A BRAIN CRYING OUT LOUD FOR PLAY

An immunohistochemical study on how social play deprivation affects the orbitofrontal cortex

Master's research project report

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"States Parties recognize the right of the child to rest and leisure, to engage in play and recreational activities appropriate to the age of the child and to participate freely in cultural life and the arts."

UN Convention on the Rights of the Child. *Article 31.1*

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ABSTRACT

Play is important for an optimal development in childhood, yet how it shapes the development of the prefrontal cortex (PFC) is not fully understood. Social play behavior, the most common type of play in mammals, is critical for the development of the brain. We have previously observed that GABAergic synapses onto layer 5 neurons in the adult medial prefrontal cortex (mPFC) are reduced when rats are subject to juvenile social play deprivation (SPD). In addition, we found indications that reward processing may be altered in SPD rats. We have therefore assessed how social play shapes the orbitofrontal cortex (OFC), a region of the PFC known to be involved in processing stimulus-reward association. This region is next to the mPFC, but appears to follow a different developmental trajectory. Given that the OFC mediates reward-processing behavior, we hypothesized a significant reduction of the inhibitory synaptic activity following SPD. We made use of juvenile rats that had been subject to SPD during a critical period followed by resocialization. This allowed us to perform an immunohistochemical study to quantify the perisomatic inhibition and determine if there was a change following SPD. Surprisingly, our results did not show relevant alterations when both hemispheres were taken together, nonetheless, when the hemispheres were assessed separately, we found altered inhibitory synapse asymmetry in terms of size and intensity for all immunohistochemical markers. This study not only shows the relevance of social play for the proper development of reward- and decision-related structures, but also, for the first time, the crucial role it takes in the development of the hemisphere asymmetry.

Keywords: Social play, Social play deprivation, Prefrontal cortex, Medial prefrontal cortex, Orbitofrontal cortex, Perisomatic inhibition, Immunohistochemistry, Hemisphere asymmetry

LAYMEN'S SUMMARY

Everybody loves to play. And most of us also love playing with other people. It is a rewarding activity, whether it is playing card, telling jokes, tickling your siblings or at a football match with friends. But what happens if we do not play as kids with other children? This question is often overlooked, but severely or chronically diseases kids (cancer, cystic fibrosis and chronic fatigue patients for example) are under this situation and scientist have observed that it affects their quality of life, mental health, cognition, learning abilities and social functioning. However, behind behavioral and cognitive changes there are alterations in the brain that are less known. Previously, we have observed that the part of brain involved in social behavior and cognition, the prefrontal cortex, is greatly affected by this lack of social play. However, there are several regions within the prefrontal cortex and most research has been focused on just one, the medial prefrontal cortex, which is related to how we make decisions based on cognition. Here we have aimed to know how lack of social play affects the orbitofrontal cortex, another region of the prefrontal cortex, involved, instead, in how we take decisions based on pleasure and reward. For this, we have used a technique called immunohistochemistry that has consisted in labelling the connection between neurons, which are called synapses. In this way, we have imaged with fluorescence these connection under a microscope in order to analyse the synapses and quantify the effect of lack of social play on them. Our study shows that lack of social play at early ages truly has a lasting effect in the orbitofrontal cortex.

ABBREVIATIONS

5-CSRTT: Five-choice serial reaction time task

AAC: Axo-axonic cells

CB1R: Endocannabinoid 1 receptor

DA: Dopamine

IHC: Immunohistochemistry

IN: Interneuron

mIPSC: miniature IPSC

sIPSC: spontaneous IPSC

L5: Layer 5

OFC: Orbitofrontal cortex

lOFC: Lateral orbitofrontal cortex

mOFC: Medial orbitofrontal cortex

PC: Pyramidal cell or neuron

PFC: Prefrontal cortex

L-PFC: Lateral prefrontal cortex

mPFC: Medial prefrontal cortex

PND: Post-natal day

PV: Parvalbumin

R&T play: Rough and tumble play

SPD: Social play deprivation

VGAT: Vesicular GABA transporter

1. INTRODUCTION

1.1. Let's play!

"My god! Why did you have to bring the video camera?" Liz asked Isaac as they were watching the tape of the birth of their twins Norah and Max. The kids have just turned 1 year old and that video camera has witnessed their first year of life and so will be in the upcoming years.

Videos of both crawling and playing. However, play slowly develops and changes as these kids grow up. Max and Norah will start touching and grasping things, then fantasizing, creating their own imaginary world and then they will start playing between them and with other kids, and at some point, it will evolve to language play, trying to understand how to actually use language to play (**Figure 1**).

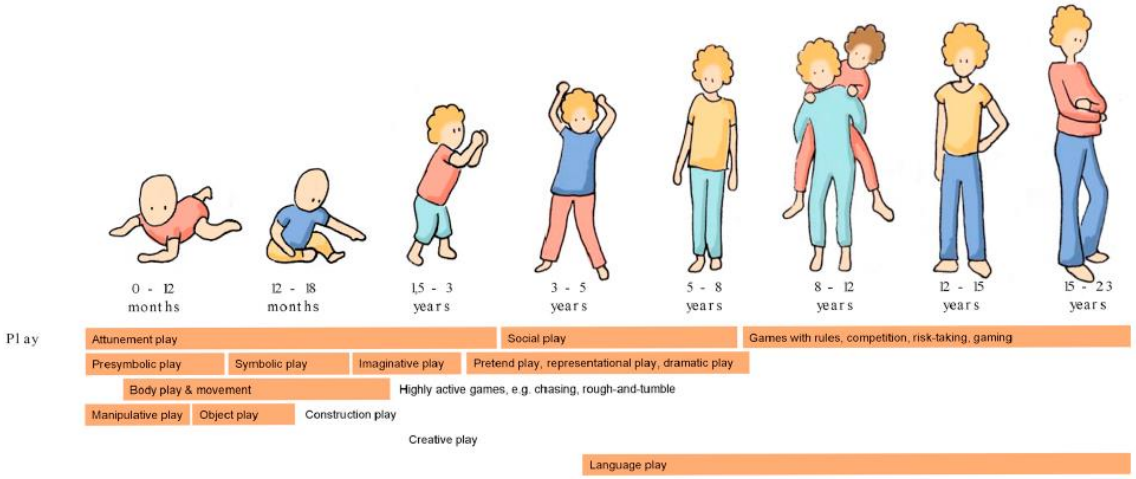

Figure 1. Stages of human play during development (Nijhof et al., 2018).

Yet, the typical play development will only take place in Max, because Norah at five years of age is diagnosed with Leukemia. Norah will not be able to play as her brother will, she will be hospitalized for three years now and thus unable to play with other kids.

But, what do we mean when we say play?

Even although it is a recognizable activity play is not an easy word to define because it covers many behavioral categories, varies considerably between and within species, its single or multiple functional significance is still being debated (Held and Špinka, 2011; Bekoff and Byers 1998; Power, 2000; Špinka et al. 2001; Burghardt, 2005) and various categories are evoked in the literature with little standardization across animals and humans (Ahloy-Dallaire et al., 2018) leading to no scientific consensus regarding its definition. Up until now, the five

criteria developed by Burghardt (2005, 2010) are the most useful tool to recognize play in all species, including the human one:

1. Performance of play is not fully functional in the context in which it is expressed.
2. Play behavior is spontaneous, voluntary, intentional, pleasurable, rewarding, reinforcing, or autotelic (done for its own sake).
3. Play differs from more serious performance of behavior structurally or temporally in at least one respect: it is incomplete (generally through inhibited or dropped final elements), exaggerated, awkward, or precocious; or it involves behavior patterns with modified form, sequencing, or targeting
4. Play is behavior performed repeatedly in a similar, but not rigidly stereotyped, form
5. Play is initiated when an animal is free from acute or chronic stress and intense levels of competing

Thus, play is usually seen as an activity for enjoyment and recreation rather than for serious or practical purposes; nevertheless, it certainly serves a purpose for those who play and allows children to use their creativity while developing their imagination, dexterity and physical, cognitive, social and emotional strength (Nijhof et al., 2018; Ginsburg, 2007). Nevertheless, play is not only present in humans, but it is well-developed as well in primates, rodents, carnivorans, ungulates, elephants and cetaceans (Graham and Burghardt; 2010). In general, play will enable individuals to generate in a rather low-cost manner, a repertoire of innovative behaviors that may be adaptative to their specific niche (Pellegrini et al., 2007).

Play is typically grouped in three different categories: locomotor-rotational play, object play and social play. Despite distinct terms, the boundaries are often blurred, and individual categories are often subdivided (Graham and Burghardt, 2010; Burghardt, 2005). Other forms of play can also be found in humans such as mimic play, free play or narrative play. However, this broad variety in forms of play poses a profound challenge to study play behavior objectively and consistently in humans (Nijhof et al., 2018).

In short, locomotor-rotational play consists of vigorous motor acts that are typically performed alone, for example, body twisting, jumping and running. Object play can be either solitary or social (Tanner and Byrne, 2010) and involves the playful use or manipulation of inanimate objects such as a dog retrieving a stick — it appears to be the most common type of play for predatory animals and scavengers (Graham and Burghardt, 2010). Lastly, social play has been defined as play directed at conspecifics and one of the earliest non-mother

directed social behaviors to contain behavioral patterns related to social, sexual and aggressive behavior (Vanderschuren et al., 1997).

As reported by Graham and Burghardt (2010), whilst locomotor play is performed alone and object play also offers such possibility, social play involves two or more players that are usually, but not always, conspecifics (in humans it could be argued that social play can take place even without peers, for instance, through videogames. But, would the effect of videogames be similar to play with peers? And which should the characteristics of these videogames be so they can induce social play?). Numerous social benefits have been conferred to social play, including enhancing social skills, strengthening social bonds, reducing aggression and refining social assessment for instance (Graham and Burghardt, 2010). In fact, it was reported by Pellis and Pellis (1998) that a form of social play, play fighting or rough-and-tumble play (R&T play), is the most common form of play in mammals. Additionally, R&T play of laboratory rats has been extensively used for studies investigating the neurobiology of mammalian play (e.g., Trezza et al., 2010; Vanderschuren et al., 2016; Achterberg et al., 2016).

In rats, R&T play (**Figure 2**) starts with one rat approaching a conspecific and attempting to rub or touch its neck with the snout. This behavior is called pouncing and is the most important parameter of play initiation. The most characteristic response to this play initiation is when the recipient rat fully rotates onto its dorsal surface, which is also known as pinning (Vanderschuren et al., 2016) and hence, indicating that social play has been consummated (Panksepp and Beatty, 1980). From here, the supine animal might initiate the whole process again by pouncing on the other subject. Pinning and pouncing frequencies can be easily quantified and these are considered the most characteristic parameters of social play behavior in rats (Panksepp and Beatty, 1980; Achterberg et al., 2016).

Figure 2. Left panel: a rat pouncing another by rubbing the nape of its peer. Right panel: the pounced animal may respond by rotating and lying its back on the floor in a process known as pinning (Trezza et al., 2010)

In rodents, social interactions (and social play among them) are also a characteristic of the post-weaning juvenile period (Bicks et al., 2020). Even although the effects of lack of social play or social play deprivation (SPD) vary considerably depending on the age of isolation and the time spent in isolation, it was described in rats that isolation during this post-weaning period, in particular from post-natal days (PND) 21 to 35 when play is acquired and later maintained, have more social deficits and cannot be counteracted by rehousing with non-isolated reared rats (Hol et al., 1999; Vanderschuren and Trezza, 2014). Similar results had been reported previously as well by Panksepp (1981): he reported that social play is increased during PND 18 to 28 and then it peaks between PND 32 and 40 until it declines gradually. These reports have helped in the development of an effective social play deprivation range. This particular range takes place during PND 21 and 42 and during this time the rats are separated by a Plexiglas wall (**Figure 3**). It has been employed satisfactorily in several studies that have addressed the behavioral effects of SPD, Baarendse et al. (2013) and Omrani et al. (2020) for instance.

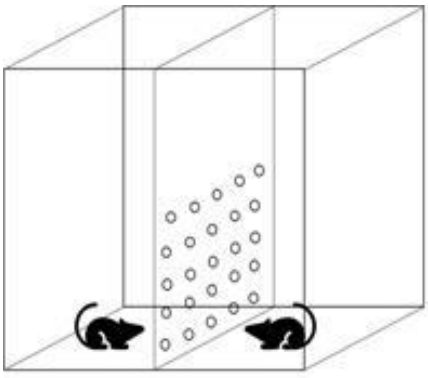

Figure 3. the rats that are subject to SPD are caged together but separated through a transparent Plexiglas wall that contains holes in order that the rats can see, hear and smell each other, but cannot play.

On the one hand, Baarendse et al. (2013) reported that after the deprivation period, it resulted in disrupted impulse control and impaired decision making. Rats were subject to the five-choice serial reaction time task (5-CSRTT) (**Figure 4**) in order to assess impulsivity (Robbins, 2002). Impulsive action, measured as premature responses in the 5-CSRTT, was enhanced in isolated rats when test conditions were unexpectedly made more demanding.

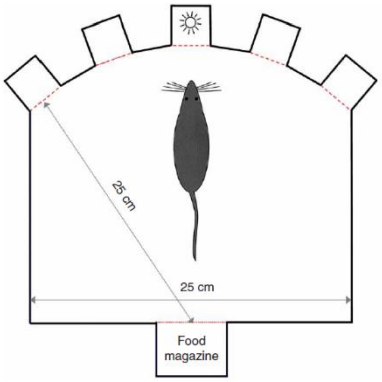

Figure 4. Schematic diagram of the 5-choice serial reaction time task chamber showing the spatial arrangement of the five response apertures in relation to the food magazine (Bari et al., 2008)

Rats were also subject to the rat-gambling task (**Figure 5**), used to assess decision making (Zeeb et al., 2009), showing how the control group developed a clear acquisition towards more advantageous choices, in comparison to the SPD group that did not. These results were backed by Vanderschuren and Trezza (2014) through an extensive review in which they stated (among other conclusions) that social isolation, during the period in life when social play behavior peaks, induces long-lasting social impairments that range from subtle changes in affiliative behavior to a profound impairment in dealing with a challenging social situation and has long lasting effects on cognitive control. At the same time, these conclusions upheld the idea previously proposed by Špinka et al. (2001) that cognitive deficits are most apparent in challenging or difficult situations.

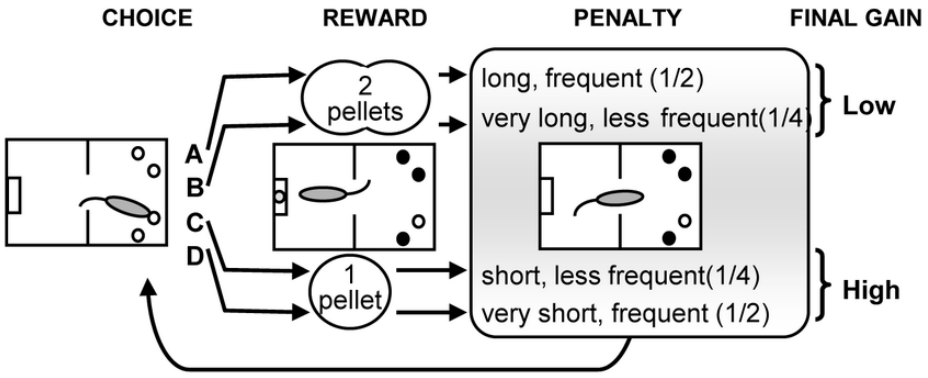

Figure 5. Principle of the Rat Gambling Task. Rats can nose-poke among four different holes in an operant cage, to earn food reward. Two options (C, D) are rewarding and are equally more advantageous than the other two (A, B), which are equally disadvantageous in the long term (Rivalan et al., 2013).

On the other hand, Omrani et al. (2020) studied the impact of SPD on cognitive flexibility in rats through the performance in a probabilistic reversal learning task (**Figure 6**). Even if the control group and the SPD group achieved the same number of rewards, trial-by-trial analysis of the computational data showed that the control group was best described by a

learning-based model whereas the SPD group completed more reversals due to a simpler, heuristic approach.

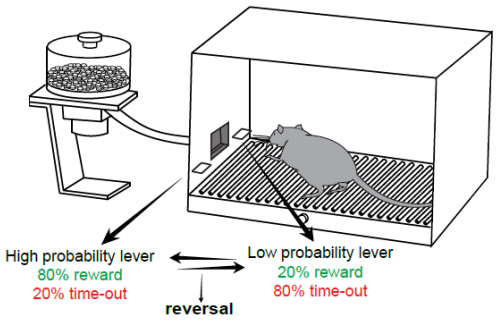

Figure 6. Probabilistic reversal learning task design. After 8 consecutive responses on the high probability lever the reversal takes place (Omrani et al., 2020).

These studies demonstrate that SPD disrupts impulse control, impairs decision making, simplifies cognitive strategies and increase responsiveness towards adulthood in rats. But now I cannot help wondering one thing: what will happen then with Norah?

Well, unlike with rats, no study has assessed directly the effects of SPD on humans. Nevertheless, from an evolutionary perspective, social play is highly conserved as social activity is indispensable for survival (Fattore et al., 2010), in fact, if the cost of play outweighed the benefits, playful individuals would be at an evolutionary disadvantage to conspecifics that were not playing at all (Held and Špinka, 2011). Out of the living 19 placental orders 16 are known to be engaged in any sort of play, and 14 in social play (Burghardt, 2005) and it is a predominantly juvenile behavior regardless of the mammal species (Graham and Burghardt, 2010).

Therefore, it is possible to argue that despite the notorious physiological differences among rodents and humans, it is not such a big step to compare social play behavior between them. In fact, rats are particularly useful model for mammalian R&T play, since this rodent species shows ample social play that is easy to recognize and quantify (Vanderschuren and Trezza, 2014), as noted before through pinning and pouncing.

Despite the fact that we do not know the exact damage that social play deprivation will have on Norah, it has been reported that survivors of childhood cancer, among others, have a higher propensity to develop neurocognitive problems and learning disabilities (Peckham, 1991) as well as difficulties in social functioning (Nijhof et al., 2016; Northman et al., 2015). These problems are, in part, thought to be the result of the (chronic) stress associated with the disease and its consequences. In short, not being able to participate in various social

playful activities puts a strain on a child's adaptative capacity and resilience (Nijhof et al., 2018) and this is probably the fate that awaits Norah.

1.2. Brains out

The research focused on lack of social play and its effect on behavior and, especially, the way in which different brain areas such as the prefrontal cortex (PFC) are implicated have not been properly conducted until recent years. The lack of knowledge in this regard has, probably, led to the inability to develop therapies to improve the deleterious effects that SPD causes in socialization and cognition in chronic diseased kids like Norah.

Nevertheless, we know today that the PFC in rodents is altered by SPD (as I explain in further detail in sections **1.3** and **1.4**), in addition, it has also been known for a long time the PFC plays a fundamental role in behavior and cognition.

Despite the fact that there are no assessments of SPD in humans, if the hypothesis that SPD leads to neurobiological changes in the PFC, it would not be the only way to "obtain" those deficits, as any damage to the PFC during the adulthood has been proven to lead to this kind of deficits. In particular, Phineas Gage's unfortunate accident, and to some extent Moniz's controversial surgery, played a relevant role in setting the bases to understand the relation between the prefrontal cortices and personality and cognition, and nowadays the neurobiology of SPD as well.

Phineas Gage (1823-1860) was a foreman for a railway development in Vermont, USA, when a metallic bar went through his brain provoking a major brain injure in the frontal lobe (Harlow, 1848). He survived, nevertheless, the most striking feature was his profound personality change after his recovery — without other apparent neurological deficits, apart from a somewhat diminished intelligence — that led his friends to say he was "no longer Gage" (O'Driscoll and Leach, 1998). He would die 13 years later in status epilepticus probably by the consequences of his accident.

This case suggested for the first time that the prefrontal cortex was involved in some way in emotion and personality, and that these functions are dissociable in the brain from many other types of function (Rolls, 2019). Indeed, measurements on the injured brain of Gage by Damasio et al. (1994) showed that the area known as prefrontal cortex, and in particular its subarea the orbitofrontal cortex (OFC), had been the most affected area as a consequence of the accident.

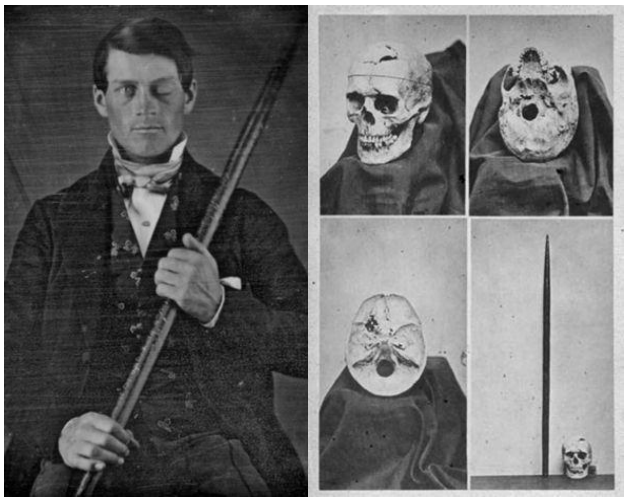

Figure 7. On the left, Phineas gage along with the iron rod that went through his skull. On the right, Gage's skull with the visible holes in the frontal lobe as a consequence of the accident.

With regard to the relevance of the prefrontal cortex in cognition, it was highlighted about 90 years later when Antonio Egas Moniz (1874-1955) in 1936 shed light on a new neurosurgical procedure that apparently would help treat several medical conditions such as anxiety, irrational fears and emotional hyperexcitability: the lobotomy, which according to Encyclopaedia Britannica (2020) is a surgical procedure in which the nerve pathways in a lobe or lobes of the brain are severed from those in other areas and this typically takes place in the prefrontal cortex. Alongside the deleterious effects in the social capacities of the patients, several researchers such as Rosvold and Mishkin (1950) and Vaysettes (1976) reported a reduction of intellect after the surgery was performed. In addition, Vaysettes also addressed that, by the time Moniz had proposed his technique, it was already known that mutilation of the frontal lobe risked undermining intellect.

Therefore, it cannot be negated the long-lasting intellectual and social consequences of any alteration upon the prefrontal cortex, whether these alterations occur due to an accident, a surgical proceeding or, potentially, because kids like Norah cannot play with peers and thus, their PFC cannot properly develop. Perhaps, all in all, Norah should consider herself lucky since neither an iron bar nor an icepick went through her skull.

1.3. Our prefrontal cortex wants us to play

The PFC is one of the few basic cortices present in all mammals that comprise the neocortex (Kaas, 1987), the part of the brain responsible for the execution of higher-order brain functions, including cognition, sensory perception and sophisticated motor control (Lodato and Arlotta, 2015). The PFC is also one of the latest to fully develop: the dendritic spine density does not get reversed until the third decade of life (Petanjek et al., 2011) and

overproduction of spines is greatest in the PFC, which then shows the slowest rate of synapse elimination (Elston et al., 2009).

The PFC is particularly known for mediating the so-called executive functions which are known to be highly correlated with both academic and life success (Gibb and Kolb, 2015). There is a general agreement that there are three core executive functions: (1) inhibitory control, that enables to control one's behavior to avoid being at mercy of impulses and therefore to do what is more appropriate; (2) working memory, that consists in holding information and mentally working with it; and lastly (3) cognitive flexibility which builds on the other two and allows us to overcome inertial tendencies so we can switch between mental sets or ways of thinking about the stimuli. From these, higher order executive functions are built such as reasoning, problem solving and planning (Diamond, 2013).

Anatomically, the PFC can be regarded as the region of the cortex that receives its principal thalamic inputs from the mediodorsal nucleus of the thalamus, located somewhere at the anterior end of the cerebral hemisphere (Kolb et al., 2012). Regardless of the mammal species, it is typically accepted that the PFC is subdivided in three functional areas**:** the medial prefrontal cortex (mPFC) is involved in processing and integration of affective and social information (Grossman, 2013), the lateral prefrontal cortex (L-PFC) appears to play more relevant roles in integrating cognitive and motivational context (Watanabe and Sakagami, 2007), and the OFC is known to be involved in processing stimulus-reward association (Kobayashi et al. 2009).

Notwithstanding, most of the research regarding the PFC and SPD has been focused on the mPFC and its role in cognitive flexibility. Since we are not in a period of history where we can ignore human rights, and hence, assess Norah's or other diseased kids' brains directly, the use of laboratory rats as models is the main source of knowledge regarding the neurobiology of SPD.

Baarendse et al. (2013) reported that early post-waning social experience is critical for the development of both cognitive capacities and mPFC function in rats. SPD during PND 21-42 caused long-lasting cellular and synaptic changes in the pyramidal cells (PCs) in the mPFC that continued even after re-socialization periods: whole-cell recordings in slice from adult animals showed that early social isolation caused mPFC PCs to lose sensitivity to modulation of synaptic response amplitude by dopamine (DA) and compromising the DA function required for coping with sudden changes in task requirements.

This phenomenon can be explained due to the essential role DA, a neurotransmitter widely known for being involved in rewarding and motivational processes, plays on cognitive control functions in the PFC (Ott and Nieder, 2019) as it is a critical modulator of the efficacy of both excitatory and inhibitory synaptic activity in this brain area (Seamans and Yang, 2004). Furthermore, social play has been described as a highly pleasurable and rewarding activity (e.g., Trezza et al., 2010) and an optimal level of dopamine is required for the expression of play behavior, as either stimulation or reduction in DA neurotransmission can disrupt social play behavior (Vanderschuren et al., 2016).

Two independent studies backed the results regarding biological changes in the PCs in the mPFC. First, Bicks et al. (2020) demonstrated that juvenile social experience is required in the parvalbumin (PV) interneurons (IN) in mouse dorsomedial PFC to develop typical adult activity patterns, demonstrating that social play plays a major role in the maturation of the morphology and plasticity of neurons in the mPFC. Second, Omrani et al. (2020) reported that the PFC undergoes analogous experience-dependent maturation and that the experience of social play behavior is important for this process, as they encountered that inhibitory synaptic input to the layer 5 (L5) PCs of the mPFC is decreased and persistent, along with cognition changes.

1.4. Tales about the orbitofrontal cortex

In this master's thesis I have focused my interest in the very area that got torn apart in Gage's case, the OFC. In contrast to the ample mPFC research in SPD, the effect of SPD in the OFC has been considerably less studied. However, given its role in stimulus-reward association and rewarding nature of social play, we consider that this area of the brain is of interest.

The OFC lies in the anterior, ventral surface of the frontal lobe, and receives connections from all sensory modalities, from limbic circuitry, and weakly, from motor areas (Kolb, 1984). As said, it is known the OFC is in charge of the rewarding and motivational aspects of decision-making, statement that was upheld by Wallis and Miller (2003) when they confirmed that the OFC is the first prefrontal region to receive the information about a rewarding stimulus**.** One important function of the OFC is olfaction in which information about the identity and reward value of odors is represented (Rolls, 2000) and this information is used to guide behavior and especially social behavior (e.g. Schoenbaum et al., 2009). In humans, functional activity of the OFC is crucial for flexible decision-making behavior (Hornak et al., 2004) and it has been observed that neural activity in the OFC also inhibits impulsive

aggressive behavior in rats, monkeys and humans (Burleson et al. 2016). In addition, while stress has been shown to reduce spine density in the OFC (Murmu et al., 2006) Burleson et al. (2016) ruled out the fact that SPD disrupts the dendritic morphology of OFC.

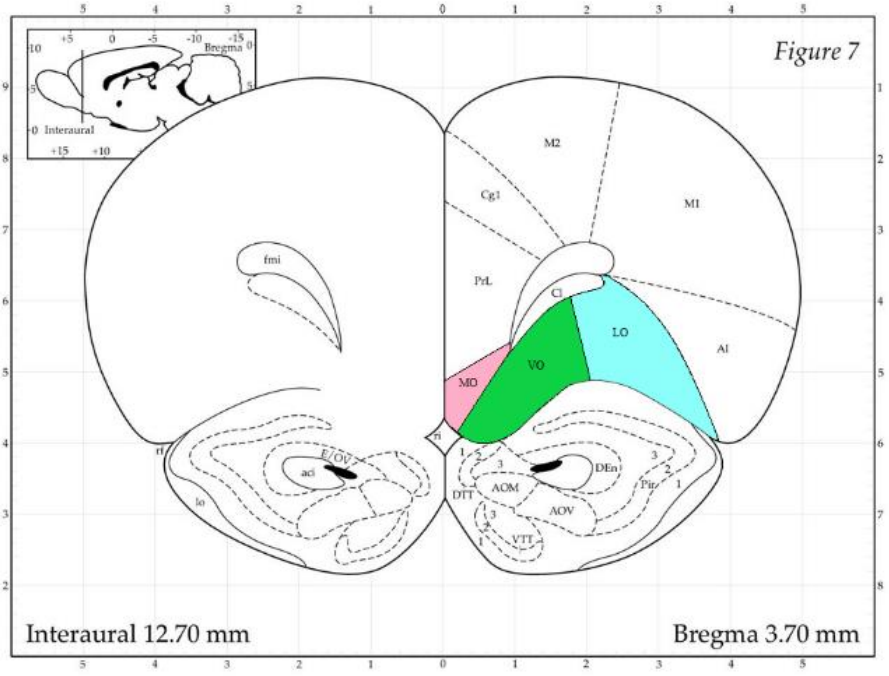

Figure 8. Stereotactic depiction of the brain at Bregma 3.7mm. MO: medial OFC, VO: ventral OFC and LO: lateral OFC (Paxinos et al., 2006).

In 2006, Pellis et al. in a series of experiments explored at different ages the effects OFC lesions on the defensive response of two different types of behavior. Through neonatal lesions the researchers assessed the effects during the juvenile period and adulthood in play fighting. On the contrary the lesions performed in adults were not only used to assess the effects play fighting, but in non-playful social interactions as well. They showed that damage to this section of the brain is more likely to interfere with how behavior patterns are used rather than with the ability to produce them regardless of the type of social behavior in which they might be involved.

Later, in 2010, Bell et al. reported that multiple peer play promotes the proliferation of basal dendrites in the OFC. In addition, being raised with multiple adults or multiple juveniles was equivalent and therefore the complexity of the OFC was the same because the OFC was sensitive to experiencing with multiple partners and not to the specific content of the interactions with these animals. Their findings suggested that the OFC contributes to the production of appropriate social actions by pairing one's behavioral responses with the identity of one's social partners, and that experiencing a variety of social partners facilitates

this ability. The OFC requires exposure to a variety of animals in order to develop the ability to distinguish among a variety of social partners. What is more, Himmler et al. (2018) showed that among non-isolated rats, those that live with more partners during the juvenile period had more complex dendrites at PND 60, even though the complexity was diminished with age so that by PND 100 the OFC cells no longer differed between rats. The studies show that social interaction is necessary so that the OFC develops properly. In particular, a higher number of interactive peers is directly related to a greater complexity at dendritic level in the OFC, although the effects disappear in the adulthood.

Verharen et al. (2020) also assessed how different regions of the PFC contribute to component processes of value-based decision-making behavior, including the medial OFC (mOFC) and lateral OFC (lOFC). Inactivation of the mOFC and lOFC impaired task performance: in the case of mOFC this was driven by reduction in punishment learning and response persistence, and in the case of lOFC it was a result of a combined reduction in reward and punishment learning.

By and large, all these studies demonstrate that in rats there is a close link between behavior and OFC and that perturbances in the OFC will also cause impairments in social domains, yet different to those observed in the mPFC, a fact that could be potentially explained by it appears that both areas follow different developmental trajectories (Hill et al., 2010; Fuster et al., 2002; Chugani and Phelps, 1991). In addition, we cannot still assure that SPD will cause deleterious effect in the OFC, however voltage clamp recordings (**Figure 9)** performed by Bijlsma (2021) points in this sense, indicating a potential decrease of the GABAergic synaptic inputs.

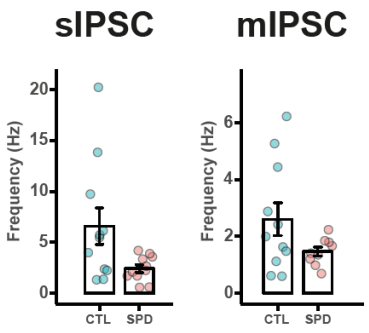

Figure 9. both spontaneous IPSC (sIPSC) and miniature IPSC (mIPSC) show a frequency decrease onto the L5 PCs of the OFC in the SPD group.

Nevertheless, the way in which rodent and primate brains are structured is not the same and there is no real consensus on how the PFC of rats relates to that of monkeys and humans

(Lambach et al., 2018). The PFC has undergone great development in primates and the OFC, in particular, is very little developed in rodents. Yet, it is one of the major brain areas involved in emotion and motivation in primates including humans (Rolls, 2019). The lack of the cortical layer 4 (also known as the internal granular layer) in rodents and presence of it in primates may account for these differences (Seamans et al., 2008).

Despite these profound neurobiological differences; as noted before, play is very conserved throughout mammals and since human research is very much limited and rodents express R&T play in a similar and measurable way, the use of rodents has enabled us to learn a lot about not only social play behavior, but also about the relation between SPD and PFC as well.

So far, while the behavioral effects of PFC damage in humans cannot be negated, the extent to which SPD affects the human PFC (and OFC) development, and behavior in consequence, remains unknown. Although play research on rats may not be the most adequate in terms of translation to humans, it is definitely showing us that play is important for proper behavior development and brain maturation as it provides the scientific reasoning that play should not be taken for granted. This research, most likely, will not set the bases for new treatments for Norah, however the interventions inspired by it will.

1.5. People matter, money too.

The exact toll lack of social play takes on chronically or severely diseased kids in the adulthood is difficult to quantify. Even though a lower quality of life (Stam et al., 2016), greater risk for mental health (Nijhof et al., 2018), neurocognitive problems and learning disabilities (Peckham 1991) and difficulties in social functioning (Nijhof et al., 2016; Northman et al., 2015) have been observed, no study has been conducted as for the concrete consequences of SPD in humans. Nevertheless, for both ethical and technical considerations, it is unviable to conduct either behavioral or invasive experiments on humans and this is where animal research, like the one carried in this project, plays a fundamental role in stating which are the behavioral, cognitive and neurobiological consequences of it. In this way, we can gear up ourselves with sound arguments as to why early childhood interventions are necessary and we, as a society, need to invest in them.

Yet, the need of these interventions should not blind us about the fact they should be focused more on emotional development through play, integrating skills such as speech, motor and cognitive skills, rather than focusing on these skills in isolation, as addressed by Nijhof et al (2018). Based on this, videogames, for instance, hold a huge potential as interventive tools.

Despite the fact that most videogame research has been focused on the negative aspects of them, it turns out that playing videogames have several benefits: videogames promote (1) a wide range of cognitive skills such as spatial, problem-solving and creativity skills; (2) motivational abilities such as persistence in the face of failure; (3) emotional ability to deal with frustration and anxiety; and (4) social abilities as certain videogames are designed to reward effective cooperation, support and helping behaviors (Granic et al., 2014). However, there are no studies as to the effects of applied games on depressive or anxiety symptoms of children with chronic diseases (Nijhof et al., 2018), there are very few longitudinal studies, limiting greatly the acquisition of knowledge and due to the difficult task of measuring play in kids it is rather complicated to measure the success of these interventions. In any case, video-games are promising tools that can aid diseased kids as the well-known case of Re-Mission (Kato et al., 2008).

What is more, investing in early childhood interventions such as videogames to prevent children from suffering social play deprivation not only is relevant to increase their quality of life but it is also useful to reduce the economic burden they will be for the state. Sentenac et al. (2018) observed that high school completion and post-secondary education enrollment was lower among children with neurodisabilities compared to those without neurodisabilities. This causes a reduction in their chances of future success and can lead to the necessity of the welfare state. According to the cognitive capital, an emerging paradigm for the investing in children whist neural development and reorganization are at their peak, investing in childhood interventions will result in significant return given the plasticity of the developing brain; similarly, the inverse also happens with negative stimuli leading to depreciation of the cognitive capital (Noble et al., 2017). In fact, brain plasticity and the ability to change behavior decrease over time, so getting things right the first time is less costly to society and individuals, than trying to fix it later (Shonkoff et al., 2011).

1.6. Playing with brains

In order to carry out the study of the effect that SPD has on the OFC, we have focused our efforts in the L5 of the OFC, by using staining of neural tissues and confocal microscopy.

L5 is particularly interesting for us due to the presence of large PCs which send many long-range projections to other cortical and sub-cortical structures (Harris and Shepherd, 2015; Naka and Adesnik, 2016). Traditionally, L5 has been considered primarily as an output layer, nevertheless L5 PCs also receive important thalamocortical inputs and from all cortical layers (Naka and Adesnik, 2016).

The first of the techniques to be employed in this project is immunohistochemistry (IHC). Sometimes also referred as immunostaining, IHC is a technique that utilizes antibodies to analyse histological tissues under the microscope (Kalyuzhny, 2016). When Albert H. Coons attached for the very first time a fluorescent antigen in a tissue section it meant the starting point for IHC. Ever since his initial publications in the early 40s (Coons, 1941; 1942) hundreds and thousands of scientific IHC articles have been published and IHC, alongside confocal microscopy, has become an indispensable technique for research and diagnostic applications (Papaccio and Desiderio, 2018; Buchwalow and Böcker, 2010).

Nevertheless, it should be borne in mind IHC has limitations, such as the requirement of a new sample or serial section for each analyte set, which limits multiplexing, and non-linear relationships between analyte abundance and staining intensity (when fluorescence is not used), which limits quantification (McCarthy and Birtwistle, 2019).

The second tool employed is the confocal microscope. Confocal laser scanning microscope was developed and patented by Marvin Minsky in the 50s. His innovative design included a pinhole, a small hole located in the specimen plane that allowed the removal of the out-of-focus reflected fluorescent light, improving the contrast, the definition and resolution. In consequence, confocal microscopy became rapidly a success and it is still used today (Renshaw, 2017; Montero-Miralles et al., 2017; Murphy and Davidson, 2013).

Nowadays confocal microscopy not only improves resolution, but allows as well to display the specimen in a variety of ways and to adjust the magnification electronically without having to change the objective. However, the slow imaging, and long-waiting times in consequence, can pose a major drawback that some researchers might try to avoid (Murphy and Davidson, 2013; Montero-Miralles et al., 2017).

Employing the images obtained at the confocal microscope following the stainings, we can perform the analysis of inhibitory synapses. Since the L5 is uniquely positioned to integrate nearly every local and afferent pathway in the cortex, inhibition onto L5 PCs is crucial for nearly every aspect of its function (Markram et al., 2015; Naka and Adesnik, 2016). For this analysis, we have assessed perisomatic synapses which are mostly made of PV and CCK basket cells (Whissell et al., 2015; Omrani et al., 2020) and the latter co-expresses the endocannabinoid receptor 1 (CB1R) (Katona et al., 1999; Omrani et al., 2020). The reasons behind this selection can be read in the following section (**Section 2**).

2. AIMS, HYPOTHESIS AND POTENTIAL OUTCOMES OF THIS PROJECT

Social play is necessary for brain development including the OFC. Similarly, the OFC is necessary for a proper performance of social behavior (e.g., Pellis et al., 2006; Bell et al., 2010). However, the concrete effect of SPD upon the OFC is rather unknown and this is the gap we aim to bridge: by carrying out the previously mentioned inhibitory synapse analysis in the cortical L5 of the OFC by immunohistochemical and microscopical means on these rodents' brains we aim to observe if there is a change in the number of inhibitory synapses in the L5 of the OFC of the SPD rats in comparison to the controls.

Previously, Omrani et al. (2020) reported a mIPSC frequency reduction accompanied by an increase in the rise time, which suggested that synapses at perisomatic locations were specifically affected. Although no analysis in the rise time has been conducted yet in the OFC, the already shown frequency reduction in the mIPSCs of the OFC lead us to consider that perisomatic synapses are mostly affected by SPD. Since perisomatic synapses are mostly made of PV and CCK basket cells we are focusing our efforts on these two subtypes.

Based on these previous analyses along with several reports that show the relevant role of the OFC in social behavior, we hypothesize that SPD will have a profound effect on the OFC leading to a significant reduction regarding perisomatic inhibition, which is presumed to be responsible of the electrophysiological differences.

Regardless of the outcome of these experiments, these analyses will offer a better understanding of the role of social play behavior in brain development. It will build up on previously acquired knowledge about the behavioral effects of SPD alongside the effects in the mPFC.

3. METHODS

3.1. Animals and isolation procedure

Male Lister Hooded Rats (Charles River) are used in this experiment, housed in a 12 h reversed day/night rhythm (lights on from 7pm to 7am) and continuous radio, with *ad libitum* access to food and water. Pups (n=12) arrived at the facility at PND 14 with nursing mothers. After an acclimatization period of 7 days, pups were weaned at PND 21 and randomly divided into a control group (n=6) or SPD group (n=6).

In the social isolation group, rats were pair-housed with a rat from a different mother. During PND 21-42 a transparent Plexiglas wall containing small holes was placed in the middle of their home cage in order to create two separate but identical compartments. This enabled the rats to see, smell and hear one another but they were unable to socially engage. During this same period, control rats were also housed in pairs. On PND 42, the screens were removed from cages of rats in the SPD group, allowing for resocialization until adulthood.

Rats were weighed and handled at least once a week throughout the course of the experiment. All experiments were approved by the Animal Ethics Committee of the Utrecht University and were conducted in agreement with Dutch laws (Wet op de Dierproeven, 1996) and European regulations (Guideline 86/609/EEC).

3.2. Tissue collection

On PND 75, rats were euthanized with Nembutal (i.p. 240mg/kg) and perfused with paraformaldehyde. The brains were extracted, cryoprotected with 30% saccharose solution and stored at -80ºC until slicing. Brains were cryosectioned with a Cryostat Leica CM 3050 S, cutting 20 micrometer OFC sections between bregma levels 4.7 and 2.7 mm. These sections were stored at -80ºC until IHC was performed.

3.3. Immunohistochemistry

A session of IHC **(Table 1)** is carried out in two days, as the incubation of the primary antibody takes place overnight. Once the staining is performed, the slices can be stored at 4ºC until image acquisition.

Table 1 – Step by step explanation of a IHC session.

DAY 1	
STEP	TIME REQUIREMENT
Slices are taken out from the freezer and let dry at room temperature.	1 hour
The slices are washed three times in phosphate buffer solution (PBS) three times for 15 minutes.	45 minutes
Slices are immersed in sodium citric acid buffer (SCAB) with tween and cooked in the microwave at 97 degrees.	13 minutes
Slices are let cool down at 4ºC.	30 minutes
The slices are washed three times in PBS for 15 minutes.	45 minutes
The slices are blocked with 400 µl of blocking buffer (10% goat-serum and 0,2% triton in 1x PBS) per slide at RT in a wet chamber and afterwards dried.	2 hours
The slices are washed three times in PBS for 15 minutes.	45 minutes
250 µl primary antibody solution in blocking buffer is added per slide and incubated overnight in a wet chamber at 4 degrees.	Overnight
DAY 2	
STEP	TIME REQUIREMENT
The slices are washed three times in PBS for 15 minutes	45 minutes
260 µl secondary antibody solution in blocking buffer is added per slide and incubated for two hours in a wet chamber at RT.	2 hours
The slices are washed three times in PBS for 15 minutes.	45 minutes
The stained slides are mounted and let dry.	30 minutes

On the present project, we carried out the IHC for inhibitory synapse analysis. The antibodies employed can be read in **Tables 2** and **3**. NeuN, was used to identify PCs and determine their perisomatic area. VGAT (vesicular GABA transporter) is a membrane protein expressed in all inhibitory synapses, thus used as a general inhibitory synapse marker. Lastly, PV and CB1R were used to select their respective synaptic subtype, due to their presence in inhibitory perisomatic synapses as mentioned earlier in **Sections 1.5.** and **2**.

TABLE 2 – Primary antibodies for inhibitory synapse analysis

Host	Epitope	Concentration	Company	Order number
Chicken	VGAT	1:1000	Synaptic system	131006
Rabbit	PV	1:1000	Life technologies	PA1933
Guinea Pig	NeuN	1:500	Milipore	ABN90
Mouse	CB1R	1:1000	Synaptic system	258011

TABLE 3 – Secondary antibodies for inhibitory synapse analysis

Host	Epitope	Concentration	Company	Order number	Fluorophore
Goat	Anti-rabit	1:500	Life technologies	A31556	Alexa fluor – 405
Goat	Anti-mouse	1:500	Life technologies	A11029	Alexa fluor – 488
Goat	Anti-guinea pig	1:500	Life technologies	A11075	Alexa fluor – 568
Goat	Anti-chicken	1:500	Life technologies	A21449	Alexa fluor – 647

3.4. Image acquisition and analysis

A Zeiss laser scanning confocal microscope (LSM700) was employed to collect images of the brain slices for the analysis. The analysis conducted has been inhibitory synapse analysis that allows the quantification and comparison of the inhibitory synapses. Z-stacks (102x102μm^2; 1024x1024 pixels) were acquired at 63x in L5 of the ventral and lateral OFC (**Figure 11)** on steps of 0.4μm for a total of 12μm.

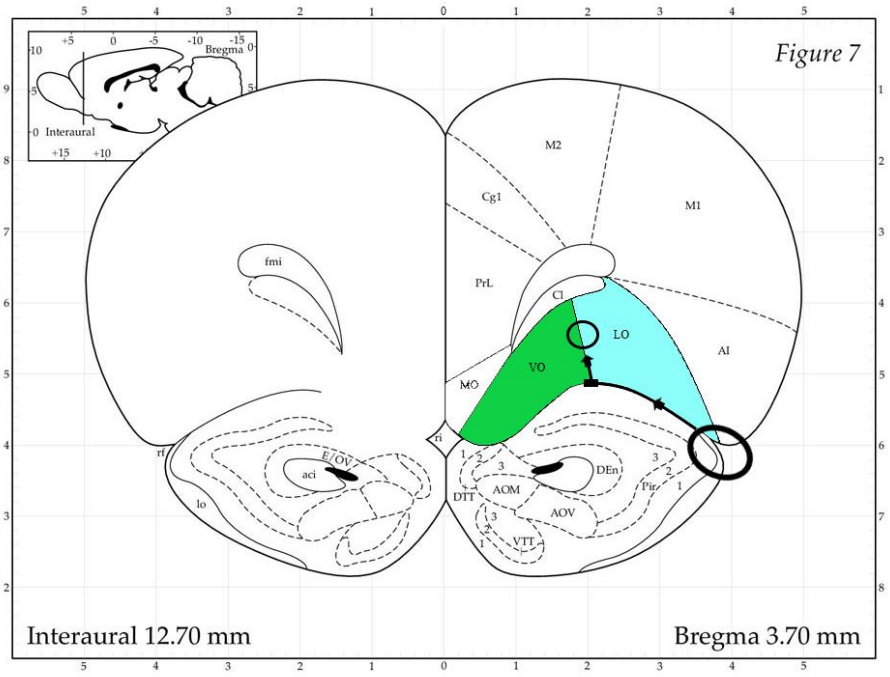

Figure 11. Schematic outline to reach the L5 of the OFC. The fissure between the cerebellum and the cortex is, first, localized, then the objective needs to be moved towards the highest point in the fissure and lastly, it must be moved upwards and stopped once large PCs are present, as they are mostly common in L5 **(Paxinos et al., 2006).**

For each of the rat brains 2 z-stacks were obtained, one for each hemisphere. Representative confocal images of the synapse stainings in the OFC can be found on **Figure 12.**

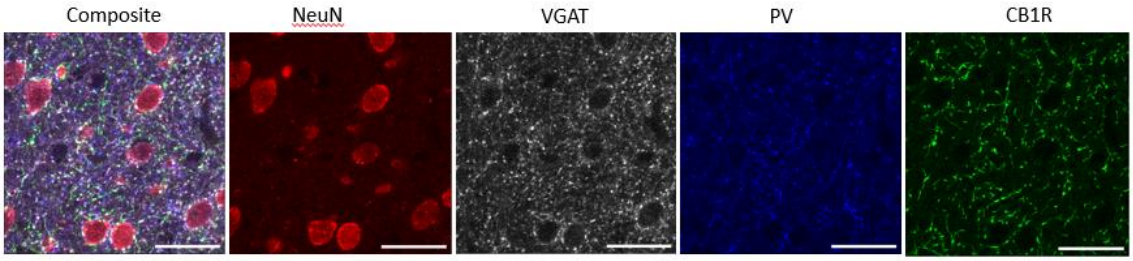

Figure 12. Representative confocal images of OFC cortical L5 synapse immunostainings used for synapse analysis. Objective used: 63x. Scale bar is 3µm.

As shown in **Figure 13**, once we obtained the z-stacks from the OFC, an average intensity projection was constructed from 5 z-stacks slices for each ROI analysed, analysing 3 to 4 ROI per z-stack and a 1.5µm-wide band around the NeuN outline was made. Subsequently, the image was median filtered (radius of 2 pixels) and then thresholded in the VGAT channel (threshold value=2200 pixels) in order to identify all inhibitory synapses. PV and CB1R puncta were only considered as synapse when colocalized with VGAT as synapse (**Figure 14**). Thresholds were also applied to PV and CB1 channels (7000 and 4500 respectively). Nevertheless, these three thresholds were indicative tools purely, relying on the eye of the investigator for this task.

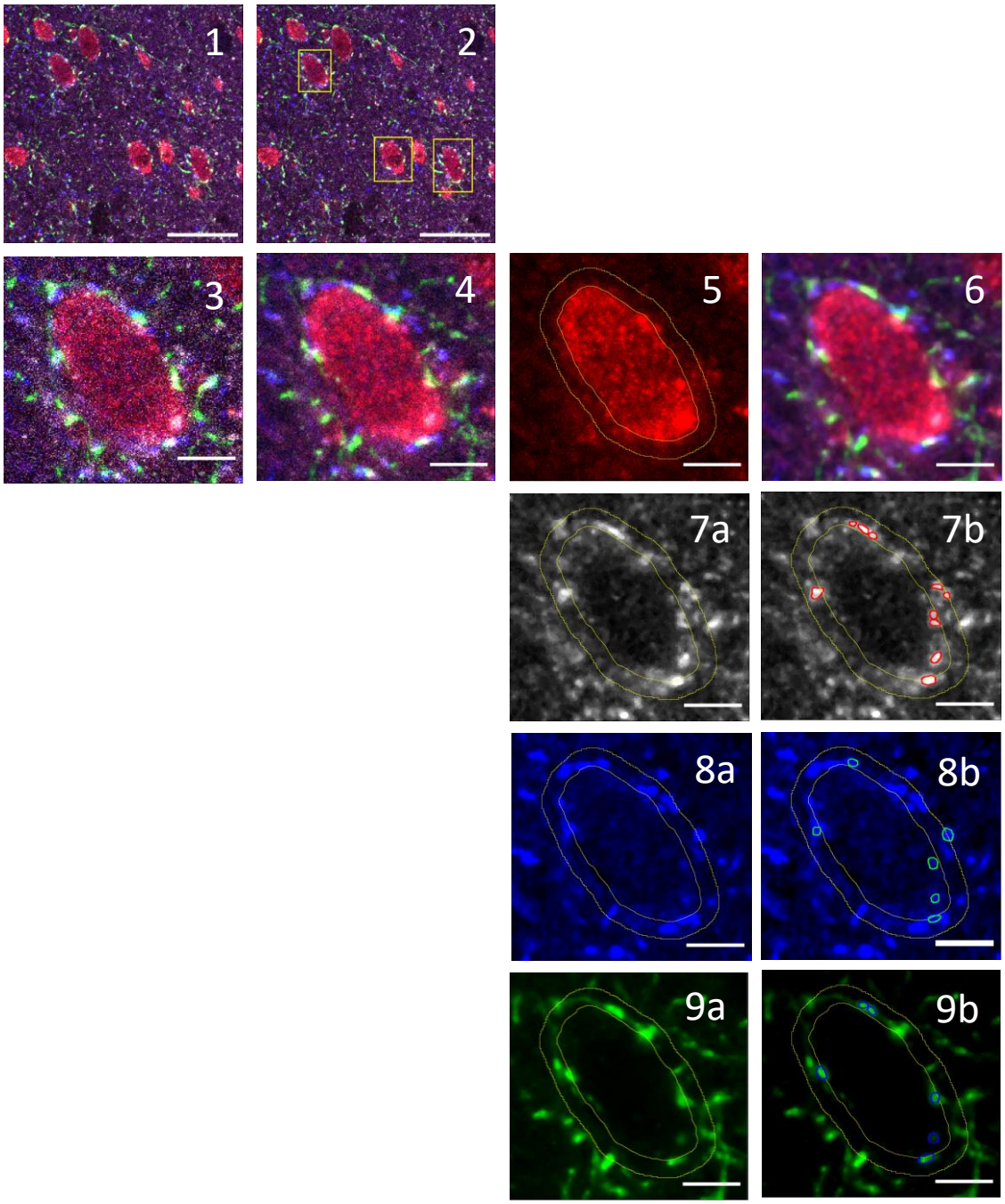

Figure 13. Synapse count analysis workflow. 1: Representative composite image of a z-stack. 2: selection of the neurons to be analysed. 3: Composite close-up of a cell of interest. 4: Average projection of 5 z-stack slices. 5: Construction of the 1.5µm-wide band around the cell of interest using NeuN channel. 6: Composite close-up of a cell of interest following medial filtering and formation of the band. 7: VGAT channel close-up and selection of the VGAT+ synapses. 8: PV channel close-up and selection of the PV+ synapses. 9: CB1R channel close-up and selection of the CB1R+ synapses. Only, colocalized CB1R puncta with VGAT synapses are selected. Scale bar in images 1-2 is 30µm. Scale bar in images 3-9 is 5µm.

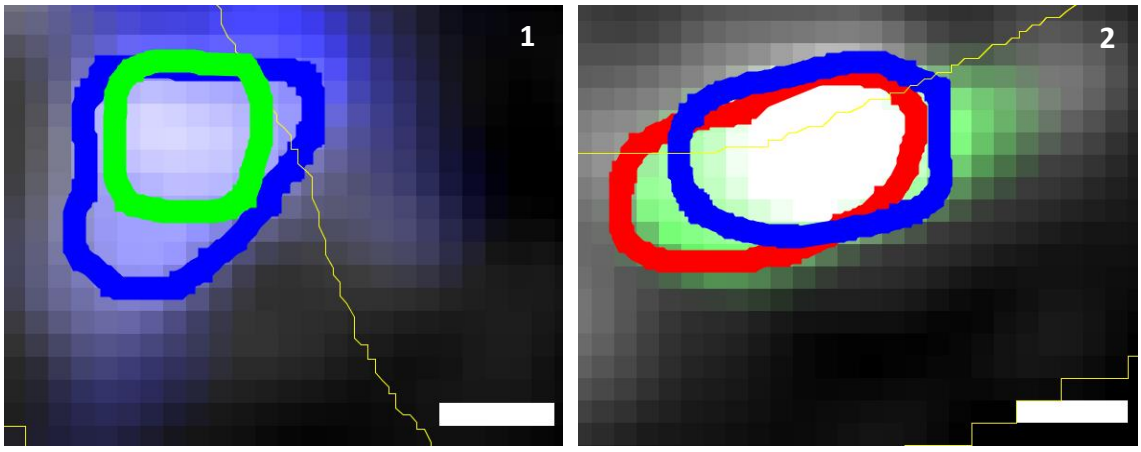

Figure 14: Synapse colocalization close-ups. 1: VGAT (blue line) and PV (green line) colocalization. 2: VGAT (blue line) and CB1R colocalization (red line). Scale bar is 0.5µm.

For both the image acquisition and analysis the researcher was blinded.

The inhibitory synapse analysis has been conducted by the analysis of the following parameters and the statistical comparison of their means for each marker between both groups.

a) Synapse density analysis: It shows if there is a significant difference regarding the concentration of perisomatic synapses in the band of the control and SPD groups. Synapse density (D) is calculated as follows:

$$D = \frac{D\ ROI_1 + \cdots + D\ ROI_n}{Total\ number\ of\ ROIs}$$

$$D\ ROI_x = \frac{Number\ of\ synapses}{Area\ of\ the\ perisomatic\ band}$$

b) Synapse intensity analysis: It shows if there is a significant difference in the intensity of the perisomatic synapses of the control and SPD groups. Synapse intensity (I) is calculated as follows:

$$I = \frac{I^1_{ROI_1} + I^2_{ROI_1} + \cdots + I^n_{ROI_1} + \cdots + I^n_{ROI_m}}{Total\ number\ of\ synapses}$$ [1]

c) Synapse size analysis: It shows if there is a significant difference regarding the size of synapses between the control and SPD groups. Synapse size (S) is calculated as follows:

$$S = \frac{S^1_{ROI_1} + S^2_{ROI_1} + \cdots + S^n_{ROI_1} + \cdots + S^n_{ROI_m}}{Total\ number\ of\ synapses}$$ [2]

[1] $I^x_{ROI_y}$ = Synapse intensity of the x[th] synapse to be analysed in the y[th] ROI.

[2] $A^x_{ROI_y}$ = Synapse area of the x[th] synapse to be analysed in the y[th] ROI.

d) Synaptic area analysis: It shows if there is a significant difference regarding the sum of the area that synapses in a ROI take up on the band between control and SPD groups. Synaptic area percentage (A) is calculated as follows:

$$A = \frac{A\ ROI_1 + \cdots + A\ ROI_n}{Total\ number\ of\ ROIs}$$

$$A\ ROI_x = \frac{Sum\ of\ the\ area\ of\ all\ synapses}{Area\ of\ the\ perisomatic\ band}$$

e) Synapse number analysis: It shows if there is a significant difference regarding the total amount of synapses in a ROI between control and SPD groups. Synapse number (N) is calculated as follows:

$$N = \frac{N\ ROI_1 + \cdots + N\ ROI_n}{Total\ number\ of\ ROIs}$$

$$N\ ROI_x = Sum\ of\ all\ synapses$$

The statistical analyses were performed in SPSS Statistics by using Welch t-test or Mann-Whitney U test depending of the normality of each parameter for each synapse marker.

4. RESULTS

4.1. Inhibitory synapse analysis

To determine the effect of SPD in the perisomatic inhibition, we have carried out the following analyses. The statistical data may be found in **Annex 1**.

4.1.1. Synapse density analysis

Synapse density is not altered following SPD. Neither the OFC L5 overall nor the separate assessment of the hemispheres separately show a significant alteration in any of the synaptic markers following SPD (**Figure 15**).

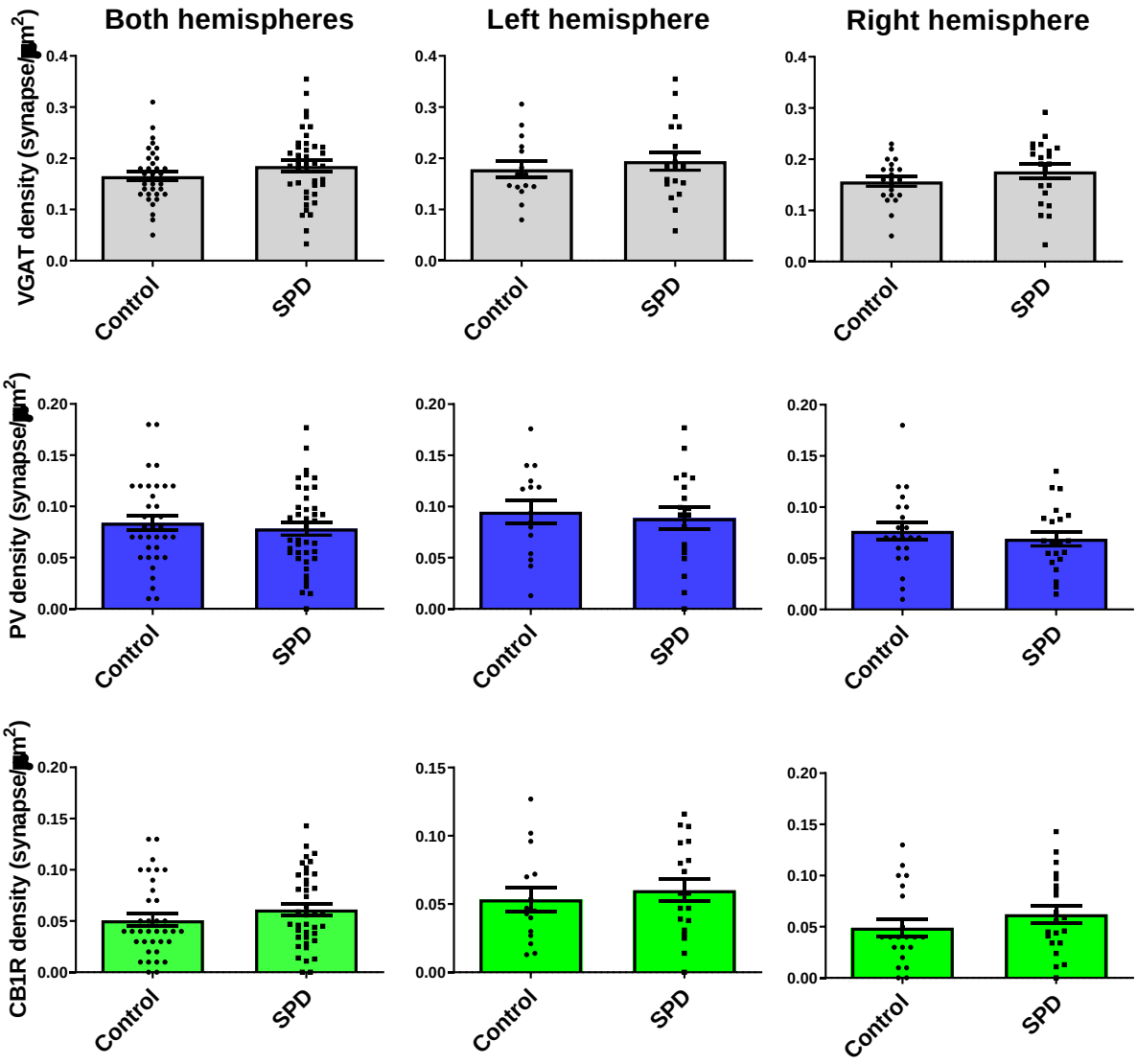

Figure 15. Results of the synapse density analysis. No significant alteration was observed in any condition. Statistical analysis performed with Welch test for every condition following a normal distribution with unequal variances, except for CB1R comparison of both hemispheres together and right hemispheres alone following a non-normal distribution.

4.1.2. Synapse intensity analysis

The intensity of the fluorescence emitted by the secondary antibodies (**Figure 16**) is significantly reduced in VGAT+ synapses ($p<0.001$) of the SPD group for a value of 23% when assessing the OFC L5 overall. On the other hand, PV+ and CB1R+ synapses do not suffer any alteration following SPD, when assessing the OFC L5 overall. Nevertheless, PV+ and CB1R+ synapses, as well as VGAT+ synapses, suffer significant alterations when assessing the hemispheres separately.

In the assessment of the left hemisphere, we can observe that VGAT+ intensity is reduced a 56%, PV+ intensity is reduced a 30% and CB1R+ intensity a 29% ($p_{VGAT}<0.001$, $p_{PV}<0.001$ and $p_{CB1R}<0.05$). However, in the assessment of the right hemisphere we can observe intensity increase for all synaptic markers: VGAT+ intensity is increased a 21%, PV+ intensity is increased a 19% and CB1R a 24%. The opposite effect of SPD in the hemisphere renders the intensity differences found in PV+ synapses and CB1R+ synapses insignificant. However due to the strong decrease in VGAT+ intensity in the left hemisphere, the slight increase in the right hemisphere is not strong enough to render it insignificant.

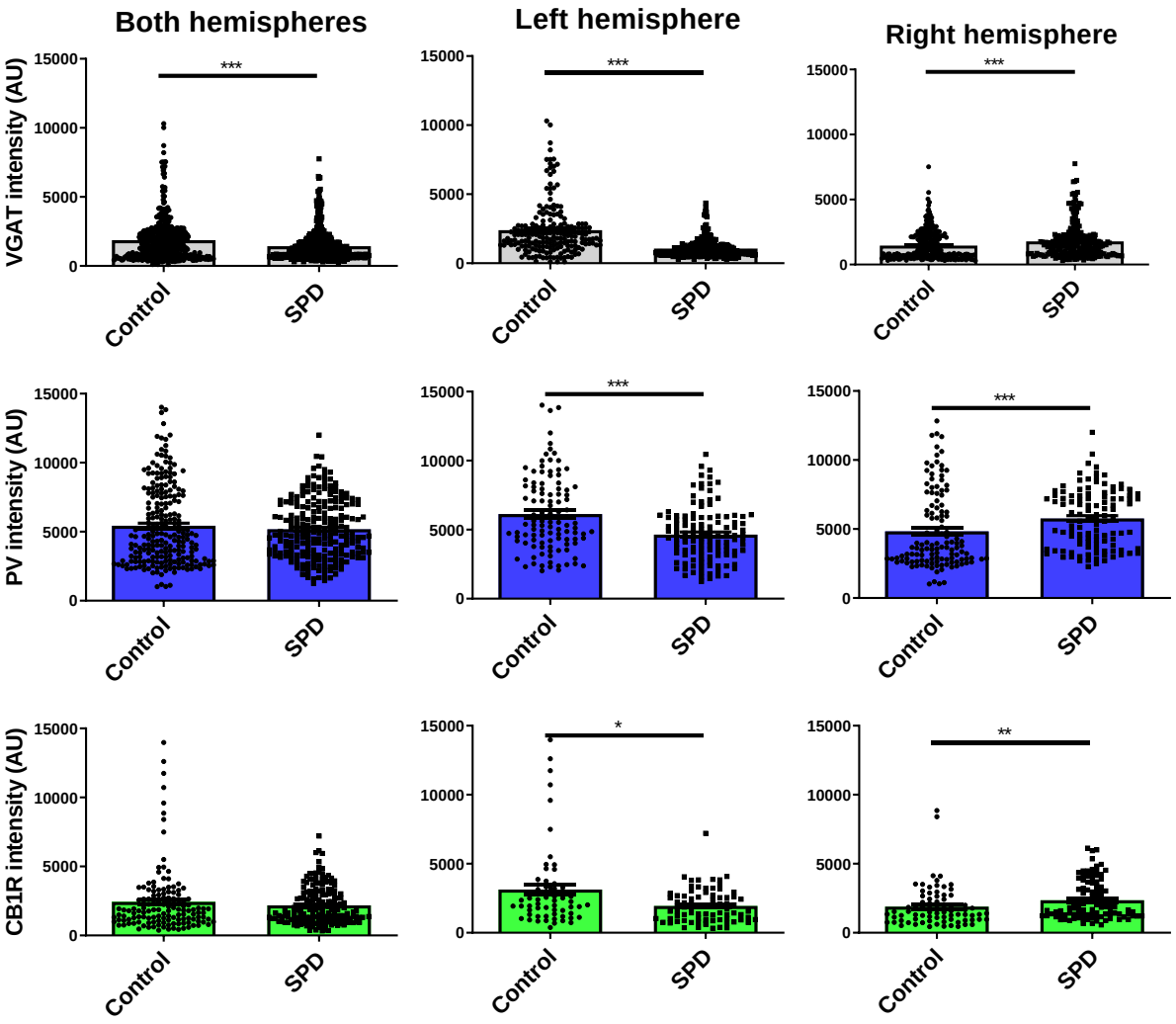

Figure 16. Results of synapse intensity analysis. Several significant alterations observed. Statistical analysis performed with Mann-Whitney U test following non-normal distribution in all conditions. Difference between control and SPD: *p<0.05, **p<0.01, ***p<0.001.

4.1.3. Synapse size analysis

The area of neither VGAT+ nor PV+ nor CB1R+ synapses suffer from any significant alteration following SPD when assessing the OFC overall (**Figure 17**). Nevertheless, in a similar manner to the synapse intensity, we can observe relevant alterations in all the markers, when assessing the hemispheres independently.

In the assessment of the left hemisphere the size of VGAT+ synapses is reduced a 14%, that of PV+ synapses is reduced a 15% and the size of CB1R synapses a 19% as well ($p_{VGAT}<0.001$, $p_{PV}<0.05$, $p_{CB1R}<0.05$). Yet, the assessment of the right hemisphere sheds opposite results: the size of VGAT+ synapses increased a 20%, the size of PV+ synapses a 28% and that of CB1R+ synapses another 8%. However, it is important to address the results

were only significant for VGAT+ and PV+ synapses ($p_{VGAT}<0.001$, $p_{PV}<0.01$). Therefore, it is not possible to ensure that SPD is the cause of the non-significant increase.

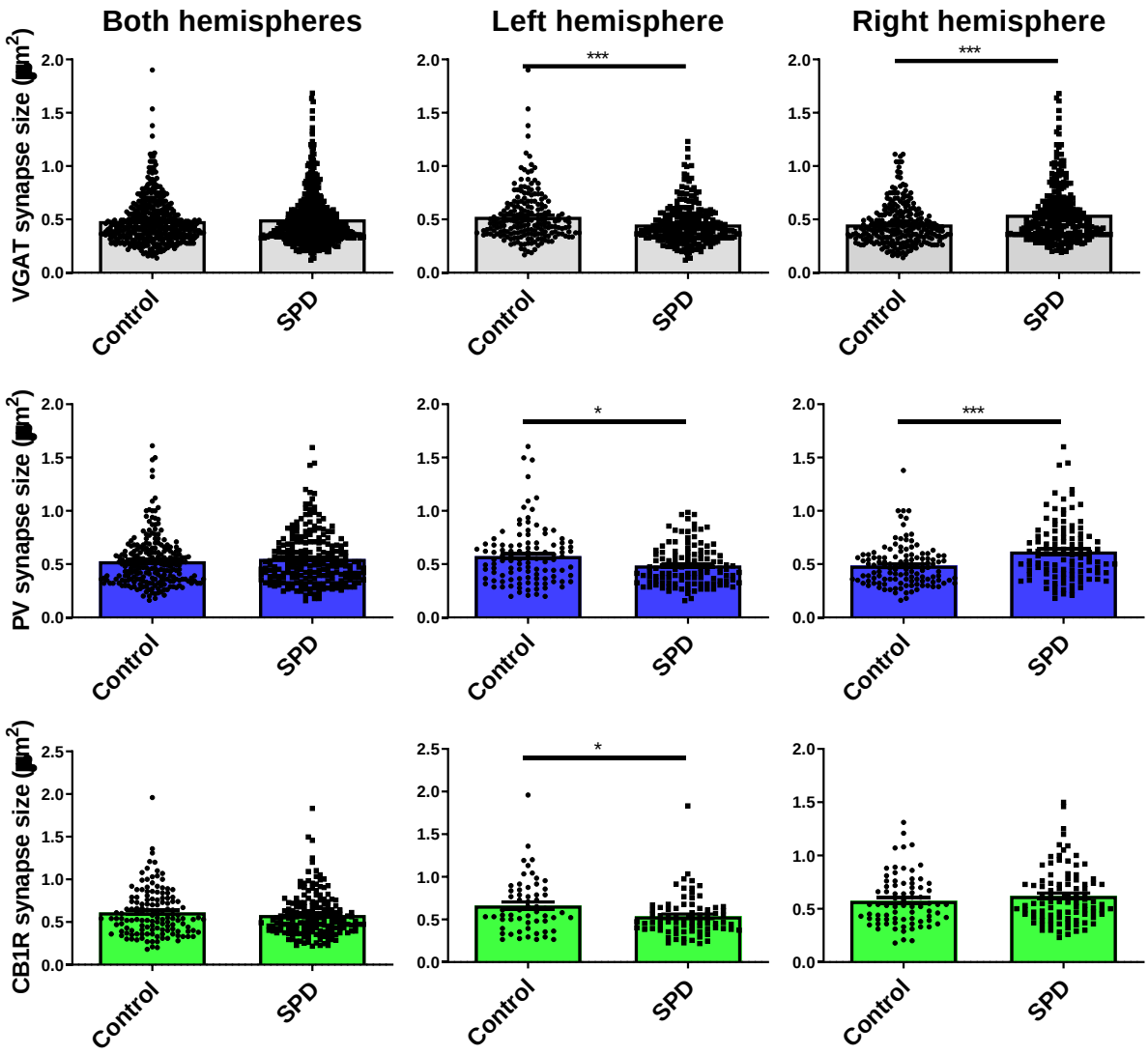

Figure 17. Results of synapse size analysis. Several significant alterations observed. Statistical analysis performed with Mann-Whitney U test following non-normal distribution in all conditions. Difference between control and SPD: *p<0.05, ***p<0.001.

4.1.4. Synaptic area analysis

While the number of synapses in a given area (synapse density analysis) may be not be altered following SPD, the percentage of band that these synapses take up is of interest to show differences that otherwise could not be observed.

When assessing the area that the synapses take up on the band in the L5 OFC overall (**Figure 18**), we can observe that there is no significant alteration in any of the synaptic markers following SPD. Nonetheless, VGAT+ synaptic area is increased from 7.7% of the band to 9.1%, for a p value of 0.075. While this value does not reach the 0.05 limit in order that it

can be considered significant, when assessing the hemispheres separately, the right hemisphere shows a significant increase ($p_{VGAT}<0.01$) in the synaptic area of VGAT synapses from 7.1% to a 9.4% of it.

On the contrary, VGAT+ synapses do not suffer from a change following SPD in this parameter on the left hemisphere. The hemisphere analysis for PV+ and CB1R+ synaptic area did not show any significant alteration following SPD in any of the hemispheres.

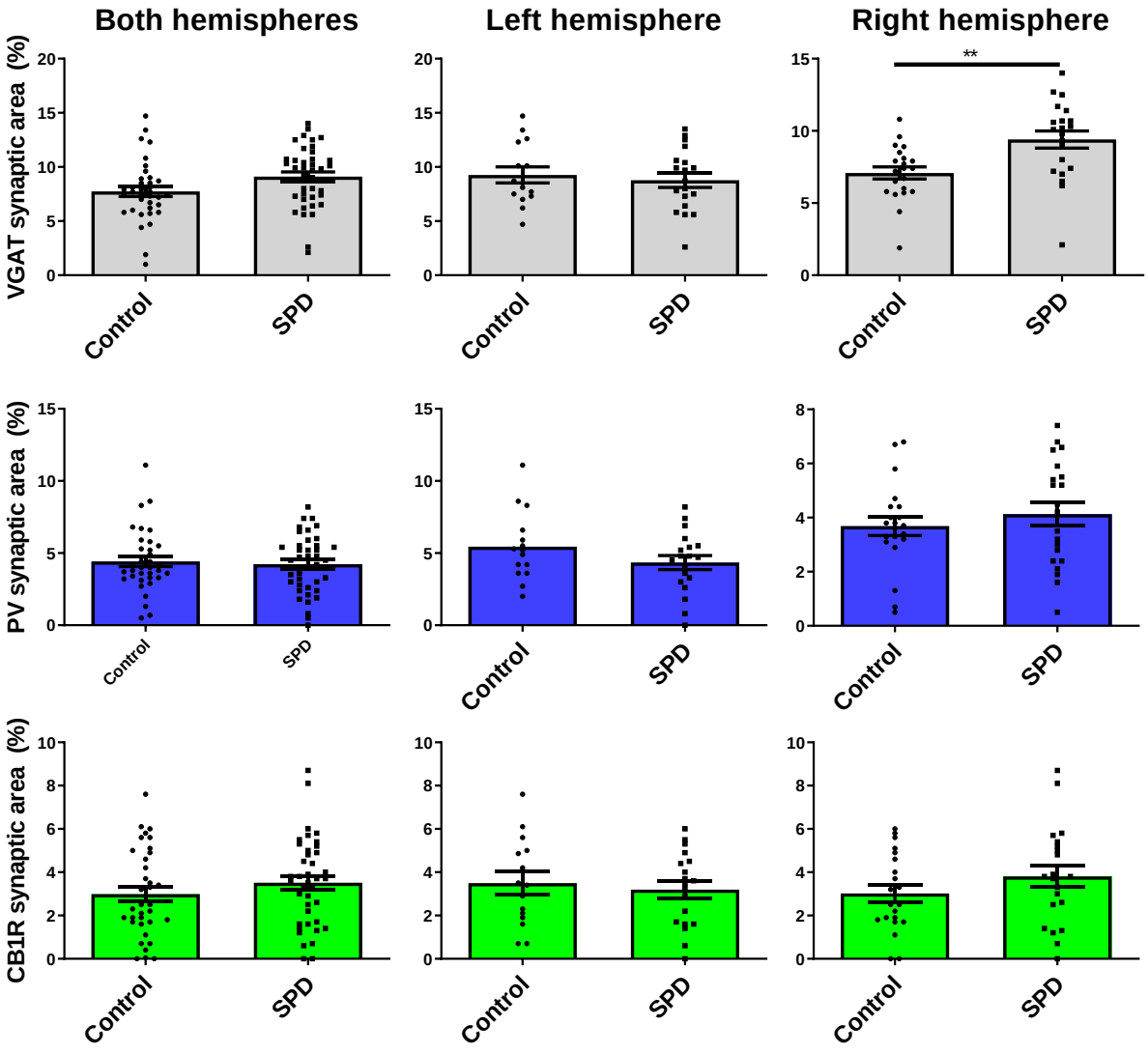

Figure 18. Results of synaptic area analysis. VGAT synaptic area is significantly increased in the right hemisphere. Statistical analysis performed with Welch test following normal distribution with unequal variances in all conditions. Difference between control and SPD: **p<0.01.

4.1.5. Synapse number analysis

Synapse number is not altered following SPD. Neither the OFC L5 overall nor the separate assessment of the hemispheres separately show a significant alteration for this parameter in any of the synaptic markers following SPD (**Figure 19**).

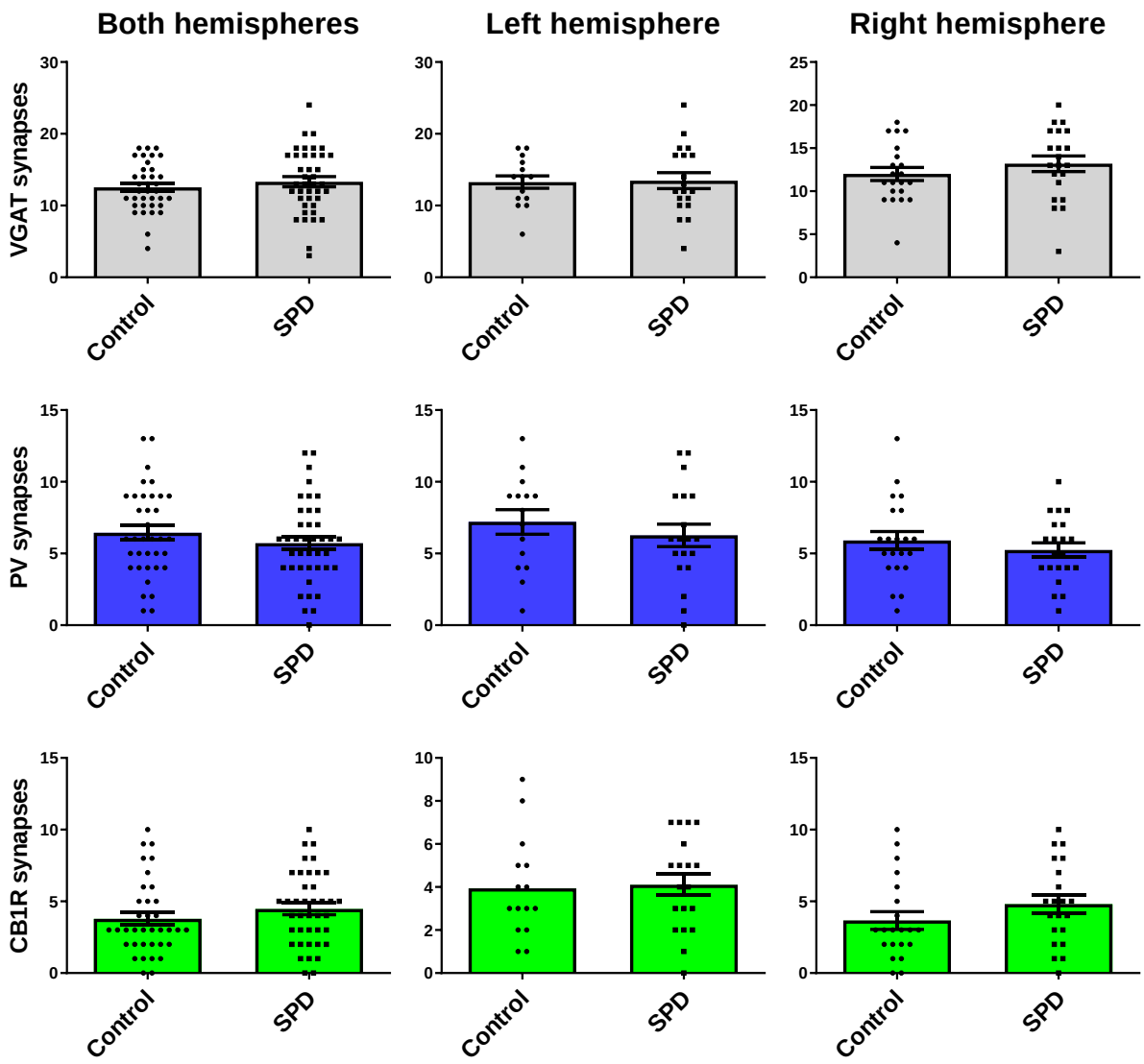

Figure 19. Results of the synapse number analysis. No significant alteration was observed in any condition. Statistical analysis performed with Welch test for every condition following a normal distribution with unequal variances, except for CB1R comparison the right hemispheres alone following a non-normal distribution.

4.2. Hemispheric comparisons

As it will be discussed in greater detail in **Section 5.1.** the alterations in size and intensity lead us to believe that SPD causes aberrant asymmetry. Following this idea, we decided to carry out the comparison between hemispheres to analyse the asymmetry in terms of synapse intensity and size. The statistical data may be found in **Annex 2**.

4.2.1. Synapse intensity comparison

In the control group, the synapses located in the left hemisphere are significantly brighter than the synapses located on the right hemisphere for a value of 64% in VGAT+ synapses ($p<0.001$); 98% in PV+ synapses ($p<0.001$) and 51% in the case of CB1R+ synapses ($p<0.05$). However, the synapses located in the left hemisphere of the SPD group are

significantly dimmer. VGAT+ synapses are 40% less bright than those located in the right hemisphere ($p<0.001$), PV+ synapses are a 45% dimmer ($p<0.001$) and CB1R+ synapses are 17% less bright in the left hemisphere when compared to the right side ($p<0.05$) (**Figure 20**).

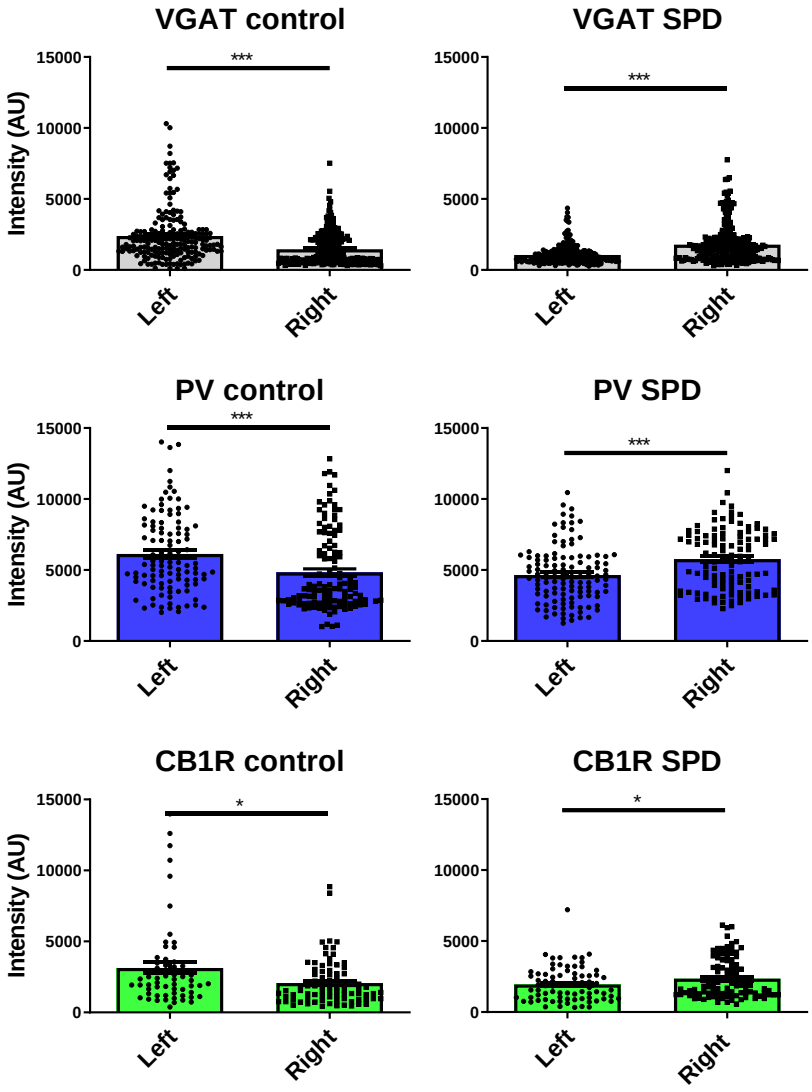

Figure 20. Results of synapse intensity comparison. All comparisons resulted significant. Statistical analysis performed with Mann-Whitney U test following non-normal distribution in all conditions. Difference between left and right hemispheres: *p<0.05, **p<0.01, ***p<0.001.

4.2.2. Synapse size comparison

In the control group, the synapses located in the left hemisphere are significantly larger than the synapses located on the right hemisphere for a value of 16% in VGAT+ synapses ($p<0.001$) and 18% in PV+ synapses ($p<0.01$). Yet, no significant difference was found regarding the size of CB1R+ synapses. On the other hand, the synapses present in the left hemisphere of the SPD group are significantly smaller. VGAT+ synapses are 17% smaller

than those located in the right hemisphere ($p<0.001$), PV+ synapses are a 21% smaller ($p<0.001$) and CB1R+ synapses are 14% smaller in the left hemisphere when compared to the right side ($p<0.05$) (**Figure 21**).

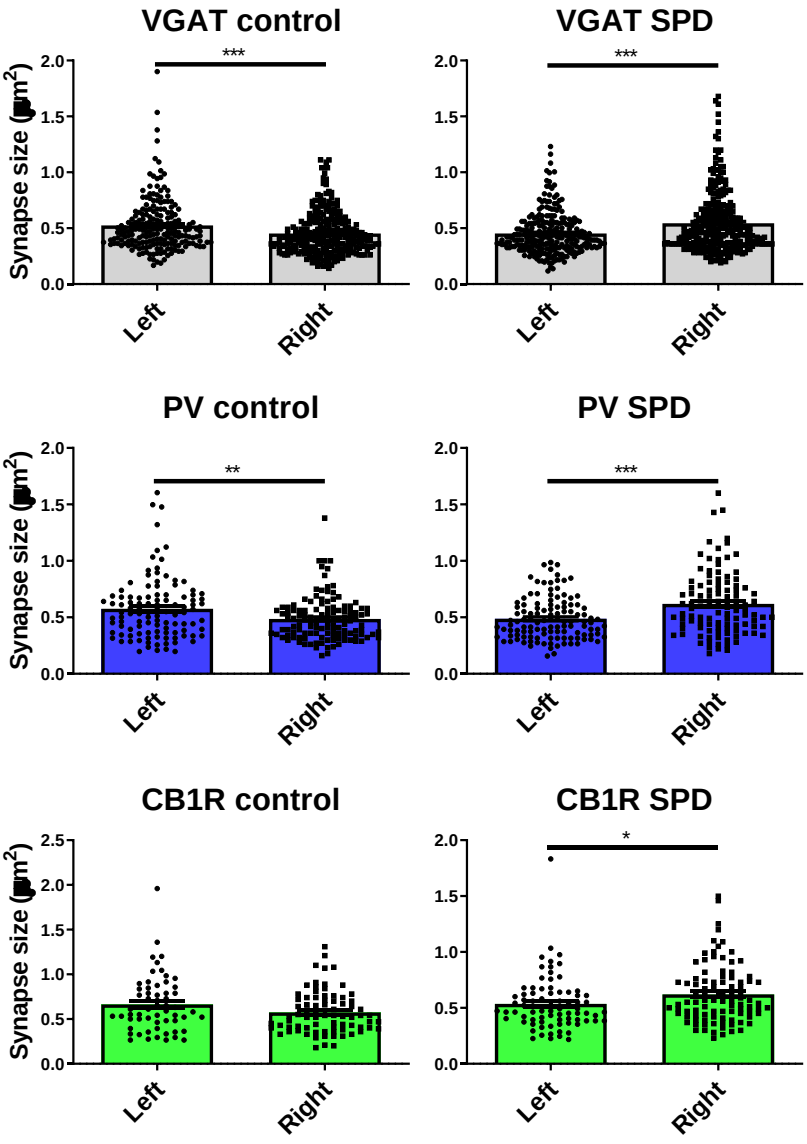

Figure 21. Results of synapse size comparison. Several significant alterations observed. Statistical analysis performed with Mann-Whitney U test following non-normal distribution in all conditions. Difference between left and right hemispheres: *$p<0.05$, **$p<0.01$, ***$p<0.001$.

5. DISCUSION

5.1. Unexpected asymmetry

The brain needs social interaction in order to develop properly. Previously, it has been reported that the GABAergic system in the L5 of the mPFC is affected (Omrani et al., 2020, Bicks et al., 2019; Baarendse et al., 2013) and the present results also demonstrate that L5 of the OFC is compromised as a result of SPD.

Based on prior literature about the OFC and the already mentioned Bijlsma's unplished data, we hypothesized a reduction in the perisomatic inhibition of the L5 of the OFC. Nonetheless, since rise time analysis has not been performed yet, we could not rule out that the inhibition took place on the dendrites instead of on the somata.

Even although the alteration takes place, the results are, to say the least, surprising. Only the intensity for VGAT+ synapses is significantly reduced when both hemispheres are studied together, but when hemispheres are studied independently, we can observe opposed development in synapse size and intensity between hemispheres. While synapses in the left hemisphere tend to be smaller and less intense following SPD, in the right hemisphere SPD causes the synapses to be larger and more intense. Santuy et al. (2017) reported that the size of synapses correlates with functional aspects such as neurotransmitter release or the number of postsynaptic receptors, therefore changes in the functional areas of the synapses have significant functional consequences. Similarly, synaptic intensity is a measurement of the relative amount of the marker (Curran et al., 2020) and both size and intensity tend to correlate with measures of synaptic strength (El-Husseini et al., 2005).

Therefore, the joint reductions in size and intensity of the synapses as it happens in the left hemisphere would show a lower functionality of the GABAergic synapses and therefore, a reduction of the inhibitory function. On the contrary, synapses in the right hemisphere increase in size and intensity implying a greater inhibition to the PCs and thus a lower activation of the L5 of the OFC.

Despite the fact that a reduction in the perisomatic inhibition of the mPFC had been reported, this is not the first time that GABAergic perisomatic synapses become larger following stressful conditions. Bueno-Fernández et al. (2021) observed that inducing stress in peripubertal mice leads to hypoactivity of the PFC as a consequence of a significant increase in the density of VGAT immunoreactive puncta, showing that stressful conditions may not only reduce the inhibition of the PFC.

This alteration in size and intensity shows that hemispheres behave differently under the same external conditions, leading us to believe that SPD causes aberrant asymmetry between hemispheres. Brain asymmetry, also known as cerebral lateralization, refers to both the relative functional differences and anatomical differences between the left and the right sides of the brain (Ocklenburg and Güntürkün, 2012), which have been observed both in humans and non-human animals and it has been suggested that hemispheric specialization increases processing abilities by reducing bilateral redundancy allowing for parallel processing, improving speed and efficiency, especially during tasks requiring behavioral flexibility (Vallortigara et al., 1999; Cohen and Wilson, 2017; López-Persem et al., 2020).

Hemisphere asymmetry can exist as a dynamic feature during behavior and development (Cohen and Wilson, 2017) and events that take place during developmental stages of the brain can severely affect lateralization processes. For instance, Pinkernelle et al. (2009) showed that paternal deprivation results in significantly altered somatosensory circuits and induces hemispheric asymmetry of PCs in somatosensory cortex.

The analysis in brain asymmetry in size and intensity shows that in the control group the intensity and size of synapses is larger in all markers of the left hemisphere and smaller in the right one, showing an asymmetrical development of inhibitory synapses in the L5 of the OFC. This in fact, not the first time that OFC lateralization is reported. Previously, it was observed lateralization in the OFC of human and non-human primates providing evidence on punishment-processing and reward-related function (Xue et al., 2013; López-Persem et al., 2020). Additionally, Cohen and Wilson (2017) also found in rodents a robust performance and task-dependent asymmetry in the rat OFC.

Lastly, our results match with several studies that reported that stress affects the PFC asymmetrically (Czéh et al., 2007; Cerqueira et al., 2008; Czéh et al., 2008), nevertheless Varga et al. (2017) did not find evidence of hemispheric asymmetry regarding GABAergic INs in the OFC following stressful conditions. However, not all stressful or adverse conditions are the same and most likely will not have the same impact upon brain development.

5.2. Nobody, nothing, never is perfect.

A common misconception in academia is that students must hand in perfect reports, ignoring the limitations there might be, and the research they perform must be flawless. However, the aim should not be such, but to be allowed to fail (to some extent) and consequently study from their own mistakes.

During the acquisition of images there has been several limitations as a consequence of lack of studies regarding OFC parcellation. On the one hand, locating the PCs in the L5 of the OFC was a hard task, and due to the high number of PCs in the layer 2/3, it cannot be discarded that some cells belong to one of these two layers. This issue, probably got accentuated, as well, due to the unintentional folds that happened during the cryoslicing. On the other hand, it is unknown if there are major differences between the lateral OFC and ventral OFC. After a lengthy discussion with the other members of the project, we decided to consider the whole area as one and ignore the potential differences that there could be between the lateral and ventral sections.

Regarding the analysis, there has also been two main issues to take into account. First, the joint number of PV+ and CB1R+ synapses does not match with that of VGAT+ synapses: in both SPD and control situations PV+ and CB1R+ only represent around 75% of the inhibitory synapses. We consider three potential explanations for this event: (1) we selected as VGAT+ synapses some that were not, and/or not all PV+ and/or CB1R+ were taken into account; (2) even though the majority of presynaptic terminals of CCK+ INs express CB1R (Rovira-Esteban et al., 2017; Eggan et al., 2010) not all of them do and the "lost" fraction of CB1R might account, to some extent, for the fact that the sum of PV and CB1R does not represent the totality of the inhibitory synapses; and (3) some of the perisomatic inhibitory synapses that were not selected as either PV+ or CB1R+, actually could be a consequence of the innervation of axo-axonic cells (AAC) onto PCs. After PV+ and CCK+ INs, AACs are the most common INs to inhibit perisomatically, as they selectively innervate the axon initial segment of PCs (Inan and Anderson, 2014). However, this looks quite unlikely as they only account for a small fraction of inhibitory INs (Yang et al., 2019). For instance, in the CA1 hippocampal region AAC were only accounted for a 4% of INs (Yang et al., 2017).

On the other side the selection of synapses was done by eye and to try to minimize the bias it would be recommendable to develop an automatized technique for their selection. This could be improved by discriminating, for instance, by employing the point spread function as anything below that value will most likely be shot noise. Similarly, it would be useful to determine the minimum average size of VGAT, PV and CB1R synapses. In this way, the selection could be carried following more objective considerations. This could be performed by electron microscopy or super-resolution microscopy.

5.3. What the future might hold

A relevant question that pops up when studying social play and SPD is the critical amount of play that is needed in order to have a successful brain, cognitive and behavioral development. Being able to define this would be particularly useful in the long term to develop and adequate the interventions to the needs of these severely or chronically diseased kids. For instance, currently, researchers at the Faculty of Veterinary Medicine at Utrecht University are assessing if a normal development is acquired in a scenario where rats are allowed to play 1 hour a day (for further information contact my supervisor A. Bijlsma). Also, it is relevant to address that not only should the OFC development be studied after the resocialization period, but right after the deprivation period, around PND 42-45, as well. In this way we could study the immediate effects of SPD in the brain and also how the resocialization period helps or to what extent reverts the effect of SPD or if SPD affects exclusively in the long term and its consequences can only be observed in the adulthood.

In a vein more related to the research carried here, it feels necessary to point out the need to study the inhibitory IN density in the L5 of the OFC to understand fully the effects of SPD on its GABAergic system, as PV+ INs had already been reported to be implicated in social behavior (Bicks et al., 2020) and PFC inhibition linked to impaired cognitive flexibility (Gruber et al., 2010; Omrani et al., 2020). In fact, PV+ INs are one of three cortical inhibitory INs (alongside somastostatin and serotonin receptor5HT3aR based on their molecular markers) (Rudy et al., 2011) and they constitute the largest sub-class of L5 INs (irrespective of the exact brain cortical region) (Naka and Adesnik, 2016) and also, the largest population of GABAergic INs in the OFC overall (Varga et al., 2017). Therefore, to further understand the alteration caused by SPD in the inhibitory system of the L5 of the OFC, it is necessary to study inhibitory IN density, with a particular focus on PV+ INs.

In addition, the development of aberrant asymmetry unlocks several paths that are worth following. While several papers have shown that there is asymmetry between both hemispheres in the OFC and that stress might induce changes, this is the very first of the studies to characterize the effect of SPD in the OFC from a synaptic point of view. Could SPD affect the rewarding circuits that connect with the OFC? Is the development of aberrant asymmetry as a consequence of SPD a phenomenon that takes place only in the OFC? Could it be that some of the non-significant result observed in the mPFC, could be so if the hemispheres are studies separately? Is the OFC the only prefrontal region prone to aberrant

asymmetry following stressful conditions? These and many more questions can be asked, however they all need of research to be answered.

Finally, if anybody tries to answer any of these questions by performing similar IHC and electrophysiological experiments, it would be useful to carry out both IHC and electrophysiological experiments with the same brains. This could be easily performed by making use of organotypic slices, since slices obtained in the cryostat are not usable for electrophysiological duties and would help minimize the number of animals to be euthanized and would allow to perform correlational studies between electrophysiological parameters and parameters analysed through IHC.

5.4. Conclusions

Our study shows that SPD causes long-lasting effects in the OFC in form of aberrant asymmetry. This information builds up on previous studies that show that the development of higher cortical areas, in particular of the PFC, is conditioned by social experience.

In addition, further research is needed, which should be addressed in two ways: on the one side the study of inhibitory IN density in the L5 of the OFC, to formally address the effect of SPD in this GABAergic system, and on the other side, asymmetry studies in order to quantify the asymmetry induced by SPD not only in the OFC but also in other cortical areas.

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> *Amaiari, nere zientzietako lehen irakaslea eta zientzietarako griña piztu zidana. Miren Bego Urrutiari, izan ere berarengatik izan ez balitz ez nintzatekeelako neurozientzietan arituko. Nere amari eta nere amoñari ere eskertu nahi diet. Era berean Arturori, hor egoteagatik denbora guztian eta neu animatu izanagatik munduan barrena ibiltzera.*

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Annex 1: Statistical data of density, intensity, size, synaptic area and synapse number analysis

Analysis type	Marker	Hemispheric considerations	N		Mean		SEM		Normality test	P-Values		Statistical test	P-Value	
			Control	SPD	Control	SPD	Control	SPD		Control	SPD			
Density	VGAT	Both hemispheres	36	40	0,17	0,18	0,01	0,01		0,76	0,98	Welch t-test	0,16	
		Left hemisphere	15	19	0,18	0,19	0,02	0,02	Shapiro-wilk	0,63	0,75	Welch t-test	0,51	
		Right hemisphere	21	21	0,16	0,18	0,01	0,01		0,77	0,41	Mann-Whitney	0,25	
	PV	Both hemispheres	36	40	0,08	0,08	0,01	0,01		0,36	0,83	Welch t-test	0,54	
		Left hemisphere	15	19	0,09	0,09	0,01	0,01	Shapiro-wilk	0,96	0,99	Welch t-test	0,69	
		Right hemisphere	21	21	0,08	0,07	0,01	0,01		0,35	0,77	Welch t-test	0,49	
	CB1R	Both hemispheres	36	40	0,05	0,06	0,01	0,01		0,01	0,35	Welch t-test	0,17	
		Left hemisphere	15	19	0,05	0,06	0,01	0,01	Shapiro-wilk	0,2	0,56	Welch t-test	0,57	
		Right hemisphere	21	21	0,05	0,06	0,01	0,01		0,03	0,71	Mann-Whitney	0,16	
Intensity	VGAT	Both hemispheres	451	533	1875	1434	74	48		0	0	Mann-Whitney	0	***
		Left hemisphere	199	256	2397	1062	132	42	Shapiro-wilk	0	0	Mann-Whitney	0	***
		Right hemisphere	252	277	1462	1776	71	78		0	0	Mann-Whitney	0	***
	PV	Both hemispheres	232	229	5435	5187	187	140		0	0	Mann-Whitney	0,9	
		Left hemisphere	108	119	6131	4647	265	181	Shapiro-wilk	0,01	0	Mann-Whitney	0	***
		Right hemisphere	124	110	4829	5772	251	202		0	0	Mann-Whitney	0	***
	CB1R	Both hemispheres	136	177	2432	2176	200	100		0	0	Mann-Whitney	0,69	
		Left hemisphere	59	78	3133	1946	390	136	Shapiro-wilk	0	0	Mann-Whitney	0,03	*
		Right hemisphere	77	99	1896	2356	168	142		0	0	Mann-Whitney	0,01	**
Size	VGAT	Both hemispheres	451	528	0,48	0,5	0,01	0,01		0	0	Mann-Whitney	0,33	
		Left hemisphere	199	256	0,52	0,45	0,02	0,01	Shapiro-wilk	0	0	Mann-Whitney	0	***
		Right hemisphere	252	272	0,45	0,55	0,01	0,02		0	0	Mann-Whitney	0	***

	PV	Both hemispheres	232	225	0,53	0,55	0,02	0,02		0	0	Mann-Whitney	0,46	
		Left hemisphere	108	119	0,58	0,49	0,03	0,02	Shapiro-wilk	0	0	Mann-Whitney	0,02	*
		Right hemisphere	124	106	0,49	0,62	0,02	0,03		0	0	Mann-Whitney	0	***
	CB1R	Both hemispheres	136	172	0,61	0,58	0,02	0,02		0	0	Mann-Whitney	0,3	
		Left hemisphere	59	78	0,66	0,54	0,04	0,03	Shapiro-wilk	0	0	Mann-Whitney	0,01	*
		Right hemisphere	77	94	0,57	0,62	0,03	0,03		0	0	Mann-Whitney	0,35	
Synaptic area	VGAT	Both hemispheres	36	40	7,7	9,1	0,47	0,44		0,18	0,36	Welch t-test	0,08	
		Left hemisphere	15	19	9,3	8,8	0,74	0,66	Shapiro-wilk	0,48	0,86	Welch t-test	0,63	
		Right hemisphere	21	21	7,1	9,4	0,42	0,59		0,61	0,37	Welch t-test	0	**
	PV	Both hemispheres	36	40	4,4	4,2	0,36	0,32		0,04	0,84	Mann-Whitney	0,92	
		Left hemisphere	15	19	5,4	4,3	0,62	0,49	Shapiro-wilk	0,63	0,97	Welch t-test	0,17	
		Right hemisphere	21	21	3,7	4,1	0,35	0,43		0,12	0,53	Welch t-test	0,44	
	CB1R	Both hemispheres	36	40	3	3,5	0,33	0,32		0,41	0,25	Welch t-test	0,39	
		Left hemisphere	15	19	3,5	3,2	0,53	0,4	Shapiro-wilk	0,76	0,74	Welch t-test	0,65	
		Right hemisphere	21	21	3	3,8	0,4	0,5		0,21	0,68	Welch t-test	0,15	
Synapse number	VGAT	Both hemispheres	36	40	12,5	13,3	0,57	0,7		0,18	0,68	Welch t-test	0,38	
		Left hemisphere	15	19	13,3	13,5	0,86	1,11	Shapiro-wilk	0,68	0,83	Welch t-test	0,88	
		Right hemisphere	21	21	12	13,2	0,75	0,91		0,23	0,55	Welch t-test	0,32	
	PV	Both hemispheres	36	40	6,4	5,7	0,51	0,46		0,33	0,4	Welch t-test	0,3	
		Left hemisphere	15	19	7,2	6,3	0,85	0,79	Shapiro-wilk	0,85	0,52	Welch t-test	0,43	
		Right hemisphere	21	21	5,9	5,2	0,62	0,5		0,43	0,79	Welch t-test	0,41	
	CB1R	Both hemispheres	36	40	3,8	4,5	0,43	0,4		0,01	0,37	Welch t-test	0,17	
		Left hemisphere	15	19	3,9	4,1	0,61	0,5	Shapiro-wilk	0,17	0,24	Welch t-test	0,83	
		Right hemisphere	21	21	3,7	4,8	0,62	0,63		0,03	0,54	Mann-Whitney	0,15	

Annex 2: Statistical data of the hemispheric comparisons

Analysis type	Marker	Group	N		Normality test	P-Values		Statistical test	P-Values	
			Left hemisphere	Right hemisphere		Control	SPD			
Intensity	VGAT	Control	199	252	Shapiro-wilk	0,00	0,00	Mann-Whitney	0,00	***
		SPD	256	277		0,00	0,00	Mann-Whitney	0,00	***
	PV	Control	108	124	Shapiro-wilk	0,01	0,00	Mann-Whitney	0,00	***
		SPD	119	110		0,00	0,00	Mann-Whitney	0,00	***
	CB1R	Control	59	77	Shapiro-wilk	0,00	0,00	Mann-Whitney	0,01	*
		SPD	78	99		0,00	0,00	Mann-Whitney	0,04	*
Synapse size	VGAT	Control	199	252	Shapiro-wilk	0,00	0,00	Mann-Whitney	0,00	***
		SPD	256	272		0,00	0,00	Mann-Whitney	0,00	***
	PV	Control	108	124	Shapiro-wilk	0,00	0,00	Mann-Whitney	0,01	***
		SPD	119	106		0,00	0,00	Mann-Whitney	0,00	***
	CB1R	Control	59	77	Shapiro-wilk	0,00	0,00	Mann-Whitney	0,12	
		SPD	78	94		0,00	0,00	Mann-Whitney	0,02	*