BEHAVIORAL AND PLASTICITY-RELATED EFFECTS OF POST-WEANING SOCIAL ISOLATION IN RATS AND MICE

PhD thesis

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1. Introduction

Early-life social adversities, incluing sexual abuse, physical abuse and neglect, and emotional abuse and neglect leave a devastating impact on both the individual and its environment. Early-life maltreatment (ELM)-associated psychopathologies are clinically distinct, characterized by earlier onset, increased severity of symptoms and increased resistance to treatment, which warrants the development of novel treatment methods.

Human neuroimaging studies show that early life is a dynamic period of large-scale changes. Notably, brain regions of the social circuitry go through active remodeling in adolescence as reintegration into new social groups takes place. The prefrontal cortex (PFC) is a central hub in the regulation of social behavior and is characterized by protracted development that peaks in adolescence, making it vulnerable to social adversities experienced in early life.

Critical periods (CP) are finite time windows of high plasticity during development that are dynamically regulated by experience. Experience-driven neuronal activity induces the expression of critical period onset regulators, such as BDNF, which promote the maturation of inhibition. Cortical information processing becomes more efficient as mature PV+ INs increase the signal-to-noise ratio. Finally, experience-dependent changes made during the CP are consolidated with the appearance of PNNs and other factors that promote CP closure

Research suggests that adolescence is a CP for PFC maturation and is probably governed by similar processes as those described in sensory CPs. Still, little is known about social learning and PFC maturation in the context of CP mechanisms and how maltreatment in early life may derail these. Additionally, PV+ INs and the PNNs that enwrap them are critical orchestrators of CP timing, and PV+ INs have been established to play a role in social information processing. But whether the two major PV+ IN subpopulations are affected differently by early-life social adversities remains to be investigated.

PWSI is a robust laboratory model of childhood neglect and its effects on social behavior have been well-established in rats, making it an ideal candidate to study social learning in the context of CP plasticity. Fluoxetine has been shown to affect plasticity in sensory and even emotion-related paradigms in rats, and research suggests that social, PFC-related tasks would be similarly affected.

Still, despite the advantages of rats in social behavioral paradigms, the eventual need for transgenic strains that facilitate the manipulation of selective circuits and neuronal populations necessitate the use of mouse models. Unfortunately, a detailed behavioral characterization of PWSI has not been performed yet in mice. Additionally, while PWSI has

been shown to induce disturbances in the structure and function of the PFC, little is known about how PWSI might impact PV+ INs and the PNNs that enwrap these, CP regulators specifically associated with the closure of cortical network development.

Here, I hypothesize that PWSI in rodents leads to social behavioral abnormalities in adulthood, accompanied by changes affecting CP plasticity regulators, which potentially contribute to the emergence of abnormal social behavior. Plasticity of social behavior, i.e. the capacity for social learning presumably decreases by adulthood, rendering social abnormalities resistant to change. Inducing CP plasticity in adulthood with chronic fluoxetine treatment could allow for social learning beyond adolescence, carrying exciting therapeutic implications in the treatment of childhood maltreatment-induced psychopathologies.

2. Objectives

In two sets of experiments, we aimed to investigate PWSI-induced social disturbances, their association with social learning in adulthood, and PWSI-induced changes in critical period regulators, i.e. PV+ interneurons and PNNs, in the PFC.

Experiment 1 - Fluoxetine and social learning in adulthood in PWSI rats:

In the first set of experiments described in the thesis, we aimed to investigate whether fluoxetine, by inducing CP-like plasticity in adulthood, can provide capacity for social learning.

- Since PWSI takes place during the presumed critical period of social learning, we wished to verify that PWSI, i.e. social deprivation from weaning to adulthood, disrupts the development of social behavior, leading to the emergence of abnormal social behavior during social interactions.
- We wished to investigate whether PWSI-induced social abnormalities are resistant to resocialization in adulthood, possibly reflecting critical period closure of social behavioral development and prefrontal network maturation.
- We aimed to study whether reinstating critical period-like plasticity in adulthood with chronic fluoxetine treatment could enable the possibility of social learning and thus ameliorate PWSI-induced abnormalities in social behavior.

Experiment 2 – Behavioral characterization of PWSI in mice and the effects of PWSI on prefrontal PV+ neurons and PNNs:

The second set of experiments investigates the behavioral output of PWSI in mice including a detailed social behavioral characterization, and studies how PWSI affects PV+

interneurons and perineuronal nets, established regulators of CP plasticity, in the PFC. We differentiated between the two main regions of the PFC, the ventromedially located OFC and the dorsomedially located mPFC, as they subserve different tasks and could be impacted by PWSI in distinct ways.

- We aimed to investigate PWSI-induced behavioral changes via an extensive behavioral test battery. We assume that PWSI-induced behavioral disturbances in mice parallel those seen in rats. Notably, since PWSI takes place during the presumed critical period of social behavioral development, we propose that disturbances induced by PWSI will be specific to the social domain.
- The maturation of PV+ interneurons and the deposition of PNNs around PV+ INs are tied to critical period closure, and it has been shown that prefrontal PV+ interneurons are involved in the regulation of social behavior. Therefore, PWSI-induced disturbances in social behavior must be reflected in changes affecting PV+ interneuronal networks. Here, we aimed to characterize changes in PV+ INs and PNNs by measuring fluorescent intensity, studying input and output properties of PV+ neurons and investigating whether PWSI-induced changes in PV+ neurons are dependent on the presence of PNNs, i.e. whether PWSI affects PV+PNN- and PV+PNN+ interneurons differently.

3. Methods

3.1. Fluoxetine and social learning in adulthood in PWSI rats

3.1.1. Animals and experimental design

Male Wistar rats were used in the first experiment. Following weaning on PN21, rats were randomly assigned to social rearing (4/cage), n = 40, or isolation (1/cage), n=80, for 8 weeks, until reaching adulthood. To investigate the social behavioral effects of PWSI, rats underwent an RI test right after the isolational period (RI Test 1).

After RI Test 1, PWSI rats either remained in individual housing (isolation; n = 40), or were placed into social groups consisting of four PWSI rats (resocialization; n = 40). Half of both groups, i.e. isolation-reared and resocialized, received chronic fluoxetine (FLX) treatment over the three-week treatment period, while the other half of animals were used as controls and received normal drinking water. FLX was administered via drinking water, at a concentration of 0.2 mg/ml. PWSI rats were allocated into the following four treatment groups: (**I**) '**Isolation**', maintained in isolation following the first RI test, without receiving FLX treatment (**IsoVEH**); (**II**) '**Resocialization**'; resocialized into groups of 4 after the first RI test, without FLX

treatment (**ResVEH**); (**III**) '**Isolation+fluoxetine**'; maintained in isolation after the first RI and received FLX treatment (**IsoFLX**) and (**IV**) '**resocialization+fluoxetine**'; resocialized after RI Test 1 and received FLX treatment (**ResFLX**). Resocialization and FLX administration lasted for 3 weeks. The social behavior of the newly created groups (resocialization and resocialization+fluoxetine) was monitored throughout.

Due to lack of space, only an excerpt of the methods is shown.

3.1.2. The resident-intruder (RI) test

3 days prior to the test rats were placed into individual cages to establish territorial behavior. The test took place at the beginning of the dark period under dim red illumination. During the test, a smaller-sized male intruder was placed into the subject's homecage for 20 mins. Behavior was recorded and analyzed by an experimenter blinded to the conditions. We investigated the total number of biting attacks, their type (i.e. soft bite, hard bite or skin pulling), and whether they were aimed at vulnerable (i.e. head, throat or belly) or non-vulnerable targets.

3.1.3. Group monitoring during resocialization

To investigate behavior during cohabitation, we studied huddling during sleep in the light/inactive phase, which is thought to indicate social cohesion. Webcameras were placed above the cages and automatically took pictures 1, 2, 4, 6, and 10 hours after the beginning of the light/inactive period of each day. Huddling during sleep denoted direct physical contact with cagemates while sleeping. Absence of contact was scored as "0", whereas at least one contact was scored as "1" irrespective to the number of contacted cagemates. Scores coming from the 5 timepoints were averaged for each individual daily to give their huddling score. Huddling was investigated every day of the 3-week treatment/resocialization period.

3.1.4. Statistical analysis

Values shown in the text and figures indicate mean ± SEM. For main effects we used two-way or repeated-measures ANOVA, as described in the text. When necessary, square-root-transformation was used on behavioral data to fulfill ANOVA requirements. Post hoc analysis was performed by the Duncan test unless otherwise specified. The StatSoft 12.0 software was used for regression analysis. Comparison between the treatment groups and socially-reared groups was done by Student's t-test. P-values lower than 0.05 were considered statistically significant.

3.2. Behavioral characterization of PWSI in mice and the effects of PWSI on prefrontal PV+ neurons and PNNs

3.2.1. Animals and experimental design

Male Crl:CD1 mice were used in the second series of experiments. Intruders used in the resident-intruder test were also male Crl:CD1 mice housed socially (in groups of 4-5 mice per cage), and kept under similar conditions as the test subjects.

Following weaning on PN21, mice were housed in groups of four (social rearing) or underwent PWSI (single housing) for 6 weeks. Mice from all litters were randomly assigned to social or PWSI rearing to eliminate litter effects. Mice were subjected to a behavioral test battery after reaching adulthood (PN63), which lasted from PN64 until PN109. Mice underwent a resident-intruder test on PN67 (**RI1**) in order to verify the appearance of PWSI-induced social disturbances and abnormal aggression described in our previous studies in rats, and another RI test (**RI2**) was conducted after the behavioral battery (PN109), to verify whether the PWSI-induced abnormal aggressive phenotype remained stable over time and to investigate fighting-induced changes in the PFC.

Mice were perfused on PN109 under resting conditions or 90 minutes after the second RI test. Sample sizes were n = 22 for PWSI mice, and n = 20 for social mice.

Due to lack of space, only an excerpt of the methods is shown.

3.2.2. Resident-intruder test

All test subjects were transferred into individual cages with fresh corn cob bedding 3 days prior to the first RI test on PN67 (**RI1**). This was done to ensure the emergence of territorial behavior. In the early hours of the dark cycle (09:00AM) under dim red illumination, a smaller-sized novel male intruder was placed into the homecage of the resident for 10 minutes. Behavior was video-recorded and analyzed as described below.

On PN109, mice were either perfused under baseline (resting) conditions or 90 minutes after aggressive interaction, i.e. the second RI test (**RI2**). Behavior was video-recorded and videos were analyzed by an experimenter blinded to the conditions. Test subjects were differentiated by marks placed with permanent hair-dye. Parameters investigated were the latency to the first biting attack, the total number of bites, the intensity of biting attacks (categorized as soft or hard), and whether attacks were aimed at the vulnerable body parts of the opponent, such as the head, throat, or belly. We also analyzed the duration and frequency of each behavioral type using the Solomon Coder event-recording software (RRID:SCR_016041). The following behavioral types were differentiated: exploration (rearing

and any exploratory activity that is not directed towards the conspecific), sniffing (non-aggressive sniffing of any body part of the opponent), grooming (self-grooming movements), offensive behavior (attack bouts, chasing, tail-rattling, aggressive grooming, mounting, punching and kicking), defensive behavior (defensive upright posture, fleeing from the opponent), submission (being unmoving while being sniffed or aggressively groomed by the conspecific, usually crouched low with eyes shut) and vigilance (continuous tense or agitated observation of the intruder from a distance, with the body constantly being directed towards the opponent.

3.2.3. Immunohistochemistry and confocal imaging

3.2.3.1. Fixation and tissue processing

Mice were placed under anesthesia using a mixture of ketamine and xylazine (16.6 and 0.6 mg/ml, respectively). Mice were then transcardially perfused using ice-cold 0.1 M phosphate-buffered saline and 4% paraformaldehyde in 0.1-M phosphate-buffered saline solution (PBS), with a pH of 7.4. Brains were removed and post-fixed for 3 hours. Following that, brains were cryoprotected in 30% sucrose in PBS for 48 hours at 4℃. After sucrose cryoprotection, a freezing sliding microtome was used to cut 30 µm sections. Sections were collected in a six-well plate with cryoprotectant (50% sodium phosphate buffer, 30% ethylene glycol, 20% glycerol) for storage, and were kept at −20°C.

3.2.3.2. Fluorescent immunohistochemistry, confocal imaging, analysis of perisomatic puncta densities and fluorescent intensities

The PFC-containing sections were incubated for 4 hours at room temperature, in a solution containing 2% Triton X-100 and 10% normal donkey serum (NDS) in 0.1 M PB. Then, sections were incubated at 4°C for four days in a solution containing 2% Triton X-100, 10% NDS, 0.05% sodium azide, and different combinations of the following primary antibodies and reagents: mouse anti-bassoon, rabbit anti-parvalbumin, mouse anti-parvalbumin, guinea pig anti-parvalbumin, guinea pig anti-vesicular GABA transporter (vGAT), guinea pig anti-vesicular glutamate transporter 1 (vGluT1), rabbit anti-vesicular glutamate transporter 2 (vGluT2), mouse anti-potassium voltage-gated channel, Shab-related subfamily, member 1 (Kv2.1) and biotinylated *Wisteria floribunda* agglutinin (WFA). The following secondary antibodies were used to visualize the primary antibodies: DyL405-conjugated streptavidin, Alexa Fluor 488-conjugated donkey anti-guinea pig, Alexa Fluor 488-conjugated donkey anti-rabbit, Cy3-conjugated donkey anti-mouse IgG, Cy3-conjugated donkey anti-guinea pig IgG,

Alexa647-conjugated donkey anti-rabbit IgG and Alexa647-conjugated donkey anti-mouse IgG.

Confocal images were taken at 12-bit depth with 1024 X 1024 pixel resolution using a Nikon A1R microscope fitted with an oil-immersion apochromatic lens (CFI Plan Apo VC60x Oil, NA 1.40; z step size: 0.5 μm; xy: 0.11 μm/pixel). The density of puncta surrounding the soma of PV neurons and Kv2.1 immunoreactive putative pyramidal neurons was analyzed using a similar methodology. In the mPFC, 40 to 50 putative pyramidal neurons were imaged per mouse in three different sections. 10-15 PV+ neurons were counted in each animal per subregion, since the density of PV+ interneurons was more limited. We used the NIS Elements Software (Nikon Europe; RRID:SCR_014329) for image processing. The outline of every soma (i.e. the cell membrane) was delineated manually, and this manual selection was further enlarged by 0.25 μm to form a "ring" that covers the area surrounding the soma. vGluT1+Bassoon+ and vGluT2+Bassoon+ puncta in close proximity to PV+ or Kv2.1+ somata were considered to establish an apposition (i.e. form putative perisomatic synapses). Bassoon is a presynaptic release site marker, while vGluT1 and vGluT2 indicate excitatory intracortical and extracortical inputs, respectively, and Kv2.1 immunoreactivity was used to identify pyramidal cells in layer 5 of the mPFC.

Using custom scripts in Fiji, labeling intensities of the perisomatic region and PV somata were quantified in arbitrary units as the mean of all selected pixels. Intensities of single neurons were plotted. Previously drawn regions of interest (ROIs) were used to measure mean fluorescent intensity and integrated density in the area ±0.25 μm from the cell border with the use of custom scripts.

3.2.4. Statistical analysis

Data are expressed as mean ± standard error of the mean (SEM). Differences between groups were analyzed in the Prism (GraphPad Prism Software Inc. San Diego, California, USA) or Statistica 13.5 (Tibco, Palo Alto, CA, USA) softwares using Student's t-test, or Mann-Whitney U test when the requirements for t-tests were not fulfilled (i.e. when data did not follow a normal distribution, as verified through the D'Agostino and Pearson normality test. We used two-way ANOVA for c-Fos activation and PNN-dependent analysis of puncta densities, followed by Fisher's post hoc analyses. During the statistical analysis of individual cell numbers as a function of group, we implemented both the factor "housing" and "animal identity" in an additive fashion to a two-way ANOVA model. This way, the explanatory effects of the social environment and individual variability of animals could be independently determined. When

performing the latter analyses, we used the R Statistical Environment. The significance level was set at $p < 0.05$ throughout, all p values are indicated with exact numbers.

4. Results

4.1. Fluoxetine and social learning in adulthood in PWSI rats

4.1.1. PWSI induces abnormal aggression in the resident-intruder test

In order to study the effects of PWSI on aggressive behavior, a resident-intruder test was performed after the 8-week isolational period (**RI Test 1**). Similarly to earlier studies from our research group, PWSI induced abnormal aggression compared to rats reared in social groups: PWSI rats displayed a higher number of biting attacks ($F(1,121) = 25.21$, $p < 0.0001$) and a greater portion of these attacks were aimed at vulnerable body parts of the opponent (i.e. the head, throat or belly; $F(1,121) = 12.19$, $p < 0.001$). Additionally, the number of bites delivered shifted from less damaging attack types in social rats to the more damaging hard bites in PWSI rats (rearing x bite type interaction, $F(2,242) = 4.23$, $p < 0.05$).

4.1.2. PWSI rats are capable of social learning

Huddling during sleep was measured as an indicator of social cohesion during the 3-week treatment period. On the first day, PWSI rats displayed low levels of huddling, but the differences between the huddling behavior of social and PWSI animals gradually disappeared over 1 week (day x rearing interaction, $F(20, 580) = 6.88$; $p < 0.0001$; fluoxetine did not show any effects alone or in interaction; data not shown). Beginning with the second week of treatment, huddling no longer differentiated the 4 groups (i.e. RegVEH, RegFLX, ResVEH and ResFLX).

4.1.3. Social learning diminishes PWSI-induced abnormal aggression only when combined with fluoxetine

To investigate the impact of treatments on aggressive behavior, rats reared in PWSI underwent a second RI-test (**RI Test 2**) after the 3-week treatment period, on PN98. The treatments altered aggressive behavior when compared to RI Test 1 (bite counts: $F(4,90) = 4.26$, $p < 0.01$; vulnerable bites: $F(4,90) = 3.13$, $p < 0.02$), but each treatment showed prominent differences in their efficacy. While resocialization alone decreased the number of biting attacks compared to RI Test 1 (repeated-measures ANOVA, test x resocialization interaction: $F(1,48) = 9.18$, $p < 0.01$), the percentage of vulnerable bites delivered was decreased only in the group

that received the combined treatment (fluoxetine x resocialization interaction: $F(1,48) = 7.98$, $p < 0.01$). Chronic administration of fluoxetine in itself did not affect any form of aggressive behavior studied here. Importantly, the combined treatment ameliorated all three measures of excessive and abnormal aggression: bite counts (4.2 ± 0.7) and the percentage of bites aimed at vulnerable targets ($14.1 \pm 5.5\%$) decreased to reflect the levels seen in socially-reared rats ($p > 0.05$ for both parameters, two-tailed unpaired t-test), while attack types shifted to less damaging ones, i.e. skin pulls became more dominant than hard bites.

The effects of chronic fluoxetine treatment in adulthood in socially-reared rats were studied separately from PWSI rats because the social learning component of resocialization was absent during the regrouping of socially-reared rats. In socially-reared rats that were regrouped for 3 weeks to mimic the resocialization of PWSI rats, chronic fluoxetine treatment decreased the number of attack bites ($F(1,23) = 4.03$, $p < 0.05$), but did not affect the percentage of vulnerable attacks ($F(1,23) = 1.26$, $p > 0.05$). When compared to RI Test 1, neither treatment (regrouping alone or regrouping+fluoxetine) affected behavior in RI Test 2 (number of attack bites: $F(2,62) = 0.91$, $p > 0.05$); percentage of vulnerable target bites: $F(2,62) = 0.64$, $p > 0.5$).

4.2. Behavioral characterization of PWSI in mice and the effects of PWSI on prefrontal PV+ neurons and PNNs

4.2.1. PWSI induces abnormal aggression, disrupted behavioral organization and behavioral fragmentation

In the second set of experiments, we sought to characterize the long-term behavioral changes induced by PWSI in mice via subjecting them to post-weaning social isolation for 6 weeks followed by a behavioral test battery designed to investigate social and emotional domains.

PWSI mice displayed heightened aggression compared to socially-reared mice as shown in the second RI test. Particularly, PWSI mice showed a decreased latency of attack ($U = 28$, $p = 0.005$) and delivered more biting attacks overall ($U = 25.5$, $p = 0.001$). Detailed analysis of aggressive interactions also revealed that PWSI mice directed more attacks toward the vulnerable body parts of the opponents, i.e. the head, throat or belly ($U = 24.5$, $p = 0.002$). Moreover, PWSI mice delivered more violent hard bites compared to social mice ($U = 27$, $p = 0.003$).

PWSI mice exhibited higher frequencies of offensive ($U = 12$, $p < 0.001$) and defensive ($U = 36.5$, $p = 0.010$) behavior and displayed less submissive behavior ($U = 20$, $p < 0.001$)

compared to the social group. Interestingly, we also observed that PWSI mice often engaged in vigilance-like behavior (i.e. agitated observation of the intruder from a distance, with the body constantly being directed toward the intruder) compared to social mice ($U = 6$, $p < 0.001$). A reduction in non-aggressive social sniffing revealed that PWSI mice spent less time with social investigation ($U = 22$, $p = 0.001$). In turn, there was an increase in grooming behavior ($U = 35.5$, $p = 0.011$) compared to social mice. Importantly, the observed changes in offensive and sniffing behaviors considerably increased the ratio of offense/sniffing events in PWSI mice ($U = 16$, $p < 0.001$). PWSI also induced a fragmented behavioral phenotype, shown by the increased number of behavioral transitions (i.e. PWSI mice displayed rapid shifting between behavioral elements) ($U = 16$, $p < 0.001$).

In order to verify the PWSI-induced aggressive phenotype previously reported in our studies of PWSI rats and to see whether PWSI-induced aggression remained present after the behavioral battery, we subjected mice to an RI test after the end of the post-weaning social isolation period and to another RI test following the behavioral battery, before perfusion. Abnormal aggressive behavior was already present during the first RI, conducted on PN67 (significant differences between PWSI and social mice in total attack bites during first RI, $U = 95$, $p = 0.0152$; percentage of vulnerable target bites, $U = 70$, $p = 0.0008$) and PWSI-induced abnormal aggression was persistent over time, with the number of attack bites ($U = 25.5$, $p = 0.001$) and share of vulnerable target bites ($U = 33$, $p = 0.0048$) remaining significantly elevated compared to social controls even following the behavioral battery.

4.2.2. PWSI increased PV+ soma intensity and PNN intensity in the mPFC

Our previous studies of PWSI in rats and current study in mice (data not included in the the present thesis) highlight that PWSI induces neuronal hyperactivation in response to aggressive interaction within selective subregions of both main areas of the PFC: the mPFC and the OFC. The OFC and mPFC are both heavily involved in emotion-regulation and behavioral flexibility, but subserve distinct roles within these processes. In our scientific paper of which this experiment forms a part of, we have demonstrated that while both regions show marked neuronal activation (as revealed by c-Fos expression) in response to aggressive interaction (RI), in PWSI mice aggression-induced hyperactivation of the PFC was specific to the mPFC when compared to social controls. This suggests that PWSI might impact the OFC and mPFC in distinct ways, warranting the investigation and comparison of both areas in PWSI-related neurobiological changes.

In the PFC, PV+ neurons play an essential role in the maintenance of synaptic excitatory/inhibitory balance during social behavior and their function is disrupted by early-life adversity. Additionally, exposure to adverse experiences has also been linked to aberrant PV soma and PNN intensities in the PFC.

Therefore, using high resolution confocal microscopy, we investigated how PWSI affects PV soma and PNN intensities of PV+ neurons and PV+PNN+ neurons, respectively, in the mPFC and OFC. Our results display that OFC and mPFC PV+ neurons in PWSI mice had significantly greater PV soma intensity (OFC, $U = 3992$, $p = 0.028$; mPFC, $U = 8612$, $p < 0.001$) compared to social mice. Intriguingly, PV+ neurons that are enwrapped by PNNs also showed greater PNN intensity in PWSI mice ($U = 1663$, $p < 0.001$). This change was specific to the mPFC, as no significant differences in the intensity of PNN was observed between groups in the OFC ($U = 1788$, $p = 0.078$).

4.2.3. PWSI increased the density of excitatory cortical (vGluT1+) and subcortical (vGluT2+) boutons surrounding the perisomatic region of PV+ neurons in the mPFC

Early-life adversity has been shown to affect the density of inhibitory synapses, therefore we tested whether PWSI affected the density of vesicular GABA transporter (vGAT+) immunoreactive inputs targeting the perisomatic region of PV+ neurons. PWSI did not affect the density of inhibitory inputs (OFC, $t(90) = 0.693$, $p = 0.490$; mPFC, $t(323) = 0.16$, $p = 0.873$).

Excitatory synaptic input can regulate the deposition of PNNs around PV+ interneurons. Since we found that, as revealed by c-Fos staining, PWSI leads to an increase in the recruitment of PV+ neurons following aggressive interaction in the mPFC but not OFC (data not included in the thesis), and that PNN fluorescence intensity also increased around PV+ neurons in the mPFC, we asked whether PWSI affected the excitatory synaptic drive arriving onto the perisomatic region of PV+ neurons. Therefore, we investigated the excitatory glutamatergic inputs targeting the somata of cortical PV+ interneurons: intracortical and subcortical inputs which use presynaptic vesicular glutamate transporter 1 (vGluT1) and 2 (vGluT2), respectively. By applying multiple labeling immunostaining and confocal microscopy, we assessed the density of vGluT1 immunoreactive (vGluT1+) and vGluT2 immunoreactive (vGluT2+) synaptic puncta apposing the somata of PV+ neurons in combination with the presynaptic release site marker Bassoon+. There was a significant increase in the density of both cortical and subcortical inputs targeting the somata of PV+ neurons in the mPFC of PWSI mice compared to social mice (vGluT1+Basson+ puncta, $U = 5945$, $p < 0.002$; vGluT2+Basson+ puncta, $U = 6455$, $p<0.001$). The observed increase in the excitatory drive was specific for the

mPFC since no such changes were detectable at the level of the OFC (vGluT1+Basson+ puncta, U = 6450, p = 0.273; vGluT2+Basson+ puncta, U = 4445, p = 0.273).

Based on our data, one might suggest that aggressive interaction-associated recruitment of PV+ neurons in PWSI mice could have been due to an enhanced excitatory drive arising from both cortical and subcortical sources.

4.2.4. PWSI increased the density of excitatory inputs targeting PV+ neurons in the mPFC, irrespective of the presence of PNN

In the following, we asked whether the observed PWSI-evoked synaptic imbalances in mPFC PV+ neurons are PNN-dependent. In line with previous observations, PV+PNN+ neurons received more vGAT+ inputs compared to PV+PNN- neurons ($F(1, 310) = 11.46$, $p < 0.001$). Accordingly, we also observed increased perisomatic vGAT fluorescence intensity around PV+PNN+ neurons ($F(1, 311) = 10.62$, $p = 0.001$), however, PWSI had no effect on the density of inhibitory inputs ($F(1, 310) = 0.054$, $p = 0.823$). In addition, we observed that the PNN intensities of PV+ neurons showed a positive correlation with perisomatic vGAT+ puncta intensities ($r = 0.768$, $p< 0.001$, data not shown).

In the case of cortical excitatory inputs, our results display that PV+PNN+ neurons received higher densities of vGluT1+ Bassoon+ inputs compared to PV+PNN- neurons ($F(1, 240) = 8.884$, $p = 0.003$). PWSI resulted in higher densities of vGluT1+ Bassoon+ inputs targeting mPFC PV+ neurons ($F(1, 240) = 12.14$, $p < 0.0001$), which did not depend on PNN coverage and housing interaction, ($F(1, 240) = 1.143$, $p = 0.286$). No differences were seen in the intensity of vGluT1+ staining (all p values > 0.05, data not shown). Additionally, PNN intensities of PV+ neurons positively correlated with perisomatic vGluT1+ puncta intensities ($r = 0.6182$, $p < 0.0001$, data not shown).

In the case of subcortical excitatory inputs, we detected increased vGluT2+ Bassoon+ puncta densities in the perisomatic regions of PV+PNN+ neurons compared to the PV+PNN- population ($F(1, 299) = 12.36$, $p < 0.001$). As with cortical excitatory inputs, the significant increase seen in the densities of vGluT2+Bassoon+ inputs of PWSI mice relative to social mice ($F(1, 299) = 29.94$, $p < 0.001$) did not depend on PNN coverage (PNN and housing interaction, $F(1, 299) = 0.005$, $p = 0.941$). In addition, PNN intensities of PV+ neurons positively correlated with perisomatic vGluT2+ puncta intensities ($r = 0.2425$, $p < 0.003$, data not shown).

4.2.5. PWSI led to a reduced density of PV+ inhibitory boutons (baskets) targeting the perisomatic region of pyramidal cells in the mPFC

PV+ basket interneurons are one of the main sources of perisomatic inhibition onto excitatory neurons, ensuring synchronized modulation of cortical activity via the regulation of oscillations. To investigate whether PWSI affected the output features of PV+ interneurons, we studied the vesicular GABA transporter (vGAT+) immunoreactive inhibitory synapses of PV+ basket interneurons targeting the soma of pyramidal cells in layer 5 of mPFC, identified by Kv2.1 immunoreactivity. Overall, PWSI induced fewer vGAT+ puncta ($t(480) = 4.272$, $p < 0.001$) and PV+ puncta ($t(480) = 2.268$, $p = 0.024$) around pyramidal neurons. mPFC layer 5 (L5) pyramidal cells in PWSI mice received fewer inhibitory inputs from PV+ neurons, since there was a reduction in the density of PV+vGAT+ puncta surrounding Kv2.1 immunoreactive pyramidal neuron somata ($t(480) = 2.895$, $p = 0.004$).

Together, our data displays that PV+ inhibitory synaptic inputs to L5 pyramidal cells are severely reduced after PWSI, suggesting less effective perisomatic inhibition in the mPFC, which could contribute to the observed hyperactivation of the mPFC (data not included in the thesis).

5. Conclusions

The main conclusions of our two experiments are as follows:

- ***PWSI induces abnormalities in social behavior and reward-related behavior***
    - PWSI leads to abnormal aggression, increased defensive and offensive behaviors and behavioral fragmentation as shown by the resident-intruder, social instigation and social interaction tests
    - PWSI leads to mild deficits in reward-related tasks as shown by the delay discounting and sucrose preference tests
    - Non-social anxiety was not present in PWSI mice, as revealed by the open field test and elevated plus-maze
- ***Social learning decreases in adulthood, rendering PWSI-induced abnormal aggression robust and enduring***
    - PWSI-induced abnormal aggression is resistant to resocialization in adulthood
    - PWSI-induced abnormal aggression is resistant to multiple testing (i.e. multi-domain behavioral battery of) in adulthood
- ***PWSI-induced abnormal aggression could only be successfully ameliorated by the combination of plasticity-inducing chronic fluoxetine treatment and resocialization***
    - Chronic fluoxetine treatment alone did not affect abnormal aggression in the resident-intruder test
    - Resocialization decreased quantitative (number of attack bites) but not qualitative (attacks aimed at vulnerable targets, dominance of hard bites among attack types) aspects of aggression
    - The combination of chronic fluoxetine treatment and resocialization successfully decreased both quantitative and qualitative aspects of aggression
- ***PWSI leads to altered properties of medial prefrontal parvalbumin interneurons***
    - PWSI increases PV fluorescent intensity of PV+ interneuron somata
    - PWSI increases fluorescent intensity of PNNs surrounding PV+ interneurons
    - PV+ neurons of PWSI mice receive a higher number of intracortical and subcortical excitatory inputs
    - PV+ neurons of PWSI mice send fewer inhibitory synapses onto medial prefrontal pyramidal neurons

6. Bibliography of the candidate's publications

6.1. List of publications used for the thesis

Biro L*, **Miskolczi C***, Szebik H, Bruzsik B, Varga ZK, Szente L, Toth M, Halasz J, Mikics E.
Post-weaning social isolation in male mice leads to abnormal aggression and disrupted network organization in the prefrontal cortex: Contribution of parvalbumin interneurons with or without perineuronal nets. Neurobiol Stress. 2023 May 29;25:100546.

Miskolczi C, Halász J, Mikics É.
Changes in neuroplasticity following early-life social adversities: the possible role of brain-derived neurotrophic factor. Pediatr Res. 2019 Jan;85(2):225-233.

Mikics É, Guirado R, Umemori J, Tóth M, Biró L, **Miskolczi C**, Balázsfi D, Zelena D, Castrén E, Haller J, Karpova NN.
Social Learning Requires Plasticity Enhanced by Fluoxetine Through Prefrontal Bdnf-TrkB Signaling to Limit Aggression Induced by Post-Weaning Social Isolation. Neuropsychopharmacology. 2018 Jan;43(2):235-245.

6.2. List of publications not used for the thesis

Balázsfi D, Zelena D, Demeter K, **Miskolczi C,** Varga ZK, Nagyváradi Á, Nyíri G, Cserép C, Baranyi M, Sperlágh B, Haller J.
Differential Roles of the Two Raphe Nuclei in Amiable Social Behavior and Aggression - An Optogenetic Study. Front Behav Neurosci. 2018 Aug 2;12:163.

Zelena D, Mikics É, Balázsfi D, Varga J, Klausz B, Urbán E, Sipos E, Biró L, **Miskolczi C**, Kovács K, Ferenczi S, Haller J.
Enduring abolishment of remote but not recent expression of conditioned fear by the blockade of calcium-permeable AMPA receptors before extinction training. Psychopharmacology (Berl). 2016 Jun;233(11):2065-2076.