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The influence of SSRIs on the myelination of the auditory brain regions

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ABSTRACT

18% of women experience depression during pregnancy. Nowadays, this is often treated with SSRIs. Both depression and SSRIs have a negative influence on the neurodevelopment of the unborn child and can cause mental and physical illness. Serotonin receptors are prevalent in the auditory brain regions, which makes it important to investigate whether exposure to SSRI during gestation influences these regions. Because serotonin also plays a role in the myelination of the brain, it would be interesting to further investigate the influence of SSRIs on the myelination in the inferior colliculus and the auditory cortex. Here, the influence will be tested on male rats, who were treated with fluoxetine or methylcellulose, and were sacrificed at PND 21 or PND 35. The brains were double stained for myelin associated glycoprotein (MAG) and myelin basic protein (MBP) with immunofluorescent histochemistry. It was found that the expression of MAG was reduced after PND 21 in both the inferior colliculus as in the auditory cortex, but the decrease between the control group and the fluoxetine group was only visible in the inferior colliculus at PND 35. This implies that the expression of MAG decreases as the myelination process decreases, and the process might go a bit faster since MAG is lower in fluoxetine PND 35 compared to control PND 35 in the inferior colliculus. There is a trend that the MAG expression decreases when the rats were exposed to fluoxetine, but further investigation must be needed to fully state that SSRIs influence the myelination of the auditory brain regions.

1 Introduction

Not for all women pregnancy will be a joyful time. Approximately 18% of pregnant women are experiencing depression during pregnancy (Szegda et al., 2014). Particularly women with a history of depression have a ~43% chance of relapse during pregnancy. Perinatal depression can cause preterm birth and a lower birthweight. These deficits are the leading cause for infant mortality and morbidity, and thus should be prevented at all costs. Depression is often treated with selective serotonin reuptake inhibitors (SSRI). Prescription of SSRIs during pregnancy differs worldwide between 2-15% (Fischer Fumeaux et al., 2019). SSRIs can cross the placental barrier, where it then targets the serotonin transporter (SERT). This potentially affects the development of social and behavioural neural circuitry and increases the risk of autism like behaviour since serotonin is used as a neurotrophic factor during gestation (Ramsteijn et al., 2022) (Gemmel et al., 2018). This makes the consideration of SSRI prescription a lot more difficult, knowing that both depression and SSRIs have an increased risk on mental and physical illness. In research of Ramsteijn et al. (2022) it was even found that, in rats, the perinatal use of SSRIs can influence the myelination of the foetus brain (Ramsteijn et al., 2022). For this paper we wanted to further investigate the influence on the myelination.

Myelination in the brain is essential for fast transduction of electric signals through neurons. The maturating process occurs mostly postnatally. It starts around Post Natal Day (PND) 10 and the maximum of myelin accumulation is around PND 20 (Downes & Mullins, 2014). After PND 20, myelination continues until adulthood but in a decreasing manner. The myelination process starts in the spinal cord and precedes rostrally. Important for myelination are the myelin basic protein (MBP) and the myelin-associated glycoprotein (MAG). MBP is mostly important for the adhesion of the cytosolic surfaces of the multi-layered compact myelin. It can also interact with other protein like actin, tubulin and clathrin (Boggs, 2006). MAG is a transmembrane glycoprotein which is found in periaxonal Schwann cells and oligodendrocytes.

MAG is important in the formation and maintenance of the myelin sheaths around the axon, although it is also an inhibitor for the regeneration of myelin sheaths (Quarles, 2007).

The role of serotonin in the myelination process already was acknowledged in 1983 when Gromova et al. (1983) published a paper about the growth of hippocampal cell differentiation when it is grown in a medium with serotonin. Gromova et al. (1983) investigated the growth of hippocampal tissue cultures *in vitro* of new-born rats. These cultures were grown in a medium including serotonin. The analysis of these cells revealed that the hippocampal cells had an earlier cell maturation than the control group, especially in the processes of myelination and synaptogenesis of the cells.

Later, in 2018, Lugo-Candelas et al. (2018) published an article about the perinatal exposure to SSRI in the development of human baby brains. It showed an increased connectivity in white matter areas. The areas with significant increase were the right amygdala-right insula, left anterior cingulate cortex-left thalamus, right precentral gyrus-right cuneus, and left insula-right precuneus.

Serotonin also plays a role in the auditory regions of the brain. In research of Hurley and Hall (2011) they showed that

during behavioural context, like stressful or social events, higher levels of serotonin are available in the auditory regions. This implies a link between serotonin and behavioural linked auditory processes. They showed that hearing loss and social isolation is followed by to a decrease in serotonergic fiber density in the auditory regions in the brain (Keesom & Hurley, 2020).

Because of minimal knowledge between serotonin and the myelination of the auditory regions, this study will further investigate this topic. We will determine the degree of myelination by fluorescent labelling of MAG and MBP in the most important auditory regions, the inferior colliculus (IC), and the auditory cortex (AU) in male rats. These regions are dependent on serotonin, which was especially shown for the auditory cortex, where SERT knock-out mice had a decreased dendritic length in the auditory cortex (Pan, 2021). It was hypothesized that the myelination of the brain will proceed faster when there is perinatal SSRI exposure. This means that the more caudal part of the brain will be myelinated faster which possibly causes a lower myelination in the caudal parts of the brain than in the rostral parts.

2 Methods

Experimental animals

The rats were housed and raised at the University of Trømso under supervision of Eelke Snoeren. The exact circumstances are still unknown, but it is assumed that the rats were housed similar to other experiments of the same research group. This includes housing in Makrolon cages on a reversed 12:12 h light/dark cycle at a temperature of 21 ± 1°C and the relative humidity was 55 ± 10 %. Food pellets, water and nesting material were available ad libitum (Sylte, 2021). The dams were treated daily with 10 mg/kg fluoxetine or 1% methylcellulose during gestation and lactation.

Histology

The rat brains were cut on a MICRON freezing microtome in slices of 40 µ. The brains were kept in a 0.01 M phosphate buffered saline (PBS) with 0.1% sodium azide.

Fluorescent histochemistry

The brain slices were washed 3 times for 5 minutes with PBS before staining them. The brain slices were then put in a blocking solution consisting of a natural serum and triton X-100 0.2% for 2 hours. Thereafter the slices were stained in the primary antibodies, the anti-MAG from a rabbit host in a dilution of 1:10.000 and the anti-MBP from a mouse host in a dilution of 1:500. They were then incubated at 4°C overnight in a blocking solution.

The second day, the slices were washed again 3 times for 5 minutes with PBS. The slices were stained with the secondary antibodies, Alexafluor 488 (donkey anti-mouse) in a dilution of 1:500 and Alexafluor 555 (goat anti-rabbit) also in a dilution of 1:500. Incubation was done for 2 hours in the dark at room temperature. Thereafter they were washed 3 times for 5 minutes in PBS. The slices were then ready to be mounted from PBS onto SuperFrost slides, around 6 slices per slide. 60 µl of prolonged gold DAPI mounting medium was used to close the slides, and base coat nail polish was used for the edges of the slide. They were stored in the dark at around 4°C.

Analysis

Fluorescent staining's were visualised and photographed using TissueFaxs microscope (Zeiss, Oberkochen, Germany) equipped with a Monochrome camera (PCO Pixelfly II 1.4 megapixels) at 2.5X and 10X magnification, TissueFaxs acquisition software and using an ET Dapi filter, ET FITC/GFP filter and ET Cy3 filter (all: Chroma, Bellows Falls, USA). Three images were obtained, one image with Cy3 as focus channel, one image as GFP as focus channel and one image with both focus channels.

Images were analysed with ImageJ 2.9.0/1.53t. MAG expressing cells were quantified by means of cell counting. Cells were semi-automatically counted by first converting images to 8-bit (Image > 8-bit), setting a reliable threshold (Adjust > Threshold) and making binary (Process > Binary > Make Binary > Convert to mask > Watershed). Then cells were counted (Analyse > Analyse Particles) while manually selecting the specific region of interest (IC or AU) and recording the region's size (Analyse > Measure in mm²). Left and right values were collected separately and then averaged for each image. Values were converted to cell count per mm² before exporting. For MBP a quantitative analysis was not possible since the density of the myelin sheaths in the images could not be determined with ImageJ. Here a qualitative analysis for the MBP staining was chosen.

GraphPad prism v9 was used for statistical analysis of the data. Data was normalised by means of a log transformation. The Shapiro-Wilk normality test and Q-Q plot conformed normality. A nested one-way ANOVA was performed on the transformed data and Tukey's multiple comparisons test was performed as a post hoc. Statistical significance was set at $p < 0.05$ and error bars in graphs represent SEM.

3 Results

Experimental groups

The brains consisted of 24 male rat brains which were separated into 4 groups.

- 7 rats with fluoxetine, brains harvested at PND 21
- 5 rats with fluoxetine, brains harvested at PND 35
- 7 rats with methylcellulose, brains harvested at PND 21
- 5 rats with methylcellulose, brain harvested at PND 35

Inferior Colliculus

All the collected data was selected per region. In *figure 1*, different images are shown of the inferior colliculus, from the different groups. The cells that are stained for MAG are now visible as small, lighter, and round dots. Half of the inferior colliculus area is circled in FLX 2. On these areas, the semi-automatically cell counting was performed. After all data collecting, the log transformed data was tested with a nested one-way ANOVA to calculate the group effect on MAG-stained cell count per mm² in the inferior colliculus. The results demonstrated a significant difference ($p=0.001$) between groups. Next, post Tukey's multiple comparisons test was performed as a post hoc test to identify which groups significantly differed from each other. Cell count per mm² was significantly decreased in the CTRL 35 group (74.20 cells/mm^2) compared to the CTRL 21 group (270.45 cells/mm^2) ($p=0.001$) and in the FLX 35 group (63.20 cells/mm^2) compared to the FLX21 group (205.02 cells/mm^2) ($p=0.005$) (*Figure 3.A*).

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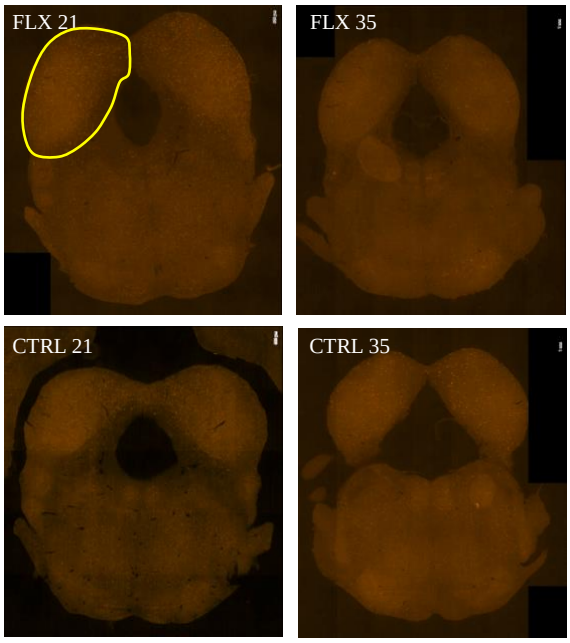

Figure 1: Images from the 4 different groups, all containing the inferior colliculus. The circled area is the inferior colliculus of one hemisphere.

Auditory cortex

All the collected data was selected per region. In *figure 2*, different images are shown of the auditory cortex, from the different groups. The cells that are stained for MAG are now visible as small, lighter, and round dots. Half of the auditory cortex area is circled in FLX 2. On these areas, the semi-automatically cell counting was performed. After all data collecting, the log transformed data was tested with a nested one-way ANOVA to calculate the group effect on MAG-stained cell count per mm² in the inferior colliculus. The results demonstrated a significant difference (p=0.003) between groups. Next, post Tukey's multiple comparisons test was performed as a post hoc test to identify which groups significantly differed from each other. Cell count per mm² was significantly decreased in the CTRL 35 group (42.78 cells/mm^2) compared to the CTRL21 group (113.45 cells/mm^2) (p=0.001) (*Figure 3.B*)

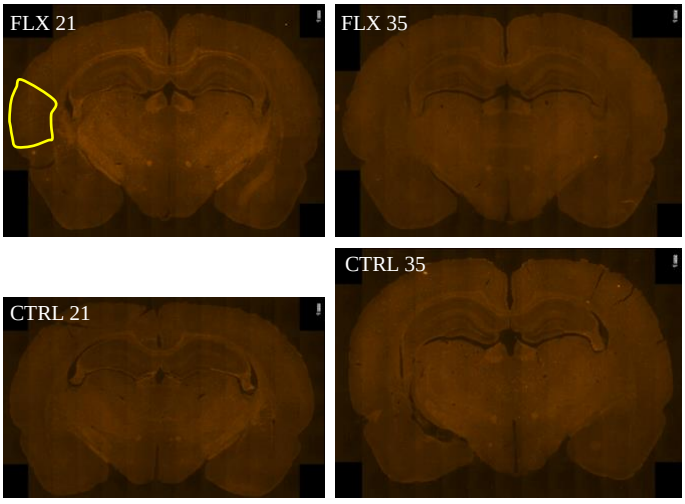

Figure 2: Images from the 4 different groups, all containing the auditory cortex. The circled area is the auditory cortex of one hemisphere.

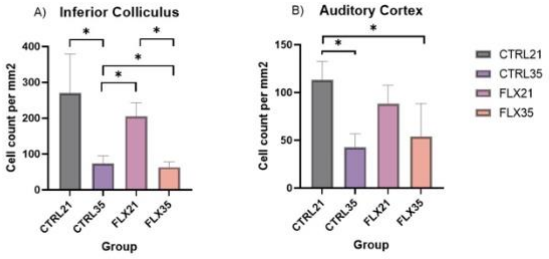

Figure 3: Bar chart of the cell count per mm² of MAG expressing cells in the IC and AU. Error bars represent SEM. Visual evaluation shows a decrease of cells between day 21 and day 35 of all groups in both regions. Moreover, a small decrease of cell count between CTRL21 and FLX21 is presented in both regions. Abbreviations: MAG = myelin associated glycoprotein, IC = inferior colliculus, AU = auditory cortex, SEM = standard error of the mean.

Myelin basic protein expression

For the cortical myelin basic protein (MBP), a qualitative analysis was done. By looking at the raw images, the brains were categorized in low, medium, or high MBP density. *Figure 4* shows the differences between the three groups.

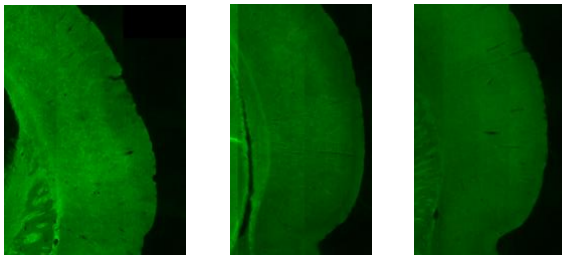

Figure 4: From left the right; relatively high, medium, and low density per mm^2 of MBP staining in the myelin sheaths.

All the brains were ranked, and the collection of data is made visible in table 1.

	FLX21	FLX35	CTRL21	CTRL35
HIGH DENSITY	2	1	1	2
MEDIUM DENSITY	3	0	3	2
LOW DENSITY	2	4	2	1

Table 1: Data from the MBP staining.

4 Discussion

In this research we wanted to investigate the influence of SSRIs on the myelination process of the auditory regions in male rats. This was investigated by staining the brain of young male rats who were treated with fluoxetine or methylcellulose (control) and were sacrificed either on PND 21 or PND 35. With use of immunofluorescent histochemistry for the proteins MAG and MBP, expression could be determined. After data retrieval and analysis, it was found that the expression of MAG was significantly decreased in the control group between PND 21 and PND 35, for both the inferior colliculus as for the auditory cortex. Also, in the fluoxetine group there is a significantly decrease in MAG expression between PND 21 and PND 35, within the inferior colliculus. This is in line with the research of Owens &

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Bunge (1989), where the main findings were 1) MAG expression occurs very early, prior to development of the myelin sheaths, 2) MAG expression persists on the surface of myelinating Schwann cells but is reduced on the surface of mature myelin segments, 3) MAG expression is continuous along the length of the developing myelin sheaths. As well in the research of Baba et al. (1987) they found that the MAG expression decreased after PND 21. Since the peak of the myelination process in rats is around PND 20, it seems logical that the expression of MAG would decrease after this day, because the myelination process decreases.

More interesting for this research is the significantly difference between CTRL 35 and FLX 35 in the inferior colliculus. This result shows that, with the treatment of fluoxetine, the MAG expression is lowered at PND 35 in the inferior colliculus. According to the research of Owens & Bunge (1989), a reduced MAG expression can be linked to a maturation of myelin segments. This could imply that with the fluoxetine treatment the rostrally proceeding myelination process is speed-up slightly, because of the lowered MAG expression in the FLX35 group in the inferior colliculus. This difference was not found in the auditory cortex.

For the staining of MBP, less conclusive results were found. Determining the MBP density was not possible with the images in the ImageJ software, a qualitative analysis was performed instead. The data retrieved from this was not useful for further statistics and no conclusion could be made form the results.

Limitations

The strength of this research is that it gives a good new overview over two combined concepts. The known linkage between serotonin and the auditory regions, is here coupled to the linkage between serotonin and myelination.

However, as every research, there also are some limitations that should be acknowledged.

First, for the data analysis of MAG fluorescent, a threshold must be applied to determine which should be counted as cells and which should not. This threshold setting was different for every image, and just by slightly adjusting the threshold, a big change could be seen in the cells that were counted. Since the data analysis was performed separately between 3 students, some variance could have occurred. For further research it could be more convenient to leave the analysis to one person, to reduce the variance.

Secondly, there was limited research to be found of MBP. This made it difficult to determine how the stained MBP could be best analysed. This problem could not be overcome, and a qualitative analysis was done instead. With the qualitative analysis it wasn't possible to draw any conclusions out of our MBP results. With more knowledge about analysing software and tools, a better estimation could be made about the density of the MBP fibres in the regions.

Furthermore, the harvesting day of the pup's brains could be doubted. It was known from the literature research that the start of the myelination is around PND 10, and the peak of the myelination was around PND 20. So, it could be more interesting to look at the myelination of the brain before PND 20 and after PND 20, where this research only investigated PND 21 and PND 35.

Finally, it would be interesting to increase the groups size. By increasing the group size, more data will be collected and the difference within groups could be lowered. This can cause a bigger and maybe even significantly difference between groups, which would give new conclusions.

In further research it would be interesting to overcome these limitations, since it could lead to more convincing results where more defined conclusion could be made.

Conclusion

To conclude this research, there is a trend in decreased MAG expression between the control group and the fluoxetine group. Important is that this does not mean that fluoxetine influences the myelination of the brain since there are no significant differences. Further investigation would be interested when the limitations of this research are considered.

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6 Appendix

TUKEY'S MULTIPLE COMPARISONS TEST	MEAN DIFF.	95.00% CI OF DIFF.	BELOW THRESHOLD?	SUMMARY	ADJUSTED P VALUE
CTRL21 VS. CRTL35	0.4289	0.1655 to 0.6923	Yes	**	0.0010
CTRL 21 VS. FLX21	0.2091	-0.03001 to 0.4483	No	ns	0.1000
CTRL21 VS. FLX35	0.4366	0.1784 to 0.6948	Yes	***	0.0007
CTRL35 VS. FLX21	-0.2197	-0.4846 to 0.04513	No	ns	0.1264
CTRL35 VS. FLX35	0.007721	-0.2745 to 0.2899	No	ns	0.9998
FLX21 VS. FLX35	0.2274	-0.03226 to 0.4872	No	ns	0.0993

Table 2: Values of Tukey's multiple comparisons test of the cell count of MAG expressing cells per mm2 in the AU, showing significance between CTRL 21 and CTRL 35 and significance between CTRL 21 and FLX 35. Abbreviations: MAG = myelin associated glycoprotein, AU = auditory cortex.

TUKEY'S MULTIPLE COMPARISONS TEST	MEAN DIFF.	95.00% CI OF DIFF.	BELOW THRESHOLD?	SUMMARY	ADJUSTED P VALUE
CTRL21 VS. CRTL35	0.5585	0.2171 to 0.8988	Yes	**	0.0010
CTRL 21 VS. FLX21	0.1038	-0.2102 to 0.4179	No	ns	0.7896
CTRL21 VS. FLX35	0.5647	0.2181 to 0.9114	Yes	**	0.0011
CTRL35 VS. FLX21	-0.4547	-0.7834 to -0.1259	Yes	**	0.0050
CTRL35 VS. FLX35	0.006271	-0.3538 to 0.3663	No	ns	>0.9999
FLX21 VS. FLX35	0.4609	0.1266 to 0.7952	Yes	**	0.0051

Table 3: Values of Tukey's multiple comparisons test of the cell count of MAG expressing cells per mm2 in the IC, showing significance between CTRL 21 and CTRL 35, CTRL 21 and FLX 35, FLX 21 and FLX 35 and between FLX 21 and CTRL 35. Abbreviations: MAG = myelin associated glycoprotein, IC = inferior colliculus.

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