental group compared to the control group.

The aim of this study is to investigate if perinatal fluoxetine exposure accelerates the gene expression of *Mag, Mbp, Clnd11, and Cnp* during early brain development of the rat. The peak myelin-gene expression in the basolateral amygdala will accelerate to before PND21, while peak myelin-gene expression in the prefrontal cortex with accelerate to around PND21. To determine whether myelination is accelerated due to perinatal fluoxetine exposure, gene expression of the four myelin-related genes mentioned above in the basolateral amygdala and prefrontal cortex at postnatal day 1, 7, and 14 were measured. The offspring was developmentally exposed to fluoxetine by injecting fluoxetine to the dam during pregnancy and lactation. Male and female offspring brains were collected at postnatal day 1, 7, and 14 for molecular analysis. First, based on previous findings, we hypothesize that fluoxetine exposure from gestation until weaning accelerates myelination. At PND1, it is expected to find no differences in myelin-gene expression between the fluoxetine and control group. At PND7,

it is expected to find higher myelin-gene expression in the BLA in the fluoxetine group compared to the control group, while no difference of the myelin-gene expression in the PFC will be expected. At PND14, it is expected to find higher levels of myelin-gene expression in the BLA and PFC in the fluoxetine group compared to the control group. Second, we hypothesize that the effect of fluoxetine on myelin-related gene expression is stronger in males than in females.

Method

Experimental animals

The experimental animals used in this research are Wistar rats. The animals were supplied ad libitum with standard lab chow (RMH-B, AB Diets; Woerden, the Netherlands) and water. They were kept on a 12:12 h light-dark cycle (lights off at 11:00 h), with an ambient temperature of 21 ± 2 °C and humidity of 50 ± 5%. The cages were provided with bedding and nesting material (Enviro-dri™, Shepherd Specialty Papers, Richland, MI, USA) and wooden stick for gnawing (10 x 2 x 2 cm). The cages are cleaned weekly. From gestational day 0, dams were housed individually in type III Makrolon cages (38.2 x 22.0 x 15.0 cm) and started to be treated with a daily dose of either fluoxetine (5mL/kg) or vehicle (autoclaved water, 5 mL/kg) until postnatal day 21. The same housing conditions applies to the offspring of the experimental animals. One male and one female per litter per period were taken for molecular analysis. Non-sacrificed animals were weaned at postnatal day 21 and group housed in same-sex cages of 3-5 animals in type IV (55.6 x 33.4 x 19.5 cm) Makrolon cages until brains were collected for further periods. All experimental procedures were approved by the Institutional Animal Care and Use Committee of The University of Groningen and were conducted in agreement with the Law on Animal Experiments of The Netherlands.

Brain collection

At different postnatal day timepoints brains were collected for molecular analysis. The different postnatal day (PND) timepoints were PND1, PND7, PND14, PND21, PND35, >PND70. Brains of PND1, PND7, and PND14 were used in this research. Animal from PND14 until >PND70 were partly sedated by exposing them to CO_2 and killed with the guillotine. For PND1 and PND7, no sedation was possible and therefore the head was removed with sharp scissors as fast as possible. Scissors were used to remove some of the skin and muscles and bone-cutter forceps were used to cut the skull. After removal, the brain was poured into dry-ice chilled isopentane for a few seconds. When frozen, the brain was wrapped into aluminium foil and stored in the -70 °C freezer. For PND1 and PND7, the brains were not removed from the skull, but instead the whole head was frozen. Because the bones are quite soft at those postnatal days, it was possible to cut through the bones while cutting the brain in the cryostat.

Collecting brain material

Myelin gene expression in the offspring was established by slicing and punching the brains and performing qPCR for *Mbp and Cnp* genes on the collected brain material. Protocols for the collection of brain material (*Appendix 2A and 2B*) and molecular analysis - RNA isolation (*Appendix 2C*), reverse transcription from mRNA to cDNA (*Appendix 2D*), and qPCR (*Appendix 2E*) - can be found in appendix 2.

For molecular analysis, brain material from specific brain regions needed to be collected with the use of the cryostat (Leica CM3050). The following brain regions were collected: prefrontal cortex (PFC), corpus callosum (CC), hypothalamus (HYPO), hippocampus (HIPP), amygdala (AMY), and dorsal raphe nucleus (DRN). Two days before slicing, the brain was transferred from the -70 °C freezer to the -20 °C freezer. At the beginning of the slicing, the cryostat, tools, and blade were cleaned with 70% ethanol. For PND1 and PND7 brain samples, the brain was trimmed by cutting part of the head in front of the eyes and part of the cerebellum with the razor to make it easier to fix onto the chuck (*Figure 2*). The brain or full head was fixed to the chuck by using a generous amount of Tissue-Tek (Sakura). When

the chuck was put into the cryostat, the chuck was adjusted in the vertical and horizontal plane as needed to make the brain as straight as possible. First, slices of 50 µm of the brain were made until the olfactory bulbs end and the prefrontal cortex could be collected. For PND1 and PND7 brain samples, the eyes were removed before making 200 um slices to avoid having problems with putting the slice on the glass slide. After every cut, the slices were placed onto an RNA-free glass slide. For PND1 and 7 brains, small incisions were made into the skin of the head to avoid the skin from pulling the slice together (*Figure 2*). A brain atlas of postnatal day 1, 7, and 14 was used to determine the location of the punches of the brain regions to be collected (Khazipov et al., 2015) (*Appendix 2A and 2B*; *Appendix 3*). The use of the 1.2 mm or 2.0 mm punching tool depended on the brain region to be collected and which postnatal day material is taken from (*Table 2*). The punching tool was cleaned with 70% ethanol between every new brain region. The punched brain material from the slices was collected in labelled Eppendorf's and stored in the -70 °C freezer upon further use.

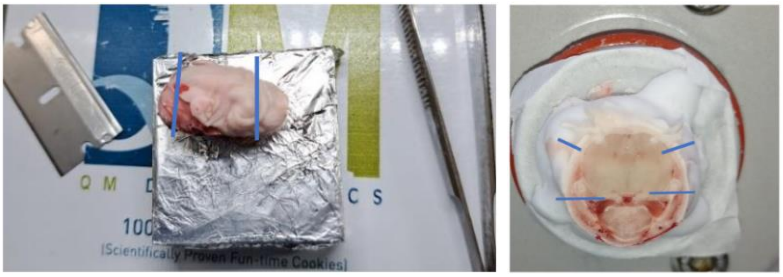

Figure 2: Example of trimming of the head and the incisions made into the skin of the head.

Table 2a: Bregma points and punching tool size of different brain region for postnatal day 1

Postnatal day 1		
Brain regions	Bregma points	Punching size
Prefrontal cortex	2.6 → 2.0	1.2 mm
Hypothalamus	0.2 → -2.6	1.2 mm
Hippocampus	-0.4 → -2.8	1.2 mm
Amygdala	-0.6 → -2.0	1.2 mm
Dorsal raphe nucleus	-3.2 → -4.6	1.2 mm
Corpus callosum	1.4 → -0.8	1.2 mm

Table 2b: Bregma points and punching tool size of different brain region for postnatal day 7

Postnatal day 7		
Brain regions	Bregma points	Punching size
Prefrontal cortex	3.2 → 1.6	1.2 mm
Hypothalamus	-0.4 → -4.0	1.2 mm
Hippocampus	-1.4 → -4.0	1.2 mm
Amygdala	-1.8 → -2.4	1.2 mm
Dorsal raphe nucleus	-5.2 → -6.8	1.2 mm
Corpus callosum	1.0 → -1.0	1.2 mm

Table 2c: Bregma points and punching tool size of different brain region for postnatal day 14

Postnatal day 14		
Brain regions	Bregma points	Punching size
Prefrontal cortex	3.4 → 1.2	1.2 mm
Hypothalamus	-0.2 → -4.0	2.0 mm
Hippocampus (1st round)	-1.6 → -2.8	1.2 mm
Hippocampus (2nd round)	-3.0 → -4.0	1.2 mm
Amygdala	-1.6 → -3.4	1.2 mm
Dorsal raphe nucleus	-5.4 → -7.6	2.0 mm
Corpus callosum	0.8 → -3.6	1.2 mm

Myelin-related gene expression

Quantitative polymerase chain reaction (qPCR) was performed to determine the concentration of the two myelin-related genes, *Mbp* and *Cnp*, at postnatal day 1, 7, and 14.

First, RNA was isolated from the collected brain tissue (for protocol see Appendix 1C). Samples were lysed using TRIzol (Ambion, Life Technologies), 5 mm stainless steel beads, and a TissueLyser II (Qiagen) 2 times for 2:00 at 30 Hz. Chloroform (Sigma-Aldrich) and Isoproponal (99,5%; Acros Organics) were added to isolate the RNA from DNA and proteins and from free nucleotides, respectively. 75% ethanol (Merkc, Boom Lab) was used to wash away the chemicals from the RNA pellet and GlycoBlue glycogen (1:10 dilution; Invitrogen, Thermo Fisher Scientific) was used to make the RNA pellet visible. The RNA pellet with UltraPure DNase/RNase-free distilled water (Invitrogen, Life Technologies) was incubated at 55 °C for 15:00 and afterwards stored in the -70 °C freezer. RNA concentration, purity, quality, and degradation was determined by using the Nanodrop 2000 (Thermo Scientific) and 1.2% agarose gel electrophoresis (for protocol see Appendix 1D). For the nanodrop, the 260/280 ratio gives an indication on how pure the sample is from contaminating protein and for RNA this ratio should optimally be higher than 1.8. However, a ratio < 1.65 is seen as a poor ratio for RNA. The 260/230 ratio gives an indication on how pure the sample is from salts and other contaminants and should be 2.0 or higher. RNA is intact if the gel electrophoresis shows two sharp bands (28S and 18S rRNA bands) and the 28S band is twice as intense as the 18S band. Degraded RNA will appear as a smear over the whole band and presence of genomic DNA will be visible as a band at the top of the column.

Second, cDNA and qPCR were produced using the two-step reverse transcriptase reactions. Oligo (dT) 18 primer (100 µM; Thermo Scientific, Life Technologies) was used the initiate the synthesis of the first cDNA strand by binding to the 3'end of the poly A tail of the mRNA. RevertAid H minus RT reverse transcriptase (200 U/µL; Thermo Scientific, Life Technologies) with 5x reaction buffer (Thermo Scientific, Life Technologies), RiboLock RNase inhibitor (40 U/µL; Thermo Scientific, Life Technologies), and dNTP SET (10mM each; Thermo Scientific, Life Technologies) were added to synthesize cDNA from mRNA. The samples were run in the thermocycler for 25 °C for 10 minutes (oligo annealing), 45 °C for 60 minutes (promotion of reserve transcriptase), and 70 °C for 15 minutes (inactivation of reverse transcription). Afterwards, the cDNA was stored in the -20 °C freezer.

Third, qPCR was performed by using specific forward primers, reverse primers, and probes for each of the two myelin-related genes (more details in *Table 3*). For initial denaturation, the reaction temperature is raised to 95 °C for 3 minutes. In the first part of the cycle, the reaction temperature was raised to 95 °C for 15 seconds. In the second part of the cycle, the reaction temperature was lowered to 60 °C for 1 minute. This process is repeated until in total 40 cycles were completed. The qPCR efficiency per gene should be around 1.9 and 2.0. Furthermore, an optimal Cq value is between 20 and 35 cycles. The efficiency of the primers and probe should be ≥ 90% and the R^2 should be ≥ 0.99.

Table 3: Name, sequence, used quencher of the primers and probes, efficiency, and R^2 values for the four myelin-related genes and two housekeeping genes. The efficiency should be ≥ 90% and the R^2 value should be ≥ 0.99.

Name	Sequence (5'→ 3')	Probe / quencher	Efficiency (%)	R^2
MBP			94,6	0,99
MBP-30 F	AGCATCTGAGAAGGCCAGTA			
MBP-137 R	AACACATCACTGTCTTCTGAGG			
MBP-91 P	FAM-AGCGTTGGCAAGCTTTCTCAGACCG-BHQ-1	FAM-BHQ-1		
CNP			96,2	0,99
CNP-605 F	GAAAAGCCGGCCAGGTCTTT			
CNP-704 R	AGCTTCTCCTTGGGTTCATCC			
CNP-626 P	VIC-TGGAGGAGCTGGGAAATCACAAGGCT-TAMRA	YY-BHQ-1		
Cldn11			84,6	0,99
Cldn-366 F	GGCTGGGGTGCTCCTTATTC			
Cldn-465 R	GCCAAAGCTCACGATGGTG			
Cldn-298 P	Cy3-GCGCCATTGTCGCCACCATCTGGT-BHQ-2	FAM-BHQ-1		
MAG			85,0	1,00
MAG-384 F	CTTCTCAGAGCACAGCGTCC			
MAG-483 R	CACCATGCAGCTGACCTCTA			
MAG-419 P	CCCCCAACATCGTGGTGCCCCC	YY-BHQ-1		
Actin-β			83,5	1,00
Actin-β F	ACCCGCGAGTACAACCTTC			
Actin-β R	ATCGTCATCCATGGCGAACTG			
Actin-β P	FAM-CGTCGCCGGTCCACACCCGC-BHQ-1	FAM-BHQ-1		
GAPDH			92,6	0,99
GAPDH-525 F	CACCACCAACTGCTTAGCCC			
GADPH-624 R	CTGAGTGGCAGTGATGGCAT			
GAPDH-573 P	Cy5-TGGCATCGTGGAAGGGCTCATGACCA-BHQ-3	Cy3-BHQ-2		

Data analysis

The qPCR data was generated by the ABI Fast 7500 qPCR machine. Analysis of the qPCR data was performed by using the programmes 7500 Software v2.3 and LinRegPCR. The programme LinRegPCR performed a linear regression to determine the starting concentration of each sample. Samples were excluded if there is no amplification, no plateau phase, too low Cq value or if the PCR efficiency of the samples lies 5% outside the group median. All statistical analysis were performed by R Studio, version R 4.1.3. Normality of the data was checked by Shapiro-Wilk tests and homogeneity of the variances was checked by a Bartlett's test. Data was separated per gene tested (*Mbp* and *Cnp*) and per brain region (PFC and BLA). The response variable, gene expression, was multiplied by 1^{10} to make the data easier visible. Data was analysed by using ANOVA, followed by a pairwise comparison. A p-value < 0.05 was considered statistically significant.

Results

The myelin-related genes, *Mag* and *Cldn11*, are not tested, because the efficiency of the primers and probe for these genes was below 90%. We continued optimising these gene-probe combinations, but this was not in time for this report. Further, the data is not normalised due to the lack of the reference/housekeeping genes. The reference gene *Actin*-β had an efficiency was below 90%. The reference gene *GAPDH* had an efficiency above 90%. However, there was amplification in the NO-RT sample for both *Actin*-β and *GAPDH*. This NO-RT sample does not contain the reverse transcriptase enzyme and should therefore not contain any cDNA that can be amplificated. The amplification of the NO-RT sample is likely due to genomic DNA that is always present after RNA isolation. The reference genes, *Actin*-β and *GAPDH*, contain lots of pseudogenes that will cause amplification with the presence of genomic DNA in the samples. Furthermore, there are no sex differences observed between the treatment groups and over time (*Mbp* in BLA: F=0.6832, ns; *Mbp* in PFC: F=0.0978, ns; *Cnp* in BLA: F=0.0255, ns; *Cnp* in PFC: F=0.0076, ns). Therefore, the sexes are not separately shown in the graphs. Further, the gene expression has been multiplied by 1^{10} to have easier readable numbers on the y-axis.

Gene expression of myelin basic protein (*Mbp*)

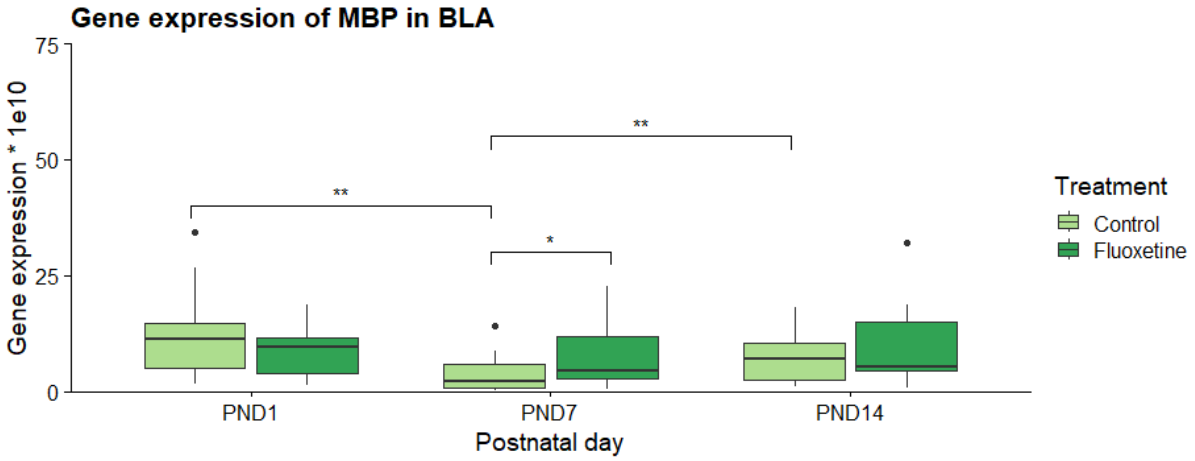

*Figure 3: Boxplots of the gene expression of Mbp at PND1 , PND7, and PND7 per treatment group in the basolateral amygdala. ** p-value < 0.01, * p-value < 0.05. Removed four samples with gene expression of 0 (PND7 control group (n=1), PND7 fluoxetine group (n=1), and PND14 fluoxetine group (n=2)). Group size per postnatal day and treatment group: PND1 (n_{Ctr} =12; n_{FLX} =12), PND7 (n_{Ctr} =17; n_{FLX} =13), and PND14 (n_{Ctr} =18; n_{FLX} =10).*

Figure 3 shows the gene expression of *Mbp* per postnatal day and treatment group in the basolateral amygdala. There is a significant effect of age on the gene expression (F=6.6003, p=0.0023) but no significant effect of treatment (F=1.1084, ns) on the gene expression. Pairwise comparison shows that in the control group, gene expression is decreased at PND7 compared to PND1 (t=3.565, p=0.0018) and PND14 (t=-3.150, p=0.0065). Further, the pairwise comparison shows no significant differences in gene expression levels in the fluoxetine group with age ($t_{PND1\text{-}PND7}$=0.900, ns; $t_{PND1\text{-}PND14}$=-0.026, ns; $t_{PND7\text{-}PND14}$=-0.883, ns). At PND7, there are higher levels of *Mbp* gene expression in the fluoxetine group compared to the control group (t=-2.063, p=0.0425).

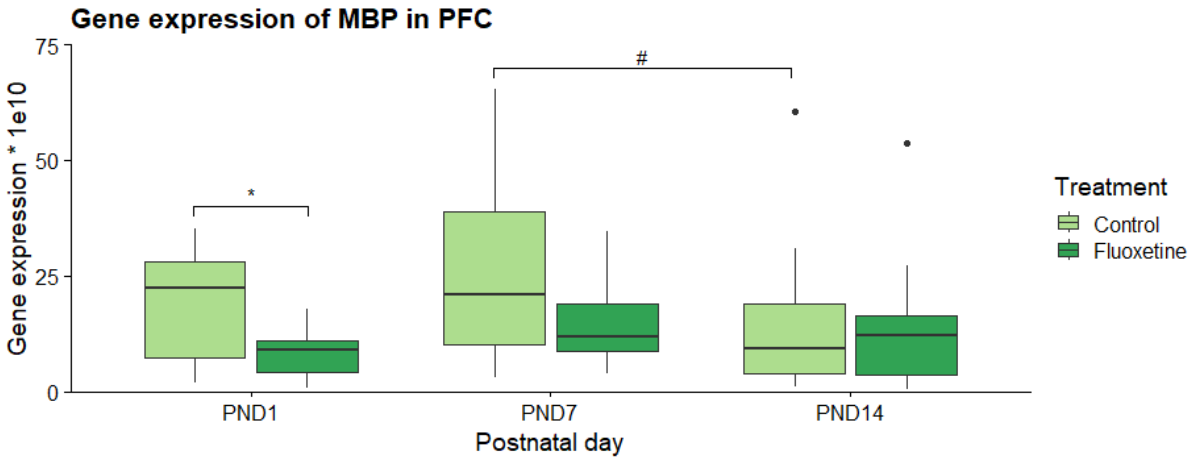

*Figure 4: Boxplots of the gene expression of Mbp per postnatal day and treatment group in the prefrontal cortex. * p-value < 0.05, # tendency for significance. Removed three samples with gene expression of 0 (PND14 control group (n=2) and PND14 fluoxetine group (n=1)). Group size per postnatal day and treatment group: PND1 (n_{Ctr} =12; n_{FLX} =12), PND7 (n_{Ctr} =19; n_{FLX} =14), and PND14 (n_{Ctr} =17; n_{FLX} =13).*

Figure 4 shows the gene expression of *Mbp* per postnatal day and treatment group in the prefrontal cortex. There is a significant effect of age on the gene expression (F=3.4875, p=0.0352) and a tendency for significant effect of treatment on the gene expression (F=3.6176, p=0.0607). Pairwise comparison shows that there is a tendency for a decrease in gene expression from PND7 to PND14 in the control group (t=2.261, p=0.0673). In the fluoxetine group, the pairwise comparison shows no significant differences in gene expression levels with age ($t_{PND1\text{-}PND7}$=-1.706, ns; $t_{PND1\text{-}PND14}$=-0.362, ns; $t_{PND7\text{-}PND14}$=1.366, ns). At PND1, there are lower levels of *Mbp* gene expression in the fluoxetine group compared to the control group (t=2.042, p=0.0444). At PND7 and PND14, there are no significant gene expression differences between the control and fluoxetine group (t_{PND7}=1.010, ns; t_{PND14}=0.344, ns).

Gene expression of 2',3'-cyclic-nucleotide 3'phosphodiesterase (*Cnp*)

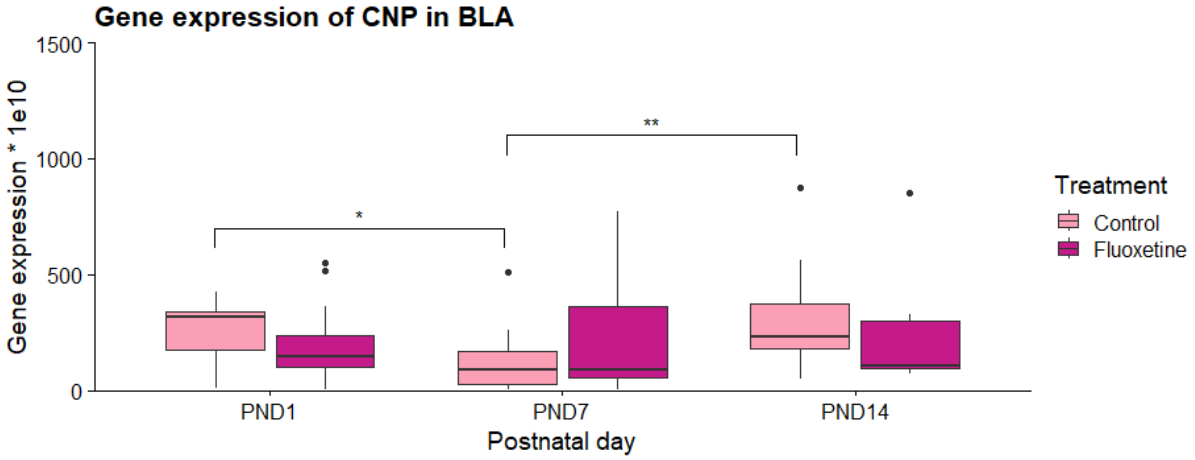

*Figure 5: Boxplots of the gene expression of Cnp per postnatal day and treatment group in the basolateral amygdala. ** p-value < 0.01, * p-value <0.05. Removed four samples with gene expression of 0 (PND7 control group (n=2) and PND14 fluoxetine group (n=2)). Group size per postnatal day and treatment group: PND1 (n_{Ctr} =12; n_{FLX} =12), PND7 (n_{Ctr} =16; n_{FLX} =14), and PND14 (n_{Ctr} =18; n_{FLX} =10).*

Figure 5 shows the gene expression of *Cnp* per postnatal day and treatment group in the basolateral amygdala. There is a significant effect of age on the gene expression (F=6.3182, p=0.0029) but no significant effect of treatment on the gene expression (F=0.0024, ns). Pairwise comparison shows a decrease in gene expression in the control group at PND7 compared to PND1 (t=2.612, p=0.0289) and

PND14 (t=-3.177, p=0.0060). In the fluoxetine group, no significant differences are found in gene expression with age ($t_{PND1\text{-}PND7}$=0.817, ns; $t_{PND1\text{-}PND14}$=-0.795, ns; $t_{PND7\text{-}PND14}$=-1.599, ns). At each postnatal day, there are no significant differences in gene expression between the fluoxetine and control group (t_{PND1}=0.789, ns; t_{PND7}=-0.966, ns; t_{PND14}=0.193, ns).

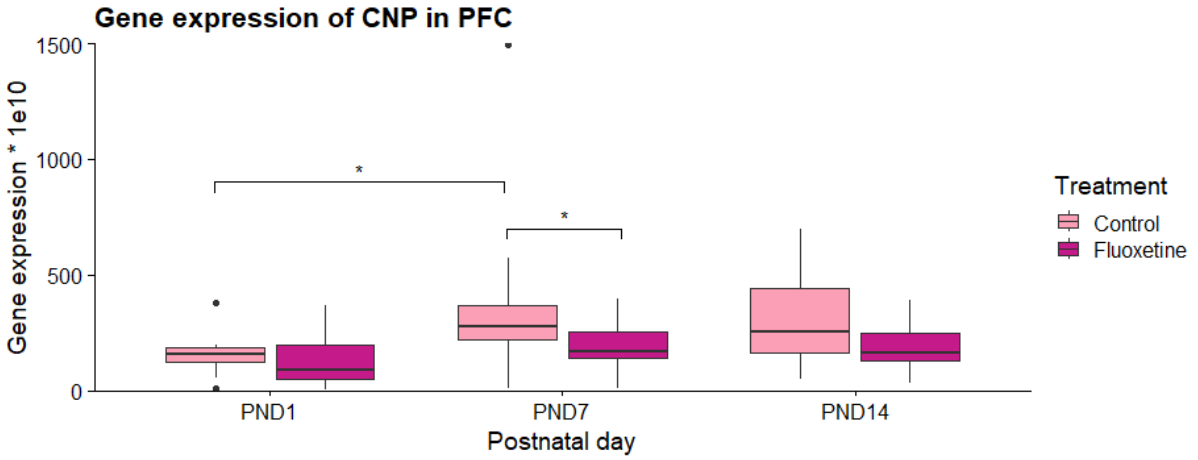

*Figure 6: Boxplots of the gene expression of Cnp per postnatal day and treatment group in the prefrontal cortex. * p-value < 0.05. Removed four samples with gene expression of 0 (PND1 control group (n=1), PND14 control group (n=1), and PND14 fluoxetine group (n=2)). Group size per postnatal day and treatment group: PND1 (n_{Ctr} =12; n_{FLX} =12), PND7 (n_{Ctr} =19; n_{FLX} =14), and PND14 (n_{Ctr} =17; n_{FLX} =13).*

Figure 6 shows the gene expression of *Cnp* per postnatal day and treatment group in the prefrontal cortex. There is a significant effect of treatment (F=6.2419, p=0.0145) and age (F=3.6038, p=0.0317) on gene expression levels. Pairwise comparison shows that for the control group there is a significant increase in gene expression from PND1 to PND7 (t=-2.636, p=0.0269). In the fluoxetine group, there are no significant differences in gene expression with age ($t_{PND1\text{-}PND7}$=-1.048, ns; $t_{PND1\text{-}PND14}$=-0.925, ns; $t_{PND7\text{-}PND14}$=0.108, ns). At PND7, there are lower levels of gene expression in the fluoxetine group compared to the control group (t=2.206, p=0.0302). At PND1 and PND14, there are no significant differences in gene expression between the control and fluoxetine group (t_{PND1}=0.457, ns; t_{PND14}=1.524, ns).

Discussion

The aim of this study is to investigate if perinatal fluoxetine exposure accelerates the gene expression of myelin-related genes *Mag* and *Cnp* in the PFC and BLA during early brain development in rats.

Results show that for both *Mbp* and *Cnp* in the BLA, a decrease in gene expression at PND7 compared to PND1 and PND14 was found in the control group. Gene expression in the fluoxetine group increases over time, although this is not significant. Ibarrola and Rodríguez-Peña (1996) measured myelin gene expression in different brain regions at different postnatal timepoints. Nothern blot/analysis was performed with specific probes for *Mbp* and *Cnp* to determine the optical density of the genes at multiple timepoints. In their research, gene expression is measured in the hippocampus not the amygdala, but is still of interest, as the amygdala and hippocampus are myelinated at the same period, and therefore have quite similar gene expression patterns (Downes & Mullins, 2014). In the hippocampus, Ibarrola and Rodríguez-Peña found that *Mbp* gene expression starts at PND10 and increases from PND10 to PND14. For *Cnp*, they found that gene expression is low at PND1 and PND7 and there is an increase in gene expression from PND7 to PND14. These results are not in line with our results of gene expression in the amygdala. Our results show already some levels of gene expression at PND1 and PND7 for both genes. However, the increase of gene expression at PND14 in our results is in line with the reported levels of gene expression in the hippocampus.

The results of the current study show that for both genes in the PFC, there is an increase in gene expression from PND1 to PND7 in the control group, although this is not significant for *Mbp*. Further, there is a decrease in gene expression from PND7 to PND14 in the control group, although this is not significant for *Cnp*. In the fluoxetine group, a non-significant increase in gene expression is found from PND1 to PND7 and no difference in gene expression from PND7 to PND14. The research by Ibarrola and Rodríguez-Peña (1996) also measured *Mbp* and *Cnp* gene expression levels in the cerebral cortex at different postnatal timepoints. They showed that *Mbp* gene expression starts at PND10 and increases with high levels from PND10 to PND14. For *Cnp*, they showed that gene expression starts around PND7 and increases from PND7 to PND14. All our observed results are not in line with the reported gene expression levels in the cerebral cortex. Our results show levels of gene expression at PND1 and PND7 and no increase in gene expression from PND7 to PND14. In addition, in the current study, gene expression even decreases from PND7 to PND14 in the control group.

There are some limitations to the current study and the study by Ibarrola and Rodríguez-Peña (1996). First, it is not clear what the level of gene expression is in our results. Because we did not have data for reference genes to normalize the data, the level of gene expression cannot be determined. However, there are higher levels of *Cnp* gene expression in both the BLA and PFC compared to the *Mbp* gene expression. This difference in gene expression between *Cnp* and *Mbp* will remain the same after normalization. Second, in the research by Ibarrola and Rodríguez-Peña (1996), gene expression is not measured at PND1 and PND7, meaning that it is not clear what levels of gene expression are present at those postnatal days. Their results show that *Mbp* gene expression probably starts around PND10, as gene expression levels are still very low. Further, their results show that *Cnp* gene expression is detectable at low levels at PND7 and may therefore also be detectable at PND1. Third, in the research by Ibarrola and Rodríguez-Peña (1996), gene expression is measured using Northern blot, which is much less sensitive compared to qPCR (Dean et al., 2002). Therefore, lower levels of gene expression can be detected using qPCR compared to Northern blot. The observed levels of gene expression in the current study at PND1 and PND7 which are not present in the study by Ibarrola and Rodríguez-Peña (1996) can be due to the higher sensitivity of the qPCR which is used in the current study. The reason for the unexpected pattern of gene expression at PND7 in the control group is not clear. After normalization with the reference genes, it is possible that this pattern disappears. Also, it is important to see if the same pattern is seen for the other myelin-related genes, *Mag* and *Cldn11*.

Overall, there is no difference in gene expression found between the fluoxetine and control group. However, at three postnatal days there is a significant difference in gene expression between the groups. At PND7 for *Mbp* in the BLA, there are higher levels of gene expression in the fluoxetine group compared to the control group. At PND1 for *Mbp* in the PFC and at PND7 for *Cnp* in the PFC,

there are lower levels of gene expression in the fluoxetine group compared to the control group. It is not clear if the significant difference in gene expression between the treatment groups will remain after normalization of the data. Furthermore, the study by Ramsteijn et al. (2022) found only a significant difference in gene expression between the fluoxetine and control group for *Cnp* in the BLA. At an individual gene expression level, differences in gene expression between the treatment groups can be small early in development. Therefore, no significant differences in gene expression levels between the treatment groups may be observed early in development. In addition, Ramsteijn et al. (2022) only found a significant difference in gene expression between the fluoxetine and control group when testing many myelin-related genes together at PND21. This means that when more myelin-related genes (such as *Mag* and *Cldn11*) are tested, significant differences in gene expression may become apparent. In addition, a study by Simpson et al., (2011) shows that exposure to the SSRI citalopram postnatally has a larger effect on abnormal myelin formation compared to exposure to citalopram gestationally. This indicates that exposure to citalopram after birth may affect myelin gene expression more than exposure to citalopram during gestation. This could explain the fact that there are differences in myelin-related gene expression between the treatment groups at PND21 and not at earlier timepoints. At earlier timepoints, the effect of citalopram may be smaller than at a later timepoint, leading to smaller differences in myelin-related gene expression between the treatment groups early in development.

The output from the Nanodrop 2000 (*Appendix 1*) shows that for all samples the ratio 260/230 is lower than 2.0. This means that the RNA is contaminated with salts and other contaminants. This contamination comes from both TRIzol, which contains guanidium salts, and from Glycoblue, which is used to visualize the RNA pellet. Furthermore, many samples, especially from the amygdala, have a ratio 260/280 lower than 1.65. This means that the RNA is contaminated with proteins, including the phenol component of TRIzol. In addition, the 260/280 ratio was lower in samples which yielded low amount of RNA. On average, the amount of contamination should be the same in all samples, because the protocol is done in the same fashion each time. Nevertheless, in samples with low RNA yield, the amount of contamination is relatively higher compared to samples with high RNA yield. The contamination of RNA can lead to inhibition of the qPCR, resulting in higher Cq values and a reduction in accuracy of the gene expression level. For future research, an additional washing step with 70% ethanol could increase both ratios by reducing the amount of TRIzol contamination, thereby improving the qPCR. The removal of genomic DNA will also improve the qPCR. Genomic DNA contamination can be removed using DNase treatment. However, DNase treatment can lead to loss of RNA amount, lower mRNA integrity, and more contaminants (Tavares et al., 2011). By removing the genomic DNA, reference genes with lots of pseudogenes, such as *Actin-β* and *GAPDH*, can also be used. However, the loss of RNA amount due to DNase treatment will lead to even lower level of RNA yield, making it harder to test more myelin-related genes. Therefore, it is not recommended to use the DNase treatment when working with low levels of RNA, as in the current study. Instead, other RNA isolation techniques can also be used, for example, the AxyPrep Multisource Total RNA Miniprep kit. This method uses no aggressive organic solvents and removes genomic DNA, without an DNase treatment step (Tavares et al., 2011). The ratios 260/280 and 260/230 will be improved by this method and less genomic DNA will be present, leading to more accurate qPCRs.

A study by Teissier et al., (2020) shows that maternal separation leads to an increase in myelin-related gene expression levels at PND15 in the prefrontal cortex. Maternal separation can lead to alterations in the serotonergic systems in the brain just as SSRIs, although in a different fashion (Lieb et al., 2019). Further, this study shows that maternal separation can lead to an earlier differentiation of oligodendrocyte progenitor cells during development, leading to a reduction of oligodendrocyte progenitor cells in adulthood. The results from the study by Teissier et al. (2020) may indicate that an increase in myelin-related gene expression early in development, leads to hypomyelination and reduced myelin-related gene expression in adulthood. Therefore, in further research myelin-related gene expression should also be measured in adulthood, creating a full picture of the influence of perinatal fluoxetine on myelination over time.

Another implication for further research is that next to testing myelin-related genes, gene expression levels of reelin are also tested. Reelin is expressed by Cajal-Retzius cells in the cortex and hippocampus and is responsible for proper neuronal migration. Reelin levels usually decrease during development (Brummelte et al., 2013). A study by Brummelte et al. (2013) measured reelin levels in the umbilical cord serum in SSRI-exposed newborns and non-exposed newborns. Newborns that were exposed to SSRI during pregnancy showed significantly lower reelin levels at birth compared to non-exposed newborns. This effect was especially found in females, even after controlling for gestational age at birth and maternal prenatal mood. Further, animal studies have found that pups exposed to increased serotonergic tone have lower cortical and blood serum reelin levels (Janušonis et al., 2004). Thus, it can be argued that SRRIs induce an increase in activity of the serotonergic system, leading to lower levels of reelin and an indication for accelerated neurodevelopment. These findings show that it could be beneficial to test the gene expression of reelin in addition to the myelin-related genes.

Conclusion

We hypothesized that fluoxetine exposure from gestation until weaning accelerates myelination in the brain and that this effect of fluoxetine on myelin-related gene expression would be stronger in males than in females. For both *Mbp* and *Cnp*, no significant differences were found in gene expression between the fluoxetine and control group and no sex differences were observed. After normalization with the reference genes, more clear conclusions about treatment effects and sex differences can be drawn, but for now our hypothesis was not confirmed. This suggests that fluoxetine does not accelerate myelination from PND1 to PND14 in the BLA and PFC.

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Appendix

1. Output from nanodrop

Appendix table 1: Amount of RNA (ng/µL), ratio 260/280, and ratio 260/230 for each sample derived from the Nanodrop 2000. The ratio 260/280 measures the amount of contaminating protein and this ratio should be ≥ 1.65. The ratio 260/230 measures the amount of contamination by salts and other contaminants and this ratio should be ≥ 2.0. All sample have a ratio 260/230 lower than 2.0. Samples in yellow also have a ratio 260/280 lower than 1.65.

Sample	Amount of RNA (ng/µL)	Ratio 260/280	Ratio 260/230	Sample	Amount of RNA (ng/µL)	Ratio 260/280	Ratio 260/230
27-1	622,7	1,95	1,66	26-6	32,4	1,55	0,31
26-1	346,8	1,91	1,52	35-6	49,8	1,65	0,54
35-1	436,2	1,96	1,67	34-6	44,7	1,52	0,19
34-1	184,1	1,84	1,21	37-6	37,7	1,4	0,29
37-1	88,6	1,8	1,04	36-6	42,7	1,6	0,4
36-1	339,4	1,89	0,49	38-6	46,1	1,62	0,45
38-1	236,8	1,89	1,36	40-6	77,6	1,41	0,3
40-1	141,2	1,86	0,6	39-6	46,8	1,6	0,43
39-1	119,4	1,79	0,81	42-6	45,9	1,7	0,63
42-1	135,2	1,75	0,36	41-6	52,0	1,54	0,14
41-1	97,9	1,74	0,22	43-6	39,8	1,68	0,58
43-1	171,3	1,87	1,24	46-6	83,6	1,61	0,38
46-1	165,3	1,84	0,84	44-6	48,0	1,54	0,38
44-1	140,1	1,79	0,81	45-6	43,4	1,52	0,27
45-1	192,5	1,89	0,72	47-6	52,8	1,55	0,41
47-1	155,3	1,84	1,01	48-6	35,3	1,56	0,35
48-1	182,8	1,85	1,36	49-6	50,7	1,68	0,67
49-1	70,4	1,79	0,95	50-6	28,1	1,61	0,29
50-1	196,6	1,87	1,66	51-6	39,5	1,57	0,37
51-1	133,9	1,83	1,34	52-6	43,5	1,66	0,28
52-1	214,7	1,83	1,24	53-6	45,7	1,63	0,51
53-1	133,8	1,82	1,26	54-6	33,7	1,64	0,42
54-1	209,5	1,8	1,02	55-6	36,0	1,55	0,19
55-1	102,4	1,8	0,88	56-6	38,5	1,63	0,47
56-1	135,0	1,84	1,31	58-6	55,7	1,7	0,51
58-1	132,4	1,83	1,27	57-6	55,2	1,72	0,51
57-1	105,9	1,85	1,1	60-6	66,8	1,73	0,48
60-1	110,1	1,85	1,05	59-6	48,7	1,76	0,88
59-1	65,3	1,69	0,58	63-6	42,1	1,77	1,3
63-1	187,9	1,85	0,95	64-6	54,1	1,77	0,81
64-1	155,9	1,87	0,78	91-6	25,5	1,72	0,61
91-1	156,9	1,88	1,13	92-6	50,1	1,74	0,77
92-1	73,0	1,66	0,51	1-6	38,7	1,75	0,73
1-1	77,6	1,82	1,13	2-6	50,5	1,75	0,28
2-1	106,8	1,82	0,47	3-6	47,7	1,78	0,7
3-1	123,4	1,72	0,59	4-6	54,8	1,77	0,37
4-1	120,7	1,83	0,95	5-6	71,0	1,72	0,65
5-1	143,6	1,8	1,06	6-6	40,3	1,76	0,68
6-1	47,8	1,53	0,31	8-6	49,0	1,71	0,84
8-1	104,0	1,76	0,31	9-6	39,4	1,69	0,65
9-1	84,6	1,64	0,53	10-6	45,7	1,69	0,69
10-1	150,6	1,84	0,68	11-6	49,2	1,71	0,97
11-1	171,7	1,83	1,37	16-6	49,5	1,72	0,6

16-1	113,6	1,78	1,06	17-6	28,3	1,64	0,52
17-1	96,0	1,79	0,93	18-6	50,7	1,7	0,55
18-1	86,2	1,76	0,77	19-6	42,7	1,67	0,47
19-1	123,7	1,81	0,78	20-6	64,3	1,77	0,82
20-1	116,7	1,74	0,6	21-6	40,1	1,76	0,22
21-1	86,3	1,74	0,91	22-6	53,8	1,75	0,52
22-1	101,8	1,77	0,68	23-6	41,9	1,7	0,6
23-1	101,4	1,79	0,87	24-6	43,5	1,75	0,28
24-1	270,8	1,85	1,44	25-6	60,2	1,78	0,87
25-1	110,2	1,76	0,87	28-6	64,2	1,79	1,12
28-1	149,5	1,82	0,8	29-6	57,9	1,74	0,66
29-1	195,8	1,86	1,25	30-6	38,1	1,77	0,3
30-1	79,0	1,68	0,48	31-6	49,2	1,74	0,99
31-1	133,8	1,81	1,09	97-6	81,0	1,82	0,74
97-1	42,2	1,64	0,77	108-6	149,0	1,91	1,13
108-1	196,9	1,81	1,16	109-6	108,5	1,9	1,37
109-1	110,4	1,83	0,92				

2. Protocols

A) Protocol for slicing and punching

Materials

- Leica CM3050 cryostat, settings:
  - Object Temperature: -10°C
  - Chamber Temperature: -12°C
  - Slicing thickness: 50 µm (advance it 4 times before slicing to make it 200 µm)
  - Angle of blade: 2°
- 70% ethanol and tissues
- Chuck and OCT/Tissue-Tek, Sakura
- Sharp blades; use a new part of the blade for every brain (about 2 brains per blade).
- Some tools like tweezers and autoclaved cotton swabs
- Plain, untreated microscope slides, 76x26 mm, Waldemar Knittel, Germany
- Glass staining jar filled with RNAse away
- 2 metal slide-racks
- Styrofoam box with dry ice
- RNAse/DNAse free 2mL Safe-lock tubes, Sarstedt + permanent pen ⇒ **2 ml Eppendorf's with round bottom**
- 1.2 mL tube (vial) filled with ethanol to clean the punching tools
- 1.2 mm punch, Harris, Uni-Core
- Lab coat and gloves
- Extra metal box to fill it with dry ice (something like the metal box below):

Notes about the Cryostat:

- Always check temperature and thickness
    - Object Temperature: -10°C
    - Chamber Temperature: -12°C
    - 50µ for first slides; 200µ to TRIM
- Cleaning: 70%
- Retraction: 200µ
- Quick frozen: ON
- Object frozen: ON

Protocol

*Wear clean gloves for the entire procedure. Clean them with 70% ethanol anytime is needed.

Preparation

1. Two days before slicing, transfer the brain from the -70°C freezer to the -20°C freezer.
2. Before slicing, transfer the brain to the cryostat.
3. Wear clean gloves. 'Shake' the right number of 2mL tubes out of the bag onto some clean tissues. Label them with the area name or abbreviation, sample number and date. Put the Eppendorf's on dry ice and weigh the Eppendorf's when they are cooled down.
4. Clean the inside of the cryostat, especially all the tools and the blade, with 70% ethanol, and place and tighten the blade.
5. Put 10 glass slides in the jar with RNAse away. Remove them, put them on some tissues. Then rub them dry and dust-free with clean tissues. Place them in a metal rack in the cryostat.
6. Cut part of the cerebellum with a razor to make it easier to fix onto the chuck.
7. Put a generous amount of OCT on a chuck at room temperature; let it cool down in the cryostat for a bit. Mount the brain onto the chuck with OCT; use forceps to hold the brain until it's stable. Make sure that it's as straight as possible, in a 90° angle on the chuck. When the OTC has hardened, apply a second layer around the brain as needed.
8. Fix the chuck in the cryostat. Adjust it in the vertical or horizontal plane as needed. Then make sure all the screws and the knife are fastened tightly.

Slicing

1. With the anti-roll slip on top of the blade and the knife holder, make slices at 50µ for several times until the plane has a little 'butterfly' shape and is big enough to collect. Then clean the knife holder and brain with the cotton tip to wipe away the first slices.
2. Make a slice at 200µ.
3. Ideally the slice should slide smoothly underneath the anti-roll slip (the position of the slip can be adjusted with the screw if necessary). If there's a lot of resistance at the start, lift the anti-roll slip over the beginning of the slice so that the rest can slide underneath smoothly. Slice at a steady, moderate pace for the best quality.
4. Lift the anti-roll slip from the slice carefully. If the slice sticks to the anti-roll slip, try to carefully release it with one of the tools.
5. If necessary: position the slice straight at the edge of the knife and flatten it a bit. If it's broken, assemble it now using the tools.
6. Take one of the clean glass slides and warm up the spot where the brain slice should stick with a finger at the back of the slide. Slowly place the slide on top of the brain slice and apply just enough pressure to make it stick. Turn the slide around and warm it up a little bit more if necessary to make the slice stick properly.
7. Place the glass slide back in the cryostat. If necessary, clean the knife holder and anti-roll slip with the cotton swab. Repeat steps 2-7 until the last area is collected.

Notes

- Always lock the slicing mechanism when not in use! Also cover the knife with the anti-roll slip.
- When a glass slide is 'full', place it on one of the metal surfaces of the coldest part in the cryostat to make the brain slices freeze completely again.
- If the brain starts curling, move the anti-slip roll more towards the brain and slowly slice the brain. If that doesn't work, you can use the plastic tool.

Punching

1. Put a layer of dry ice onto the extra metal box and close it to use the upper part as a 'cold table'. Place the extra metal box carefully into the cryostat. Clean the punch properly with some ethanol poured in the 1.2mL tube, dried with a small piece of tissue, and place it in the holder in the cryostat.
2. Place the glass slides that cover the brain area to be collected on the 'cold table'. Using a brain atlas, determine the location of the punches to be collected.
3. With the punch, collect all the tissue of one area. Then deposit the tissue in the tube (wipe it on the side, as far as possible into the tube).
4. Clean the punch properly with ethanol, wipe it, and place it back in the holder. Repeat steps 2-4 until finished.
5. After getting all samples, weigh each Eppendorf again.
6. When finished, place the Eppendorf's in the -70°C freezer.

Additional notes

- Punch as soon as possible after slicing, otherwise the slices will dry out.
- If the brain slices are too cold (they crack) or too warm (they melt) for punching, adjust the amount of dry ice in the extra metal box.
- Keep punched material frozen in a box filled with dry ice.

B) Protocol for slicing and punching PND1 and PND7

Materials

- Leica CM3050 cryostat, settings:
  - Object temperature: -10 °C
  - Chamber Temperature: -12 °C
  - Slicing thickness: 50 um & 200 um (TRIM function)
  - Angle of the blade: 2
- 70% ethanol and tissues
- Chuck and OCT/Tissue-Tek, Sakura
- Sharp blades; use a new part of the blade for every brain (2 brains per blade)
- Razor to cut front and back part of the head
- Tweezers and small brushes
- Plain, untreated microscope slides, 76x26 mm, Waldemar Knittel (Germany)
- Glass staining jar filled with RNAse away
- 2 metal slide-racks
- Styrofoam box with dry ice
- RNAse/DNAse free 2 mL Safe-lock tubes, Sarstedt (2mL Eppendorf's with round bottom)
- Permanent marker
- 2 mL tube filled with 70% ethanol
- 1.2 mm punching tool, Harris Uni-Core

- Extra metal box to fill with dry ice to use for punching

Notes about the cryostat:

- Always check temperature and thickness
    - Object temperature: -10 °C
    - Chamber temperature: -12 °C
    - 50 um for first slices, 200 um to TRIM
- Cleaning: 70% ethanol
- Quick frozen: ON
- Object frozen: ON

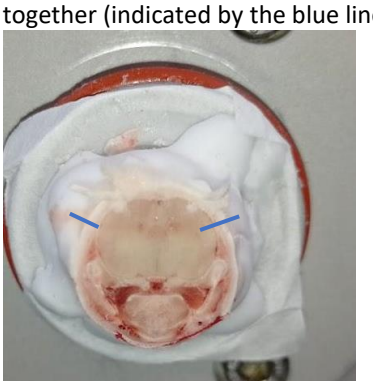
Preparation

1. Two days before slicing, transfer the brain(s) from the -70 freezer to the -20 freezer.
2. Before slicing, transfer the brain to the cryostat.
3. Put on a lab coat and clean gloves.
4. Clean the outside and everything on the inside of the cryostat, the punching tool, and brushes with 70% ethanol.
5. Shake the right number of 2 mL tubes out of the bag onto a clean tissue. Label the tubes by using the permanent marker with sample number, abbreviation of area name, and date. Put the tubes on dry ice into the Styrofoam box.
6. Weigh each tube three times. Before putting in the analysis scale, remove excessive dry ice in the tubes.
7. Put 12 glass slides in the jar with RNAse away. Remove them and put them on some tissues. Rub them dry and dust-free with clean tissues. Place them in a metal rack in the cryostat.
8. Put some tissues underneath the blade to easily remove the tissue that was not needed.
9. Put a layer of dry ice into the metal box and close it to use the upper part as a 'cold table'. Place the metal box into the left part of the cryostat.
10. Remove the front part of the head until the eyes and cut the back part at an angle by using the razor (indicated by the blue lines)

11. Put a generous amount of OCT on the chuck at room temperature, put the back of the head onto the chuck and let it cool down in the cryostat. The tweezers can be used to put the head in the right position (make it as straight as possible, with the head pointing a little bit downwards). When the OCT has hardened, apply a second layer around the head.
12. Fix the chuck in the cryostat, adjust it in the vertical and horizontal plane as needed. Make sure all the screws and knife are fastened tightly.
13. If the head is too close to the knife, cut part of the front of the head to make it fit.

Slicing

1. First slice at 50 um for several times until the plane has a little 'butterfly' shape. During the first slices, pay attention to how the olfactory bulbs appear. Adjust the horizontal plane if one bulb appears larger then the other.
2. Using the razor, remove the eyes from the head. This is done to avoid problems with sticking the brain onto the glass slide. Also make small incisions into the skin to avoid having the skin pull the slice together (indicated by the blue lines).



3. Turn on the TRIM setting on the cryostat to start making 200 um slices.
4. Slice at a steady, medium pace for the best quality. Adjust the anti-roll slip if necessary. In the beginning, the anti-roll slip should be more towards the head. When the brain becomes bigger, move the anti-roll slip away from the head. If the slice is cracking, move the anti-roll slip more away from the head or make more incisions in the skin.
5. If the slice is broken, try to carefully assemble it with the brushes.
6. Take a clean glass slide and warm up a spot with your finger at the back of the slide where the brain slice should stick. Slowly place the slide on top of the brain slice and apply just enough pressure to make it stick. Turn the slide around and warm it up a little bit more if necessary to make the slice, especially the brain part, stick properly.
7. Place the glass slide on the coolest part of the cryostat (quick freezer part).
8. Clean the knife holder, tissue, and anti-roll slip with the brushes.
9. Repeat steps 3-8 until two glass slides are filled (approximately 4 slices on 1 glass slide).

Punching

1. Place the two glass slides on the 'cold table'. Using the brain atlas, determine the location of the punches to be collected.
2. Punch all the tissue of one area from all the two slices and deposit the tissue in the right 2 mL tube from the Styrofoam box with dye ice. Wipe it on the side as far as possible into the tube.
3. Clean the punching tool properly by using the tube filled with 70% ethanol and some tissues and place it back into the cryostat.
4. Repeat steps 1-3 until all areas of the two slices are collected.
5. Repeat step 3-8 of the slicing and step 1-3 of the punching until all brain areas are collected.

Cleaning

1. When finished, clean the inside of the cryostat, the blade, the punching tool, the brushes, and the outside of the cryostat.
2. Weigh the Eppendorf's after collecting material by using the analysis scales.
3. Clean the chuck with warm water and some tissues.

Transfer the Eppendorf's to the -70 freezer.

C) RNA ISOLATION FOR SMALL SAMPLES, CLEANING OF BEADS, AND 1.2% GEL ELECTROPHORESIS

RNA Isolation Protocol Small Samples (max. 50 mg.)

Materials:

- Filter tips; 10, 20, 100, 300 & 1250 µL
- Micropipettes in range 0.2 µL to 1000 µL (P100, P100, P10)
- RNA/DNA-free tubes (1.5 mL)
- Dry ice & wet ice
- Cooling centrifuge
- Smaller centrifuge for short spinning
- Water bath, heat block, or incubator at 55°C
- Stainless steel homogenizing beads 5mm
- Tissuelyser

Reagents:

- RNase Away reagent (Thermo Scientific)
- TRIzol reagent (Ambion, Life Technologies)
- Chloroform (Sigma-Aldrich)
- Isopropanol, 99.5% (Acros Organics)
- UltraPure™ DNase/RNase-Free Distilled Water (Invitrogen, Life Technologies)
- 75% molecular grade ethanol: mix 37.5 mL 100% ethanol (Merkc, Boom Lab) with 12.5 mL UltraPure water
- GlycoBlue™ glycogen 1:10 dilution (1.5µg/µL) (Invitrogen, Thermo Fisher Scientific)

Notes:

- TRIzol can be kept in the chemical cabinet at room temperature for long term storage, when using often it can be kept in the 4°C fridge
- UltraPure™ DNase/RNase-Free Distilled Water should be found in the 4°C fridge
- GlycoBlue™ glycogen should be found in the -20°C freezer

Protocol

The day before:

- Storage TRIzol at 4°C
- Prepare clean stainless steel homogenizing beads
- Calculate the weight for all samples to be processed the next day
- Check glycoblue 1:10 dilution

Things to prepare before to proceed:

- Clean the hood as well as racks, pipettes, bottles with chloroform, ethanol, and isopropanol with RNAse away and locate them into the hood.
- Prepare the cooling centrifuge at 4°C.
- Prepare the water bath for incubation at 55°C.

Procedure:

1. Transfer samples from -80°C freezer directly onto dry ice to prevent melting. Then, transfer them to a clean rack after cleaning the outer part of the tube to prevent contamination.
2. Add 500µL TRIzol to sample and close tube immediately after.
3. Add one bead per tube. Place all tubes into the wells of the Tissuelyser blocks as evenly as possible and homogenize the sample using a Tissuelyser 2 times 2:00, 30 Hz. The 2nd time, swap the Tissuelyser blocks to make homogenization as evenly as possible.
4. Incubate for 5:00 at room temperature.
5. Spin sample at 12,000 x *g* and 4 °C, radius 9.5, for 10:00. Place tubes as evenly as possible into the centrifuge.
6. Transfer supernatant to a new 1.5 mL tube without disturbing the pellet.
7. Add 100µL Chloroform to the supernatant. Chloroform evaporates quickly, so keep the tube closed.

8. Shake samples vigorously by hand for 0:15.
9. Incubate at room temperature for 2:00-3:00.
10. Spin sample at 12,000 x *g* and 4 °C for 15:00.
11. Carefully, transfer 250µL of the upper aqueous phase to a new 1.5mL tube.
12. Add 6.7µL GlycoBlue to aqueous phase
13. Add 250µL 99.5% isopropanol to the aqueous phase.
14. Mix by inverting 10x and incubate at room temperature for 10:00.
15. Spin sample at 12,000 x *g* and 18 °C for 10:00.
16. Remove supernatant by decanting the tube. Prevent losing the pellet, this is your RNA isolated.
17. *Add 500µL 75% ethanol to the pellet.*
18. *Mix by inverting 10x.*
19. *Spin sample at 12,000 x g and 18 °C for 5:00.*
20. *Remove supernatant by decanting the tube. Prevent losing the pellet.*
21. *Spin the tubes shortly (i.e., 10sec) to collect traces of ethanol and remove ethanol by pipetting carefully.*
22. Repeat steps 17-21.
23. Air-dry pellet for max. 10:00.
24. Add 27µL UltraPure water.
25. Incubate at 55 °C for 15:00, then put on wet ice.
26. Determine RNA concentration and purity (260/280 and 260/230 ratios) of 2µL sample using Nanodrop 200. Remember to pipet with filtered tips.
27. Determine RNA quality by running 200ng RNA in 10µL H2O + 2µL Loading Dye on 1.2% agarose gel by electrophoresis at 90V.
28. Store samples at -80°C or proceed directly to reverse transcriptase.

Extra Notes:

- Once procedure finished, clean the hood, pipettes, and bottles with RNAse away, close the hood and switch the hood light off.
- Leave the dirty rack into the bath with hypochlorite.
- Collect the beads in a 50mL tube for cleaning (see protocol below).
- Dispose the wet ice into the sink after melting with hot water.

Cleaning and RNase removal of 5mm stainless steel homogenizing beads for RNA isolation

Materials:

- 500mL glass bottles
- Pipette balloon/pipetteboy
- 1000µL micropipette
- 200-1000µL tips, blue
- 5mL pipettes
- 50mL conical centrifuge tubes
- 90mm petridishes
- 70°C Incubator

Reagents:

- NaOH, 10M (100 mL);
- 0,1M NaOH (500mL); Add 5mL 10M NaOH to 495mL MilliQ H2O. Incubate 12hrs at 37°C and autoclave for 20min at 121°C.

Cleaning of beads after RNA Isolation:

1. Collect beads in a 50mL tube containing 25mL demi-H2O.
2. Vortex for several seconds and pour H2O is an appropriate waste container.
3. If tissue residues are present in the beads, wipe beads clean with Kim-wipe tissue.
4. Wash the beads overnight with 20ml 0.1M NaOH in MilliQ.
5. Pour out the solution, while preventing losing the beads.
6. Wash the beads 3x with MilliQ. Pour out solution after each wash step.
7. Transfer beads in a clean petridish and incubate beads at 70°C with the lid slightly opened. Beads can also be dried at room temperature, but it will take much longer.

8. Beads can be stored in the petridish or transferred to a bigger stock until next usage.

1.2% agarose gel electrophoresis

Materials:

- Small/medium gel holder
- Scales
- Micropipette (P10 & P2)
- Erlenmeyer
- Cilinder
- 0.2 mL 8 tube strip
- Centrifuge

Reagents:

- Agarose
- TAE (Tris base, acetic acid and EDTA) buffer
- SyberSafe
- Loading dye (4 °C fridge)
- DNA ruler (4 °C fridge)

Settings:

Size	max wells	Volume	75%	SyberSafe	Voltage	Run time
small	2 x 15	50 mL	37,5 mL	2,5 µL	90 V	30 min
medium	2 x 30	75 mL	56,25 mL	3,75 µL	90 V	25 min

Prepare samples:

1. Calculate needed amount of µL for 200 ng/20 ng of RNA. Pipette the calculated amount in a 2 mL 8 tube strip.
2. Calculate the amount of UltraPure water to add to the sample. The total volume should be 10 µL.
3. Add 2 µL of loading dye to each sample. Centrifuge the tubes shortly.

Prepare gel:

1. Fill a gel holder with TAE
2. Ready preferred size gel clamp with combs
3. Measure desired amount of agarose by using the scales in an Erlenmeyer
- 1.2% Small: 0,6 g agarose
- 1.2% Medium: 0.9 g agarose
4. Measure desired amount of TAE in a cylinder:
- 1.2 % Small: 50 mL TAE
- 1.2 % Medium: 75 mL TAE
5. Put 75% of the needed TAE into the Erlenmeyer with agarose
- 1.2 % Small: 37.5 mL TAE
- 1.2 % Medium: 56.25 mL TAE
6. Set scale to 0,00 (Tare)
7. Dissolve agarose completely by using microwave
8. Add desired amount of SyberSafe and mix:
- 1.2 % Small: 2.5 µL
- 1.2 % Medium: 3.75 µL
9. Add rest of TAE and mix

10. Pour agarose in the corner of the clamp to prevent bubbles. Use pipette tip to move bubbles to the side
11. Let the gel get solid

Loading of the samples:

12. Load 12 uL of loaded RNA mix in every well in the gel (start from the second well)
13. Add 2 uL of DNA ladder to the first well

D) Reverse transcription mRNA to cDNA

Reagents:

- **Oligo(dT)$_{18}$ primer** (Thermo Scientific, Life Technologies)
- **RevertAid H minus RT reverse transcriptase, including 5x reaction buffer** (Thermo Scientific, Life Technologies)
- **dNTP SET 10 mM each solutions** (Thermo Scientific, Life Technologies)
- **RiboLock RNase inhibitor** (Thermo Scientific, Life Technologies)
- **UltraPure ™ DNase/RNase-free distilled water** (Invitrogen, Life Technologies)

1. Clean table with RNase away and the pipettes with 70% ethanol
2. Take the Oligo (dT)$_{18}$, 5x reaction buffer, dNTP mix, and RiboLock RNase inhibitor at of the -20 freezer and put on ice.
3. Prepare on ice per RNA sample the following mix in a 0.2mL 8 tube strip:

Total RNA can be calculated the following way: 1000 divided by ng of RNA /µL from the nanodrop. UltraPure water should be added to make the whole reaction 13.5 µL.

★ First add UltraPure water, then the right amount of RNA, and lastly the Oligo (dT)$_{18}$

Reagent	**13,5 µL reaction**
Oligo(dT)$_{18}$ (100µM)	0.5µL
total RNA	1000ng
Ultrapure water	to 13.5µL

4. Shortly use the vortex to mix the samples.
5. Incubate at 65 ℃ for 5 min. in a thermocycler and transfer immediately to wet ice.

➔ Biorad: CDNAone & CDNAtwo

➔ Veriti: Gerrit - RTone & RTtwo

6. Incubate at least for 1 min. on ice
7. Add the following to each RNA/oligo (dT)$_{18}$ mix:

★ It is also possible to prepare a mix by calculating all reagents needed for the 6.5 uL reaction. Composition of the mix = volume of each reagents x (the number of samples + 1). After making the mix, add 6.5 uL to every sample.

➔ Spin every reagent tube shortly before preparing the mix

Reagent	**6,5 µL reaction**
5x Reaction buffer	4.0 µL
dNTP mix (10 mM each)	1.0 µL
RiboLock RNase inhibitor (40U/µL)	0.5 µL

RevertAid H minus RT (200U/µL)	1.0 µL

8. Mix reagents by gently vortexing and spin strips for several seconds
9. Run samples at the following program in a thermocycler:
10. 25 ℃ for 10 min (oligo annealing);
11. 45 ℃ for 60 min (reverse transcription);
12. 70 ℃ for 15 min (inactivation reverse transcriptase);
13. 4 ℃ forever
14. Dilute cDNA with Ultrapure water to 10 ng/ µL for the qPCR protocols. There is 1000 ng in 20 µL = 50 ng/µL. Therefore, sample needs to be diluted 5 times, which means adding 20 x 4 = 80 µL UltraPure water to the cDNA.
15. Use the cDNA directly as template for qPCR or store at -20 ℃

Extra calculations for samples which contain less than 100 ng/uL to make 10 ng/uL after converting to cDNA:

- Calculate total amount of RNA in 13 uL.
  - *70.4 ng/uL → 13 uL x 70.4 ng/uL = 915.2 ng in total*
- To make the cDNA, there is 20 uL in total. To calculate the amount of ng per uL, divide the total amount of RNA by 20.
  - *915.2 ng / 20 uL = 45.76 ng/uL*
- For the qPCR, the sample needs to have a concentration of 10 ng/uL. First, divide 10 by the concentration of RNA in the sample.
  - *10 / 45.76 = 0.218531…*
- Then divide 1 by the previous number. 1 part is RNA, the (number - 1) is part UltraPure water
- *1 / 0.218531… = 4.576.. → 4.576… - 1 = 3.576…*
- Multiply the previous number by 20 uL to get the amount of UltraPure water to add to the 20 uL of cDNA.
  - *3.576… x 20 = 71.52 uL of UltraPure water*

E) qPCR

Materials:

- Filter tips: 10, 20, 100, 300 & 1250 µL
- Micro pipettes in range 0.2 µL to 1000 µL
- Eppendorf tubes
- 96-well plate and plate seals
- Cooling centrifuge
- Wet ice

Reagents:

- Taqman Multiplex Mastermix (Applied Biosystems)
- Ultrapure DNase/RNase-free distilled water (Invitrogen, Life technologies)
- Forward primer (different per gene tested) 1:10 dilution 10 µM
- Reverse primer (different per gene tested) 1:10 dilution 10 µM
- Probe (different per gene tested) 1:10 dilution 10 µM
- 5 ng/ µL cDNA samples

1. Clean table and pipettes with 70% ethanol and DNase away
2. Make premix (per sample):

iTaq mastermix	10 µL
Ultrapure H_2O	2,5 µL
Forward primer	1 µL
Reverse primer	1 µL

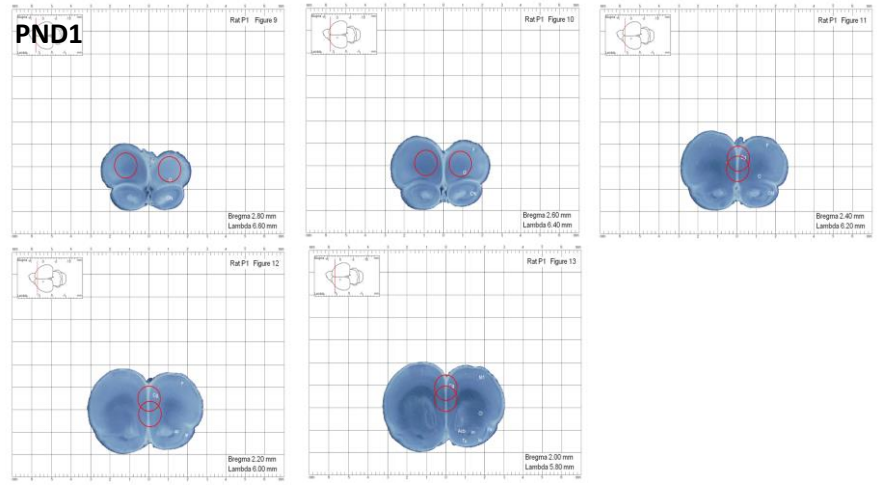
Probe	0,5 µL
Total	15 µL

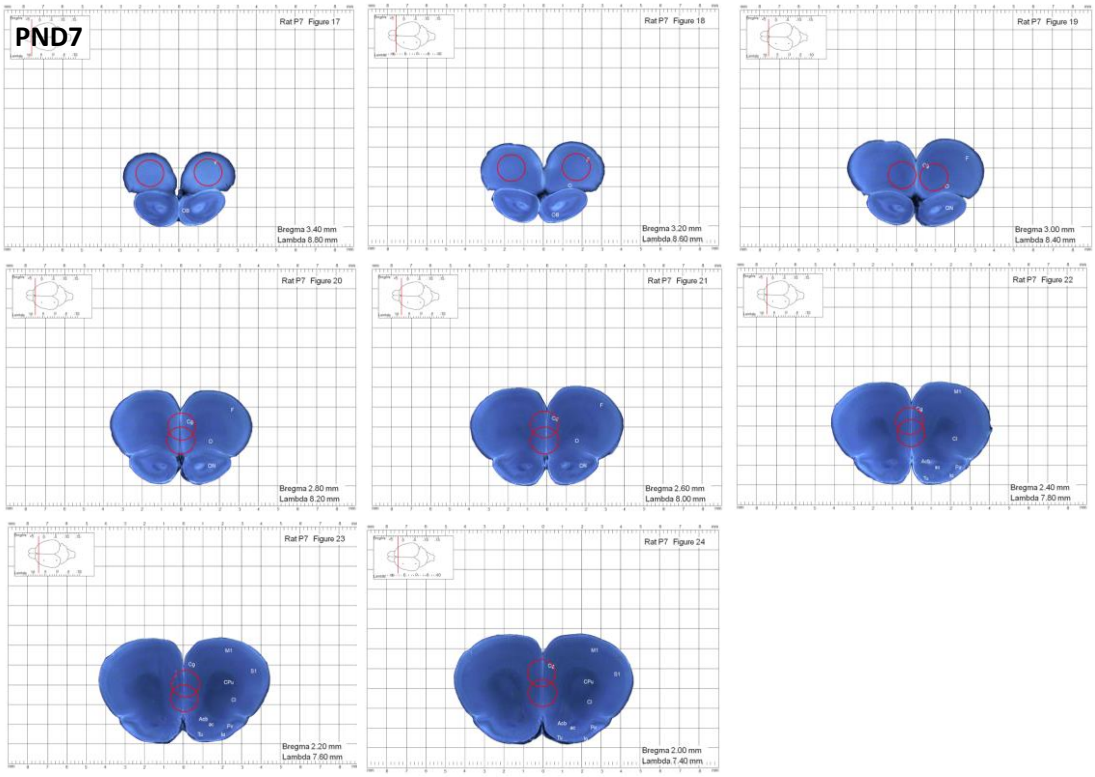
3. Pipette 15 µL of the premix into every well
4. Pipette 5 µL of cDNA (5 ng/µL) into every well
5. Seal the plate, and spin 1 minute at 1000g at 20°C
6. Run the following program on the qPCR machine (ABI 7500)

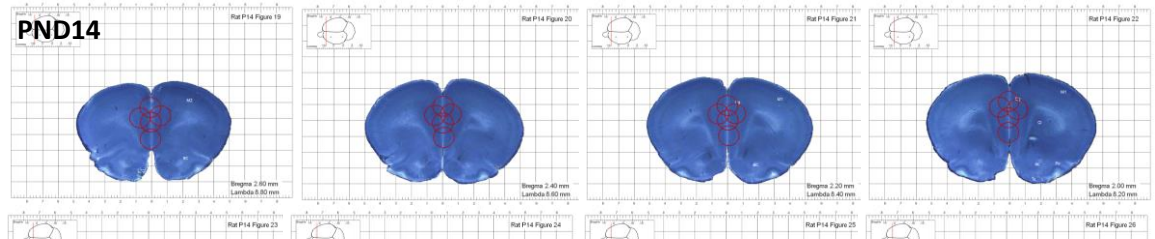
a) 3 min. at 95 °C
b) 40 cycles of 15 sec. at 95 °C and
c) 1 min. at 60 °C

3. Location of punches

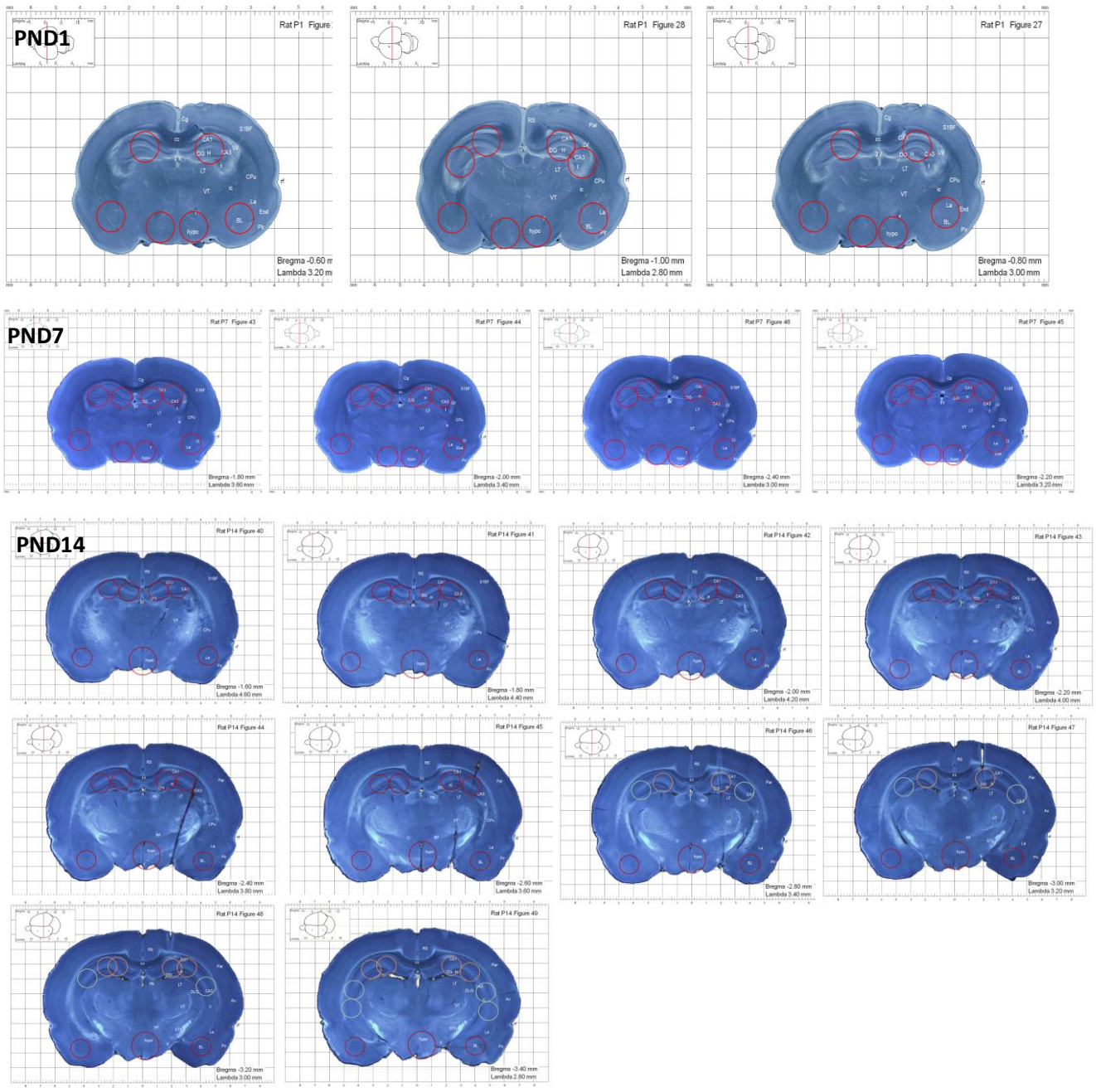
A) Location of punches of prefrontal cortex (PFC) of PND1, PND7, and PND14







B) Location of punches of basolateral amygdala (BLA) of PND1, PND7, PND14



***Appendix Figure 1**: Location of the punches for the collection of brain material, depicted in red circles: A) The PFC at PND1 (1), PND7 (2), and PND14 (3). B) The BLA at PND1 (1), PND7 (2), and PND14 (3). Pictures adapted from: Khazipov, R., Zaynutdinova, D., Ogievetsky, E., Valeeva, G., Mitrukhina, O., Manent, J.-B., & Represa, A. (2015). Atlas of the Postnatal Rat Brain in Stereotaxic Coordinates. Frontiers in Neuroanatomy, 9. https://doi.org/10.3389/fnana.2015.00161*