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Perinatal fluoxetine exposure and myelination:

are MBP and MAG protein levels altered?

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Abstract

Background

During the perinatal period, many women experience symptoms of depression and up to 8% of pregnant women use antidepressants. Selective serotonin reuptake inhibitors (SSRIs) are the most commonly used antidepressants. SSRI treatment during pregnancy is considered to be safe as it has no gross neuroteratogenic effects. Yet, recent studies show that perinatal SSRI exposure may have side-effects after all. Perinatal fluoxetine (FLX) exposure, a well-known SSRI, resulted in altered brain gene expression in juvenile rats in for instance myelination-related genes. Yet, expression of such genes was found to be upregulated in the prefrontal cortex (PFC) and downregulated in the basolateral amygdala (BLA) at postnatal day (PND) 21. Myelination occurs rostrally, meaning myelination of the BLA is finished earlier than that of the PFC. An explanation for the observed differences in RNA levels could be that the peak of the myelination rate, which is normally around PND 21, has shifted, with shifted RNA expression as a result. It is thus hypothesised that perinatal SSRI exposure accelerates myelination of the offspring.

Objective

This research aims to unravel the levels of myelination-related proteins in the BLA and the PFC as a consequence of perinatal FLX exposure, and compare these protein levels with the previously observed differences in RNA expression. Additionally, two time points will be taken into account to test whether at these timepoints myelination is accelerated.

Methods

Female rats were administered either FLX or a vehicle during pregnancy and lactation. The brains of their male offspring were collected at PND 21 or PND 35. Upon these brains, fluorescent immunohistochemistry was performed. Proteins of interest were myelin basic protein (MBP) and myelin-associated glycoprotein (MAG). Regions of interest were the BLA and PFC. For MAG, the number of positive cells were counted in FIJI. For MBP, the percentage of the area stained positively was measured using the thresholding function of FIJI.

Results

No significant differences due to perinatal exposure to FLX were found for MAG and MBP at both timepoints in the PFC and BLA. In the FLX exposed animals as well as the control animals, the number of MAG positive cells decreased significantly over time. For MBP, no significant differences between PND 21 and PND35 were observed.

Discussion and conclusion

FLX did not alter the protein levels of MBP and MAG at PND 21 and PND 35. This could suggest the presence of a posttranscriptional process that compensates for the previously observed changes in RNA expression. The decrease of MAG positive cells at PND 35 compared to PND 21 might be due to its periaxonal location. Limitations of this study include the type of microscopy used, resulting in less reliable data for MBP. Ultimately, these results suggest that myelination is not accelerated between PND 21 and PND 35, but additional research is needed to further elucidate the effects of perinatal SSRI exposure on the levels of myelination-related proteins.

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1. Introduction

1.1 Depression and pregnancy

Major Depressive Disorder (MDD) is one of the most prevalent mental disorders with an estimated 280 million people worldwide suffering from this disease (World Health Organization, 2022). The main criteria for being diagnosed with MDD are either having a depressed mood or anhedonia for at least two consecutive weeks. In addition to this, at least four of several other symptoms should be present such as insomnia, recurrent thoughts of death or feelings of worthlessness (American Psychiatric Association, 2022).

Approximately 20% of women experience symptoms of depression during and/or after pregnancy (Marcus et al., 2003). When these symptoms progress into MDD, we speak of perinatal depression, which can be subdivided into two categories. Depression during pregnancy is called prenatal depression and depression up until one year after pregnancy is called postnatal depression (American Psychiatric Association, 2022; Gaynes et al., 2005). Globally, 10.9% of all women develop depression during the perinatal period (Woody et al., 2017). There are many factors contributing to the risk of developing perinatal depression, including but not limited to having a low income, a lack of social support or having previous negative life experiences (Lancaster et al., 2010).

Perinatal depression is not only a burden for the mother, it also has negative consequences for the unborn child. Correlations have been found between the exposure to maternal depression during the perinatal period and higher anxiety levels of the offspring (Davis & Sandman, 2012; Kingston et al. 2018). Furthermore, it has been found that children born from depressive mothers are at a higher risk of developing depression themselves later in life (Pawlby et al. 2008; Pearson et al., 2013).

1.2 Antidepressant treatment and pregnancy

Because of the high burden on both the mother and child, depression should not be left untreated. Therapy is the first approach, while more severe cases may require additional treatment through antidepressant medication. The most common type of antidepressants is Selective Serotonin Reuptake Inhibitors (SSRIs) with a little over 50% of all prescribed antidepressants belonging to this category (Bogowicz et al., 2021; Verhaak et al., 2019). Many patients suffer from reduced brain serotonin (5-HT) levels. SSRIs increase 5-HT levels by blocking the transporter responsible for 5-HT reuptake by the presynaptic neurons (Commons & Linnros, 2019). However, it must be mentioned that as research uncovers more about the causes of depression, it becomes evident that it is a complex mental disorder in which many other different factors besides 5-HT are involved. Regardless, SSRIs are still included in the first-line treatment of depression.

In the United States, approximately 8% of pregnant women are prescribed an SSRI (Andrade et al., 2008). A benefit for the mother is that in addition to this, continuing with the SSRI prescription

during pregnancy lowers the chances of relapsing into depression (Cohen et al., 2006). However, with so many pregnant women having an SSRI prescription, it is of utmost importance that perinatal SSRI usage is safe for the unborn child. Overall, SSRI exposure during the perinatal period does not seem to have any gross neuroteratogenic effects, such as cardiac birth defects, on the child (Furu et al., 2015).

Recent studies, however, start to question this view. Reason for this is the finding that SSRIs are able to cross the placenta. Research by Heikkinen et al. (2003) has for instance found that fluoxetine (FLX), which is one of the main types of SSRIs, as well as its active metabolite norfluoxetine (NFLX) crosses the placenta. At the time of childbirth, concentrations of FLX and NFLX in the infant plasma were 65% and 72% of the maternal plasma levels. Albeit at a much lower level of 3.8%, FLX and NFLX have also been found to be present in breast milk even after 2 months post-delivery.

Whilst during adulthood 5-HT acts as a neurotransmitter, it acts as a neurotrophic factor early in life. A few of the many neuronal developmental processes 5-HT is involved with is corticogenesis; it aids in neuronal migration and positioning (Riccio et al., 2011). Further, 5-HT contributes to processes including dendrite formation, neuronal connectivity and synaptic formation (Hanswijk et al., 2020; Vitalis et al., 2007). With the SSRIs in the infant's blood circulation, these processes could possibly be altered.

1.3 The influence of perinatal SSRI exposure

Taking SSRIs during pregnancy has been associated with poorer pregnancy outcomes, such as lower birth weight and a higher chance of preterm birth (Eke et al., 2016; Oberlander et al., 2006). When looking at the effects of SSRIs on the children, however, findings are conflicting, for example concerning anxiety (Hanley et al., 2015; Hermansen et al., 2016; Misri et al., 2006; Oberlander et al., 2007) or when considering correlations with mental disorders such as autism spectrum disorders (ASD) or attention-deficit/hyperactivity disorder (ADHD) (Clements et al., 2015; Malm et al., 2016; Man et al., 2015).

These discrepancies might partly arise due to how difficult it is in human studies to disentangle the effects of SSRIs from the effects of maternal depression. To resolve this issue, animal models can be used. One systematic review evaluated studies using either mice- or rat models, analysing the effects that SSRIs had on the behaviour of the rodents. This review found that overall, SSRI exposure in the perinatal period reduced activity and exploration behaviour, as well as caused more passive stress coping mechanisms in the animals (Ramsteijn et al., 2020). Furthermore, the same systematic review found that perinatal SSRI exposure significantly caused sensory processing to occur less efficiently in the animals (Ramsteijn et al., 2020). Another recent study found social behaviour to be altered in rats as a result of perinatal SSRI exposure. That is, five week old rats showed a reduced amount of social play behaviour, a finding more pronounced in male than in female offspring (Houwing et al., 2019).

Consequences of perinatal SSRI exposure can further be observed on the molecular level, such as in the lower hippocampal BDNF levels or in the alterations of the organisation of the barrel cortex, a region of the somatosensory cortex of rodents (Boulle et al., 2016; Xu et al., 2004). This latter finding supports the hypothesis that perinatal SSRI exposure results in altered sensory perception and processing (Van Der Knaap et al., 2021).

1.4 A possible influence of SSRIs on myelination?

Another process which is suggested to be altered as a result of SSRI exposure is myelination; the production of an insulated sheath around axons to facilitate saltatory neuronal signal transduction (Martinsen & Kursula, 2021). In humans, the development of myelin sheaths starts during gestation, around 5 months after conception. Myelination continues after birth, rapidly during the first two to three years of life and at a slower pace during young adulthood (Lebel & Deoni, 2018; Morell, 1999). In rats, myelination occurs mainly after birth, with the initiation of myelin formation around postnatal day (PND) 10 and the highest rate of the process being around PND 20. Comparable to humans, the rate of the myelination process continues at a slower pace into adulthood. In both species, myelination of the brain is initiated in the spinal cord and proceeds rostrally (Downes & Mullins, 2014). Because the myelination process of humans is easily translatable to that of rats, these animals are an adequate animal model for studying possible changes due to perinatal SSRI exposure (as reviewed by Houwing, 2020).

A study of Simpson et al. (2011) found callosal connectivity to be altered in response to perinatal exposure of the SSRI citalopram. Axons of rats perinatally exposed to citalopram were more often either hyper- or hypo-myelinated. The cells responsible for myelination of the CNS, oligodendrocytes (OL), showed more malformations, a finding which is reaffirmed by a follow up study by Fan et al. (2015), who additionally showed an increased rate of cell death of precursor OLs.

Not only does the corpus callosum seem to be altered, the hippocampus is likely to be affected as well. In the study of Kroeze et al. (2015), female rats were neonatally (PND 1 - PND 21) exposed to FLX via the milk of the dams. In the long term, when the rats were 28 weeks old, the myelin related genes transferrin and ciliary neurotrophic factor were found to be downregulated in the hippocampus.

Further evidence that perinatal SSRI exposure alters gene expression comes from a previous study by Ramsteijn et al. (2022) investigating a.o. the myelin related genes myelin basic protein (MBP) and myelin associated glycoprotein (MAG). MBP is one of the most abundant myelin proteins and is necessary for myelin production in the CNS. This protein ensures that the multiple layers of the myelin sheath are held close together, resulting in myelin being tightly packed around the axon (Martinsen & Kursula, 2021). MAG is a transmembrane protein located on the cell membrane of oligodendrocytes and periaxonal on the target axon. It stabilises contact between the oligodendrocyte and the neuronal axons, as well as maintains the axons. Consequently, MAG aids in the attachment of myelin to the axon (Quarles, 2007). In the study of Ramsteijn et al. (2022), it is found that perinatal exposure to FLX,

independently of maternal depression, increases RNA levels of MBP and MAG in the prefrontal cortex (PFC) of 21 day old male pups and simultaneously decreases RNA levels of these genes in the basolateral amygdala (BLA). From this, and the fact that myelination occurs rostrally, it is hypothesised that these changes in RNA expressions are because fluoxetine accelerates the myelination processes. This results in the peak rate of myelination occurring earlier during development (the theorised process is visualised in Figure 1).

1.5 Research question and hypotheses

A limiting factor of the study of Ramsteijn et al. (2022) is that only RNA levels were investigated. Consequently, it remains unknown whether aforementioned changes are also reflected in the protein levels. Moreover, only one time point was taken into consideration in this study. In order to strengthen the acceleration hypothesis, more time points ought to be taken into account. In the scope of the present study, we aimed to resolve these two limitations, with as main research question: How does perinatal FLX exposure influence the protein levels of the myelination genes MAG and MBP? To answer this question, we studied the brain areas PFC and BLA in male rat pups at PND 21, similar as in the study by Ramsteijn, and at PND 35, when play behaviour is known to be altered by perinatal fluoxetine exposure (Houwing et al., 2019). It was hypothesised that the changes of protein levels are in line with the previously described changes in RNA levels. That is, MAG and MBP levels are expected to be lower in both brain areas in the control group, compared to the group perinatally exposed to FLX. Since myelination is largely finished by PND 35, no significant differences were expected between the control- and FLX-exposed animals at that point in time.

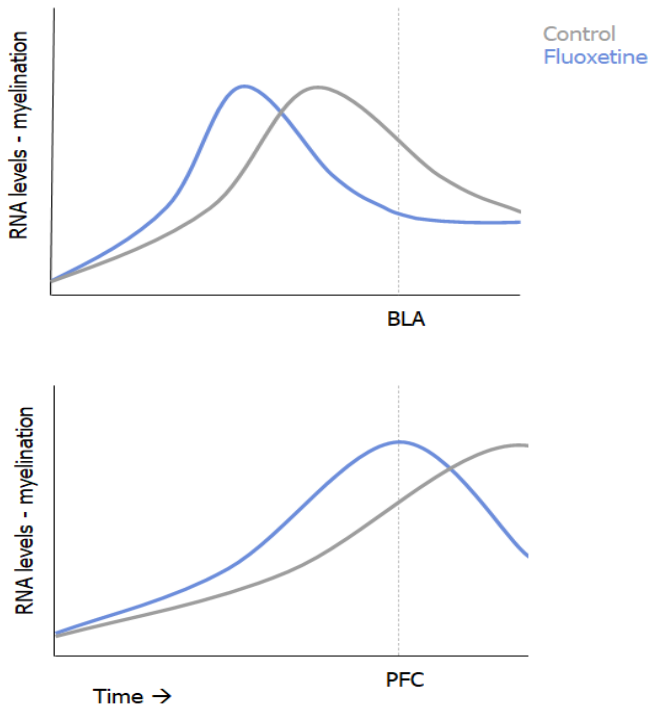


Figure 1: Theoretical visualisation of accelerated myelination. Lines represent the process of myelination. The difference in RNA levels at PND 21 (the dashed line) results from the accelerated myelination, with the peak rate of myelination shifted to the left, earlier in time.

2. Materials & methods

2.1 Animals

Animals were housed, euthanized, intracardially perfused and consequent brain collection was performed at the Arctic University of Tromsø under the supervision of Eelke Snoeren. The animals used in this study were Wistar rats. Dams were treated orally either with 10 mg/kg fluoxetine or with a 1%methylcellulose solution (control) once a day during the entirety of the pregnancy up until postnatal day 21. When their pups were either 21 days old or 35 days old, perfusion of the male offspring took place. This results in the following four groups, with corresponding sample sizes (Table 1).

Table 1: Overview of experimental groups

Age	FLX	Control
PND 21	6	7
PND 35	8	6

The brains were collected and stored in a sucrose solution (30% in Phosphate Buffered Saline). Consequently, they were shipped to the Olivier lab and stored at 4 °C for approximately 8 months.

2.2 Tissue sectioning

After storage, the final end of the spinal cord was removed from the brains and they were frozen using liquid nitrogen. The brains were stored at -70 °C, ranging from 16 days up until 38 days. In further preparation for the cutting procedure, brains were placed into -20 °C freezers, for times ranging from 18 hours till slightly more than 72 hours. The machine used to slice the brains was a sliding microtome (MICROM International GmbH, Walldorf, Germany) type HM 450. Brains were sliced into 40 µm thick coronal sections and collected from around bregma 4.20 until around bregma -8 (according to the atlas of Khazipov et al., 2015). The slices were stored in tubes containing 0.01 M PBS + 0.1% sodium azide (pH = 7.4) at 4 °C. Cutting occurred in series and one sixth of each brain was assigned for immunohistochemistry.

2.3 Immunohistochemistry

Brain sections were washed 3 times for a duration of 5 minutes with 0.01 M PBS, after which they were submerged in a blocking solution (0.01 M PBS, 5% BSA, 0.2% Triton X-100) for two hours at room temperature. Subsequently, sections were incubated with anti-MAG (Abcam ab277524, 1:10.000) and anti-MBP (Biolegend #808401, 1:500) primary antibodies overnight at 4 °C. Then, the

sections were washed 3 times with 0.01 M PBS and were consequently incubated for two hours with the secondary antibodies (donkey anti-mouse, Invitrogen, Alexa fluor 488, 1:500 and goat anti-rabbit, Invitrogen, Alexa fluor 555, 1:500) at room temperature. Sections were mounted using ProLong Gold Antifade with DAPI (ThermoFisher, P36931). For the full protocol, see Supplementary information 1.

2.4 Microscopy and image analysis

The microscope used is a Tissuefaxs, model Zeiss AxioObserver Z1. For a detailed protocol, see Supplementary information 2. Pictures were acquired with a magnification of 10x, using Cy3 and GFP filters. The obtained pictures spanned from bregma 2.8 till 1.8 (medial PFC) and -1.6 till -3 (BLA) for the PND 21 brains (according to the atlas of Khazipov et al., 2015). For the PND 35 brains, pictures spanning from bregma 3.72 till 2.76 (medial PFC) and bregma -1.92 till -3.24 (BLA) were made (according to the atlas of Sherwood and Timiras, 1970). The resulting images were analysed using the computer programme FIJI, 2.9.0/1.53t, running with Java 1.8.0_322 (64 bit version).

The MAG protein was analysed by manually counting MAG positive cells. Initially, a threshold was established through visual assessment to determine which cells should indeed be considered as cells and which should not. The threshold was adjusted accordingly. The cell counter plugin was used for the cell-counting. Additionally, the area of the regions of interest were measured, resulting ultimately in the measure of the number of MAG positive cells per mm2. For the BLA, four to six regions were measured including either both the anterior BLA and the posterior BLA or only the anterior BLA. The average of these six regions was taken. For the exact location, see an example in Supplementary information 3. Four to six measurements of the medial prefrontal cortex were taken and averaged. Since the mPFC consists of three areas, that is, the cingulate cortex (CG), the prelimbic cortex (PL) and the infralimbic cortex (IL), these areas were separately measured, as well as totalled into one measurement for the PFC. For an example of the regions of interest, see Supplementary information 3.

A visual assessment was used to determine a threshold for the MBP analysis. This was founded upon the fibre-like pattern that arises as a consequence of MBP staining. These fibres ought to be visible in the resulting thresholded image as well. After setting the threshold, the percentage of area that was positive for the MBP staining was measured. For both the PFC and BLA, four till six regions of interest were chosen, measured and averaged. As mentioned before, the three areas of the PFC will be measured separately, as well as totalled. Due to the quality of the pictures taken, for a few animals only two or three regions of interest were measured either for the PFC or the BLA.

2.5 Statistics

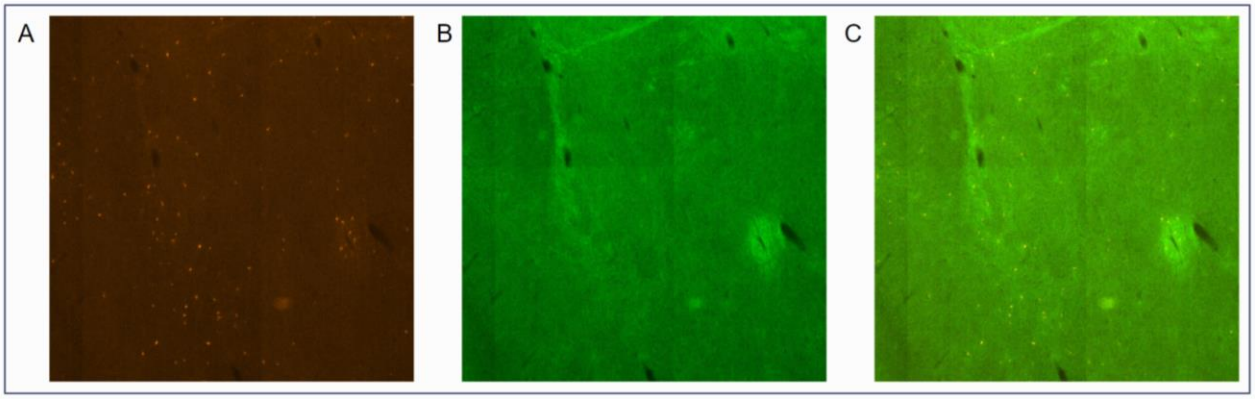
The collected data from imageJ was statistically analysed using R (version 4.2.2) and R-studio (version 2023.06.0-421). The data was checked for normality and equality of variances by using the Shapiro-Wilk's and Bartlett's tests. When $p > 0.05$, normality and equality of variances was assumed.

When these requirements were not met, the data was transformed. With a two-way ANOVA (Analysis of Variances), measures for MBP and MAG were analysed in respect to treatment group and age. The independent variables were age and treatment. The dependent variable was for MAG the number of positive cells and for MBP the % of area positively stained. Post-hoc Tukey tests were performed when the ANOVA showed significant results. $p < 0.05$ was considered to be significant.

3. Results

Immunohistochemical staining was performed to assess the protein levels of MAG and MBP in the BLA and the PFC. The immunohistochemical protocol resulted in successful targeting of both proteins, an example of the consequent obtained pictures can be seen in Figure 2. In the following two sections, the results of the MAG and MBP will be shown.

Figure 2: Immunohistochemical staining of the BLA. 10x magnification, Tissuefaxs. A) MAG, cy3 filter. B) MBP, GFP filter. C) Merged image of A and B.



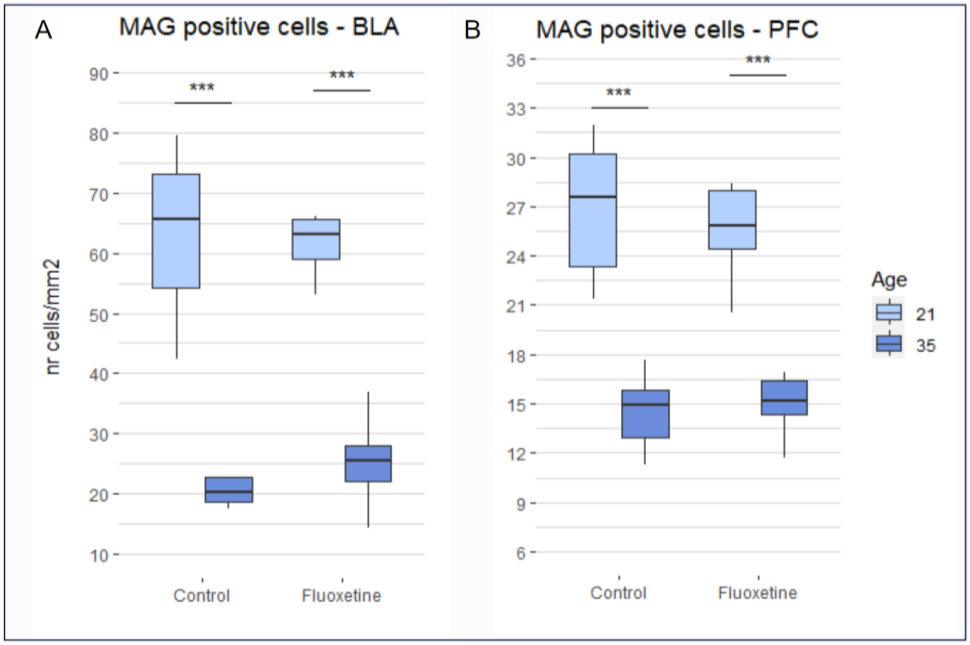
3.1 MAG data

To test the hypothesis that the number of cells positive for MAG change due to FLX exposure and slightly increase as a result of age, a two way ANOVA and post-hoc Tukey's test were performed for the BLA (Figure 3A) and the PFC (Figure 3B). For the BLA data, the assumptions of a normal distribution and an equality of variances were met. Tukey's test for the BLA revealed significant differences between PND 21 and PND 35 for both the control group and the FLX group ($p < .0001$ for both). No significant differences were found between the control and FLX groups at PND 21 as well as at PND 35 (p 0.9936 and 0.9727 respectively).

For the PFC data, the assumption of equality of variances was not met, with a p value of 0.0473 in the Bartlett's test. After a squared root transformation, Bartlett's test allowed for the assumption of equality of variances. Consequent two-way ANOVA and Tukey's test revealed a similar pattern in significance as in the BLA. The number of MAG positive cells was both in the FLX group as well as

the control group significantly lower at PND 35 compared to PND 21, with respective p-values of 0.0001 and < 0.0001. When comparing MAG positive cells of the control group with those of the FLX group, neither at PND 21 nor at PND 35 significant differences were observed (p values of 0.8302 and 0.9563). All three areas of the PFC (the CG, PL and IL) showed the same pattern regarding the significance between the four groups. Graphs for these areas can be found in Supplementary Information 4.

Figure 3: Number of MAG positive cells as a result of treatment and age. Quantity of MAG positive cells in the BLA (A) and the PFC (B). For both A and B, the number of animals used from left to right as depicted in the plot: n=7, n=6, n=6, n=8. *** p < 0.001.

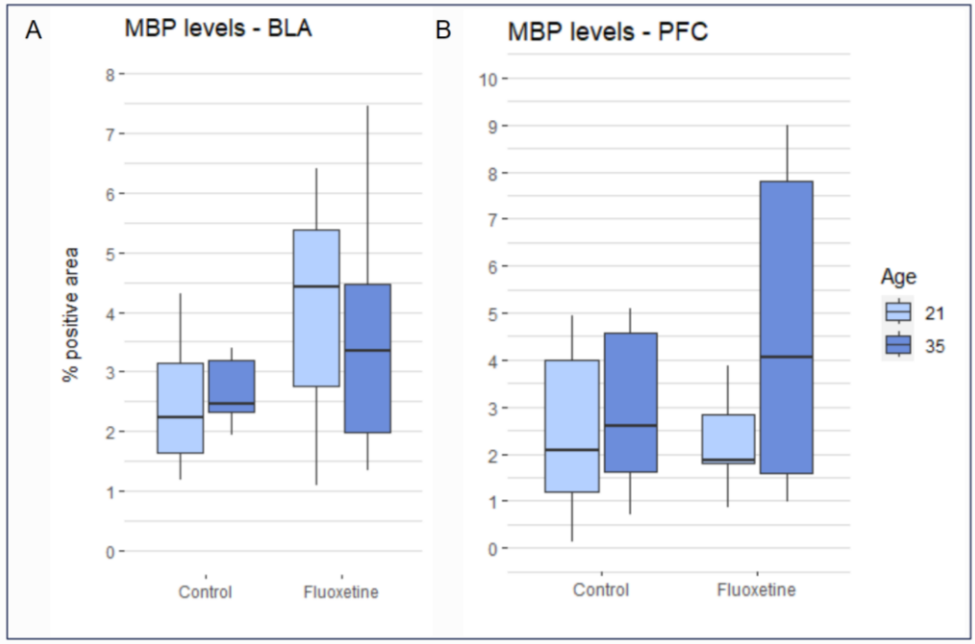


3.2 MBP data

As mentioned in the methods section, MBP levels were assessed by measuring the percentage of the area positively stained. The statistical tests used for this analyses were a two-way ANOVA and subsequent Tukey's test. For the BLA (Figure 4A), data was non-normally distributed. A square root transformation was performed upon which the Shapiro-Wilk's test indicated that normality could be assumed (p = 0.6716). The following two-way ANOVA did not reveal any significant differences due to the treatments (p = 0.4708) or the two age groups (p = 0.6462).

A similar lack of significant differences is found in the PFC (Figure 4B). Normality and equality of variances could be assumed without transformation of the data. The two-way ANOVA revealed again no effects of the treatment (p = 0.73155) and the age (p = 0.09578). When looking at the three areas of the PFC separately, no significant differences between any of the groups was found for all three areas (Supplementary information 6).

Figure 4: Percentage of target area stained positively for MBP as a result of treatment and age. Percentage of area positive for MBP in the BLA (A) and the PFC (B). For both A and B, the number of animals used from left to right as depicted in the plot: n=7, n=6, n=6, n=8. No significant differences between any of the groups.



4. Discussion

The aim of this study was to investigate whether levels of the proteins MBP and MAG were altered after perinatal exposure to fluoxetine and whether these possible changes were in line with previous RNA data of these proteins. The approach was to study protein levels in the BLA and PFC of male rats aged 21 PND and 35 PND. For MBP and MAG, no differences were observed between the control and FLX animals. When looking at the protein levels between the different ages, no significant differences were observed for MBP. The results do indicate that the number of cells expressing MAG decreased from PND 21 to PND 35. In the next few paragraphs, these main findings will be discussed.

4.1 No effects of FLX on MAG and MBP

For both the BLA and the PFC, perinatal FLX exposure does not seem to influence MAG and MBP levels. Previous studies have focussed on the RNA levels of these proteins as a consequence of perinatal FLX exposure. Whilst they investigated a different brain area (namely, the hippocampus), one study did not find any significant difference for MAG in adult rats (Kroeze et al., 2015). Present results of protein levels are in line with this, as MAG levels are similar between the control and FLX group. On the other hand, another study found a significant difference in MAG RNA levels for the BLA and the PFC. Additionally, a strong trend for altered MBP RNA levels due to perinatal FLX treatment was

observed in the BLA and the PFC Ramsteijn et al., 2020). These observed differences do not seem to translate to a difference in protein levels, when taking the data of our study into account. Generally, it is known that protein levels do not necessarily correlate with mRNA levels (Liu et al., 2016). There are other mechanisms that contribute to establishing the protein expression, in addition to RNA expression. It might be the case that such mechanisms also compensate for the lack of significant differences in protein levels between the treatment groups here.

Campagnoni et al. (1990) reviewed multiple of these other, posttranscriptional mechanisms regarding MBP. For instance, the structure of the mRNA of MBP influences the translation speed. At least five isoforms of MBP are known as a result of alternative splicing (Newman et al., 1987). Each of these isoforms have a slightly different structure and as a consequence also different translation efficiencies, as described in detail by Campagnoni et al. (1987). In the context of accelerated myelination due to perinatal FLX exposure, it is speculated in our study that as a response to altered RNA levels, different isoforms are more produced than others. Via this, MBP protein levels could be regulated. For MAG, less is known regarding the translation speed and mRNA structure. However, we do know that MAG has two isoforms (Pedraza et al., 1991). These two isoforms differ mainly in their C-termini (Erb et al., 2003), differences in which are known to influence translation efficiency (Weber et al., 2020). It is therefore not unlikely that the different MAG isoforms could potentially influence the final protein levels.

A second post transcriptional mechanism that might influence final protein levels is translocation. MBP protein production takes place in free ribosomes. These free ribosomes are either located in the oligodendrocytic cell body or at the site where myelination actively occurs. MBP protein synthesis occurs more rapidly when produced by the ribosomes located near the active site (Campagnoni et al., 1991). It might be possible that when RNA levels are higher due to the acceleration caused by FLX, MBP production might switch to taking place in the cell body. Yet, as of the time of writing, this has not been investigated.

The aforementioned information remains relatively unknown and further research is needed to investigate whether these suggestions hold truth. Nevertheless, there could be cellular mechanisms in the oligodendrocyte such as the two described here, which compensate for the previously observed RNA differences due to perinatal FLX exposure, leading to a lack of significant differences in corresponding protein levels.

4.2 Decreased MAG positive cells from PND 21 to PND 35

As observed, the levels of MAG decrease from PND 21 to PND 35 for both the BLA and the PFC. This finding is in line with the previous research of Baba et al. (1987). They found MAG protein levels to be highest at PND 18 and already decreasing at PND 21, which indicated a degradation of MAG proteins. At a first glance, however, these findings seem to be unexpected. MAG is located

periaxonal where it facilitates glial-axon interactions and maintains myelin integrity (Trapp, 1990). Loss of MAG, such as in knock-out models for multiple sclerosis, does not hinder myelin formation, but in the long term does lead to degradation of the formed myelin sheaths (Fruttiger et al., 1995). Therefore, the degradation of MAG observed in this study seems to be counterintuitive. The exact localisation of MAG sheds a different light on the present findings. That is, MAG is not located in all myelin sheaths of the CNS, but only in non-compact myelin sheaths (Bartsch et al., 1989). It is hypothesised that because myelin formation has not yet completed, relatively large quantities of myelin are non-compact and that during development, more myelin becomes compacted (Baba et al., 1987). This could explain the decreased MAG levels from PND 21 to PND 35 as observed in this study.

From the previous paragraph, a limitation of this study becomes apparent that might also contribute to the explanation as to why we observe decreased MAG levels. In this study, we performed cell counting of MAG positive cells. Consequently, we did not look at the quantity of expressed MAG proteins. More importantly, the periaxonal localised MAG has not been measured, making it impossible to draw conclusions regarding the exact amount of expressed MAG. It can only be said that there are fewer oligodendrocytes expressing MAG at PND 35 than at PND 21. Considering that the peak rate of myelin development lies around PND 21, most of the oligodendrocytes have already produced MAG and incorporated it into the sheaths surrounding the axons, which we were unable to include in our protein level measurements.

4.3 No differences in MBP between PND 21 and PND 35

Another unexpected finding was the lack of increase of MBP levels from PND 21 to PND 35. From previous research it has become apparent that MBP levels increase during development, up until myelination is finished (Delassalle et al., 1981; Han et al., 2022; Matthieu en Amiguet, 1990; Zeller et al., 1985). This ought not to come as a surprise, since MBP exerts its function of ensuring myelin is tightly packed by being situated in between the multiple layers of the myelin sheath and thus is a constituent of the myelin sheath. Consequently, at time points later during the development, more myelination has taken place and as a result there should be more MBP.

Yet, the present findings are in contradiction to this. The reason being the main limitation of this study, namely the microscopic techniques used. Due to technical issues, we had to switch from the microscope initially meant for this study (Leica 4000B), to the currently used Tissuefaxs. An advantage of this microscope is that it produces a complete overview of the brain slice, indicating where at a certain bregma the target protein can be found. It does so by generating 10x magnified pictures of brain areas and by mending these individual photos together to create one overview of the complete brain slice. Between these separate photos, lightning conditions differ. As a consequence, some parts of the brain slice were better lit than others and thresholding was very difficult. For instance, the fibres were nicely

visible in one individual picture, but were not visible in the adjacent photo, because the lighting was very dark.

Further, when looking at the brain slice picture as a whole, some parts were clearer than others. This occurred because the primary focus channel was Cy3, for MAG. Sometimes, a second focus channel for MBP could not be set, for then the quality of the MAG pictures would be diminished. Because of the resulting unclarity, the MBP fibres were not visible and after thresholding it would appear that there was no MBP present at all.

For the MAG stainings, these two limitations were no issue, as the cells were still clearly visible and cell counting was not impaired by the thresholding beforehand. On the other hand, the microscopic technique used renders the resulting data unreliable. It is known that around PND 21 around 60% of the brain oligodendrocytes are positive for MBP (Zeller et al., 1985), meaning that a lot of myelination occurs and that consequently a relatively large area should be positive for MBP staining. However, when performing the data analysis, sometimes the observed brain areas were < 1% positive for MBP. Since this is lower than is to be expected at these timepoints, it is likely that the MBP data do not resemble the true protein levels.

An alternative explanation is that the protocol for MBP staining was suboptimal, resulting in a lower quality of the pictures. However, prior to executing the experiment, a pilot has been conducted to establish the optimal concentration of the primary antibodies. Data analysis for this pilot occurred with a different microscope, a Leica 4000B. Pictures were produced with this microscope that did not have the same disadvantages as those produced with the Tissuefaxs (Supplementary Information 6). Further, additional examination of the slides produced in the final experiment were also analysed with a confocal microscope (Leica SP8). From this, it also became apparent that our protocol produced nice pictures of MBP, see Supplementary Information 6.

Thus, due to the disadvantages of the used microscope, not the immunohistochemical protocol, consequent data analysis seems to be unreliable. It might therefore be premature to conclude that MBP levels do not increase after PND 21 in the BLA and PFC.

4.4 Implications and future research

Despite the limitations regarding the microscopic methods used, the importance of our findings should not be overlooked. Whilst several studies have focussed on myelination-related gene expression in the context of perinatal SSRI exposure, it still remains unknown whether these observed changes are also translatable to the protein levels. The present study aimed to get more insight into this knowledge gap since ultimately, possible altered myelination processes should be reflected by the levels of the involved proteins.

In order to elucidate how protein levels are affected, the limitations of this study should be resolved first. That is, it is suggested to investigate MBP levels with a different type of microscope.

Further, more insight can be gained by expanding the target proteins to other myelin proteins such as Cnp and Cldn11, which also have been shown to have altered RNA levels in response to perinatal FLX exposure (Ramsteijn et al., 2022). In addition to this, more brain areas can be taken into account. Moreover, the present study focuses merely on the male brain. To gain knowledge as to how women are affected by perinatal SSRI exposure, future studies should also include females. Lastly, this study has only looked at PND 21 and 35, whilst MAG and MBP expression occurs early during development, even before onset of myelination. In fact, MAG expression occurs even earlier than MBP expression (Keita et al., 2002). Therefore, the protein levels should also be investigated before PND 21, a suggestion that is further supported by the statement of a previous study that MAG protein levels reach their peak at PND 18 in the brainstem (Baba et al., 1987).

If future studies have broadened the scope by implementing aforementioned suggestions, and if it becomes apparent that perinatal SSRI exposure does not accelerate myelination on the protein level, it is worth looking further into which compensatory mechanisms are activated. For example, it might be worth looking further into the two processes discussed previously, that is, translocation and mRNA structure.

If, on the other hand, myelination indeed is accelerated, the question arises whether accelerated myelination is sufficient for producing the observed behavioural differences when being perinatally exposed to SSRIs. For this, behaviour should be observed without the context of perinatal SSRI exposure, but with the accelerated myelination occurring. Recently, a model has been developed using the thyroid hormone T4 to accelerate myelination, in which MBP protein synthesis has successfully been accelerated (Lariosa-Willingham et al., 2022). Of course, this introduces the risk of hyperthyroidism, which can also have behavioural symptoms such as anxiety and hyperactivity (National Health Service UK, 2023). As this possibly confounds behavioural data, further development of this model is therefore advisable. Nevertheless, it is a promising option for looking into the behavioural consequences of accelerated myelination outside the context of perinatal SSRI exposure.

Ultimately, studies investigating the possibility of accelerated myelination contribute to the growing knowledge of consequences of perinatal antidepressant exposure. As 8% of pregnant women in the U.S. alone are being prescribed SSRIs (Andrade et al., 2008), a lot of children are exposed. Their choice of whether to take antidepressants or not ought to be as completely informed as possible. Further research should therefore investigate possible side-effects for the offspring to optimise treatment, balancing necessary treatment for the mother while keeping the risk for unwanted effects on the children minimally.

4.5 Conclusion

The central question of this study was whether the protein levels of MBP and MAG reflect the previously observed changes in RNA levels as a consequence of perinatal FLX exposure. These

previously observed changes were the downregulation of these genes in the BLA at PND 21 and the upregulation observed in the PFC at PND 21. In addition to this, the present study also took into consideration the protein levels at PND 35. Here, no differences in the protein levels were found as a result of perinatal FLX treatment. This holds for the two timepoints in both the PFC and the BLA. Further, no significant differences have been found in MBP protein levels between PND 21 and PND 35. Contrarily, protein levels of MAG were significantly decreased from PND 21 to PND 35. The findings presented here show that myelination is not accelerated from PND 21 to PND 35. If myelination indeed is accelerated, it occurs already before the time points investigated here.

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6. Supplementary information

6.1 Immunohistochemistry protocol

Primary antibody dilutions:

- anti-MAG 1:10.000 (host = rabbit)
- anti-MBP 1:500 (host = mouse)

Secondary antibody dilutions:

- Alexa Fluor 555, goat anti-rabbit, 1:500
- Alexa Fluor 488, donkey anti-mouse, 1:500

Method:

Day 1

- Divide the tube with 1/6th of a brain into two, each tube should contain slices of equal size.
- Wash → 3*5 min PBS
- Block → neutral serum + detergent → 2 hours
    - Neutral serum, 5% = BSA (Bovine Serum Albumin)
    - Detergent = Triton X-100, 0.2%
    - Each well contains 2 ml of liquid for all the following incubation steps.
- Primary antibodies → MAG 1:10.000 MBP 1:500, incubate at 4 °C overnight + in blocking solution

Day 2

- Wash → 3*5 min PBS
- Secondary antibody → Incubate for 2 hours at room temperature, in the Dark, Keep slices as much in the dark afterwards as possible. Dilutions for both 1:500
- Wash → 3*5 min PBS
- Mounting + dapi staining
    - PBS for mounting
    - Prolong gold antifade + dapi ~ 60 ul per slide
    - nail polish to close the slides
- Store slides in the dark at 4 degrees

6.2 Microscope details and settings

Type microscope: Zeiss AxioObserver Z1. The equipped cameras are Monochrome camera system PCO Pixelfly II 1.4 Megapixels and a CMOS-colour camera PL-B623 Pixelink 3.1 Megapixels. Tissue FAXS cell analysis system version 6.0, build 6245.0136 is used to acquire the pictures.

Firstly, a Zeiss- EC “Plan-Neofluar” 2.5x/0.075 Dry lens made a quick prescan with a dapi-filter (Chroma ET Dapi-filter set). Then, regions of interest were manually traced. Next, the pictures were acquired using a Cy3 filter as first focus channel and a GFP filter as the second focus channel (Filter sets: Chroma ET FITC/GFP-filter set Chroma ET CY3-filter set) with the following lens: Zeiss- EC "Plan-Neofluar" 10x/0,3 Dry, Ph1. For this, the exposure time was set at 20 ms. Lower and upper sensitivity thresholds were set at “auto”. Further, the TL lamp had a voltage of 7.64. Condenser aperture was set at 0.09 and the FL lamp attenuator was set at 12.

6.3 Regions of interest

Figure 5: Indication of the region of interest: BLA. Magnification 10x, cy3 filter used. BLA is delineated in both hemispheres in yellow.

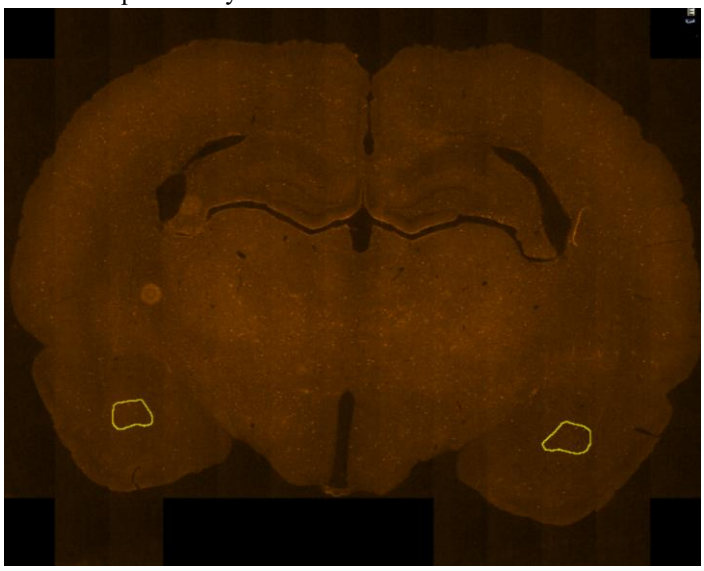
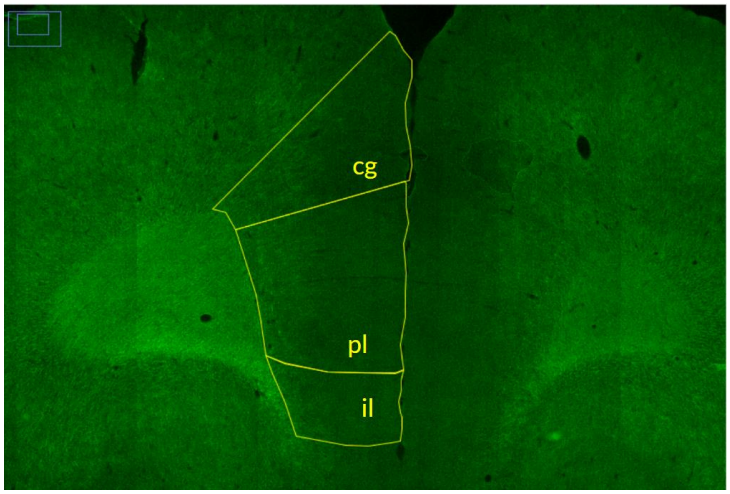
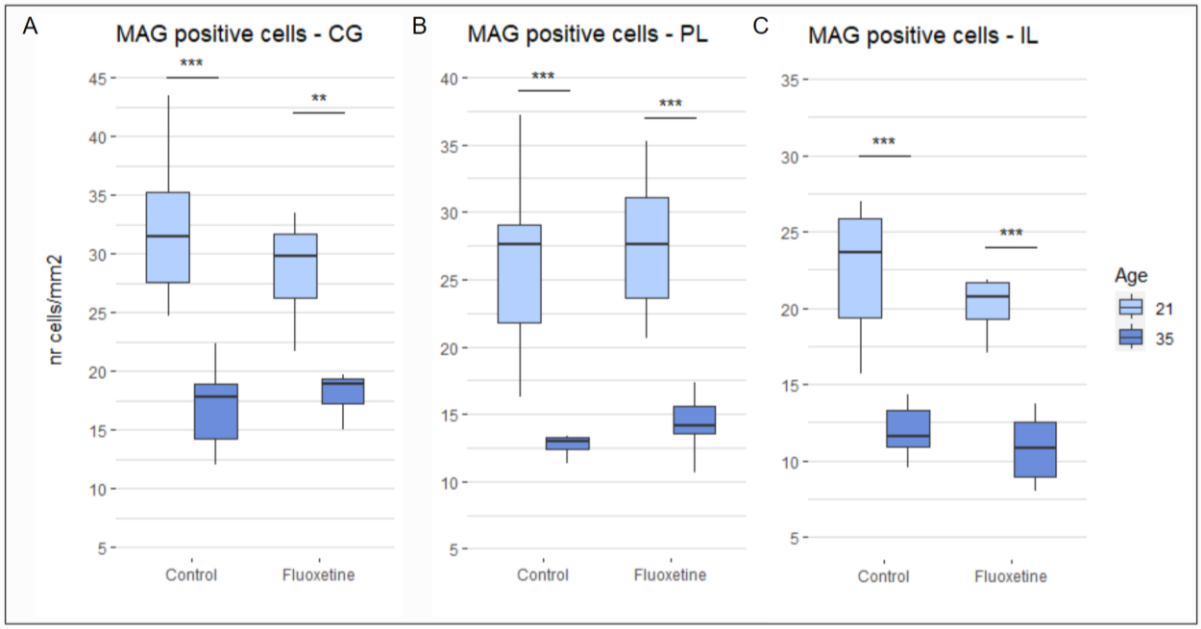


Figure 6: Indication of the region of interest: PFC. Magnification 10x, GFP filter used. PFC is subdivided into three regions: CG, PL and IL.



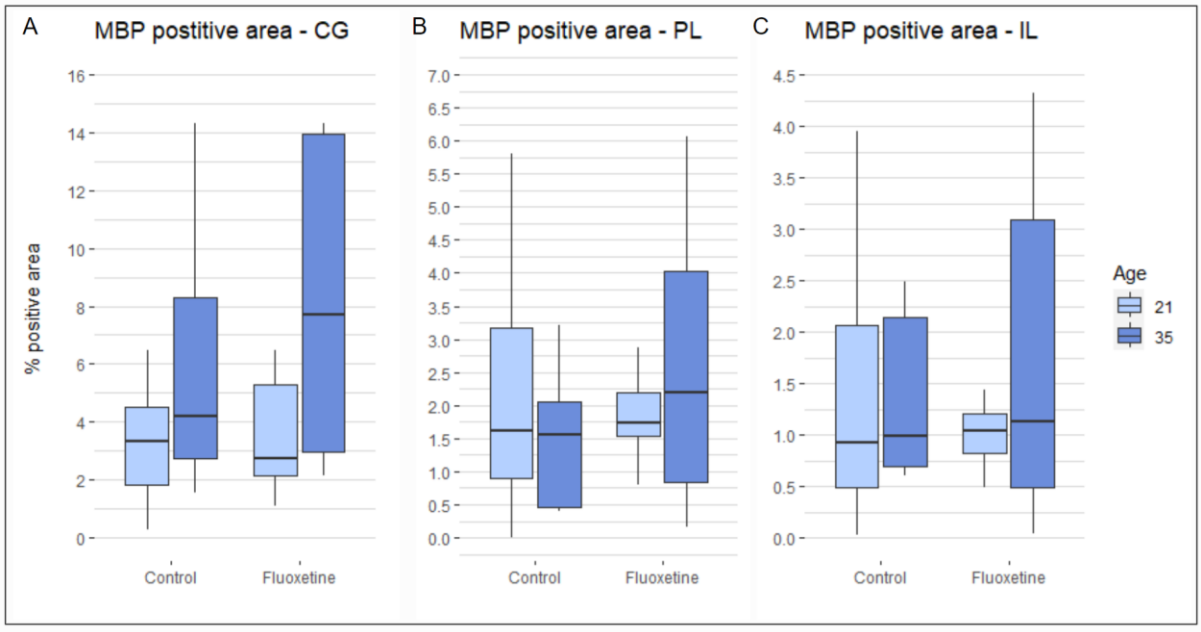
6.4 MAG positive cells CG, PL and IL

Figure 7: MAG data for each of the subregions of the PFC. A) MAG positive cells in the CG. p values: control 21 and FLX 21 0.5421, control 21 and control 35 < 0.0001, control 35 and FLX 35 0.8772, FLX 21 and FLX 35 0.0039. B) MAG positive cells in the PL, square root transformation is performed. p values: control 21 and FLX 21 0.9334, control 21 and control 35, 0.0003, control 35 and FLX 35 0.9836, FLX 21 and FLX 35 0.0001. C) MAG positive cells in the IL, 1/data transformation. p values: control 21 and FLX 21 0.9573, control 21 and control 35 0.0002, control 35 and FLX 35 0.5161, FLX 21 and FLX 35 < 0.0001. In A, B, and C, the number of animals used from left to right as depicted in the boxplot: n=7, n=6, n=6, n=8. ** p < 0.02, *** p < 0.001.



6.5 MBP data CG, PL and IL

Figure 8: MBP data for each of the subregions of the PFC. A) MBP positive area of the CG. Two-way ANOVA revealed an effect of age (p = 0.03163). Resulting p values of Tukey's test: control 21 and FLX 21 0.9995, control 21 and control 35 0.6479, control 35 and FLX 35 0.77676, FLX 21 and FLX 35 0.2000. B) MBP positive area of the PL, square root transformation is performed. Two-way ANOVA showed no significant effects of treatment (p = 0.9744) and age (p = 0.6132). C) MBP positive area of the IL, square root transformation. Again, the two-way ANOVA showed no significant effects of treatment (p = 0.7859) and age (p = 0.9789). In A, B, and C, the number of animals used from left to right as depicted in the boxplot: n=7, n=6, n=6, n=8.



6.6 Alternative microscopes - pilots

Figure 9: MPB and MAG staining in the auditory cortex. Visualisation with Leica 4000B, 5x magnification. Red is MAG, green is MBP.

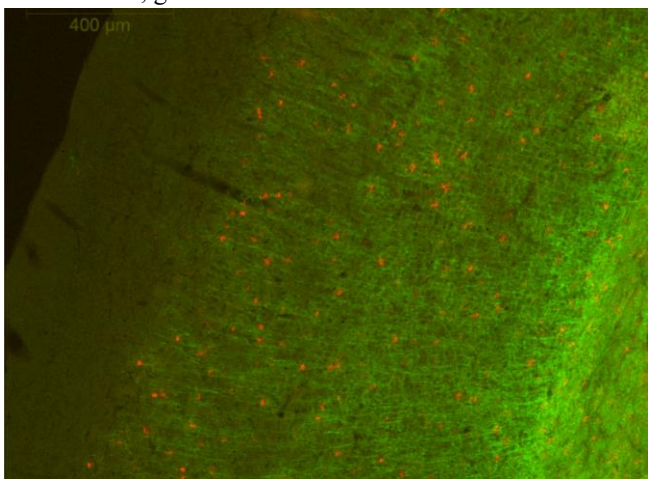


Figure 10: MBP and MAG staining in the cortex. Visualised with a confocal microscope, 40x magnification. Red is MAG, green is MBP.