HOW SOCIAL PLAY EXPERIENCE SHAPES DEVELOPMENT OF HIGHER BRAIN FUNCTION: FROM SYNAPSES TO BEHAVIOUR

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How social play experience shapes development of higher brain function: From synapses to behaviour

Hoe sociale spelervaring de ontwikkeling van een hogere hersenfunctie vormt:
Van synapsen tot gedrag
(met een samenvatting in het Nederlands)

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TABLE OF CONTENTS

CHAPTER 1

General introduction

Social behaviour is a fundamental activity in animal life, which includes a variety of behaviours comprising reproduction and parenting as well as a wide range of sophisticated social interactions outside of the setting of mating. For the most part, these social behaviours are crucial for the survival of the individual, group or species. During life, the behavioural repertoire develops from simple interactions between infant and mother to complex interactions within social networks. The focus of this thesis will be on a specific social behaviour that is expressed across all stages of life but is mostly seen in juvenile and early adolescent mammals and which has been extensively studied in the rat, i.e. social play behaviour (Panksepp et al., 1984; Vanderschuren et al., 1997; Pellis and Pellis, 2009).

The laboratory rat is undeniably important in biomedical research. Almost all human genes that are associated with diseases have a counterpart in the rat genome and rats have been proven to be useful in a large range of biomedical areas such as neuroscience, immunology, toxicology, oncology, and cardiovascular science. It is one of the most widely used animals in behavioural research as they are well capable of learning complex cognitive tasks and have an extensive social repertoire. From birth until around three weeks of age rat pups will stay with their mother after which the weaning process starts. Weaning is a developmental process that is unique to mammalian young during which the infant is converting from a dependent (the mother's milk) to an independent diet (solid food). A few weeks later, rats begin to reach puberty and undergo sexual maturation, which is defined by the separation of the foreskin from the glans penis in males and the opening of the vagina in females. Sexual maturity is reached in males around P45 – P48 and around P32 - P34 in females but large differences can be found between individuals (Lewis et al., 2002).

Puberty signals the beginning of adolescence, a phase of life commonly defined as a transitional period between being a child and becoming an adult. Adolescence is an important developmental period of critical neurobiological, physiological, and cognitive changes (La Greca et al., 2001; Hay et al., 2004). In laboratory rats, early adolescence (P30 – P42) is accompanied by high levels of play behaviour and increased exploratory activity (Spear, 2000) and it is also characterized by impulsivity and risk-taking behaviours, which suggest immaturity in decision-making processes (Spear, 2000; Laviola et al., 2003; Quan et al., 2010; Skelly et al., 2015; Tanaś et al., 2015). Current theories suggest that these behavioural patterns, that facilitate the development of independence from parents, are linked to the maturational time course of cortical and subcortical brain areas and the coordination between these areas (Casey et al., 2008; Ernst et al., 2009; Somerville and Casey, 2010). At the start of adolescence, the

production of synaptic contacts and wiring is still in process with the synaptic density in the rat brain reaching a maximum at around P35 (Aghajanian and Bloom, 1967). Newly established synaptic connections are highly dynamic and the refinement of these connections continues as development advances, which makes them sensitive to environmental experiences. The transition to adolescence is an especially sensitive period during which adverse events substantially affect behaviour and brain function that persist into adulthood (Freedman et al., 1961; Chugani et al., 2001; Andersen, 2003; Bick et al., 2017). It is important to note that in rodent studies, the effects caused by adverse effects during adolescence are typically not reversed by subsequent positive experiences (Chugani et al., 2001; Weaver et al., 2004; Eluvathingal et al., 2006; Makinodan et al., 2012; Yamamuro et al., 2018).

Social play behaviour

Play behaviour is hard to define; psychologist Peter Gray (Gray, 2017) once described it as follows: "Play is a concept that fills our minds with contradictions when we try to think deeply about it. It is serious, yet not serious; trivial, yet profound; and imaginative and spontaneous, yet bound by rules. Play is not real, it takes place in a fantasy world, yet it is about the real world and helps children cope with that world. It is childish, yet it underlies many of the greatest achievements of adults". It has been theorized that through play, children can experiment with tools, learn to solve made-up problems, think creatively, and coordinate and cooperate with others to gain a deeper understanding of the world and most importantly, of themselves (Pellegrini and Smith, 1998; Ginsburg et al., 2007; Pellegrini et al., 2007; Bento and Dias, 2017; Nijhof et al., 2018). The roots of this theory can be traced back to German philosopher Karl Groos who in 1898 published a book called "The Play of Animals" in which he argued that play is part of natural selection as certain skills need to be trained to survive. Today, this "practice theory of play" is well-accepted by most researchers as play is seen as a vital activity for social development due to the resemblance between behavioural play patterns and adult social, sexual and agonistic behaviour (Bateson, 2014). Limitations to freely expressing play are therefore thought to hinder health and well-being (Little and Eager, 2010). Indeed, a link has been suggested between the sharp decline in children's free play in developed nations and the increase in anxiety, depression, suicide, and narcissism in children, adolescents and young adults (Gray, 2011; Brussoni et al., 2012). Because of this, play was deemed critical for the development of children and it was included in Article 31 of the United Nations Convention on the Rights of the Child (Brussoni et al., 2012).

Play in animals can be roughly divided into three types, which are abundant throughout the animal kingdom: locomotor play, object play and the one that has been investigated the most, i.e. social play (Panksepp et al., 1984; Špinka et al., 2001; Pellis et al., 2010; Melotti et al., 2014; Vanderschuren and Trezza, 2014; Sgro and Mychasiuk, 2020). Social play behaviour, also termed rough-and-tumble play or play fighting, is thought to promote physical, social, cognitive, and emotional development (Špinka et al., 2001; Pellis et al., 2010; Vanderschuren and Trezza, 2014). Moreover, it allows animals that live in social groups, like humans, elephants, lions, penguins, rats and many more, to build their complex and well-organized repertoire of social behaviours needed for adulthood, a period in which the ability to respond with appropriate behaviours within the correct context at the right moment is can be crucial for survival (Lore and Flannelly, 1977).

Most of the fundamental research on social play has been performed in the laboratory rat (Panksepp and Beatty, 1980; Panksepp, 1981; Van Kerkhof et al., 2013; Lesscher et al., 2015; Burke et al., 2017; Himmler et al., 2018). Social play in rats emerges at 15 to 18 days of age after which a strong increase in play frequency is seen until a peak is reached at approximately 35 days of age in early adolescence. From this moment, the frequency of play behaviour diminishes gradually until adult levels are reached (Thor and Holloway, 1984; Vanderschuren et al., 1997). Rough-and-tumble play in rats involves the attack and defence of the nape of the neck, which, if contacted, is gently nuzzled with the snout (Pellis and Pellis, 1987; Pellis, 1988). This often results in the defending rat rotating itself on its back while being pinned down by its playmate. While comparable, there are important differences with serious fighting, during which the attack and defence involve biting of the opposing rat directed at the rump and lower flanks (Pellis and Pellis, 1987; Yamada, 1999). A play bout can also be distinguished from serious fighting through the emission of specific, high-frequency 50-kHz ultrasonic vocalisations which is thought to reflect a positive affective state of the rat (Panksepp and Burgdorf, 1999; Manduca et al., 2014; Burke et al., 2022). Moreover, when a rat lays flat on its back during a serious fight it signals the end of the fight while during a play bout, it simply promotes continued interaction by allowing the rat that is pinned down to make contact with the nape of his playmate.

The performance of a playful action is rewarding (Panksepp and Burgdorf, 1999; Klein et al., 2010) and it is associated with the experience of positive emotional states as play often takes place in the absence of fitness threats, making social play in animals a possible welfare indicator (Boissy et al., 2007; Oliveira et al., 2010; Held and Špinka, 2011). The pleasurable properties of play make it possible for play to be used as a

form of reinforcement (Humphreys and Einon, 1981; Normansell and Panksepp, 1990; Crowder and Hutto, 1992; Ikemoto and Panksepp, 1992; Achterberg et al., 2016), and these have also been studied using place conditioning, in which juvenile rats develop a preference for an environment associated with social play (Calcagnetti and Schechter, 1992; Douglas et al., 2004; Thiel et al., 2008; Trezza and Vanderschuren, 2009; Peartree et al., 2012). During play, pleasure is experienced through the activation of neural systems that have also been implicated in other types of reward such as sex, palatable food and drugs of abuse (Trezza et al., 2010; Siviy and Panksepp, 2011; Vanderschuren et al., 2016).

Figure 1. Social play deprivation paradigm used in this thesis, showing two lister hooded rats housed together but seperated from each other by a Plexiglass divider. Picture by Tara Pimentel.

In this thesis, we test the hypothesis that social play is essential for the development of prefrontal connectivity underlying behavioural flexibility in adulthood. To test this, rats needed to be isolated during post-weaning development, losing the ability to play with each other. In this thesis, we chose to use a paradigm in which rats were housed in pairs with a littermate, but a transparent Plexiglas divider containing small holes was placed in the middle of the home cage from P21 to P42, creating two separate but identical compartments (Fig. 1). These holes made it possible for the rats to see, smell and hear each other and to have limited physical interaction without the opportunity to play. Earlier studies have shown that social play-deprived rats have a comparable behavioural repertoire as other rats that grew up in a social environment but showed inappropriate reactions and responses to stressful and fearful social and non-social situations (Meaney and Stewart, 1979; Potegal and Einon, 1989; Lopes da Silva et al., 1996; Hol et al., 1999; Van Den Berg et al., 1999b, 1999a; Van den Berg et al., 2000; Von Frijtag et al., 2002; Himmler et al., 2018). Additionally, deprived rats tend to display

higher levels of impulsivity (Baarendse et al., 2013), are less competent in solving cognitive tasks (Einon et al., 1981) and are more prone to substance use (Whitaker et al., 2013; Baarendse et al., 2014; Lesscher et al., 2015). How social play is essential for the maturation of the prefrontal cortex (PFC) on a cellular/molecular level remains largely unknown, however.

The prefrontal cortex

The prefrontal cortex (PFC) is an important brain region for higher cognitive, so-called executive functions (Miller and Cohen, 2001; Dalley et al., 2004; Izquierdo et al., 2017) such as decision-making, planning, working memory, and cognitive flexibility. Additionally, the PFC plays a crucial role in neural operations required during social interactions (Frith and Frith, 2012; Rilling and Sanfey, 2012). On an anatomical level, there is evidence that various rodent PFC subregions contribute differently to various executive functions, which is consistent with studies done in primates and humans (Eichenbaum et al., 1983; Kesner et al., 1996; Bussey et al., 1997; Decoteau et al., 1997; Ragozzino et al., 1999). In rats, there are five well-established subregions in the PFC: the anterior cingulate cortex (ACC), the prelimbic cortex (PrL), the infralimbic cortex (IL), the medial orbitofrontal cortex (mOFC), and the lateral orbitofrontal cortex (lOFC). In this thesis, we will focus primarily on the medial prefrontal cortex (mPFC; consisting of the Prl and IL; Fig. 2: Red) and the lateral orbitofrontal cortex (OFC; Fig. 2: Blue). Both regions are required for social play (Schneider and Koch, 2005; Pellis et al., 2006; Bell et al., 2009; Van Kerkhof et al., 2013), social play facilitates the maturation of these regions (Pellis et al., 2010; Baarendse et al., 2013; Himmler et al., 2018), and neonatal lesions of these regions affect the structure of social play and its sensitivity to social cues (Panksepp et al., 1994; Schneider and Koch, 2005; Pellis et al., 2006; Bell et al., 2009).

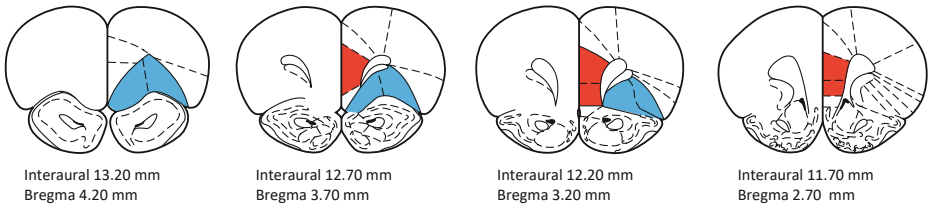

Figure 2. The PFC regions of interest in this thesis: the mPFC (red) and OFC (blue). Adapted from "The Rat Brain in Stereotaxic Coordinates" by Paxinos and Watson (1997).

The mPFC is located medially in the frontal cortex and predominantly receives afferent projections from motor areas, the insular cortex, the hippocampus, the claustrum, the amygdala and the thalamus (Kolb, 1984; Heidbreder and Groenewegen, 2003; Hoover and Vertes, 2007). It is involved in a wide range of cognitive functions, including decision-making (Kennerley and Walton, 2011), working memory (Bissonette et al., 2013), emotional regulation (Sotres-Bayon and Quirk, 2010), learning novel methods (Birrell and Brown, 2000; Floresco et al., 2006), switching between response rules (Ragozzino et al., 1999), identifying stimuli (Bussey et al., 1997), and social interaction (Li et al., 2014). Additionally, the mPFC has been suggested to be necessary for the organization of movements during play behaviour (Bell et al., 2009, 2010).

The OFC receives connections from all sensory modalities, limbic circuits, and weakly from motor areas (Kolb, 1984) and is located anteriorly on the ventral surface of the frontal cortex. It is involved in cognitive processes like decision-making (Tremblay and Schultz, 1999), signalling predicted outcomes (Schoenbaum et al., 2009), stimulus-reward linkages (McAlonan and Brown, 2003; Ghods-Sharifi et al., 2008), and reward expectancy (Howard and Kahnt, 2021). It has been demonstrated that ablation of the orbitofrontal cortex causes rats to selectively fail to alter their social behaviour, both playful and non-playful, in response to the behaviour of other animals (Pellis et al., 2006). Additionally, studies have shown that juveniles who are raised in social housing after weaning have more complex OFC dendritic fields than those who are raised in isolation (Bock et al., 2008). These findings support the hypothesis that the neurons of the OFC are altered in response to various types of social experience.

The OFC and mPFC exhibit reciprocal responsiveness in terms of dendritic complexity. For example, it has been found that treatments or events that increased the complexity of OFC dendrites induced a decrease in the complexity of the mPFC dendrites (Crombag et al., 2005; Bell et al., 2010). This suggests that there is a complex interplay between these two areas in the brain and that changes in one area can affect the other. The reciprocal connection between the subregions was verified by extensive anatomical studies (Vertes, 2004; Hoover and Vertes, 2007, 2011). Moreover, several studies indicate that communication between the two regions is required for social behaviour (Singer et al., 2009; Bell et al., 2010).

Experience-dependent plasticity

A fundamental property of our brain is the ability to continuously adapt in response to changes in the internal and external environment, so-called neuroplasticity. While our brain remains “plastic” throughout life, there is no doubt that neuroplasticity is

at its strongest during early development. It is well-established that many (if not all) brain regions have a so-called critical or sensitive period of enhanced plasticity during development. During a critical period, external input and environmental influences are essential for the fine-tuning of neuronal connectivity to ensure optimal performance of that particular brain area during the rest of life. The onset of a critical period is thought to be linked to the development of inhibitory circuitry, as accelerating or delaying the maturation of inhibitory interneurons is shown to affect the opening and closing of the critical period (Hensch, 2005; Sengpiel, 2007). Additionally, plasticity in the visual cortex was reduced in mice deficient for GAD65, an enzyme that is crucial for GABA synthesis, which could be restored by treatment of diazepam, a GABAergic signalling enhancer (Hensch et al., 1998).

Classic examples of critical periods have been described in the olfactory cortex (van der Linden et al., 2020) and the aforementioned visual cortex (Hensch, 2005). In this latter structure, complete darkness after birth to limit the number of visual experiences has been shown to lead to the development of an immature visual cortex (Levelt and Hübener, 2012). Additionally, the loss of visual experiences during only a small, but sensitive, time frame resulted in activity-dependent remodelling of the complete visual cortical circuitry in combination with long-lasting impairments in visual functioning during adulthood (Hensch, 2005). This shows that experiences during a critical period are crucial for shaping adult brain function. Furthermore, it provides an important framework for understanding how alterations in environmental experiences can have persistent effects on the functioning of the adult brain and behaviour. Understanding the neural mechanisms behind this early-life remodelling and how this is affected during critical periods has been one of the major goals in neuroscience for many years.

It has been hypothesized that similar developmental refinement also occurs in non-sensory brain regions, including the PFC. Indeed, a recent study demonstrated that cortical activity during early development played an important role in modelling PFC development, as altering this activity leads to poorer mnemonic and social abilities during adulthood (Bitzenhofer et al., 2021). A lack of this early-life remodelling and consolidation of circuits might cause dysregulated information processing within the PFC, which has been implicated in neurodevelopmental psychiatric disorders, such as schizophrenia and autism spectrum disorders (Fagiolini and Leblanc, 2011; Nelson and Valakh, 2015; Yamamuro et al., 2020).

Prefrontal development

In comparison to other brain regions, the PFC develops quite late (Huttenlocher, 1990) as significant anatomical and functional remodelling only starts around the transition from the juvenile period into adolescence (Spear, 2000; Chambers et al., 2003; Crews et al., 2007; Pokhrel et al., 2013). This suggests that the functional maturation of the PFC is driven by other cortical and subcortical regions which need to mature first, including the hippocampus, thalamus, ventral tegmental area, and striatum. The adolescent period appears to be a particularly important period during which the refinement of PFC-dependent cognitive functions occurs (Blakemore, 2008; Crone and Dahl, 2012; Larsen and Luna, 2018) and therefore represents a vulnerable period in which abnormal PFC development can have long-lasting impacts on prefrontal function and behaviour (Makinodan et al., 2012; Thomases et al., 2013; Yamamuro et al., 2018; Bicks et al., 2020), increasing the risk of mental disorders (Giedd et al., 2008; Godsil et al., 2013; Schubert et al., 2015; Sigurdsson and Duvarci, 2016; Kolk and Rakic, 2022).

As mentioned above, alterations during the critical period mainly affect the GABAergic system. Activity in the adult PFC is controlled by a variety of GABAergic interneurons, each of which has a unique connection pattern, morphology, physiology, and function (Courtin et al., 2014; Tremblay et al., 2016; Abbas et al., 2019; Bicks et al., 2020). Parvalbumin-expressing interneurons, which are well recognized for having a prolonged period of maturation (Caballero et al., 2014; Caballero and Tseng, 2016), mediate feed-forward inhibition via strong synapses at the soma of pyramidal cells (Gabernet et al., 2005; Cruikshank et al., 2007; Atallah et al., 2012). In contrast, somatostatin-expressing interneurons mediate feedback inhibition by facilitating synapses onto the dendrites (Gentet et al., 2012). There is also a high density of cholecystokinin-expressing interneurons that express cannabinoid type 1 receptors on their axon terminals (Katona et al., 1999; Wilson et al., 2001; Whissell et al., 2015). Together, these three interneuron subtypes account for over 80 % of the total GABAergic cells in the rat PFC.

In the first weeks after birth, the GABAergic landscape is dominated by somatostatin (non-fast-spiking) interneurons after which a transition can be seen to an environment in which parvalbumin (fast-spiking) interneurons gradually gain significance (Caballero and Tseng, 2016; Chini and Hanganu-Opatz, 2021). This shift is thought to be due to a gradual increase in the number and strength of excitatory inputs from the thalamus on parvalbumin interneurons in the mPFC (Caballero and Tseng, 2016). It is during this process that social experiences may have an impact on inhibitory synaptic transmission and the maturation of the local GABAergic system, which is essential for the functional

maturation of the PFC network (Caballero et al., 2016). In parallel, the development of the juvenile PFC is characterized by an increase in the expression and activity of GABA-synthesizing enzymes GAD1 and GAD2. Additionally, GABA transporters that are responsible for the regulation of the GABA concentration in the synapse are upregulated, resulting in an overall strengthening of inhibitory neurotransmission (Minelli et al., 2003a, 2003b; Willing and Juraska, 2015; Caballero and Tseng, 2016; Bicks et al., 2020).

Dopaminergic neuromodulation of the prefrontal cortex

In the adult brain, PFC-mediated executive functions like working memory, inhibitory control and attention are under the significant modulatory control of neurotransmitter systems, including dopamine. Specifically, PFC function is modulated by dopaminergic projections from the ventral tegmental area (Brozoski et al., 1979; Goldman-Rakic et al., 2000; Horvitz, 2000; Egan et al., 2001; Jay, 2003; O'Donnell, 2003; Floresco and Magyar, 2006; Willing et al., 2017). In both humans and rodents, neuromodulatory neurotransmitter systems do not mature fully until early-to-late adolescence (Insel et al., 1988; Spear, 2000; Andersen, 2003). This makes the development of these systems vulnerable to disruption by adverse experiences in early life. Within the PFC, dopamine innervation undergoes qualitative and quantitative changes in fibre density at varying rates, as these innervations continue to grow until P60.

Social play behaviour is modulated by dopaminergic neurotransmission. For example, increased (forebrain) DA release (Robinson et al., 2011) is seen during social play. Earlier studies were somewhat inconclusive regarding the precise role of dopamine in social play behaviour. That is, whereas treatment with dopamine receptor antagonists reduced play behaviour, both decreases and increases in play have been reported after treatment with dopamine receptor agonists (Beatty et al., 1982, 1984; Niesink and Van Ree, 1989; Siviy et al., 1996; Vanderschuren et al., 2008; Trezza and Vanderschuren, 2009). This suggests that during social play, dopamine neurotransmission functions at a subtle optimum, diversion of which disrupts this behaviour, or that after systemic drug treatment play-stimulatory effects of drugs in one brain structure are offset by their effects in another region (see e.g. (Cools and Robbins, 2004). More recently, nucleus accumbens dopamine has been explicitly implicated in social play, whereby stimulation of dopamine signalling in this region enhanced, and inhibition reduced social play, respectively (Manduca et al., 2016). In another study (Achterberg et al., 2016), dopamine was shown to be involved in the motivation for social play.

Scope of the Thesis

In this thesis, we have studied the development of the PFC in rats and how it is shaped by social play experience in early life. We have examined how lack of social play in young rats affects cognitive skills and behaviour later in life and we link this to PFC circuit changes at the synaptic level. In this way, this thesis provides a link between previous behavioural studies on cognitive development and neurophysiological studies on the development of the underlying PFC circuits.

In **Chapter 2** we investigated how cognitive flexibility and inhibitory signalling in the adult PFC are affected when rats are deprived from social play behaviour during post-weaning development. Young male rats were deprived from social play behaviour during the period in life when social play behaviour normally peaks (P21-P42). At P85, we recorded synaptic currents in layer 5 cells in slices from the mPFC of social play deprived (SPD) and control (CTL) rats and observed that inhibitory synaptic currents were reduced in SPD slices, while excitatory synaptic currents were unaffected. This was associated with a decrease in perisomatic inhibitory synapses from parvalbumin-positive GABAergic cells. In parallel experiments, adult SPD rats achieved more reversals in a probabilistic reversal learning (PRL) task, which depends on the integrity of the PFC, by using a simplified cognitive strategy compared to controls. We further introduced a second group of SPD rats that were allowed to play daily for 1 h with their cage mate during the deprivation period (SPD1h). Interestingly, one daily hour of play during SPD partially rescued the performance in the PRL, but did not prevent the decrease in PFC inhibitory synaptic inputs.

In **Chapter 3** we examined the impact of social play on the development of excitatory and inhibitory inputs in the mPFC and OFC. We performed whole-cell patch-clamp recordings in layer 5 pyramidal neurons of both regions from juvenile (P21), adolescent (P42) and adult (P85) CTL and SPD rats. We observed that on P21, inhibitory and excitatory synaptic input was multiple times higher in the OFC than in the mPFC. Additionally, SPD did not affect excitatory currents, but reduced inhibitory transmission in both mPFC and OFC. Intriguingly, the reduction occurred in the mPFC during the deprivation period (P21-P42), while the reduction in the OFC only became manifest after SPD (P42-P85). Our data revealed a complex interaction between social play experience and the specific developmental trajectories of prefrontal subregions.

In **Chapter 4** we tested the hypothesis that exposure to risks during play facilitates adequate responsiveness to risks and uncertainty. Furthermore, we investigated whether this was different in male and female rats. To that aim, we combined social play deprivation with opportunities for risk-taking in a play cage that was enriched

with multiple ladders, plateaus and other objects to interact with. When the rats were adults, they were tested for cognitive flexibility, behavioural inhibition and anxiety-like behaviour and we made recordings of synaptic currents in mPFC slices. We show that playing in a high-risk environment alters cognitive flexibility in both sexes in a PRL task. Behavioural control and behaviour in the elevated plus maze were only affected in males and behaviour in the open field only in females. SPD was shown to affect cognitive flexibility in both sexes while increasing anxiety of female rats in the elevated plus maze. Additionally, we found that synaptic inhibitory currents in the mPFC were increased in male, but not female rats that engaged in play in a high-risk environment, while SPD lowered synaptic inhibition in both sexes.

In **Chapter 5** we hypothesized that social play behaviour shapes the development of inhibitory PFC connections through dopaminergic neurotransmission, and also focused on the importance of social play on the development of reward mechanisms. Thus, we investigated how social play deprivation affects the motivation and preferences of rats for a reward, in our case sucrose. In parallel, to determine if dopaminergic modulation of the prefrontal cortex is altered after social play deprivation, we determined the density and intensity of Tyrosine Hydroxylase (TH)-positive fibres in the mPFC. Additionally, we recorded how inhibitory currents in the adult mPFC are modulated by D1- and D2-agonists. We report that adult rats showed an increase in sucrose intake and increased motivation to respond after SPD. In parallel experiments, we found that TH-positive fibre volume and intensity were reduced in layer 5 of the mPFC after SPD. The modulation by D1- and D2-agonists of inhibitory currents, which were shown to be affected by SPD in previous work, recorded in pyramidal neurons in the mPFC was comparable between CTL and SPD animals.

In **Chapter 6**, all results reported in this thesis and their implications will be discussed.

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CHAPTER 2

Social play behavior is critical for the development of prefrontal inhibitory synapses and cognitive flexibility in rats

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ABSTRACT

Sensory driven activity during early life is critical for setting up the proper connectivity of the sensory cortices. We ask here if social play behavior, a particular form of social interaction that is highly abundant during post-weaning development, is equally important for setting up connections in the developing prefrontal cortex (PFC). Young rats were deprived from social play with peers during the period in life when social play behavior normally peaks (postnatal day 21-42; SPD rats), followed by resocialization until adulthood. We recorded synaptic currents in layer 5 cells in slices from medial PFC of adult SPD and control rats and observed that inhibitory synaptic currents were reduced in SPD slices, while excitatory synaptic currents were unaffected. This was associated with a decrease in perisomatic inhibitory synapses from parvalbumin-positive GABAergic cells. In parallel experiments, adult SPD rats achieved more reversals in a probabilistic reversal learning task (PRL), which depends on the integrity of the PFC, by using a more simplified cognitive strategy than controls. Interestingly, we observed that one daily hour of play during SPD partially rescued the behavioral performance in the PRL, but did not prevent the decrease in PFC inhibitory synaptic inputs. Our data demonstrate the importance of unrestricted social play for the development of inhibitory synapses in the PFC and cognitive skills in adulthood and show that specific synaptic alterations in the PFC can result in a complex behavioral outcome.

INTRODUCTION

The developing brain requires proper external input to fine-tune activity and connectivity in neural circuits to ensure optimal functionality throughout life. This process has been extensively studied in the sensory cortices, and it is long known that sensory deprivation during development causes long-lasting deficits in sensory processing resulting from improper synaptic wiring (Hensch, 2005; Gainey and Feldman, 2017). However, how experience-dependent plasticity contributes to the development of other brain structures, such as the prefrontal cortex (PFC), remains largely unknown (Kolb et al., 2012; Larsen and Luna, 2018; Reh et al., 2020).

The PFC is important for higher cognitive, so-called executive functions (Miller and Cohen, 2001; Floresco et al., 2008), as well as neural operations required during social interactions (Frith and Frith, 2012; Rilling and Sanfey, 2012). By analogy of sensory cortex development, proper PFC development may require complex cognitive and social stimuli. Importantly, during the period when the PFC matures, i.e. in between weaning and early adulthood (Kolb et al., 2012), young animals display an abundance of an energetic form of social behavior known as social play behavior (Panksepp et al., 1984; Vanderschuren et al., 1997; Pellis and Pellis, 2009). Social play behavior involves PFC activity (Van Kerkhof et al., 2014), and lesions or inactivation of the PFC have been found to impair social play (Bell et al., 2009; Van Kerkhof et al., 2013). It is widely held that exploration and experimentation during social play facilitates the development of a rich behavioral repertoire, that allows an individual to quickly adapt in a changeable world. In this way, social play subserves the development of PFC-dependent skills such as flexibility, creativity, and decision-making (Špinka et al., 2001; Pellis and Pellis, 2009; Vanderschuren and Trezza, 2014).

The developmental changes in the PFC circuitry that facilitate these cognitive skills are incompletely understood. Lack of social play experience during post-weaning development has been reported to cause long-lasting changes in PFC circuitry and function (Leussis et al., 2008; Bell et al., 2010; Baarendse et al., 2013; Vanderschuren and Trezza, 2014). However, the cellular mechanisms by which social play facilitates PFC development remain elusive. It is well described that sensory deprivation induces specific alterations in inhibitory neurotransmission that affect adult sensory processing (Turrigiano and Nelson, 2004; Hensch, 2005; Rupert and Shea, 2022). We therefore hypothesized that early life social experiences specifically shape PFC inhibition. To test this, we here investigated how cognitive flexibility and inhibitory signaling in the adult PFC are affected when rats are deprived from social play behavior during development.

MATERIALS AND METHODS

Animals and housing conditions

All experimental procedures were approved by the Animal Ethics Committee of Utrecht University and the Dutch Central Animal Testing Committee and were conducted in accordance with Dutch (Wet op de Dierproeven, 1996; Herziene Wet op de Dierproeven, 2014) and European legislation (Guideline 86/609/EEC; Directive 2010/63/EU). Male Lister Hooded rats were obtained from Charles River (Germany) on postnatal day (P) 14 in litters with nursing mothers. The rats were housed under a reversed 12:12h light-dark cycle with ad libitum access to water and food. Rats were weaned on P21 and allocated to either one of the social play deprivation (SPD and SPD1h) groups or the control (CTL) group. Control (CTL) rats were housed with a littermate during the entire experiment. SPD and SPD1h rats were also housed in pairs with a littermate, but between P21 to P42 a transparent Plexiglas divider containing small holes was placed in the middle of the home cage, creating two separate but identical compartments. These holes made it possible for the rats to see, smell and hear each other and to have limited physical interaction without the opportunity to play. SPD1h animals were housed similarly to the SPD group but were allowed to socially interact with their cage mate for 1 hour per day. Social interaction sessions took place in a Plexiglas arena of 40 x 40 x 60 cm (l × w × h) with approximately 2 cm of wood shavings. The social interaction sessions were recorded and the behavior was manually scored per pair using the Observer XT 15 software (Noldus Information Technology BV, Wageningen, The Netherlands). Four behaviors were scored:

- Frequency of pinning: one animal lying with its dorsal surface on the floor with the other animal standing over it.
- Frequency of pouncing: one animal attempts to nose/rub the nape of the neck of the partner.
- Time spent in social exploration: one animal sniffing or grooming any part of the partner's body.
- Time spent in non-social exploration: the animals exploring the cage or walk around.

Of these behaviors, pinning and pouncing are considered the most characteristic parameters of social play behavior in rats (Panksepp and Beatty, 1980; Vanderschuren et al., 1996).

A total of 10 pairs were recorded during their social interaction sessions. Four of these were eventually used for the electrophysiology experiments while the other six pairs were used for behavioral testing.

On P42, the Plexiglas divider was removed and SPD and SPD1h rats were housed in pairs for the remainder of the experiment. SPD animals, but not SPD1h rats, showed enhanced and somewhat altered play upon resocialization. However, this behavior normalized within a week and after that, all animals behaved indistinguishably from each other. All rats were housed in pairs for at least 4 weeks until early adulthood (10 weeks of age) after which experimentation began. All experiments were conducted during the active phase of the animals (10:00 - 17:00). One week before the start of testing, the rats were subjected to food-restriction and were maintained at 85% of their free-feeding weight for the duration of the behavioral experiment. Rats were provided with ~20 sucrose pellets (45mg, BioServ) in their home cage for two subsequent days before their first exposure to the operant conditioning chamber to prevent potential food neophobia. Rats were weighed and handled at least once a week throughout the course of the experiment.

Probabilistic reversal learning

Apparatus: Behavioral testing was conducted in operant conditioning chambers (Med Associates, USA) enclosed in sound-attenuating cubicles equipped with a ventilation fan. Two retractable levers were located on either side of a central food magazine into which sugar pellets could be delivered via a dispenser. A LED cue light was located above each retractable lever. A white house light was mounted in the top-center of the wall opposite the levers. Online control of the apparatus and data collection was performed using MED-PC (Med Associates) software.

Pre-training: Rats were habituated once to the operant chamber for 30 min in which the house light was illuminated and 50 sucrose rewards were randomly delivered into the magazine with an average of 15 s between reward deliveries. On the subsequent days, the rats were trained for 30 min under a Fixed-Ratio 1 (FR1) schedule of reinforcement for a minimum of three consecutive sessions. The session started with the illumination of the house light and the insertion of both levers, which remained inserted for the remainder of the session. One of the two levers was the 'correct' lever rendering a reward when pressed, whereas pressing the other lever had no consequences. There was no limit other than time on the amount of times a rat could press the 'correct' lever. If the rat obtained 50 or more rewards in a session it was required to press the other lever the following day. If it obtained less than 50 rewards the rat was tested on the

same schedule the next day. Next, the animals were familiarized with lever insertion and retraction, in daily sessions that lasted 100 trials of 60 min, whichever occurred first. A trial started with an inter-trial-interval (ITI) of 5 s with the chamber in darkness, followed by the illumination of the house-light and the insertion of one of the two levers into the chamber. A response within 30 s on the inserted lever resulted in the delivery of a reward. If the rat failed to respond on the lever within 30 s, the lever retracted and the trial was scored as an omission. Rats were trained for ~ 3-4 days to a criterion of at least 50 rewards and had to perform a lever press in more than 80% of the trials before progressing to the probabilistic reversal learning.

Probabilistic reversal learning: The protocol used for this task was modified from those of previous studies (Bari et al., 2010; Dalton et al., 2016; Verharen et al., 2020). At the start of each session one of the two levers was randomly selected to be 'correct' and the other 'incorrect'. A response on the 'correct' lever resulted in the delivery of a reward on 80% of the trials, whereas a response on the 'incorrect' lever was reinforced on 20% of trials. Each trial started with a 5 s ITI, followed by the illumination of the house-light and the insertion of both levers into the chamber. After a response, both levers retracted. In case the rat was rewarded, the house light remained illuminated, whereas the house light extinguished in case the rat was not rewarded on the 'correct' lever. An 'incorrect' response or a failure to respond within 30 s after lever insertion (i.e. omission) lead to the retraction of both levers, extinction of the house light so that the chamber returned to its ITI state. When the rat made a string of 8 consecutive trials on the 'correct' lever (regardless of whether they were rewarded or not), contingencies were reversed, so that the 'correct' lever became the 'incorrect' lever and the previously 'incorrect' lever became the 'correct' lever. This pattern repeated over the course of a daily session. Daily sessions lasted for 200 trials or after 60 minutes have passed, whichever occurred first. The average session time was 29 ± 3 min (range 24-50 min) and there were no differences between groups ($p = 0.43$ and $p = 0.85$ for batch 1 and batch 2 respectively; batch 1 vs batch 2: $p = 0.16$).

Trial-by-trial analysis: This analysis was performed to assess the shifts in choice behavior between subsequent trials, in order to investigate the sensitivity to positive and negative feedback. Depending on whether the rat received a reward or not, it can press the same lever on the subsequent trial or shift towards the other lever, resulting in 4 different choices (i.e. win-stay, win-shift, lose-stay, lose-shift) for both the 'correct' and 'incorrect' lever. We calculated these choices (win-stay vs win-shift and lose-stay vs lose-shift) as percentages per session.

Normalization: The PRL task was performed twice with two different batches of animals. The first batch consisted of 12 CTL and 12 SPD animals (Fig. 3). The second batch consisted of three groups of 12 rats (CTL, SPD and SPD1h; Fig. 7). Rats in the second batch overall made a higher number of reversals performed generally better than in the first batch (CTL rats made 2.8 ± 0.6 reversals in batch 1 and 5.0 ± 1.3 in batch 2; $p<0.0001$). We verified that our conclusions were supported by each batch separately. To compare the number of reversals between the groups in both batches we used the following normalization using the minimum (*groupmin*) and maximum (*groupmax*) values per group: $x' = \frac{x-groupmin}{groupmax-groupmin}$, in which *x* is the original individual value and *x'* is the normalized value. To compare the win-stay and lose-shift choices per session for the pooled data, we normalized the SPD and SPD1h data to the average of their respective control group.

Computational Modelling

Eight different computational models were fit to the trial-by-trial responses to assess differences in task strategy between the three groups of animals. Best-fit model parameters were estimated using maximum likelihood estimation, using Matlab (version 2018b; The MathWorks Inc.) function 'fmincon' (Verharen et al., 2018). These maximum likelihood estimates were corrected for model complexity (i.e., the number of free parameters (n_f)) by calculating the Akaike information criterion (AIC) for each session:

AIC = $2*n_f - 2*log(likelihood)$,

in which a lower AIC indicates more evidence in favor of the model. These log-model evidence estimates were subsequently used to perform Bayesian model selection (Rigoux et al., 2014) using the Matlab package SPM12 (The Wellcome Centre for Human Neuroimaging), taking into account the family to which each model belonged (Penny et al., 2010). This yielded the protected exceedance probability for the 8 individual models and for each family of models (random choice, heuristic and Q learning family) , indicating the probability that each of the (family of) models was most prevalent in the group of rats.

Table 1 contains an overview of the eight computational models. The random choice model is the null model, which assumes that animals choose randomly (i.e., p = 0.5 for each choice, so that the log likelihood is given by [number of trials]*log(0.5)). The second family of models contained strategies based on 'heuristics'; simple strategies to complete the task. The third family contained Q learning models, consisting of four derivatives of the Rescorla-Wagner model (Rescorla and Wagner, 1972).

Table 1. Overview of computational models

Model family	#	Model name	n_f	Description
Random choice	1	Random choice model	0	Animal choses randomly
Heuristics family	2	Win-Stay, Lose-Switch	1	Animal stays on the same lever after winning, moves away from the lever after a loss.
	3	Win-Stay, Lose-Random	1	Animal stays on the same lever after winning, randomly picks a lever after a loss.
	4	Random choice + stickiness	2	Animal choses randomly but attributes value to the lastly chosen lever (Verharen et al., 2020).
Q learning family	5	Q learning, single learning rate for reward and punishment learning	2	Animal learns from previous decisions. Learning rate for positive and negative feedback are the same (Verharen et al., 2020).
	6	Q learning, separate learning rates for reward and punishment learning	3	Animal learns from previous decisions. Learning rate for positive and negative feedback may differ (Verharen et al., 2020).
	7	Model 6 + stickiness parameter	4	Animal learns from previous decisions. Learning rate for positive and negative feedback are separately calculated. Additionally, the animal attributes value to the lastly chosen lever (Verharen et al., 2020).
	8	Rescorla-Wagner Pearce-Hall	4	Animal learns from previous decisions. Learning rate for positive and negative feedback are the same. Additionally, the animal may learn better when task volatility is higher (e.g., after a reversal).

In all models (except for the random choice model), choice was modeled using a Softmax equation, such that the probability of choosing the left lever in trial *t* was given by:

$$p_{left,t} = \frac{\exp(\beta \cdot Q_{left,t})}{\exp\left(\beta \cdot Q_{left,t}\right) + \exp\left(\beta \cdot Q_{right,t}\right)}$$

In which β is the Softmax' inverse temperature (which was a free parameter in models 2 through 8), and Q is the value of the lever.

For models that included a stickiness parameter, the Softmax was given by:

$$p_{left,t} = \frac{\exp(\beta \cdot Q_{left,t} + \pi \cdot \varphi_{left,t-1})}{\exp\left(\beta \cdot Q_{left,t} + \pi \cdot \varphi_{left,t-1}\right) + \exp\left(\beta \cdot Q_{right,t} + \pi \cdot \varphi_{right,t-1}\right)}$$

In this equation, π is the stickiness parameter. ϕ is a Boolean that was 1 when that lever was chosen in the previous trials, and 0 otherwise.

After each trial outcome, lever values Qleft and Qright were updated according to a Q learning rule:

$$Q_{left,t} = Q_{left,t} + \alpha \cdot RPE_t$$

Here, α is the learning rate, and RPE_t indicates the reward prediction error on trial *t*. In turn, the reward prediction error was given by:

$$RPE_t = \begin{cases} 1 - Q_{s,t-1} \text{ for win trials} \\ 0 - Q_{s,t-1} \text{ for lose trials} \end{cases}$$

In which $Q_{s,t-1}$ represents the expected reward value of the chosen lever Q_s

Electrophysiological analysis

The electrophysiological experiments were conducted in two batches, which were performed by different researchers using different recording protocols with several years in between. The first batch consisted of 12 CTL and 12 SPD animals (Fig. 1). The second batch consisted of three groups of 12 (CTL, SPD and SPD1h; Fig. 9). *Slice preparation*: Rats (12-15 weeks of age) were anesthetized by intraperitoneal injection of sodium pentobarbital (batch 1) or induction with isoflurane (batch 2) and then transcardially

perfused with ice-cold modified artificial cerebrospinal fluid (ACSF) containing (in mM): 92 N-methyl-D-glutamine (NMDG), 2.5 KCl, 1.25 NaH_2PO_4, 30 $NaHCO_3$, 20 HEPES, 25 glucose, 2 thiourea, 5 Na-ascorbate, 3 Na-pyruvate, 0.5 $CaCl_2.4H_2O$, and 10 $MgSO_4.7H_2O$, bubbled with 95% O_2 and 5% CO_2 (pH 7.3–7.4). For batch 2 NMDG was replaced by choline chloride and thiourea was left out. Coronal slices of the medial PFC (300 µm) were prepared using a vibratome (Leica VT1200S, Leica Microsystems) in ice-cold modified ACSF. Slices were initially incubated in the carbogenated modified ACSF for 5-10 min at 34 °C and then transferred into a holding chamber containing standard ACSF containing (in mM): 126 NaCl, 3 KCl, 2 $MgSO_4$, 2 $CaCl_2$, 10 glucose, 1.25 NaH_2PO_4 and 26 $NaHCO_3$ bubbled with 95% O_2 and 5% CO_2 (pH 7.3) at room temperature for at least 30 minutes (2 $MgSO_4$ was replaced by 1.3 $MgCl_2$ in batch 2). They were subsequently transferred to the recording chamber, perfused with standard ACSF that is continuously bubbled with 95% O_2 and 5% CO_2 at 28–32°C.

Whole-cell recordings and analysis: Whole-cell patch-clamp recordings were performed from layer 5 (L5) pyramidal neurons in the medial PFC. We chose to study L5 cells, because these are the main output neurons of the PFC (Douglas and Martin, 2004). These neurons were visualized with an Olympus BX61W1 microscope using infrared video microscopy and differential interference contrast (DIC) optics. Patch electrodes were pulled from borosilicate glass capillaries and had a resistance of 3-6 MΩ when filled with intracellular solutions. Excitatory postsynaptic currents (EPSCs) were recorded in the presence of bicuculline (10 µM) and with internal solution containing (in mM): 140 K-gluconate, 1 KCl, 10 HEPES, 0.5 EGTA, 4 MgATP, 0.4 Na_2GTP, 4 Na_2phosphocreatine (pH 7.3 with KOH). Inhibitory postsynaptic currents (IPSCs) were recorded in the presence of 6-cyano-7- nitroquinoxaline-2,3-dione (CNQX) (10 µM in batch 1; 20 µM in batch 2) and D,L-2-amino-5-phosphopentanoic acid (D,L-AP5) (20 µM in batch 1; 50 µM in batch 2), with internal solution containing (in mM): 125 CsCl, 2 $MgCl_2$, 5 NaCl, 10 HEPES, 0.2 EGTA, 4 MgATP, 0.4 Na_2GTP (pH 7.3 with CsOH; batch 1) or 70 K-gluconate, 70 KCl, 10 HEPES, 0.5 EGTA, 4 MgATP, 0.4 Na_2GTP, 4 Na_2phosphocreatine (pH 7.3 with KOH; batch 2). Action-potential independent miniature IPSCs (mIPSCs) were recorded under the same conditions, but in the presence of 1 µM tetrodotoxin (TTX; Sigma) to block sodium channels. The membrane potential was held at -70 mV for voltage-clamp experiments. Signals were amplified, filtered at 3 kHz and digitized at 10 kHz using an EPC-10 patch-clamp amplifier with PatchMaster v2x73 software (batch 1) or MultiClamp 700B amplifier (Molecular Devices) with pClamp 10 software (batch 2). Series resistance was constantly monitored, and the cells were rejected from analysis if the series resistance changed by >20% or exceeded 30 MΩ. No series resistance compensation

was used. Resting membrane potential was measured in bridge mode (I=0) immediately after obtaining whole-cell access. The basic electrophysiological properties of the cells were determined from the voltage responses to a series of hyperpolarizing and depolarizing square current pulses. Input resistance was determined by the slope of the linear regression line through the voltage-current curve. Passive and active membrane properties were analyzed with Clampfit 10 (Axon Instrument) or Matlab (Mathworks). Miniature and spontaneous synaptic currents (IPSCs and EPSCs) data were analyzed with Mini Analysis (Synaptosoft Inc., Decatur, GA). All events were detected with a criterion of a threshold >3× root-mean-square (RMS) of baseline noise. The detected currents were manually inspected to exclude false events.

Immunohistochemistry

Tissue preparation: Rats were anesthetized with Nembutal (i.p. 240mg/kg) and transcardially perfused with 0.1 M phosphate-buffered saline (PBS, pH 7.3-7.4) followed by 4% paraformaldehyde in 0.01 M PBS. The brains were removed from the skull and post-fixed overnight in the same paraformaldehyde solution at 4◦C and subsequently cryoprotected in 30% sucrose for three days at 4◦C. Thereafter, the brains were rapidly frozen in aluminum foil on dry ice and stored at −80◦C until further use. Brain sections (20 µm thick) from the PFC between Bregma levels of 4.2 - 2.2mm (Paxinos and Watson, 2007) were made with a Cryostat Leica CM 3050 S. Sections were stored at -80°C until immunohistochemistry was performed. Brain slices were thawed and let dry for 1h at room temperature (RT). Slices were washed in PBS three times for 15 min (3x15min) at RT. Sections were cooked in sodium citric acid buffer (SCAB, 10mM sodium citric acid in demi water, pH6) for 10 min at 97°C in a temperature controlled microwave, cooled for 30 min at 4°C and washed again (3x15min in PBS). Slices were blocked with 400 µl of blocking buffer (10% normal goat-serum, 0,2% triton-X 100 in PBS) for 2h in a wet chamber at RT. Slices were incubated overnight at 4°C in the wet chamber with 250 µl of primary antibodies in blocking buffer. Sections were washed (3x15min in PBS), followed by incubation with the secondary antibodies in blocking buffer for 2h at RT in a wet chamber. After another wash step (3x15min in PBS), slides were mounted and stored at 4°C until image acquisition. Primary and secondary antibodies are listed in Table 2 (for cell density analysis) and Table 3 (for synaptic puncta analysis).

Table 2A. Primary antibodies for interneuron analysis

Host	Epitope	Concentration	Company	Order number
Rat	Ctip2	1:1000	Abcam	ab18465
Rabbit	PV	1:1000	Life technologies	PA1933
Mouse IgG1	NeuN	1:500	Millipore	MAB377
Mouse IgG2a	GAD67	1:500	Millipore	MAB5406

Table 2B. Secondary antibodies for interneuron analysis

Host	Epitope	Fluorophore	Concentration	Company	Order number
Goat	Anti-rat	Alexa Fluor 568	1:500	Life technologies	A11077
Goat	Anti-rabbit	Alexa Fluor 405	1:500	Life technologies	A31556
Goat	Anti-mouse IgG1	Alexa Fluor 647	1:500	Life technologies	A21240
Goat	Anti-mouse IgG2a	Alexa Fluor 488	1:500	Life technologies	A21131

Table 3A. Primary antibodies for synapse analysis

Host	Epitope	Concentration	Company	Order number
Chicken	VGAT	1:1000	Synaptic systems	131006
Rabbit	PV	1:1000	Life technologies	PA1933
Guinea pig	NeuN	1:500	Millipore	ABN90
Mouse	CB1-R	1:1000	Synaptic systems	258011

Table 3B. Secondary antibodies for synapse analysis

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

Image acquisition and analysis: Images were taken with a Zeiss Confocal microscope (type LSM700). The investigator was blinded to the groups of the sections when acquiring the images and performing the quantifications. Image analysis was performed in ImageJ (National Institutes of Health USA).

Cell density analysis: z-stacks were acquired at 20x of all layers of the mPFC. Tile scan z-stacks (1600 x 1280 μm^2, 2 µm steps, total of 10 µm) were acquired of the mPFC

in both hemispheres of control (n=6) and SPD (n=6) rats. Antibodies staining for NeuN, Ctip2, GAD67 and PV were used. NeuN (neuronal nuclei) is a nuclear protein specific for neurons and was used as a marker to identify neurons. Expression of Ctip2 (CtBP (C-terminal binding protein) interacting protein) is restricted to L5/6 and was used to facilitate identification of the cortical layers. The GABA synthesis enzyme GAD67 (glutamate decarboxylase) and the calcium-buffering protein PV (parvalbumin) were used to identify inhibitory cells.

Synapse analysis: z-stacks were acquired at 63x in layer 5 of the mPFC. For each of the rat brains (Control (n=6), SPD (n=6)) z-stacks (102 x 102 μm^2, 0.4 µm steps, total of 12 µm) were acquired in both hemispheres. Image analysis was performed semi-automatically using custom-written ImageJ macros and MATLAB scripts. NeuN was used to determine the outline of individual L5 cell somata. VGAT (vesicular GABA transporter) is expressed in all inhibitory synapses and was used as a general inhibitory synaptic marker. For each L5 cell, a maximum intensity image was constructed from 4 z-stack slices, which was median filtered and thresholded. Only synaptic puncta larger than 0.2 µm and with a circularity of 0.6-1.0 that were inside of the 1.5 µm band around the NeuN outline were included. PV and CB1-R puncta were only included when co-localized with VGAT.

Data processing and statistical analyses

Statistical analyses were performed with GraphPad Prism (Software Inc.) and RStudio 1_2_5019 (R version 3.6.1, R Foundation for Statistical Computing). Normality of the data was tested with a Shapiro-Wilk test. Differences between two groups were then tested with a nonparametric Mann-Whitney-Wilcoxon test (MW), or a parametric Welch t test (T). Differences between three groups were tested with one-way ANOVA followed up with a Tukey's test. In the electrophysiology and immunocytochemistry data sets the variance between cells or ROIs within slices was larger than the variance between slices, indicating that individual cells and ROIs can be treated as independent measurements. Behavior in the PRL was analyzed using two-way repeated measures ANOVA (with sessions as within-subjects factor and housing condition as between-subjects factor) was used for multiple comparisons followed by T-tests (with Bonferroni correction). All graphs represent the mean ± standard error of the mean (SEM) with individual data points shown in colored circles.

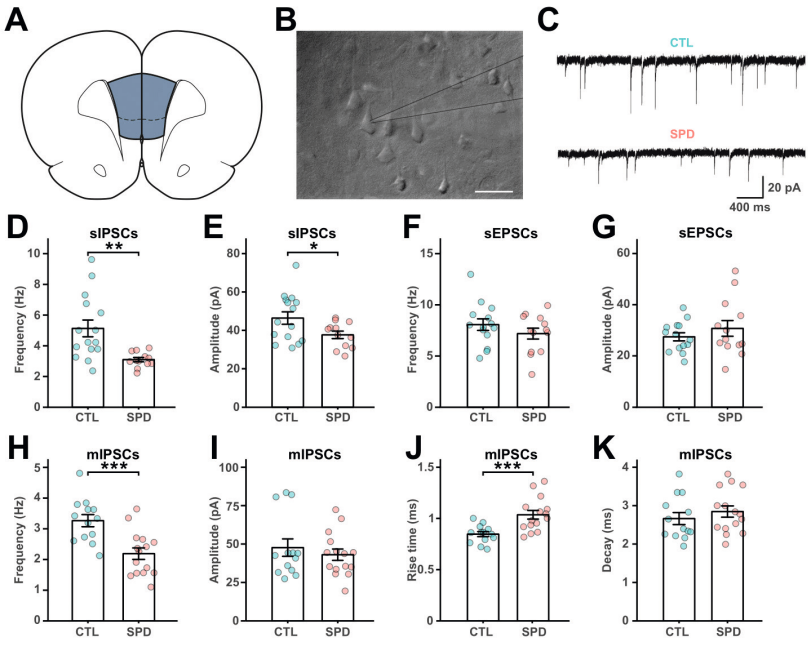

Figure 1. Reduced prefrontal inhibition in L5 pyramidal cells after social play deprivation
(A) Schematic diagram depicting the recording site in the mFPC. (B) DIC image of L5 cells in the mPFC with the recording electrode (grey lines). Scale bar is 20 µm. (C) Example traces of spontaneous IPSCs (sIPSCs) in L5 pyramidal cells in slices from control (CTL) and SPD rats. (D, E) Frequency (D) and amplitude (E) of sIPSCs in CTL and SPD slices ($p = 0.002$ and $p = 0.03$; T test). (F, G) Frequency (F) and amplitude (G) of spontaneous EPSCs ($p = 0.27$ and $p = 0.35$; T test). (H, I) Frequency (H) and amplitude (I) of miniature IPSCs ($p < 0.0005$ and $p = 0.50$; T test). (J) Rise time of mIPSCs ($p = 0.0008$; T test). (K) Decay time of mIPSCs ($p = 0.40$; T test). Data from 15 CTL and 13 SPD brain slices (6 rats per group). Statistical range: * $p \leq 0.05$; ** $p<0.01$; *** $p<0.001$

RESULTS

Rats were deprived of social play for a period of 3 weeks post-weaning (postnatal days 21-42), which is the period in life when social play is most abundant (Baenninger, 1967; Meaney and Stewart, 1981; Panksepp, 1981) . Rats in the social play deprivation (SPD) group were separated from their cage mate by a plexiglass wall, which allowed smelling, hearing, seeing and communicating, but not physical interaction and playing. After the SPD period, the wall was removed and pair-wise social housing was maintained until adulthood when experiments were performed (postnatal week 8-10). Control

(CTL) rats were housed in pairs during the entire period. We performed voltage-clamp recordings from layer 5 (L5) pyramidal cells of the medial PFC (mPFC), the main output neurons, in slices prepared from adult SPD and CTL rats to assess the impact of SPD on PFC circuitry development (Fig. 1A-C). We found that the frequency and amplitude of spontaneous inhibitory postsynaptic currents (sIPSCs) was reduced in SPD rats (Fig 1D, E). By contrast, spontaneous excitatory postsynaptic currents (sEPSCs) were unaffected (Fig. 1F, G). The frequency of miniature inhibitory currents (mIPSCs) was also reduced in SPD slices (Fig. 1H), while mIPSC amplitudes (Fig. 1I) were not affected. The reduction in mIPSC frequency in SPD slices was accompanied by an increase in the average rise time (Fig. 1J), suggesting that particularly mIPSCs with fast kinetics were lost. Decay kinetics (Fig. 1K) and intrinsic excitability (Fig. 2A-C) were unaffected. Together, these data indicate that SPD leads to selective reduction in GABAergic synaptic inputs onto L5 pyramidal cells in the adult mPFC.

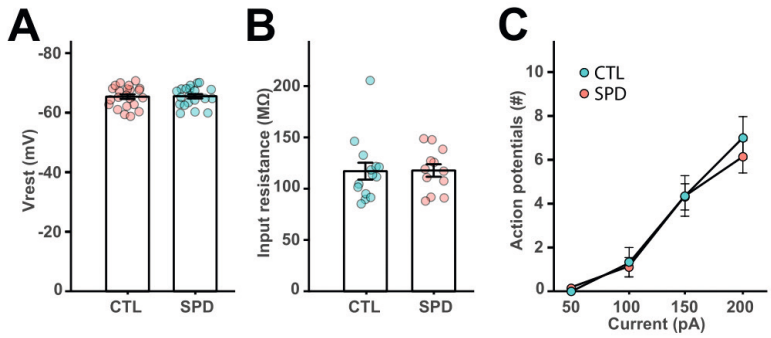

Figure 2. Passive membrane properties of L5 cells are similar in SPD and CTL slices

(A) Resting potential of L5 pyramidal neurons in CTL and SPD slices (p = 0.97; T test). (B) Input resistance (p = 0.61; MW test). (C) Number of action potentials after current injections in CTL and SPD neurons (p = 0.58; 2w ANOVA, condition). Data in A is from 20 CTL and 22 SPD cells; in B from 14 CTL and 12 SPD cells; in C from 19 CTL and 22 SPD cells.

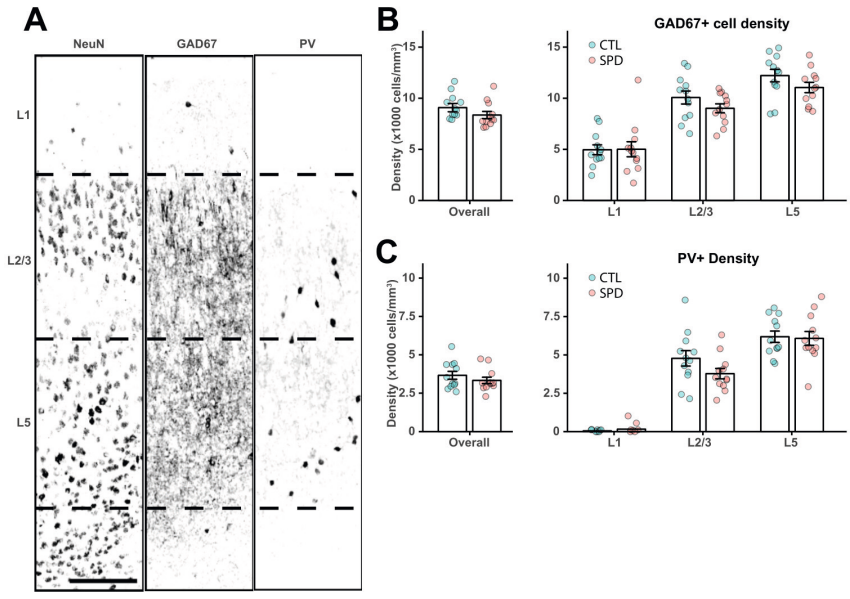

Figure 3. Interneuron density is similar in CTL and SPD tissue

(A) Representative confocal image of NeuN, GAD67 and PV positive neurons in prelimbic cortex layers. Borders between layers are denoted by the dashed lines. Scale bar is 10 µm. (B) Left: The average density of GAD67-positive cells in the mPFC over all layers (p = 0.19; T test); right: GAD67 cell density in Layer 1 (p = 0.95; T test), Layer 2/3 (p = 0.19; T test) and Layer 5 (p = 0.15; T test). (C) Left: The average density of PV-positive cells in the mPFC over all layers (p = 0.33; T test); right: PV cell density in Layer 1 (p = 0.90; MW test), Layer 2/3 (p = 0.12; T test) and Layer 5 (p = 0.85; T test). Data in B and C from 6 CTL and 6 SPD rats. For each rat two measurements (from both hemispheres) were included.

A reduction in mIPSCs with fast rise times suggests that inhibitory synapses at perisomatic locations were affected. Perisomatic synapses are made by parvalbumin (PV) and cholecystokinin (CCK) basket cells (Whissell et al., 2015), of which only the latter express the cannabinoid receptor 1 (CB1-R) (Katona et al., 1999). We performed immunohistochemistry on the mPFC of adult SPD and CTL rats and quantified the number of GAD67- and PV-positive cell bodies (Fig. 3A). The density of GAD67-positive interneurons (Fig. 3B) and PV-positive cells (Fig. 3C) in the mPFC was not different between SPD and CTL rats. We then quantified inhibitory synaptic markers around the soma of L5 pyramidal neurons, using NeuN staining to draw a narrow band around the soma of individual pyramidal neurons and analyze the synaptic PV and CB1 puncta within this band (Fig. 4A-B). The density of VGAT puncta was not different in SPD and

CTL tissue, but the density of PV synapses (colocalizing with VGAT) was significantly lower in SPD tissue compared to CTL tissue (Fig. 4C). The density of CB1-R synapses was not altered. In addition, synaptic PV and CB1-R puncta intensity was decreased in SPD tissue (Fig. 4D). This reduction was specific as VGAT puncta intensity was similar in SPD and CTL tissue (Fig. 4D). Puncta size was not much affected, although synaptic CB1-R puncta were slightly larger in SPD tissue (Fig. 4E). We verified that somata of L5 pyramidal cells had similar size in SPD and CTL tissue (Fig. 4F) and synaptic density was not correlated with cell size (Fig. 4G). Together, these data show that SPD results in a decrease in inhibitory synaptic input to L5 pyramidal cells in the adult PFC, with differential effects on perisomatic PV and CB1-R synapses.

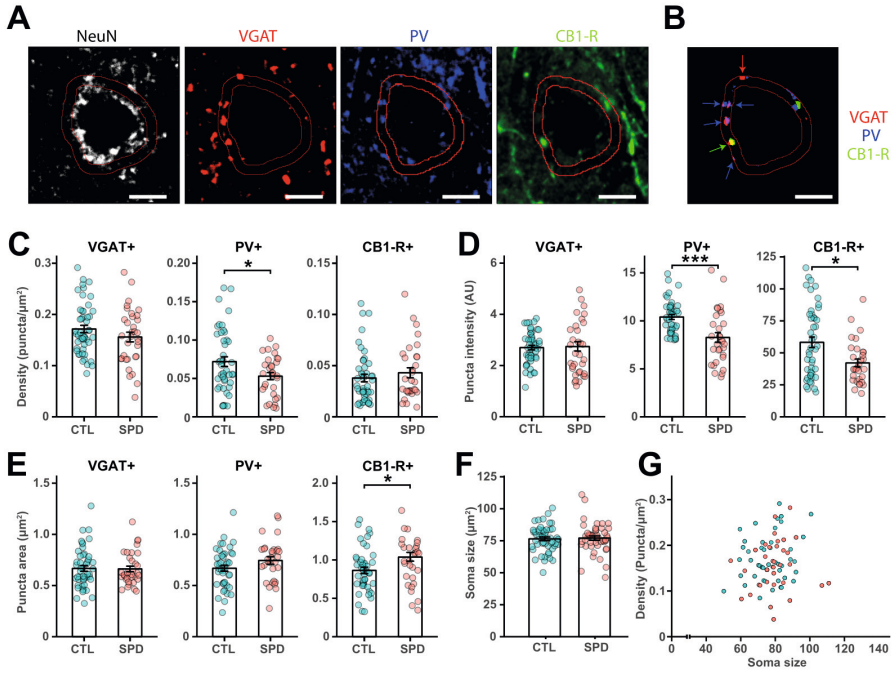

Figure 4. Reduction in perisomatic inhibitory synapses after SPD

(A) Representative confocal images for VGAT, PV, CB1-R and NeuN immunostaining. Scale bar is 1 µm. A 1.5 µm band around the soma was drawn based on the NeuN staining. Individual puncta were selected after thresholding Only PV and CB1-R puncta that co-localized with VGAT were considered synaptic puncta. (B) Summary of the selected VGAT, PV and CB1-R puncta from C. (C) The density of synaptic VGAT, PV and CB1-R puncta (VGAT p = 0.19; PV p = 0.02; CB1-R p = 0.36). (D) The mean intensity of synaptic puncta (VGAT p = 0.51; PV p < 0.0005; CB1-R p = 0.02). (E) The mean area of synaptic puncta (VGAT p = 0.93; PV p = 0.11; CB1-R p = 0.05). (F) Soma size of L5 pyramidal cells (p = 0.20; T test). (G) Correlation between L5 soma size and VGAT synaptic puncta density. Data in C-G from 49 CTL cells and 34 SPD cells (6 rats per group, 2 hemispheres). Statistical range: * p≤0.05; ** p<0.01; *** p<0.001.

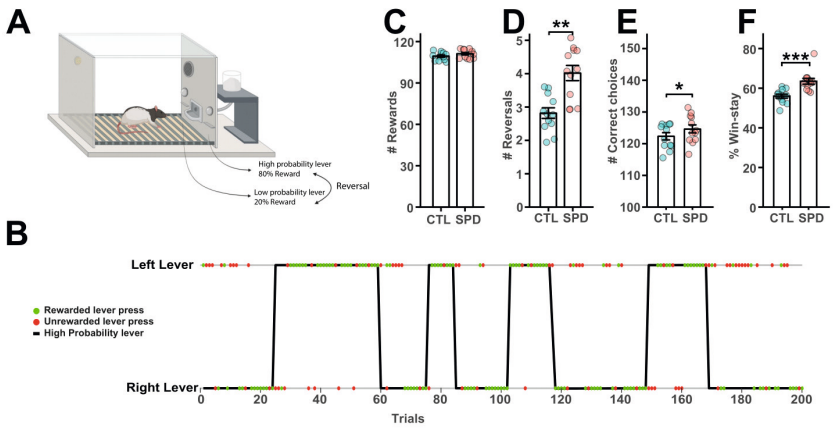

Figure 5. Altered PRL performance after SPD

(A) Probabilistic reversal learning task design. Reversals occur when the rat has pressed the high probability lever 8 consecutive times. (B) Representation of the 200 lever presses of an example session. Green and red dots represent rewarded and unrewarded lever presses respectively. A reversal is indicated by the chance of the high probability lever. (C) Average number of rewards for CTL and SPD rats ($p = 0.16$; T test). (D) Average number of reversals ($p = 0.001$; T test). (E) Average number of correct responses ($p= 0.032$; T test). (F) Average percentage of win-stay choices ($p= 0.001$; T test). Data in C-F from 12 CTL and 12 SPD rats (batch 1). Statistical range: * $p \leq 0.05$; ** $p<0.01$; *** $p<0.001$.

In order to assess the impact of SPD on cognitive flexibility, a PFC-dependent probabilistic reversal learning task (PRL) was used (Fig. 5A, B) (Dalton et al., 2016; Verharen, 2020). In this task, responding on the 'correct' and 'incorrect' levers was rewarded on 80% and 20% of trials, respectively, and position of the 'correct' and 'incorrect' levers switched after 8 consecutive responses on the 'correct' lever. Rats in the SPD and CTL groups readily acquired the task and achieved a comparable performance level in terms of rewards obtained (Fig. 5C). Remarkably, SPD rats completed more reversals (Fig. 5D) and made more correct choices (Fig. 5E) compared to CTL rats. SPD rats showed an enhanced tendency to stay at a lever after it was rewarded (so-called 'win-stay' behavior, Fig. 5F). These observations indicate that cognitive performance in adult rats was altered after SPD.

Early behavioral studies have shown that behavioral performance after social isolation can be partially rescued by allowing the rats daily play for a short amount of time (Einon et al., 1978; Potegal and Einon, 1989a). The idea behind these studies was that a daily limited, but condensed play time should be sufficient to prevent neural

and behavioral changes. We therefore introduced a second group of SPD rats that was allowed to play daily for 1 hour with their cage mate during the deprivation period (SPD1h). We quantified pinning and pouncing - the most characteristic social play behaviors in rats (Panksepp and Beatty, 1980; Vanderschuren et al., 1996) -, as well as social and non-social exploration during the play sessions of the SPD1h rats (Fig. 6A-D). SPD1h rats made ~200 pins and ~300 pounces per hour, which was comparable to rats in other studies that were isolated for 24 h (Niesink and Van Ree, 1989; Vanderschuren et al., 1995, 2008; Achterberg et al., 2016). This is 4 to 6 times higher compared to socially housed rats of the same age, which typically show 40-60 pins per hour (Vanderschuren et al., 2008; Schneider et al., 2016b). Quantification of the social interactions showed that around 75 % of pins and pounces occurred in the first half hour of the session (Fig. 6E). This indicates that SPD1h rats experienced a substantial amount of the daily social play that control rats are normally experiencing (equivalent to 4-6 hours, roughly ~50% of daily play time), but in a very condensed time.

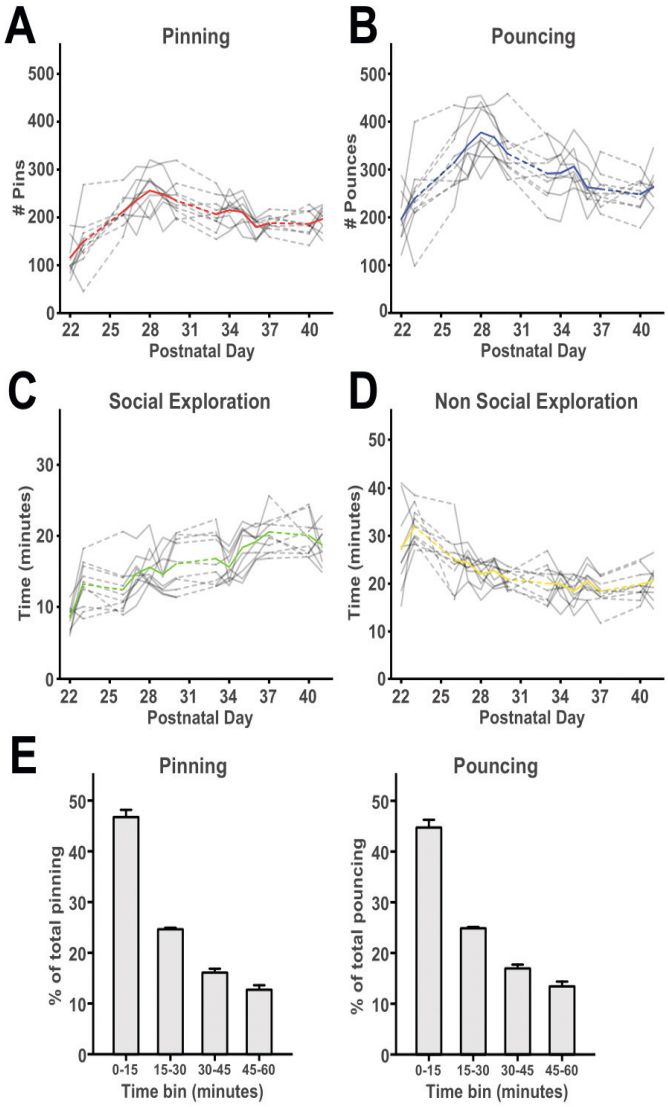

Figure 6. Social behavior during 1-hour play sessions

(A, B) The frequency of (A) pinning and (B) pouncing of the SPD1h rats during the 1-hour play sessions per day. (C, D) The time spent in (C) social and (D) non-social exploration. Each grey line represents a pair of rats with the colored line representing the groups' average. (E, F) The behavioral readouts of social play are expressed as fraction of the total. For both (E) pinning and (F) pouncing, the amount was separated in bins of 15 mins (For statistics see statistical table 1). Data from 10 pairs of SPD1h rats.

We then repeated the PRL task with three groups of rats: control, SPD and SPD1h. Rats of all three groups learned the task equally well and gained similar numbers of rewards (Fig. 7A). To examine possible differences between the groups, we analyzed the number of reversals per session (Fig. 7B). Consistent with our earlier results (Fig. 5D), SPD rats tended to complete more reversals per session than CTL rats (Fig. 7B), but this difference did not reach statistical significance. This was probably due to a ceiling effect because of the higher overall performance of all rats compared to the previous PRL experiment (see methods and compare values in Fig. 7B to Fig. 5D). However, when we normalized and pooled the data from both batches, the number of reversals in the SPD group was clearly above CTL ($p<0.001$; 2w ANOVA with Tukey posthoc). In addition, we observed that SPD rats made more correct choices compared to CTL rats (Fig. 7C), establishing an important independent confirmation of our earlier results (Fig. 5E).

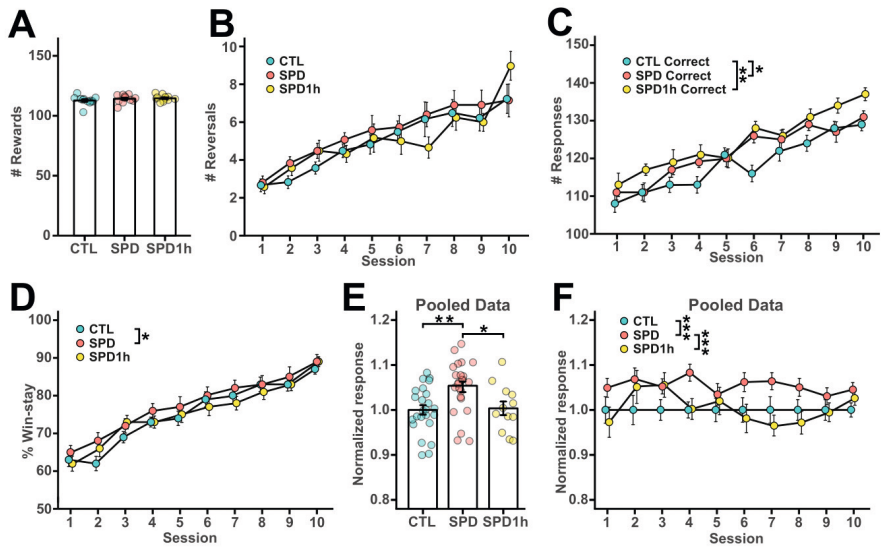

Figure 7. Behavioral analysis of altered PRL performance after SPD

(A) Number of sucrose rewards for CTL, SPD and SPD1h rats ($p = 0.33$ ANOVA). (B) Number of reversals per session ($p=0.22$ ANOVA). (C) Number of correct lever presses per session ($p<0.001$ ANOVA; CTL-SPD, $p = 0.050$; CTL-SPD1h, $p = 0.006$; SPD-SPD1h, $p = 0.72$). (D) Normalized win-stay responses per session ($p<0.001$ ANOVA; CTL-SPD, $p = 0.04$; CTL-SPD1h, $p = 0.97$; SPD-SPD1h, $p = 0.07$). (E) Normalized average win-stay responses (pooled data from both batches) ($p<0.001$ ANOVA; CTL-SPD, $p = 0.003$; CTL-SPD1h, $p = 0.97$; SPD-SPD1h, $p = 0.03$). (F) Normalized average win-stay responses per session ($p<0.001$, 2w ANOVA; CTL-SPD, $p<0.001$; CTL-SPD1h, $p = 0.74$; SPD-SPD1h, $p<0.001$). Data in A-D from 12 CTL, 12 SPD and 12 SPD1h rats (batch 2); data in E-F from 24 CTL, 24 SPD and 12 SPD1h rats (batch 1 and 2 pooled). Statistical range: * $p\leq0.05$; ** $p<0.01$; *** $p<0.001$.

At first glance, the 1-hour daily play sessions did not seem to affect PRL performance in SPD rats, as SPD1h rats made more correct choices compared to CTL rats (Fig. 7C), echoing the behavior of SPD rats during the sessions. However, the performance in reversal completion appeared qualitatively different as the SPD1h rats resembled the SPD group during the initial sessions, but performed closer to the control group in later sessions (Fig. 7B). To further explore possible differences between the groups in more detail, we assessed win-stay behavior over the course of the sessions. Consistent with Fig. 5F, we observed consistently more win-stay choices in the SPD group compared to CTL rats in all sessions, but this effect was not present in the SPD1h group (Fig. 7D). This difference between SPD and SPD1h group was even clearer when we pooled the data from both batches (Fig. 7E, F). When we normalized the win-stay responses in each group to the CTL group, SPD choices are clearly different from both other groups. The SPD1h curve resembled the SPD curve in some of the earlier sessions, but then remained closer to the CTL curve towards the later sessions. There was no difference between groups in their choices after non-rewarded trials (CTL: 45 ± 8 % SPD: 45 ± 7 %; SPD1h: 45 ± 7 %; ANOVA p = 0.86).

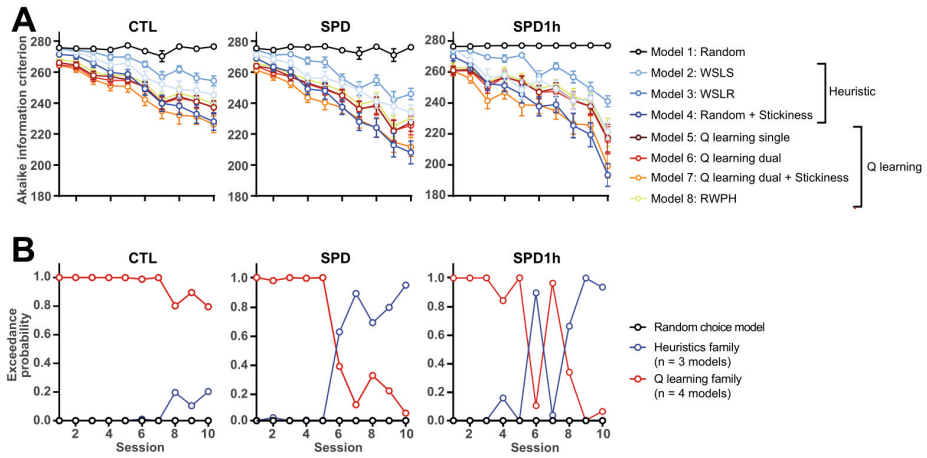

Figure 8. Trial-by-trial analysis of PRL performance

(A) Akaike Information Criterion (AIC) scores for the 7 models. Please note that lower AIC scores reflect a better fit between model predictions and behavioral data. (B) Exceedance probability for different families of computational models (Random choice, Heuristics and Q-learning families) based on Bayesian model selection for the three groups (CTL, SPD and SPD1h rats). (B) Exceedance probability for random and specific heuristic (Win-stay/Lose-shift (WSLS), Win-stay/Lose-random (WSLR), Random + stickiness) and Q-learning family models (Q-Learning single, dual, dual + stickiness, Rescorla-Wagner-Pearce-Hall (RWPH)).

We next performed computational modeling analysis of the behavioral data (Verharen et al., 2018, 2020) to reveal possible alterations in the component processes subserving probabilistic reversal learning. This model-based approach investigates task performance based on an extended history of trial outcomes, and not merely the most recent outcome, such as win- and lose-stay measures do, providing a more in-depth analysis of the task strategy used by the animals. We compared different computational models to describe the behavioral choices of the rats. The simplest random model assumes that animals always randomly choose a lever to press. A family of three heuristic models assumes simple practical strategies to complete the task (e.g. win-stay; lose-shift, etc). Finally, the four Q learning models integrate sensitivity to positive and negative feedback, and weigh exploration versus exploitation (see Methods for details). We calculated Akaike Information Criterion (AIC) scores to assess how well the behavioral choices were described by the different computational models (Fig. 8A). Although in all groups Q learning models (models 5 to 8) were generally better than the heuristic win-stay models (models 2 and 3) to describe behavioral choices, models that contained a 'stickiness' parameter (models 4 and 7) always performed best (Fig. 8A). The behavior of CTL rats was best described by a Q-learning model in all sessions (Fig. 8B left panel), but the SPD and SPD1h rats shifted towards behavior congruent with a simpler heuristic strategy as reversal learning progressed (Fig. 8B middle and right panel), showing a tendency to remain at the previously chosen lever. Thus, SPD rats, with or without daily playtimes, switched from a learning-based strategy to a more perseverative strategy, whereas CTL rats continued learning throughout training. These data indicate that juvenile SPD alters PFC function and cognitive flexibility in adulthood. Furthermore, it shows that 1hr daily play during SPD only partially restores the cognitive performance in SPD rats.

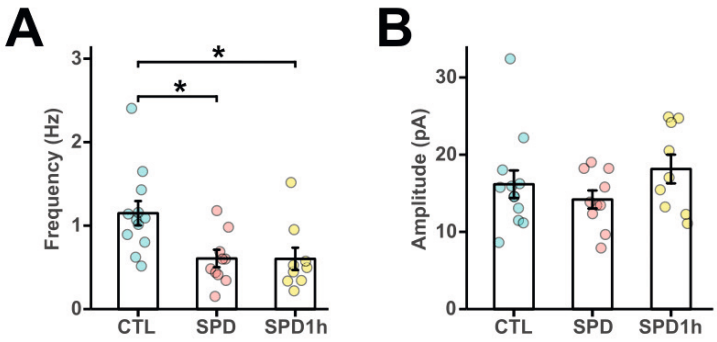

Figure 9. Reduced prefrontal inhibition in L5 pyramidal cells in SPD and SPD1h slices
(A) Frequency of mIPSCs in CTL, SPD and SPD1h slices (ANOVA p=0.0049, Tukey's: CTL vs SPD p = 0.011; CTL vs SPD1h p = 0.017; SPD vs SPD1h p = 0.99). (B) Amplitude of mIPSCs in CTL, SPD and SPD1h slices (ANOVA p = 0.29). Data from 12 CTL, 10 SPD and 9 SPD1h cells (6 rats per group). Statistical range: * $p \leq 0.05$.

To check if the partial rescue in PRL behavior of the SPD1h rats was due to a rescue of the inhibitory microcircuitry in the mPFC, we recorded miniature inhibitory currents (mIPSCs) in prefrontal slices from all 3 groups. Consistent with our earlier results (Fig 1H), a clear reduction in mIPSC frequency was found in the SPD group compared to CTL rats (Fig. 9B). This reduction was similar in the SPD and SPD1h slices, indicating that daily playtimes during the play deprivation period did not affect the development of PFC inhibition (Fig. 9B). Amplitudes of the events were not different between groups (Fig. 9C).

Our findings demonstrate that the organization of the adult GABAergic system in the mPFC of rats is robustly altered when rats are deprived of social play behavior during development, with important consequences for cognitive flexibility. The inclusion of daily play sessions during the SPD period failed to rescue the reduction in PFC GABAergic synapses and only partially restored the cognitive performance in a PFC-dependent PRL task.

DISCUSSION

Like most other mammalian species, young rats display an abundance of a particular, highly rewarding and energetic form of social behavior, termed social play behavior (Panksepp et al., 1984; Vanderschuren et al., 1997; Pellis and Pellis, 2009; Achterberg et al., 2016). Playing with peers is thought to allow young animals to experiment with their behavioral repertoire, and to provide practice scenarios to obtain the social, cognitive and emotional skills to become capable adults who can easily navigate a changeable world (Špinka et al., 2001; Pellis and Pellis, 2009; Vanderschuren and Trezza, 2014; Larsen and Luna, 2018). Here we found that social play deprivation from P21 until P42 reduces specific inhibitory connections in the PFC and affects cognitive skills in adulthood. Importantly, after the temporary deprivation when rats were young, the animals had ample opportunity for social interaction during the weeks before testing. However, even after several weeks of unrestricted social interactions, pronounced changes in PFC function and cognition were present. This emphasizes the importance of early post-weaning social play, consistent with earlier studies that identified this time window as a critical period for PFC maturation and behavioral development (Einon and Morgan, 1977; Hol et al., 1999; Van Den Berg et al., 1999; Lukkes et al., 2009; Kolb et al., 2012; Whitaker et al., 2013). Furthermore, previous studies have suggested that a daily limited, but condensed play time should be sufficient to prevent the neural and behavioral changes observed (Einon and Morgan, 1977; Potegal and Einon, 1989b). However, we found that although 1 hour of daily condensed play (roughly equivalent to 4-6 hours of 'normal'play) partially rescued cognitive performance in SPD rats, the reduction in PFC inhibition remained unaltered. Our data therefore suggest that the experience of unrestricted juvenile social play is crucial to instruct the development of specific inhibitory connections in the PFC and to shape adaptive cognitive strategies in the adult brain.

Social play enhances neuronal activity in a broad network of limbic and corticostriatal structures (Gordon et al., 2002, 2003; Van Kerkhof et al., 2014). This integrated neuronal activity likely drives PFC maturation during development, analogous to the well-described experience-dependent maturation of cortical sensory circuits, which requires appropriate sensory activation during development (Hensch, 2005; Gainey and Feldman, 2017). To our knowledge, our study is the first to report a specific and robust synaptic alteration in the adult PFC after SPD. We observed that perisomatic inhibition onto L5 cells was reduced in the adult mPFC after SPD. We found a ~30% reduction in mIPSC frequency, associated with a comparable reduction in perisomatic PV synapses, in line

with previous observations in sensory cortex showing a reduction in inhibition after sensory deprivation (Hensch et al., 1998; Morales et al., 2002; Chattopadhyaya et al., 2004; Jiao et al., 2006; Mowery et al., 2019; Reh et al., 2020). In addition, we report a reduced level of PV and CB1 expression in prefrontal L5 inhibitory synapses after SPD. This may indicate a reduced activity level in PV cells (Donato et al., 2013; Caballero et al., 2014) and an altered endocannabinoid tone (Sciolino et al., 2010; Schneider et al., 2016a).

Alterations in PV cells during development are shown to shape cognitive capacities in adulthood (Donato et al., 2015; Mukherjee et al., 2019; Canetta et al., 2022). Changes in PFC network activity directly affect cognitive and social skills (Yizhar et al., 2011; Dalton et al., 2016; Verharen et al., 2020), but it is hard to pinpoint how reduced PV innervation of L5 cells will affect PFC function. A reduction in PFC inhibition has previously been linked to impaired cognitive flexibility (Gruber et al., 2010), and a direct link between PFC PV cell activity and social behavior was recently demonstrated (Bicks et al., 2020; Sun et al., 2020). From our data it is not clear if the reduction in inhibitory drive to L5 cells will lead to enhanced activity in downstream brain regions or whether it actually compensates for a change in upstream activity or connectivity within the local mPFC network. Interestingly, recent studies showed that prefrontal PV and CCK cells, which provide the perisomatic inhibition on L5 cells, receive specific input from the ventral hippocampus (Liu et al., 2020), while the thalamic drive appears less important for PV cells in the PFC (Benoit et al., 2022). It will be important to further explore other synaptic changes in the intricate PFC circuitry after SPD and their consequences for PFC-driven modulation of behavior.

In this study we observed that adult rats which had experienced SPD displayed a different behavioral strategy in the PRL task compared to CTL animals. PRL performance depends on complex interactions between several component processes, including the sensitivity to positive and negative feedback, response persistence and exploration versus exploitation, each of which requires functional activity in distinct PFC regions (Verharen et al., 2018, 2020), as well as brain regions outside the PFC (Izquierdo et al., 2017). CTL rats seemed to follow a sophisticated cognitive strategy which was well-described by a Q-learning model. Our data indicate that SPD rats made more correct choices by using a simplified strategy, in which they relied less on feedback-driven learning, and more on perseverance (represented by a 'stickiness' parameter in our models). This is probably cognitively less demanding (Christie and Schrater, 2015), and may therefore be preferred under certain conditions, especially if this does not lead to a reduction in reward. A similar increase in reversals was also reported after prelimbic

mPFC inactivation (Dalton et al., 2016), suggesting that the mPFC may be less involved in PRL performance in SPD rats compared to CTL rats.

Our data demonstrate that social play behavior is of critical importance for the development of PFC circuitry and function, and PFC-dependent cognitive processes. At this point, we can only speculate whether it is the social, cognitive, emotional, sensory or physical aspects of social play – or their interaction – that determines proper PFC development. We observed that one hour of daily playtime during the deprivation period could not rescue the reduction in prefrontal IPSCs, but it partially rescued behavior in adulthood. Play is not displayed continuously by young rats, but appears in peaks of relatively short duration across the day (Melotti et al., 2014; Lampe et al., 2019). We observed that SPD1h rats played very intensely during the first 15-30 minutes of each play session resulting in a total amount of play that makes up a substantial fraction of the daily play that socially housed CTL rats show at this age. As this was not enough to prevent the reduction in prefrontal inhibition after SPD, it suggests the necessity of unrestricted, voluntarily elicited or repeated play for proper PFC maturation. Our observation that the behavior of SPD1hr fell in between SPD and CTL rats, while their reduction in mPFC inhibition was similar to SPD rats, suggests that the partial rescue of behavior involves compensatory changes other than L5 inhibition, and further studies will be needed to elucidate these. Together, our results demonstrate a key role for juvenile social play in the development of perisomatic inhibition in the PFC and PFC-dependent cognitive flexibility.

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CHAPTER 3

Social play behaviour shapes the development of prefrontal inhibition in a region-specific manner

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ABSTRACT

Experience-dependent organization of neuronal connectivity is critical for brain development. We recently demonstrated the importance of social play behaviour for the developmental fine-tuning of inhibitory synapses in the medial prefrontal cortex (mPFC) in rats. When these effects of play experience occur and if this happens uniformly throughout the prefrontal cortex is currently unclear. Here we report important temporal and regional heterogeneity in the impact of social play on the development of excitatory and inhibitory neurotransmission in the mPFC and the orbitofrontal cortex (OFC). We recorded in layer 5 pyramidal neurons from juvenile (postnatal day (P)21), adolescent (P42) and adult (P85) rats after social play deprivation (SPD; between P21-P42). The development of these PFC subregions followed different trajectories. On P21, inhibitory and excitatory synaptic input was higher in the OFC than in the mPFC. SPD did not affect excitatory currents, but reduced inhibitory transmission in both mPFC and OFC. Intriguingly, the reduction occurred in the mPFC during SPD, while the reduction in the OFC only became manifested after SPD. These data reveal a complex interaction between social play experience and the specific developmental trajectories of prefrontal subregions.

INTRODUCTION

The developing brain requires proper external input to fine-tune activity and connectivity in neural circuits for optimal functionality throughout life. Experience-dependent plasticity is well described in sensory cortices, but it is also essential for the development of higher-order brain regions, including the prefrontal cortex (PFC) (Larsen and Luna, 2018; Bicks et al., 2020). The PFC undergoes intensive functional remodelling during the juvenile and adolescent phases of life, roughly between postnatal day (P) 21 and 85 in rodents (Kolb et al., 2012; Thomases et al., 2013; Caballero and Tseng, 2016; Caballero et al., 2016; Larsen and Luna, 2018). Cytoarchitectonic characteristics of the PFC do not stabilize until around P30. Around this time, white matter volume increases because of myelination, and grey matter volume in the PFC starts to decrease due to synaptic pruning and apoptosis (Markham et al., 2007). In addition, at the onset of adolescence (around P30 – P42), the PFC starts to receive long-range afferents from sensory and subcortical brain regions including the amygdala, ventral hippocampus and mediodorsal thalamus (Hoover and Vertes, 2007, 2011; Murphy and Deutch, 2018; Yang et al., 2021). During adolescence (P35-P60), local interneurons are undergoing important remodelling (Caballero et al., 2014a; Cass et al., 2014; Caballero and Tseng, 2016), which is critical for the maturation of the PFC network (Tseng et al., 2008).

During the juvenile and adolescent phases of life, when the PFC is developing, most mammalians species, including rats and humans, display an abundance of a pleasurable and energetic form of social interaction, known as social play behaviour (Panksepp et al., 1984; Vanderschuren et al., 1997; Pellis and Pellis, 2009). One important characteristic of social play is that it allows animals to experiment with their own behaviour and their interactions with others. This experimentation during social play is thought to facilitate the development of a rich behavioural repertoire, that allows an individual to quickly adapt in a changeable world. In this way, social play may subserve the development of PFC-dependent skills such as flexibility, creativity, and decision-making (Špinka et al., 2001; Pellis and Pellis, 2009; Vanderschuren and Trezza, 2014). Indeed, during play the PFC is engaged (Van Kerkhof et al., 2014) and required (Bell et al., 2009; van Kerkhof et al., 2013). Moreover, limiting the time young animals can play has been shown to lead to impaired social interactions (Hol et al., 1999; Van Den Berg et al., 1999) and long-lasting changes in PFC function and circuitry in adulthood (Bell et al., 2010; Baarendse et al., 2013; Vanderschuren and Trezza, 2014).

The PFC comprises multiple subregions that display functional specialization and overlap (Miller and Cohen, 2001; Dalley et al., 2004; Izquierdo et al., 2017; Verharen

et al., 2020). Of these, both the medial prefrontal cortex (mPFC) and the orbitofrontal cortex (OFC) are required for social play (Schneider and Koch, 2005; Pellis et al., 2006; Bell et al., 2009; Van Kerkhof et al., 2013), and social play facilitates the maturation of these regions (Pellis et al., 2010; Baarendse et al., 2013; Himmler et al., 2018). Both subregions have been implicated in higher cognitive, so-called executive functions, whereby the OFC is thought to be important for emotionally influenced cognition, such as reward-based decision making (Schoenbaum et al., 1998, 2009; Rolls, 2000; O'Doherty et al., 2003), and the mPFC subserves functions in working memory and planning (Bechara and Damasio, 2005; Posner et al., 2007; Euston et al., 2012). However, there is also a substantial degree of functional overlap between PFC regions (Sul et al., 2010; Lodge, 2011; Hardung et al., 2017). During the production of social behaviours the mPFC and OFC are functionally linked to each other and the two regions are reciprocally connected (Singer et al., 2009; Hoover and Vertes, 2011).

We recently showed that deprivation of social play affects inhibitory, but not excitatory connections in the adult mPFC, emphasizing the importance of social play for PFC circuit development (Bijlsma et al., 2022). How social play contributes to the development of OFC connections is currently unknown. Additionally, how the excitatory and inhibitory inputs of the two subregions develop and how the deprivation of social play experiences affects their developmental trajectories has not been addressed. Here we report the distinct development of synaptic inputs onto layer 5 pyramidal neurons in the mPFC and OFC and describe how social play deprivation (SPD) differentially affects the developmental trajectories of these two PFC regions.

MATERIALS AND METHODS

Animals and housing conditions

All experimental procedures were approved by the Animal Ethics Committee of Utrecht University and the Dutch Central Animal Testing Committee and were conducted in accordance with Dutch (Wet op de Dierproeven, 1996; Herziene Wet op de Dierproeven, 2014) and European legislation (Guideline 86/609/EEC; Directive 2010/63/EU). Male Lister Hooded rats were obtained from Charles River (Germany) on postnatal day (P) 14 in litters with nursing mothers. All rats were subject to a normal 12:12h light-dark cycle with ad libitum access to water and food. Rats used in the P21 measurements were directly taken from the litter at P21. Rats used in the P42 and P85 groups were weaned on P21 and were either allocated to the control (CTL) group or the social play deprivation (SPD) group. CTL rats were housed in pairs for the remainder of the experiment. SPD

rats were pair-housed but during P21 to P42 a transparent Plexiglas divider containing small holes was placed in the middle of their home cage creating two separate, identical compartments. SPD rats were able to see, smell and hear one another but they were unable to physically interact. On P42, the Plexiglas divider was removed and SPD rats were housed in pairs for the remainder of the experiment. Rats were weighed and handled at least once a week until they were used for neurophysiological experiments. Experiments were performed on P21, P42 and P85 with a spread of 2 days as it was not always possible to perform measurements on the exact postnatal day.

Electrophysiological analysis

Slice preparation: Rats were anaesthetized with Isoflurane and then transcardially perfused with ice-cold modified artificial cerebrospinal fluid (ACSF) containing (in mM): 92 Choline chloride, 2.5 KCl, 1.2 NaH_2PO_4, 30 $NaHCO_3$, 20 HEPES, 25 glucose, 5 Na-ascorbate, 3 Na-pyruvate, 0.5 $CaCl_2$, and 10 $MgSO_4$, bubbled with 95% O_2 and 5% CO_2 (pH 7.3–7.4). The brain was quickly removed after decapitation and coronal slices (300 µm) of the medial PFC (consisting of the prelimbic and infralimbic cortex) and OFC (consisting of the ventral and lateral orbital cortex) were prepared using a vibratome (Leica VT1000S, Leica Microsystems) in ice-cold modified ACSF. Slices were initially incubated in the carbonated modified ACSF for 5 min at 35 °C and then transferred into a holding chamber containing standard ACSF containing (in mM): 126 NaCl, 3 KCl, 1.3 $MgCl_2$, 2 $CaCl_2$.2H2O, 20 glucose, 1.25 NaH_2PO_4 and 26 $NaHCO_3$ bubbled with 95% O_2 and 5% CO_2 (pH 7.3) at room temperature for at least 30 minutes. They were subsequently transferred to the recording chamber, perfused with standard ACSF that is continuously bubbled with 95% O_2 and 5% CO_2 at 28–32°C.

Whole-cell recordings and analysis: Whole-cell patch-clamp recordings were performed from layer 5 pyramidal neurons in the medial PFC and OFC. Neurons were visualized with an Olympus BX51W1 microscope using infrared video microscopy and differential interference contrast (DIC) optics. Patch electrodes were pulled from borosilicate glass capillaries and had a resistance of 4-6 MΩ when filled with intracellular solutions. Excitatory postsynaptic currents (EPSCs) were recorded with an internal solution containing (in mM): 140 K-gluconate, 4 KCl, 10 HEPES, 0.5 EGTA, 4 MgATP, 0.4 NaGTP, 4 Na_2-phosphocreatine (pH 7.3 with KOH). Spontaneous inhibitory postsynaptic currents (sIPSCs) were recorded in the presence of 6,7-dinitroquinoxaline-2,3-dione (DNQX) (20 µM) and D,L-2-amino-5-phosphopentanoic acid (D,L-AP5) (50 µM), with an internal solution containing (in mM): 70 K-gluconate, 70 KCl, 10 HEPES, 0.5 EGTA, 4 MgATP, 0.4 NaGTP, 4 Na_2-phosphocreatine (pH 7.3 with KOH). Action-potential

independent miniature IPSCs (mIPSCs) were recorded under the same conditions as sIPSCs, but in the presence of 1 µM tetrodotoxin (TTX) to block voltage-gated sodium channels. The membrane potential was held at -70 mV for voltage-clamp experiments. Signals were amplified, filtered at 2 kHz and digitized at 10 kHz using a MultiClamp 700B amplifier (Molecular Devices) and stored using pClamp 10 software. Series resistance was constantly monitored, and the cells were rejected from analysis if the resistance changed by >20% during the experiment or reached a value higher than 30 MΩ. No series resistance compensation was used. Resting membrane potential was measured in bridge mode (I=0) immediately after obtaining whole-cell access. The basic electrophysiological properties of the cells were determined from the voltage responses to a series of 500 ms hyperpolarizing and depolarizing square current pulses. Passive and active membrane properties were analysed with Matlab (R2019b, MathWorks) using a custom script. Miniature and spontaneous synaptic currents (IPSCs and EPSCs) data were analysed with Mini Analysis (Synaptosoft). The detected currents were manually inspected to exclude false events.

Data processing and statistical analyses

Statistical analyses and data processing were performed with GraphPad Prism (Software Inc.) and RStudio 1_2_5019 (R version 3.6.1, R Foundation for Statistical Computing). The variance between cells within slices was larger than the variance between slices, indicating that individual cells can be treated as independent measurements. Differences between time points (P21, P42 and P85) were tested with one-way ANOVA followed by a Tukey's test when significant (denoted in figures by a color-coded asterisk in blue for CTL and red for SPD). Differences between groups were tested with two-way ANOVA followed by a Tukey's test (black asterisks in figures). Percentage growth for the P21-P42 timeframe was calculated by normalizing the values of P42 (CTL and SPD) to the mean of P21. For the P42-P85 timeframe, the P85 values were normalized to the P42 mean of the same condition. All graphs represent the mean ± standard error of the mean (SEM) with individual data points shown in coloured circles.

RESULTS

We performed whole cell patch clamp recordings in layer 5 (L5) pyramidal cells in the mPFC (Fig. 1A) in slices prepared from juvenile (P21), adolescent (P42) and adult (P85) control (CTL) male rats to assess the development of their synaptic input currents (Fig. 1B,C). We found that the frequency of inhibitory inputs onto L5 mPFC pyramidal

neurons strongly increased between P21 and P85. A large, 3-fold, increase in sIPSC frequency occurred between P21 and P42 (Fig 1D,E). Between P42 and P85, a smaller ~60% increase in sIPSC frequency was observed, while large individual differences between L5 cells emerged (Fig 1D,E). Amplitudes of the sIPSCs remained stable across time points (Fig. 1F), while rise and decay kinetics were faster at P42 compared to P21, an effect that was less prominent at P85 (Fig. 1G,H). This suggests that the inhibitory synaptic inputs to L5 cells in the mPFC are undergoing intense development between P21 and P42, with a smaller rate of growth after P42 until adulthood. These findings are in agreement with previous studies showing an increase in inhibitory synaptic inputs (Cass et al., 2014; Kalemaki et al., 2020) and accelerating kinetics (Vicini et al., 2001; Hashimoto et al., 2010) during early development.

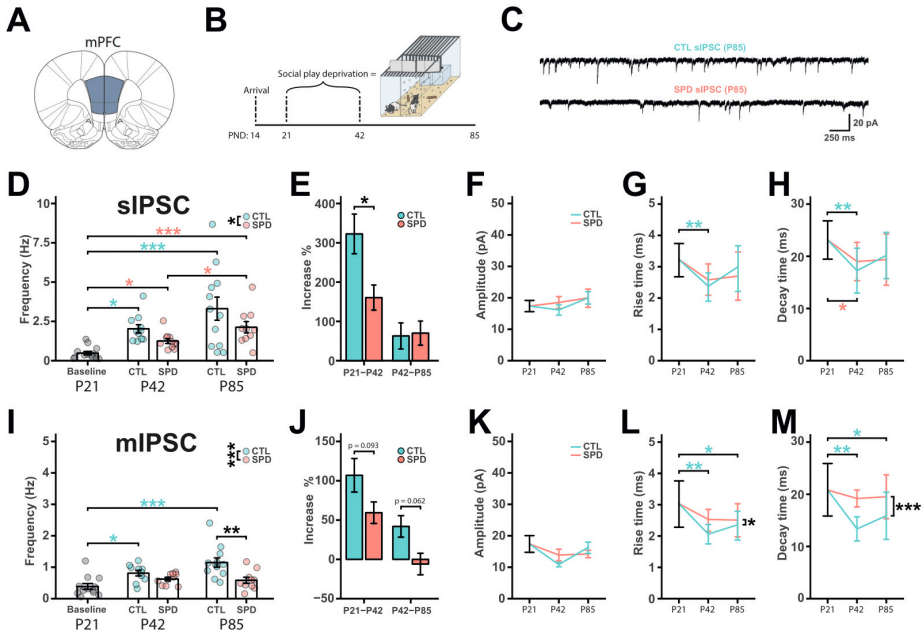

Figure 1.

(**A**) Schematic diagram depicting the recording site in the mPFC. (**B**) Social play deprivation (SPD) paradigm. (**C**) Example traces of spontaneous inhibitory postsynaptic currents (sIPSCs) in L5 pyramidal cells in slices from P85 control (CTL) and SPD rats. (**D**) Frequency of sIPSCs in Baseline (P21), CTL and SPD slices (P42 & P85) (CTL 1W-ANOVA, Time: p<0.001; SPD 1W-ANOVA, Time: p<0.001; 2W-ANOVA, Condition: p=0.031, Time: p=0.023, Interaction: p=0.67). (**E**) Percentage increase of sIPSC frequency from P21 to P42 and P42 to P85 for both CTL and SPD slices (P21-P42 T-Test, p=0.018; P42-P85 T-Test, p=0.88). (**F-H**) Amplitude (**F**) (CTL 1W-ANOVA, Time: p=0.34; SPD 1W-ANOVA, Time: p=0.72; 2W-ANOVA, Condition: p=0.63, Time: p=0.22, Interaction: p=0.56), Rise time (**G**) (CTL 1W-ANOVA, Time: p=0.005; SPD 1W-ANOVA, Time: p=0.045; 2W-ANOVA, Condition: p=0.95. Time: p=0.078, Interaction: p=0.24) and Decay time (**H**) (CTL 1W-ANOVA, Time: p=0.004; SPD 1W-ANOVA, Time: p=0.020; 2W-ANOVA, Condition: p=0.77, Time: p=0.23, Interaction: p=0.36) of sIPSC events. (**I**) Frequency of miniature inhibitory postsynaptic currents (mIPSCs) in Baseline (P21), CTL and SPD slices (P42 & P85) (CTL 1W-ANOVA, Time: p<0.001; SPD 1W-ANOVA, Time: p<0.001; 2W-ANOVA, Condition: p<0.001, Time: p=0.15, Interaction: p=0.089) (**J**) Percentage increase of mIPSC frequency from P21 to P42 and P42 to P85 for both CTL and SPD slices (P21-P42 T-Test, p=0.093; P42-P85 T-Test, p=0.062). (**K-M**) Amplitude (**K**) (CTL 1W-ANOVA, Time: p=0.072; SPD 1W-ANOVA, Time: p=0.42; 2W-ANOVA, Condition: p=0.85, Time: p=0.058, Interaction: p=0.10), Rise time (**L**) (CTL 1W-ANOVA, Time: p=0.001; SPD 1W-ANOVA, Time: p=0.081; 2W-ANOVA, Condition: p=0.042, Time: p=0.32, Interaction: p=0.34) and Decay time (**M**) (CTL 1W-ANOVA, Time: p<0.001; SPD 1W-ANOVA, Time: p=0.61; 2W-ANOVA, Condition: p<0.001, Time: p=0.20, Interaction: p=0.34) of mIPSC events. (**D-H**) Data from 13 (P21), 11 (P42 CTL), 11 (P42 SPD), 12 (P85 CTL), 10 (P85 SPD) cells. (**I-M**) Data from 12 (P21), 10 (P42 CTL), 10 (P42 SPD), 12 (P85 CTL), 10 (P85 SPD) cells. Statistical range: * p≤0.05; ** p<0.01; *** p<0.001

We previously showed that SPD during P21-42 results in a reduction of inhibitory synapses onto L5 pyramidal somata in the mPFC of adult rats (Bijlsma et al., 2022). Here we assessed how SPD (Fig. 1B) affects the developmental trajectory of the synaptic circuitry in the mPFC. We observed that the large increase in sIPSCs found in CTL animals between P21 and P42 was reduced in SPD animals, and sIPSC frequency modestly increased between P42 to P85 (Fig. 1D). Interestingly, when the developmental increase was calculated relative to the sIPSC frequency at P42, we observed that the sIPSC reduction in L5 cells was entirely attributable to the SPD period between P21 and P42, while the increase from P42 to P85 was comparable in both conditions (Fig. 1E). SPD did not affect sIPSC amplitude (Fig. 1F) and the developmental acceleration of rise and decay time (Fig. 1G,H).

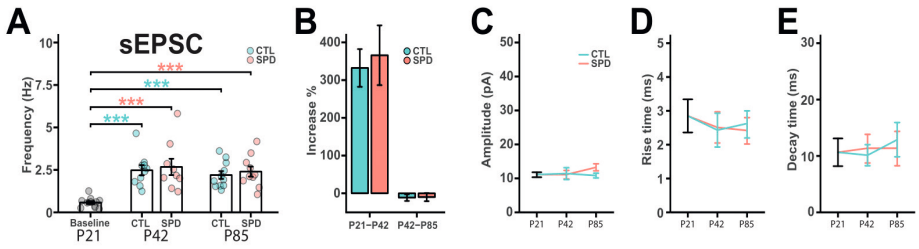

Figure 2.

(**A**) Frequency of spontaneous excitatory postsynaptic currents (sEPSCs) in Baseline (P21), CTL and SPD mPFC slices (P42 & P85) (CTL 1W-ANOVA, Time: p<0.001; SPD 1W-ANOVA, Time: p<0.001; 2W-ANOVA, Condition: p=0.55, Time: p=0.40, Interaction: p=0.99). (**B**) Percentage increase of sEPSC frequency from P21 to P42 and P42 to P85 for both CTL and SPD mPFC slices (P21-P42 T-Test, p=0.73; P42-P85 T-Test, p=0.93). (**C-E**) Amplitude (**C**) (CTL 1W-ANOVA, Time: p=0.94; SPD 1W-ANOVA, Time: p=0.22; 2W-ANOVA, Condition: p=0.36, Time: p=0.55, Interaction: p=0.27), Rise time (**D**) (CTL 1W-ANOVA, Time: p=0.15; SPD 1W-ANOVA, Time: p=0.11; 2W-ANOVA, Condition: p=0.70, Time: p=0.76, Interaction: p=0.38) and Decay time (**E**) (CTL 1W-ANOVA, Time: p=0.052; SPD 1W-ANOVA, Time: p=0.84; 2W-ANOVA, Condition: p=0.76, Time: p=0.11, Interaction: p=0.14) of sEPSC events. (**A-E**) Data from 11 (P21), 10 (P42 CTL), 9 (P42 SPD), 11 (P85 CTL), 10 (P85 SPD) cells. Statistical range: * p≤0.05; ** p<0.01; *** p<0.001

We also recorded miniature inhibitory postsynaptic currents (mIPSCs) in the presence of TTX which blocked all neuronal activity in the slices. In CTL slices, mIPSC frequency doubled between P21 and P42, followed by a smaller increase between P42 and P85 (Fig. 1I,J). Consistent with our observations for sIPSCs, mIPSC amplitudes did not change over this developmental period (Fig. 1K), while rise and decay kinetics became faster (Fig. 1L,M). The developmental increase in the frequency of mIPSCs was smaller compared to sIPSCs (compare fig. 1E and 1J), which suggests that the increase in inhibitory currents reflects the formation of new inhibitory synapses during this

period as well as an increase in activity-dependent release. Consistent with our previous findings (Bijlsma et al., 2022), mIPSC frequency was reduced in the mPFC of SPD slices at P85, but the reduction was less pronounced at P42 (Fig. 1I). In SPD rats, mIPSC frequency only increased marginally between P21 and P42 and remained stable after P42, while mIPSC frequency in CTL rats gradually increased during this entire period (Fig. 1J). The amplitude (Fig. 1K) of the mIPSCs were not affected by SPD, but the acceleration of rise and decay kinetics appeared less pronounced compared to CTL (Fig. 1L,M). Together, these results indicate that SPD interferes with the development of activity-dependent and -independent inhibitory currents in L5 cells of the mPFC and that the strongest effect is observed immediately after the deprivation period.

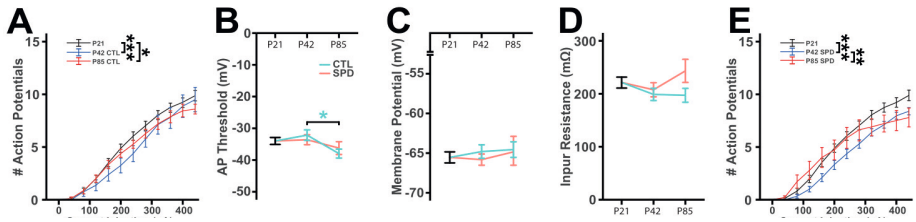

Figure 3.

(**A**) Number of action potentials during 500 ms current injections in mPFC slices from P21, P42 and P85 CTL rats (2W-ANOVA, AP: p<0.001, Current: p<0.001, Interaction: p= 0.93). (**B-D**) AP Threshold (**B**) (CTL 1W-ANOVA, Time: p=0.039; SPD 1W-ANOVA, Time: p=0.51; 2W-ANOVA, Condition: p=0.86, Time: p=0.020. Interaction: p=0.36), resting potential (**C**) (CTL 1W-ANOVA, Time: p=0.68; SPD 1W-ANOVA, Time: p=0.77; 2W-ANOVA, Condition: p=0.55, Time: p=0.53, Interaction: p=0.70) and input resistance (**D**) (CTL 1W-ANOVA, Time: p=0.26; SPD 1W-ANOVA, Time: p=0.31; 2W-ANOVA, Condition: p=0.13. Time: p=0.26, Interaction: p=0.24). (**E**) Number of action potentials during 500 ms current injections in mPFC slices from P21, P42 and P85 SPD rats (2W-ANOVA, AP: p<0.001, Current: p<0.001, Interaction: p= 0.030). (**A-E**) Data from 33 (P21), 16 (P42 CTL), 21 (P42 SPD), 15 (P85 CTL), 14 (P85 SPD) cells. Statistical range: * p≤0.05; ** p<0.01; *** p<0.001

We previously showed that excitatory synaptic currents in L5 cells were unaffected by SPD in the adult mPFC. However, SPD may influence the developmental time course of excitatory synapse formation in the mPFC. We therefore measured excitatory synaptic inputs in mPFC slices from CTL and SPD rats at all three ages. In CTL slices, we observed a large increase in sEPSC frequency between P21 and P42, but sEPSC frequency remained stable after P42 (Fig. 2A,B). This is in line with the reported developmental increase of sEPSCs onto L5 fast-spiking interneurons in mPFC slices in a similar developmental period (Caballero et al., 2014a). The amplitudes of excitatory inputs remained stable over this period (Fig. 2C). The rise and decay times of sEPSCs were comparable between all time points (Fig. 2D,E). SPD did not affect any aspect of sEPSCs (Fig. 2A-E). These data indicate that similar to inhibitory synapses, excitatory synaptic inputs to L5 neurons in the mPFC undergo strong growth between P21 and P42. However, in stark contrast to inhibitory synapses, the development of excitatory synapses is not affected by SPD.

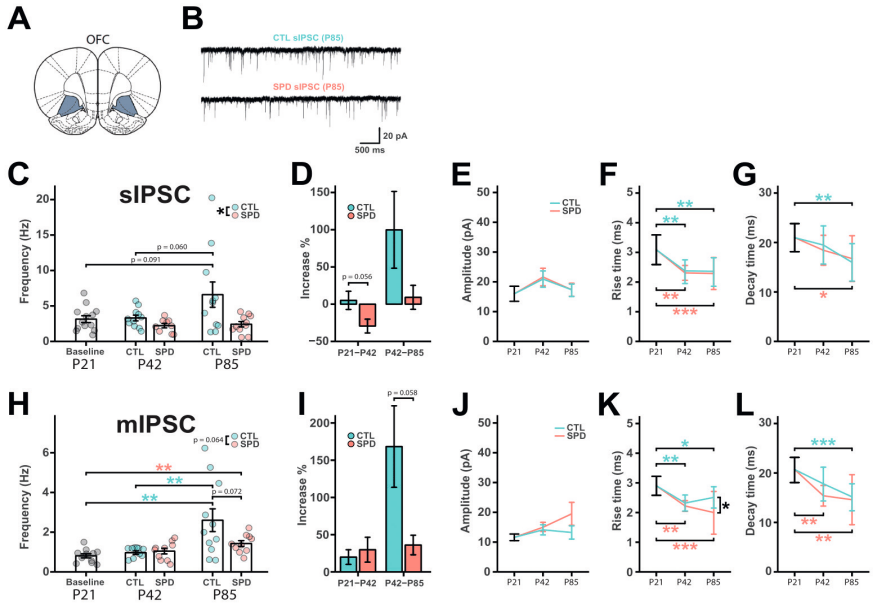

Figure 4.

(**A**) Schematic diagram depicting the recording site in the OFC. (**B**) Example traces of spontaneous inhibitory postsynaptic currents (sIPSCs) in L5 pyramidal cells in slices from P85 control (CTL) and SPD rats. (**C**) Frequency of sIPSCs in Baseline (P21), CTL and SPD slices (P42 & P85) (CTL 1W-ANOVA, Time: p=0.045; SPD 1W-ANOVA, Time: p=0.26; 2W-ANOVA, Condition: p=0.012, Time: p=0.073, Interaction: p=0.13). (**D**) Percentage increase of sIPSC frequency from P21 to P42 and P42 to P85 for both CTL and SPD slices (P21-P42 T-Test, p=0.056; P42-P85 T-Test, p=0.12). (**E-G**) Amplitude (**E**) CTL 1W-ANOVA, Time: p=0.35; SPD 1W-ANOVA, Time: p=0.310; 2W-ANOVA, Condition: p=0.93, Time: p=0.12, Interaction: p=0.92), Rise time (**F**) (CTL 1W-ANOVA, Time: p<0.001; SPD 1W-ANOVA, Time: p<0.001; 2W-ANOVA, Condition: p=0.74, Time: p=0.95, Interaction: p=0.97) and Decay time (**G**) (CTL 1W-ANOVA, Time: p=0.007; SPD 1W-ANOVA, Time: p=0.032; 2W-ANOVA, Condition: p=0.85, Time: p=0.044, Interaction: p=0.47) of sIPSC events. (**H**) Frequency of miniature inhibitory postsynaptic currents (mIPSCs) in Baseline (P21), CTL and SPD slices (P42 & P85) (CTL 1W-ANOVA, Time: p=0.002; SPD 1W-ANOVA, Time: p=0.008; 2W-ANOVA, Condition: p=0.064, Time: p=0.006, Interaction: p=0.077). (**I**) Percentage increase of mIPSC frequency from P21 to P42 and P42 to P85 for both CTL and SPD slices (P21-P42 T-Test, p=0.76; P42-P85 T-Test, p=0.058). (**J-L**) Amplitude (**J**) (CTL 1W-ANOVA, Time: p=0.51; SPD 1W-ANOVA, Time: p=0.084; 2W-ANOVA, Condition: p=0.17, Time: p=0.53, Interaction: p=0.27), Rise time (**K**) (CTL 1W-ANOVA, Time: p=0.002; SPD 1W-ANOVA, Time: p<0.001; 2W-ANOVA, Condition: p=0.035, Time: p=0.89, Interaction: p=0.16) and Decay time (**L**) (CTL 1W-ANOVA, Time: p<0.001; SPD 1W-ANOVA, Time: p=0.001; 2W-ANOVA, Condition: p=0.27, Time: p=0.15, Interaction: p=0.43) of mIPSC events. (C-G) Data from 13 (P21), 11 (P42 CTL), 9 (P42 SPD), 11 (P85 CTL), 11 (P85 SPD) cells. (**E-L**) Data from 11 (P21), 9 (P42 CTL), 10 (P42 SPD), 11 (P85 CTL), 10 (P85 SPD) cells Statistical range: * p≤0.05; ** p<0.01; *** p<0.001

We also assessed the intrinsic excitability of L5 pyramidal neurons in mPFC slices from CTL and SPD rats. We recorded action potentials (APs) during a series of increasing current injections. We observed that the intrinsic excitability of CTL cells slightly decreased from P21 to P42 and this was maintained in the P85 rats (Fig. 3A). AP threshold remained stable between P21 and P42 but was slightly lower at P85 (Fig. 3B). The membrane potential (Fig. 3C) and input resistance (Fig. 3D) were not different between time points. In slices from SPD rats, the developmental reduction in intrinsic excitability between P21 and P42 (Fig. 3E) was comparable to the CTL animals (comparing Fig. 3A and Fig. 3E, P42 CTL-SPD 2W-ANOVA, condition: p=0.097). This reduction was partly reversed, especially at lower current injections, in P85 rats. When comparing CTL and SPD cells at P85, no differences were found in AP number (comparing Fig. 3A and Fig. 3E, P85 CTL-SPD 2W-ANOVA, condition: p=0.79). The AP threshold (Fig. 3B), resting membrane potential (Fig. 3C) and input resistance (Fig. 3D) of the recorded neurons remained unaffected by SPD, in line with current literature (Baarendse et al., 2013; Bicks et al., 2020; Yamamuro et al., 2020). Our data indicate that AP firing in L5 pyramidal neurons is slightly reduced over development and that this is only mildly affected by SPD.

In contrast to the mPFC, developmental studies on circuitry development in the OFC are scarce or even absent. We therefore compared the development of the OFC and the mPFC and we assessed how SPD affects OFC development. Interestingly, the frequency of sIPSCs on OFC L5 pyramidal neurons at P21 (Fig. 4A,B) was 6-fold higher than in mPFC P21 slices in CTL rats. The sIPSC frequency remained stable between P21 and P42, and seemed to increase between P42 and P85 with large cell-to-cell variability (Fig. 4C,D). Similar to the mPFC, sIPSC amplitudes remained stable across time points (Fig. 4E) while the rise and decay kinetics became slightly faster in P42 and P85 rats compared to juvenile animals (Fig. 4F,G). This suggests that inhibitory synaptic inputs to L5 cells in the OFC only undergo growth after P42.

Similar to the mPFC, sIPSC frequency in the OFC in slices from SPD rats was reduced compared to CTL at P85 (Fig. 4C). The sIPSC frequency in SPD slices did not change much during this developmental period and the large cell-to-cell variability that we observed in CTL slices at P85 was completely absent (Fig. 4C,D). We noticed that the sIPSC frequency appeared slightly reduced immediately after the SPD period at P42, but this did not reach significance. Similar to our observations in the mPFC, SPD did not affect sIPSC amplitude (Fig. 4E) or the acceleration in rise and decay kinetics (Fig. 4F, G). These results show that SPD may not affect sIPSC frequency during the deprivation

period, but appeared to prevent the increase of inhibitory currents onto L5 neurons in the OFC afterwards.

The developmental time course of mIPSCs was comparable to that of sIPSCs, with no change in frequency between P21 and P42 and a ~2-fold increase between P42 and P85 (Fig. 4H,I). Event amplitudes remained unchanged across the different time points (Fig. 4J) with both rise and decay kinetics becoming faster after P21 (Fig. 4K,L), similar to the mPFC. We did not find any effect of SPD on the mIPSC frequency between P21 and P42 (Fig. 4H), while the increase between P42 and P85 appeared to be reduced after SPD compared to CTL (Fig. 4I). SPD did not affect mIPSC amplitude (Fig. 4J), rise (Fig. 4K) and decay time (Fig. 4L). As the frequency of sIPSCs was substantially higher than of mIPSCs at P21, it is clear that there was already an activity-dependent component in the sIPSCs in the OFC at this early age, which was different from the mPFC. The effects of SPD are comparable between spontaneous and miniature inhibitory currents, suggesting that the SPD effect does not depend on activity but reflects a reduction in the number of inhibitory synapses after P42.

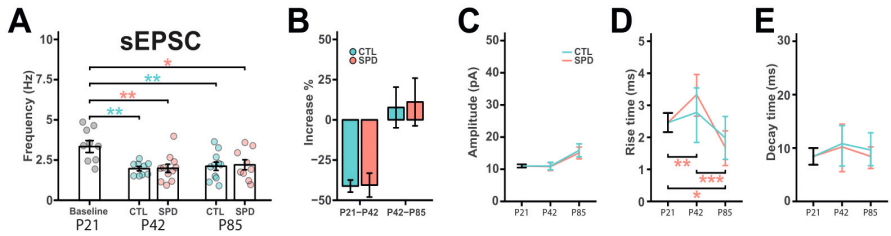

Figure 5.

(**A**) Frequency of spontaneous excitatory postsynaptic currents (sEPSCs) in Baseline (P21), CTL and SPD OFC slices (P42 & P85) (CTL 1W-ANOVA, Time: p=0.002; SPD 1W-ANOVA, Time: p=0.008; 2W-ANOVA, Condition: p=0.88, Time: p=0.47, Interaction: p=0.89). (**B**) Percentage increase of sEPSC frequency from P21 to P42 and P42 to P85 for both CTL and SPD slices (P21-P42 T-Test, p=0.95; P42-P85 T-Test, p=0.87). (C-E) Amplitude (**C**) (CTL 1W-ANOVA, Time: p=0.038; SPD 1W-ANOVA, Time: p=0.054; 2W-ANOVA, Condition: p=0.57, Time: p=0.009, Interaction: p=0.84), Rise time (**D**) (CTL 1W-ANOVA, Time: p=0.083; SPD 1W-ANOVA, Time: p<0.001; 2W-ANOVA, Condition: p=0.24, Time: p<0.001, Interaction: p=0.048) and Decay time (**E**) (CTL 1W-ANOVA, Time: p=0.44; SPD 1W-ANOVA, Time: p=0.41; 2W-ANOVA, Condition: p=0.46, Time: p=0.30, Interaction: p=0.61) of sEPSC events. (**A-E**) Data from 8 (P21), 9 (P42 CTL), 11 (P42 SPD), 11 (P85 CTL), 9 (P85 SPD) cells. Statistical range: * p≤0.05; ** p<0.01; *** p<0.001

We also assessed the development of excitatory synapses in the OFC. Similar to the inhibitory synapses, sEPSC frequency was higher at P21 in the OFC compared to the mPFC. sEPSC frequency decreased between P21 and P42 and then remained stable until P85 (Fig. 5A,B). Amplitudes showed a small increase between P42 and

P85 (Fig. 5C). sEPSC rise kinetics became faster with development (Fig. 5D), while the decay kinetics remained stable (Fig. 5E). SPD rats showed a similar decrease in sEPSC frequency between P21 and P42 and an increase in sEPSC amplitude between P42 and P85 compared to CTL rats. No differences were found in rise and decay kinetics after SPD (Fig. 5A-E). This indicates that similar to the mPFC, SPD did not affect the development of excitatory currents in the OFC.

Intrinsic excitability of layer 5 pyramidal neurons was assessed in OFC slices from CTL and SPD rats. Similar to what was observed in the mPFC, the intrinsic excitability of OFC cells decreased from P21 to P42, but then recovered at P85 (Fig. 6A). AP threshold increased between P21 and P42 after which a small decrease was found at P85 (Fig. 6B), eventually coming back at P21 levels. The membrane potential (Fig. 6C) and input resistance (Fig. 6D) did not change over this developmental period. In SPD slices, the transient reduction in intrinsic excitability between P21 and P42 was absent (comparing Fig. 6A and Fig. 6E, P42 CTL-SPD 2W-ANOVA, condition: $p<0.001$), and AP firing rates showed a gradual increase over development (Fig. 6E). AP firing rates at P85 were comparable between CTL and SPD slices (comparing Fig. 6A and Fig. 6E, P85 CTL-SPD 2W-ANOVA, condition: $p=0.56$). The membrane potential (Fig. 6B), AP threshold (Fig. 6C) and input resistance (Fig. 6D) of the recorded neurons were unaffected after SPD. These experiments show that SPD has a small, but transient, effect on the intrinsic excitability of L5 cells in the OFC.

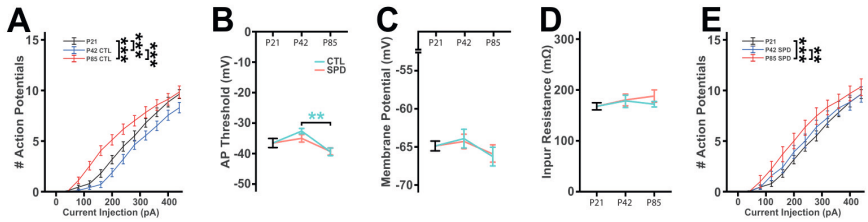

Figure 6.

(**A**) Number of action potentials during 500 ms current injections in OFC slices from P21, P42 and P85 CTL rats (2W-ANOVA, AP: $p<0.001$, Current: $p<0.001$, Interaction: $p<0.001$). (**B-D**) AP Threshold (**B**) (CTL 1W-ANOVA, Time: $p=0.002$; SPD 1W-ANOVA, Time: $p=0.16$; 2W-ANOVA, Condition: $p=0.47$. Time: $p<0.001$, Interaction: $p=0.31$), resting potential (**C**) (CTL 1W-ANOVA, Time: $p=0.31$; SPD 1W-ANOVA, Time: $p=0.60$; 2W-ANOVA, Condition: $p=0.88$, Time: $p=0.11$, Interaction: $p=0.70$) and input resistance (**D**) (CTL 1W-ANOVA, Time: $p=0.74$; SPD 1W-ANOVA, Time: $p=0.46$; 2W-ANOVA, Condition: $p=0.51$. Time: $p=0.97$, Interaction: $p=0.59$). (**E**) Number of action potentials during 500 ms current injections in OFC slices from P21, P42 and P85 SPD rats (2W-ANOVA, AP: $p<0.001$, Current: $p<0.001$, Interaction: $p= 0.99$). (**A-E**) Data from 24 (P21), 24 (P42 CTL), 27 (P42 SPD), 18 (P85 CTL), 13 (P85 SPD) cells. Statistical range: * $p\leq0.05$; ** $p<0.01$; *** $p<0.001$

Together, these data highlight the differential development of synaptic connections onto L5 pyramidal cells in the mPFC and OFC. At P21, inhibitory and excitatory synaptic inputs onto L5 pyramidal cells were already present in the OFC, while these were largely absent, or at least silent, in the mPFC (Fig. 7A,B). SPD strongly affected the development of inhibitory inputs in both brain regions (Fig. 7C) while leaving excitatory synapses unaffected (Fig. 7D) and with only a transient effect on the firing properties of L5 cells. In both brain regions, inhibitory currents were reduced in adult slices from SPD rats, but this reduction occurred at different times in the OFC and mPFC. The strongest reduction of inhibitory inputs in the mPFC was observed immediately after SPD at P42, while the reduction of sIPSCs in the OFC only became apparent at P85.

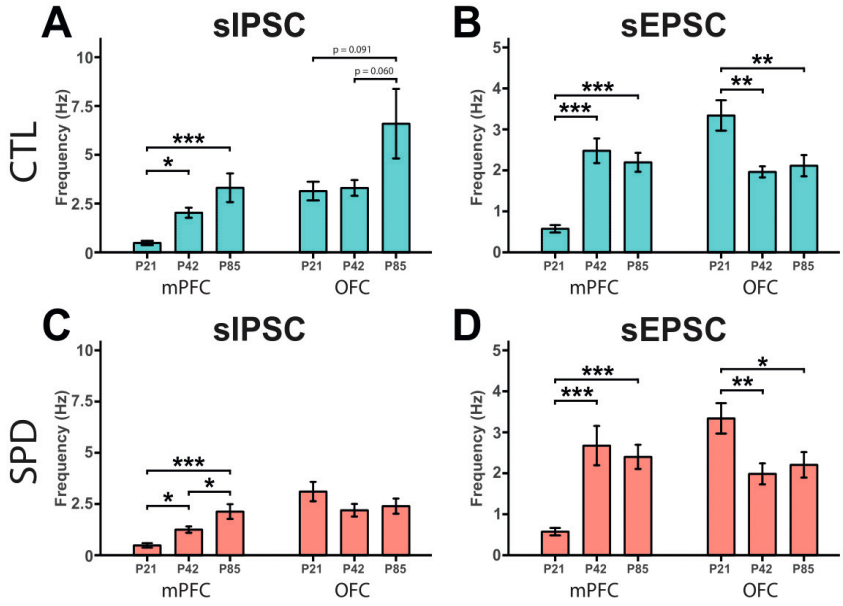

Figure 7.

(**A,B**) Frequency of sIPSCs (**A**) (mPFC 1W-ANOVA, Time: p<0.001; OFC 1W-ANOVA, Time: p=0.049) and sEPSCs (**B**) (mPFC 1W-ANOVA, Time: p<0.001; OFC 1W-ANOVA, Time: p=0.002) in mPFC and OFC slices from P21, P42 and P85 CTL rats. (**C,D**) Frequency of sIPSCs (**C**) (mPFC 1W-ANOVA, Time: p<0.001; OFC 1W-ANOVA, Time: p=0.265) and sEPSCs (**D**) (mPFC 1W-ANOVA, Time: p<0.001; OFC 1W-ANOVA, Time: p=0.007) in mPFC and OFC slices from P21, P42 and P85 SPD rats. Statistical range: * p≤0.05; ** p<0.01; *** p<0.001

DISCUSSION

In this study we present the developmental timeline of inhibitory and excitatory synaptic inputs onto layer 5 pyramidal neurons in two subregions of the rat PFC, i.e. the mPFC and OFC. We found that these subregions develop with a differential time course and that SPD affects inhibitory but not excitatory inputs onto L5 pyramidal neurons in both regions, resulting in a specific reduction of inhibitory currents in adulthood. However, the reduction in IPSCs in the two PFC subregions arose via differential developmental trajectories. In the mPFC, development was mostly affected by SPD between P21 and P42, while IPSCs in the OFC were mainly affected after P42.

Social play enhances neural activity in the PFC and in corticostriatal and limbic structures which are connected to the PFC in the adult brain (Gordon et al., 2002, 2003; Hoover and Vertes, 2011; Van Kerkhof et al., 2014). Social play is almost absent before P21 (Baenninger, 1967; Panksepp, 1981), so activity in the PFC generated by social play is expected to be low at that age. During early adolescence (~P30 - P42), play is abundant and play-induced neural activity is likely one of the driving forces of PFC maturation. Both the OFC and mPFC have been implicated in social play behaviour (Schneider and Koch, 2005; Van Kerkhof et al., 2013), but they exert a differential function in social interactions and cognitive flexibility. The mPFC in rats is important for shifting between cognitive strategies (Ragozzino et al., 1999; Birrell and Brown, 2000) and for coordination of movements during social interactions (Bell et al., 2009; Himmler et al., 2014). In contrast, the OFC may be more involved in shifting between stimulus-reward associations (Ghods-Sharifi et al., 2008) and response modulation when interacting with different social play partners (Pellis et al., 2006). Analysis of morphological development of pyramidal cells in the PFC has indicated that cellular maturation in the mPFC depends on mere social play experience, whereas OFC pyramidal cell maturation depends on interaction with multiple social partners (Bell et al., 2010; Himmler et al., 2018). As such, future studies on OFC development should also include animals raised within larger groups than two animals per cage. In any event, the current study shows that social play is one of the important driving forces of PFC maturation, and that SPD differentially affects the development of synaptic connections in the mPFC and the OFC.

To the best of our knowledge, our study is the first study that explicitly compares the developmental trajectory of inhibitory and excitatory synaptic inputs across these developmental timepoints in both the mPFC and OFC. So far, there have been a handful of studies that examined the development of postsynaptic inputs in the mPFC (Caballero et al., 2014b; Cass et al., 2014; Miyamae et al., 2017; Kroon et al., 2019; Kalemaki et al.,

2020), but the development of the OFC circuitry has not been addressed. Our data show that the synaptic connections onto L5 cells in the mPFC and OFC develop via distinct trajectories (Fig. 7A,B). In the mPFC, the frequency of inhibitory inputs increases across juvenile and adolescent development with a strong increase in activity-dependent currents between P21 and P42. This coincides well with the described transition from an inhibitory system dominated mostly by regular spiking (calretinin (CR)-positive) to fast-spiking (parvalbumin (PV)-positive) interneurons (Caballero et al., 2014a; Caballero and Tseng, 2016) and the increasing excitatory drive onto PV interneurons (Caballero et al., 2014a). The modest additional increase in sIPSC frequency between P42 and P85 is also in agreement with a previous report (Cass et al., 2014). In contrast, the frequency of inhibitory inputs in the OFC was already high at P21 and remained stable until P42 (Fig. 7A). Comparison of mIPSC and sIPSC frequencies (Figs 1 and 4) indicates a strong activity-dependent contribution to the inhibitory drive, which occurs earlier in the OFC than in the mPFC. This suggests that the transition of the inhibitory system to a PV interneuron-dominated system occurred earlier in the OFC than in the mPFC. The development of excitatory inputs to L5 cells increased in the mPFC between P21 and P42 (Caballero et al., 2014a), but decreased in the OFC to reach comparable values at P42 (Fig. 7B). In both regions, sEPSC frequency remained stable after P42 until adulthood. Together, our data demonstrates a clear difference in the development of synaptic circuitry in these two main subregions of the rat PFC, which likely influences how they are affected by early life experience.

The mPFC and OFC are reciprocally connected, which was shown by extensive anatomical studies using antero- and retrograde tracers (Vertes, 2004; Hoover and Vertes, 2007, 2011). It is therefore likely that a synaptic change in one of the regions will affect the circuit development in the other. Consistent with our previous findings (Bijlsma et al., 2022), we observed that SPD affects the inhibitory, but not the excitatory, connections in both PFC regions. SPD resulted in reduced synaptic inhibition, reminiscent of the impaired development of inhibitory connections that has been described after sensory deprivation (Mowery et al., 2019; Reh et al., 2020). The reduction in IPSCs occurred before P42 in the mPFC, while inhibitory currents in the OFC were only affected after P42. This late effect in the OFC could either reflect a specific effect of the recovery from SPD in the OFC, or an indirect consequence of the reduced IPSCs in the mPFC and possibly other regions. It will be important to determine which inputs to the OFC are responsible for the difference in excitatory and inhibitory drive of L5 cells in the mPFC and OFC at P21, before the onset of play.

Together, our results demonstrate that excitatory and inhibitory synaptic inputs in the mPFC and OFC follow distinct developmental trajectories, and that lack of social play experience disturbs this development in a region-specific manner. This study highlights the differential vulnerability of PFC subregions to developmental insults, such as the lack of social play, which likely contributes to the multifaceted impact on cognitive performance in adulthood.

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CHAPTER 4

Opportunities for risk-taking during play alters cognitive performance and prefrontal inhibitory signalling in rats

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ABSTRACT

Social play behaviour is a rewarding activity that can entail risks, allowing young individuals to test the limits of their capacities and to train their cognitive and emotional adaptability to challenges. Here, we tested in rats how opportunities for risk-taking during play affect the development of cognitive and emotional capacities and medial prefrontal cortex (mPFC) function, a brain structure important for risk-based decision-making. Male and female rats were housed socially or social play-deprived (SPD) between postnatal day (P)21 and P42. During this period, half of both groups were daily exposed to a high-risk play environment. Around P85, all rats were tested for cognitive performance and emotional behaviour after which inhibitory currents were recorded in layer 5 pyramidal neurons in mPFC slices. We show that playing in a high-risk environment altered cognitive flexibility in both sexes, and improved behavioural inhibition in males. High-risk play altered anxiety-like behaviour in the elevated plus maze in males and in the open field in females, respectively. SPD affected cognitive flexibility in both sexes and increased anxiety-like behaviour in the elevated plus maze in females. We found that synaptic inhibitory currents in the mPFC were increased in male, but not female, rats after high-risk play, while SPD lowered PFC synaptic inhibition in both sexes. Together, our data show that exposure to risks during play alters cognition and emotional behaviour and affects the development of inhibition in the mPFC. Furthermore, our study suggests that the opportunity to take risks during play cannot substitute for social play behaviour.

INTRODUCTION

Play behaviour is an intrinsically rewarding activity that is abundant among the young of humans and most other mammalian species (Pellis and Pellis, 2009; Graham and Burghardt, 2010). It is typically expressed under safe circumstances in which individuals can experiment with their behaviour and develop motor, social, cognitive and emotional skills necessary for optimal functioning later in life (Špinka et al., 2001; Pellis and Pellis, 2009; Gray, 2017; Nijhof et al., 2018; Sgro and Mychasiuk, 2020). Importantly, when exploring and experimenting with behaviour, playful situations may entail risks, such as the risk of rejection, injury or failure. Although the term "risk" often has negative connotations, risky activities provide ample opportunities to acquire new skills, explore one's physical boundaries (Brussoni et al., 2012; Lavrysen et al., 2017) and learn to cope with anxiety through exposure to fearful stimuli (Allen and Rapee, 2005; Sandseter and Kennair, 2011). Case studies, for example, suggest that play in a natural environment can increase children's problem-solving skills and the ability to successfully cope with difficult or challenging life experiences (McArdle et al., 2013), while limited risky and outdoor play have been associated with lower self-esteem and academic achievements (Tremblay, 2011; Tremblay et al., 2015). There is substantial concern that opportunities for children to engage in free and risky play have declined over the past decades in Western societies (Valentine and McKendrick, 1997; Weir et al., 2006; Gray, 2011; Brussoni et al., 2012; Little et al., 2012). However, although it is generally assumed that risk-taking during play is important to prepare the individual for challenges later in life, little empirical research has been done to explore the contribution of risks to the development of brain and behaviour.

Animal studies, especially in rats, have been valuable to investigate the functions of social play behaviour (Vanderschuren and Trezza, 2014; Pellis et al., 2023). Rats that had been deprived of social play between weaning and adolescence, a period in which social play is especially abundant, display inappropriate behaviour in a social conflict situation (Van Den Berg et al., 1999; Von Frijtag et al., 2002), altered impulse control and decision making (Baarendse et al., 2013; Bijlsma et al., 2022) and increased sensitivity to substances of abuse (Whitaker et al., 2013; Baarendse et al., 2014; Lesscher et al., 2015). At the neuronal level, altered functioning of the prefrontal cortex (PFC) (Bell et al., 2009; Wall et al., 2012; Baarendse et al., 2013; Bijlsma et al., 2022, 2023), a brain structure that is important for higher cognitive, so-called executive functions (e.g. working memory, impulse control, attention, planning and decision making), has been observed in play-deprived animals. Indeed, we have recently demonstrated that deprivation of social play

affects the synaptic connectivity in the medial prefrontal cortex (mPFC) of adult rats, and specifically led to a reduction in inhibitory synaptic inputs onto layer 5 pyramidal cells (Bijlsma et al., 2022, 2023). These findings support the notion that social play behaviour subserves the development of emotional and cognitive capacities and their PFC substrate. However, it remains unclear how the opportunity to take risks during play affects the development of emotion, cognition and PFC function.

Sandseter (2007) has described six categories of "risky" play in children: 1) play with great heights; 2) play with high speed; 3) play with harmful tools; 4) play near dangerous elements; 5) rough-and-tumble play; and 6) play where children can 'disappear'/get lost (Sandseter, 2007). Currently, social play behaviour assessment in rats entails only one of these categories of risky play, i.e. rough-and-tumble play, but not the other ones. Moreover, the typical laboratory housing settings for rodents are limited in the actual risks that the animals may encounter. Here we therefore combined social play deprivation with opportunities for risk-taking in play to create four rearing conditions in rats that reflect a continuum of play behaviour, ranging from no play to a combination of social and risky play. These conditions were used to answer the following questions: 1) Does the opportunity for risk-taking behaviour during play alter cognitive flexibility, behavioural inhibition and anxiety-like behaviour?; 2) How does the opportunity for social play affect cognitive flexibility, behavioural inhibition and anxiety-like behaviour?; 3) How does play manipulation affect the maturation of the mPFC?; and 4) Do the consequences of high-risk play and social play deprivation differ between males and females?

MATERIALS & METHODS

Animals and housing conditions

Three batches of 48 male and one batch of 48 female Lister Hooded rats were obtained from Charles River (Sulzfeld, Germany). They arrived on postnatal day (P)14 in litters of eight with nursing mothers. All rats were subjected to a reversed 12:12h light-dark cycle (lights on 07:00, lights off 19:00) with ad libitum access to water and food. All experiments (Fig. 1A) were conducted during the active phase of the animals (9:00 - 17:00). Rats were weighed and handled at least once a week throughout the experiment. One week before the start of behavioural testing, the rats were subjected to a restricted diet of 4.5 grams of chow per 100 grams of body weight. In this way, animals were kept at 90% of their body weight for the duration of behavioural testing. Body weights did not differ significantly between the three male batches. Rats were provided with 30

sucrose pellets (45mg, BioServ) in their home cage before their first exposure to the pellets in the operant conditioning chamber to reduce potential food neophobia. The age at the start of behavioural experimentation was comparable for each task and batch. All experiments were approved by the Animal Ethics Committee of the Utrecht University and the Dutch Central Animal Testing Committee. They were conducted in agreement with Dutch (Wet op de Dierproeven, 1996; Herziene Wet op de Dierproeven, 2014) and European legislation (Guideline 86/609/EEC; Directive 2010/63/EU).

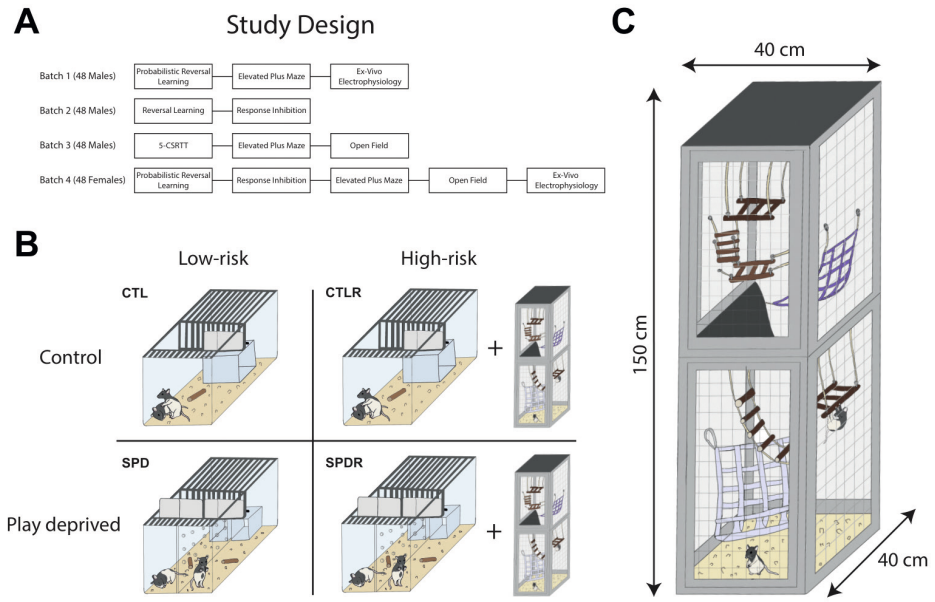

Figure 1. Study design.

(**A**) Study overview. (**B**) Combination of a social play deprivation paradigm and high-risk play enrichment resulting in four rearing conditions that reflect a continuum of play behaviour: control (CTL-LR), social play deprivation (SPD-LR), control risky play (CTL-HR) or social play deprivation risky play (SPD-HR). (**C**) The risky play cage was enriched with multiple ladders, plateaus and other objects to interact with.

Social play deprivation and risky play

Rats were weaned on P21 and were either subjected to the control (CTL-LR), social play deprivation (SPD-LR), control risky play (CTL-HR) or social play deprivation risky play (SPD-HR) group, resulting in 4 groups of 12 rats (Fig. 1B). All rats were housed in pairs with a littermate during the entire experiment. From P21 to P42, a transparent plexiglass divider containing small holes was placed in the middle of the home cage

of SPD-LR and SPD-HR rats creating two separate but identical compartments. Social play-deprived rats were therefore able to receive visual, olfactory and auditive cues from one another. The holes in the plexiglass allowed the rats to have limited physical interaction but no opportunity to physically engage in play. The divider was removed on P42 and SPD-LR/SPD-HR rats were housed in the same pairs for the remainder of the experiment. CTL-HR and SPD-HR were housed under the same conditions as the CTL-LR and SPD-LR groups but were transferred to a "risky play cage" twice a day for 30 minutes during the deprivation period (P21 – P42). The risky play cage measured 150 x 40 x 40 cm (H x W x D) and was enriched with multiple ladders, plateaus and other objects to interact with (Fig. 1C). CTL-HR rats were placed together in the risky play cage, while SPD-HR rats were alone. The risky play cages were designed to allow the rats more opportunities to take risks during play, in comparison to their normal home cage.

Probabilistic reversal learning (PRL) task

Apparatus: Behavioural testing was conducted in operant conditioning chambers (Med Associates, Georgia, USA) enclosed in sound-attenuating cubicles equipped with a ventilation fan. Two retractable levers were located on either side of a central food magazine into which sugar pellets could be delivered via a dispenser. A LED cue light was located above each retractable lever. A white house light was mounted in the top centre of the wall opposite the levers. Online control of the apparatus and data collection was performed using MED-PC (Med Associates) software.

Pre-training: Rats were first habituated once to the operant chamber for 30 min in which the house light was illuminated and 50 sucrose pellets were randomly delivered into the magazine with an average interval of 15 s between reward deliveries.

Phase 1: On the subsequent days, the rats were trained for 30 min under a Fixed-Ratio 1 (FR1) schedule of reinforcement for a minimum of two consecutive daily sessions. A FR1 session started with the illumination of the house light and the insertion of both levers, which remained inserted for the remainder of the session. A response on one of the levers resulted in the delivery of a sucrose pellet into the magazine. There was no limit other than time (max 30 min) or the number of times a rat could press the levers (max 100). To proceed to phase 2, the rat had to obtain an average of at least 50 rewards over two completed sessions. In case a rat obtained a lower number of rewards during the first two sessions, it was further trained on subsequent days until the criterion was met.

Phase 2: A trial started with the presentation of the left lever, the right lever, or both levers and pressing either lever was reinforced under a FR-1 schedule. In this

phase of training, the levers were only retracted after a response, and the animals were subjected to the same sequence of events as during a reinforced trial in the probabilistic reversal learning task. When all animals made at least 100 responses in a session during this phase they progressed as a group to the next phase.

Phase 3: Rats were familiarized with the probabilistic nature of the PRL task, in which both levers were presented and a lever press resulted in 80% reward delivery instead of 100%. Levers were presented until pressed. Rats were trained to a criterion of at least 50 rewards and to make at least one lever press in more than 80% of the trials before progressing to the probabilistic reversal learning phase; this required ~3-4 days.

Probabilistic reversal learning: The protocol used for this task was modified from those of previous studies (Bari et al., 2010; Dalton et al., 2016; Verharen et al., 2020; Bijlsma et al., 2022). At the start of each session, one of the two levers was randomly selected to be 'correct' and the other 'incorrect'. A response on the 'correct' lever resulted in the delivery of a reward on 80% of the trials, whereas a response on the 'incorrect' lever was reinforced on 20% of trials. Each trial started with a 5 s ITI, followed by the illumination of the house light and the insertion of both levers into the chamber. After a 'correct' response, both levers retracted. In case the rat was rewarded, the house light remained illuminated, whereas the house light extinguished in case the rat was not rewarded on the 'correct' lever. An 'incorrect' response or a failure to respond within 30 s after lever insertion (i.e. omission) lead to the retraction of both levers and extinction of the house light so that the chamber returned to the ITI state. When the rat made a string of 8 consecutive trials on the 'correct' lever (regardless of whether they were rewarded or not), contingencies were reversed, meaning that the 'correct' lever became the 'incorrect' lever and the previously 'incorrect' lever became the 'correct' lever. This pattern repeated throughout a daily session. Daily sessions were completed upon performing 200 (males) or 140 trials (females). Female rats were subjected to a lower maximum of trials as they became sated faster which lead to a lower response rate in later trials. The possible number of reversals made was solely limited by the number of trials in a session.

Trial-by-trial analysis: This analysis was performed to assess the shifts in choice behaviour between subsequent trials and to investigate the sensitivity to positive and negative feedback. Depending on whether the rat received a reward or not, it can press the same lever on the subsequent trial or shift towards the other lever, resulting in 4 different possibilities:

- Win-stay: Same lever press on the subsequent trial after receiving a reward.
- Win-shift: Opposite lever press on the subsequent trial after receiving a reward.

- Lose-stay: Same lever press on the subsequent trial after receiving no reward.
- Lose-shift: Opposite lever press on the subsequent trial after receiving no reward.

Response inhibition (RI) task

Apparatus: Behavioural testing was performed in the operant chambers as used for the PRL, except that they were equipped with a shock grid floor and a tone generator (4500 Hz).

Pre-training: Rats were first habituated for four days to the operant chamber for 30 minutes during which the house light was illuminated. Fifty sucrose pellets were randomly delivered into the magazine with an average of 15 s between reward deliveries. This phase was followed by a training phase in which, after an initial 20 s before the start of the first trial, rats had 40 s per trial to retrieve a sucrose pellet. When the sucrose pellet was not retrieved, a new trial started but no new reward was given. The session ended after 60 trials.

Response inhibition: The protocol used for this task was modified from Verharen et al. (2019) (Verharen et al., 2019). In contrast to the study by Verharen et al., in which the shock intensity was adjusted for each individual, we used the same shock intensity for every rat. A response inhibition (RI) session consisted of 60 trials of 40 seconds of which 30 were non-stimulus (NS) trials and 30 were shock trials. In NS trials, rats were allowed to retrieve the sucrose reward directly after its delivery. During the first 12 seconds of shock trials, two cue lights were turned on and a tone was produced by a speaker in the top right corner on the same wall as the house light. When rats collected the reward during the 12 seconds of tone and light presentation, they were punished with a footshock produced by a shock stimulator (Med Associates, USA). After the offset of the tone, the rats could retrieve the reward without consequences. When rats retrieved the reward within 40 seconds without getting shocked (i.e. after tone offset), the trial was labelled as a successful trial. A shock trial was noted if the animal retrieved the reward during the 12 second tone presentation. If the rats did not retrieve the reward within 40 seconds in either NS or shock trials, the trial was labelled as an omission. After an omission, a new trial was started but a reward was only given when the food receptacle was empty. The rats were tested in daily RI sessions for three consecutive days followed by a rest day and then another six consecutive days. A shock intensity of 0.35 mA was used during the first three days. Next, the animals were tested for three days with a shock intensity of 0.45 mA, followed by three sessions with an intensity of 0.55 mA. These shock intensities were based on our previous study (Verharen et al.,

2019), in which shock intensities were titrated for individual animals to reach a criterion of 20 success trials out of 30 stimulus trials, which required a median shock intensity of 0.50 mA (25-75th percentile in between 0.40-0.60 mA).

Parameters : Successful reward collection, the number of omissions, shock count and reward collection latency in both stimulus and non-stimulus trials were used to evaluate overall performance in this task across the three different shock intensities.

Elevated Plus Maze (EPM)

The EPM consisted of two open arms of 50 x 10 cm (L x W) and two closed arms of 50 x 10 x 40 cm (L x W x H) that extended from a central platform of 10 x 10 cm (L x W). The EPM was elevated to a height of 76 cm. It was located in a brightly lit room, with a light intensity of 300 lux on the open arms and central platform, and 240 lux on the closed arms. The test started with each rat being placed on the central platform facing an open arm. Testing lasted for 5 min and the maze was cleaned with water and soap between every trial. All trials were divided over two days, to prevent interference of short social isolation of the cage-mate. The uneven-numbered rats were tested on the first day and the even-numbered rats on the second day. The total time spent on the open arms, closed arms and central platform, the mean velocity and the total distance moved were assessed. All data was acquired through video recordings (Logitech C920 camera) and were analyzed with tracking software (EthoVision, Version 9.0.718, Noldus).

Open Field (OF)

The OF was a circular arena with a diameter of 100 cm and 33.5 cm high walls. The OF was placed in a red-light-lit room. One external white light source was placed on the floor, which resulted in a light intensity of 12 lux in the OF. At the start of the trial, a rat was placed on a marked spot in the peripheral circle facing the walls. The test lasted for 10 min and the OF was cleaned with water and soap after each test. To avoid interference of social isolation, testing was divided over two days in the same way as mentioned for the EPM. Total time in the peripheral, middle and central zone, mean velocity, and total distances moved were measured. The peripheral, middle and central zones were created digitally in EthoVision (Version 9.0.718). Both the peripheral and central zones had a width of 20 cm, while the middle zones measured 10 cm. Data was obtained using video tracking software (EthoVision, Version 9.0.718, Noldus).

Ex vivo electrophysiology

Slice preparation: Adult rats were anaesthetized after behavioural testing (P100 – P130) by induction with isoflurane and then transcardially perfused with ice-cold modified artificial cerebrospinal fluid (ACSF) containing (in mM): 92 Choline chloride, 2.5 KCl, 1.2 NaH_2PO_4, 30 $NaHCO_3$, 20 HEPES, 25 glucose, 5 Na-ascorbate, 3 Na-pyruvate, 0.5 $CaCl_2.2H_2O$, and 10 $MgSO_4.7H_2O$, bubbled with 95% O_2 and 5% CO_2 (pH 7.3–7.4). The brain was quickly removed after decapitation and coronal slices of the mPFC (300 µm) were prepared using a vibratome (Leica VT1000S, Leica Microsystems) in ice-cold modified ACSF. Slices were initially incubated in the carbonated modified ACSF for 5 min at 35 °C and then transferred into a holding chamber containing standard ACSF containing (in mM): 126 NaCl, 3 KCl, 1.3 $MgCl_2.6H_2O$, 2 $CaCl_2.2H_2O$, 20 glucose, 1.25 NaH_2PO_4 and 26 $NaHCO_3$ bubbled with 95% O_2 and 5% CO_2 (pH 7.3) at room temperature for at least 30 minutes. They were subsequently transferred to the recording chamber, perfused with standard ACSF that is continuously bubbled with 95% O_2 and 5% CO_2 at 28–32°C.

Whole-cell recordings and analysis: Whole-cell patch-clamp recordings were performed from layer 5 pyramidal neurons in the mPFC. These neurons were visualized with an Olympus BX61W1 microscope using infrared video microscopy and differential interference contrast (DIC) optics. Patch electrodes were pulled from borosilicate glass capillaries and they had a resistance of 4-6 MΩ when filled with intracellular solutions. Action-potential independent miniature IPSCs (mIPSCs) were recorded in the presence of 6,7-dinitroquinoxaline-2,3-dione (DNQX) (20 µM) and D,L-2-amino-5-phosphopentanoic acid (D,L-AP5) (50 µM), with an internal solution containing (in mM): 70 K-gluconate, 70 KCl, 10 HEPES, 0.5 EGTA, 4 MgATP, 0.4 NaGTP, 4 Na2 phosphocreatine (pH 7.3 with KOH) in the presence of 1 µM tetrodotoxin (TTX) to block sodium channels. The membrane potential was held at -70 mV for voltage-clamp experiments. Signals were amplified, filtered at 2 kHz and digitized at 10 kHz using a MultiClamp 700B amplifier (Molecular Devices) and stored using pClamp 10 software. Series resistance was constantly monitored, and the cells were rejected from analysis if the resistance changed by >20% or it reached a value >30 MΩ. No series resistance compensation was used. Resting membrane potential was measured in bridge mode (I=0) immediately after obtaining whole-cell access. Passive and active membrane properties were analysed with Matlab (MathWorks) using a custom script and synaptic currents were analysed with Mini Analysis (Synaptosoft Inc., Decatur, GA). The detected currents were manually inspected to exclude false events.

Data processing and statistical analyses

Statistical analyses were performed with GraphPad Prism (Software Inc.) and RStudio (R version 3.6.0. R Foundation for Statistical Computing). Data were analysed with a two-way ANOVA with housing condition and risky play as between-subjects factors followed by a post-hoc Tukey's test where appropriate. In the electrophysiological data, the variance between cells within slices was larger than the variance between slices, indicating that individual cells can be treated as independent measurements. All graphs represent the mean ± standard error of the mean (SEM) with individual data points shown in coloured circles. The statistical range used in all figures: * $p<0.05$; ** $p<0.01$; *** $p<0.001$.

RESULTS

Rats were weaned on P21 and were subjected to either the control (CTL-LR), social play deprivation (SPD-LR), risky control (CTL-HR) or risky social play deprivation (SPD-HR) conditions, resulting in 4 groups of 12 rats. To assess the impact of high-risk social play on cognitive flexibility, a probabilistic reversal learning task (PRL) was used, which depends on PFC function (Fig. 2A,B (Dalton et al., 2016; Verharen et al., 2020; Bijlsma et al., 2022). Male rats in the high-risk groups (CTL-HR and SPD-HR) reached a higher performance level in terms of rewards obtained compared to the low-risk groups (CTL-LR and SPD-LR) (Fig. 2C), mainly caused by a difference between the CTL-LR and CTL-HR animals. All male groups completed a comparable amount of reversals (Fig. 2D). The difference in performance could be explained by an enhanced tendency of the high-risk animals to stay at a lever after it was rewarded (win-stay behaviour; Fig. 2E). Consistent with our previous findings (Bijlsma et al., 2022), male social play-deprived rats displayed enhanced win-stay behaviour compared to their socially housed counterparts, but this was only the case within the low-risk groups. In female rats, the high-risk groups obtained more rewards (Fig. 2F), achieved more reversals (Fig. 2G) and showed a higher percentage of win-stay behaviour (Fig. 2H) in comparison to their low-risk counterparts. Additionally, social play-deprived rats (SPD-LR and SPD-HR) obtained more rewards than control rats (CLT-LR and CTL-HR) while achieving a comparable amount of reversals and showing similar win-stay behaviour. These observations indicate that cognitive performance in adult rats was altered after the opportunity to take risks during social play in both male and female rats. SPD affected win-stay behaviour of males and the number of rewards collected in females, which is in general agreement with our previous findings (Bijlsma et al., 2022).

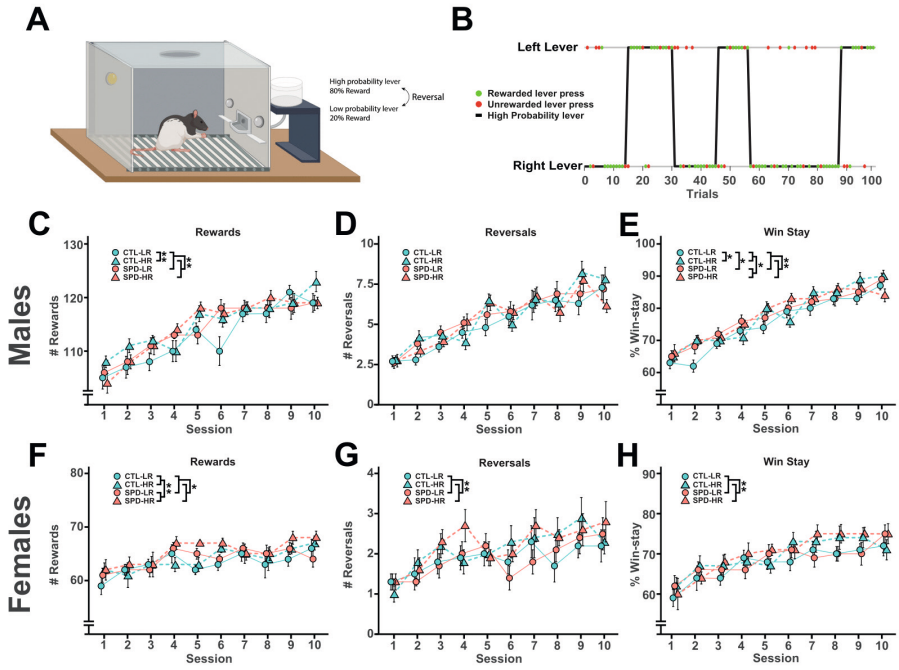

Figure 2. Performance in the PRL task after social play deprivation with or without the opportunity for risky play

(A) PRL task. (B) Representation of the 100 lever presses of an example session. Green and red dots represent rewarded and unrewarded lever presses respectively. A reversal is indicated by the change of the high probability lever. (C-E) PRL results from male rats. (C) Rewards obtained per session (2W-ANOVA, Housing: p=0.26, Risk: p=0.003, Interaction: p=0.024; TUKEY, CTLLR-SPDLR: p=0.18, CTLLR-CTLHR: p=0.005, SPDLR-SPDHR: p=0.84, SPDHR-CTLHR: p=0.95). (D) Reversals achieved per session (2W-ANOVA, Housing: p=0.41, Risk: p=0.35, Interaction: p=0.083). (E) % Win-stay behaviour per session (2W-ANOVA, Housing: p=0.035, Risk: p=0.010, Interaction: p=0.039; TUKEY, CTLLR-SPDLR: p=0.048, CTLLR-CTLHR: p=0.022, SPDLR-SPDHR: p=0.86, SPDHR-CTLHR: p=0.99). (F-H) PRL results from female rats. (F) Rewards obtained per session (2W-ANOVA, Housing: p=0.003, Risk: p=0.049, Interaction: p=0.89). (G) Reversals achieved per session (2W-ANOVA, Housing: p=0.68. Risk: p=0.006, Interaction: p=0.81). (H) % Win-stay behaviour per session (Circles, 2W-ANOVA, Housing: p=0.23, Risk: p=0.006, Interaction: p=0.99). Statistical range (p): * p<0.05; ** p<0.01; *** p<0.001.

Behavioural control was assessed using a response inhibition (RI) task, in which rats need to wait to collect a reward in half of the trials (indicated by a light and tone cue), with foot shocks of increasing intensity as a penalty (Fig. 3A; (Verharen et al., 2019)). During stimulus trials, male rats in all groups showed a higher success rate (thus avoiding punishment and consequently receiving less shocks) when the footshock intensity was increased (Fig. 3B-C). Rats in the high-risk groups succeeded more often to collect their rewards (Fig. 3B), and received fewer shocks than low-risk rats (Fig. 3C). The number of omissions increased with higher shock intensity and was comparable between the groups (Fig. 3D). Deprivation of social play behaviour did not affect RI task performance in male rats. The latency of reward retrieval was only affected by SPD during non-stimulus trials with SPD animals collecting the reward faster than CTL rats (Table 1). No differences in the number of rewards retrieved or omissions during non-stimulus trials were found between groups (data not shown). In female rats, there were no effects of SPD or the opportunity for risky play on performance in the RI task. Similar to the males, the success rate for female rats increased (Fig. 3E) and the number of shocks received decreased when the shock intensity was increased (Fig. 3F). The number of omissions did not change with shock intensity (Fig. 3G). The latency of reward retrieval was only affected by the opportunity for risky-play during non-stimulus trials (Table 1). No differences in the number of rewards retrieved or omissions during non-stimulus trials were found between groups (data not shown). Our data suggest that male, but not female rats, that engaged in risky play as juveniles (i.e., the CTL-HR and SPD-HR groups)

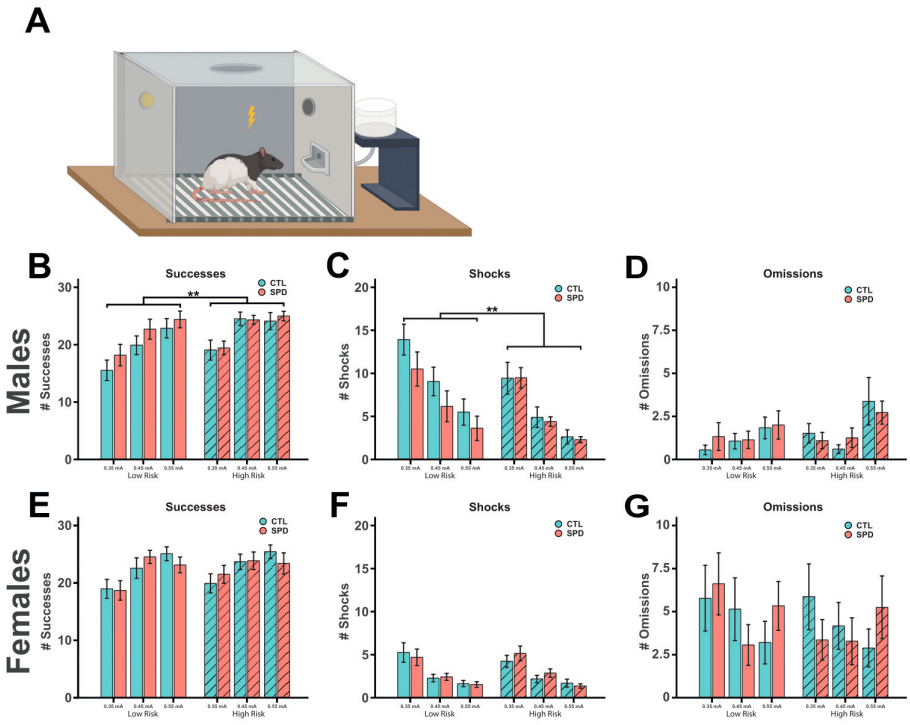

Figure 3.

Performance in the RI task after social play deprivation with or without the opportunity for risky play showed better behavioural inhibition in this task, whereas deprivation of social play did not affect RI task performance. RI task (A) results for male (B-D) and female rats (E-G). (B) Successes during stimulus trials (2W-ANOVA, Housing: p=0.084, Risk: p=0.004, Interaction: p=0.18, Shock Intensity: p<0.001; TUKEY, 0.35mA – 0.45mA: p<0.001, 0.35mA – 0.55mA: p<0.001, 0.45mA – 0.55mA: p=0.45). (C) The number of shocks received during stimulus trials (2W-ANOVA, Housing: p=0.076, Risk: p=0.001, Interaction: p=0.12, Shock Intensity: p<0.001; TUKEY, 0.35mA – 0.45mA: p<0.001, 0.35mA – 0.55mA: p<0.001, 0.45mA – 0.55mA: p=0.021). (D) The number of omissions stimulus trials (2W-ANOVA, Housing: p=0.66, Risk: p=0.072, Interaction: p=0.34, Shock Intensity: p<0.001; TUKEY, 0.35mA – 0.45mA: p=0.89, 0.35mA – 0.55mA: p<0.001, 0.45mA – 0.55mA: p<0,001). (E) Successes during stimulus trials (2W-ANOVA, Housing: p=0.88, Risk: p=0.24, Interaction: p=0.98, Shock Intensity: p<0.001; TUKEY, 0.35mA – 0.45mA: p<0.001, 0.35mA – 0.55mA: p<0.001, 0.45mA – 0.55mA: p=0.72). (F) The number of shocks received during the 30 stimulus trials (2W-ANOVA, Housing: p=0.68, Risk: p=0.84, Interaction: p=0.33, Shock Intensity: p<0.001; TUKEY, 0.35mA – 0.45mA: p<0.001, 0.35mA – 0.55mA: p<0.001, 0.45mA – 0.55mA: p=0.053). (G) The number of omissions during stimulus trials (2W-ANOVA, Housing: p=0.97, Risk: p=0.29, Interaction: p=0.64, Shock Intensity: p=0.17). Statistical range (p): * p<0.05; ** p<0.01; *** p<0.001.

Table 1. Overview of the reward retrieval latency during non-stimulus trials (Non-stim), stimulus trials in which the animal receives a shock (Shock) and stimulus trials where the animal avoid punishment (Success) for all shock intensities.

		Male				Female			
		CTL-LR	SPD-LR	CTL-HR	SPD-HR	CTL-LR	SPD-LR	CTL-HR	SPD-HR
Non-stim	0.35	4.42±0.7	3.93±0.8	4.57±0.5	3.83±0.6	8.72±1.1	7.89±0.9	7.46±1.2	6.51±0.9
	0.45	4.91±0.9	3.37±0.6	3.09±0.3	2.72±0.4	7.25±0.9	5.91±0.8	4.86±0.6	5.04±1.0
	0.55	4.72±0.8	3.90±0.7	4.26±0.6	3.35±0.4	6.49±1.2	5.53±0.8	4.17±0.6	5.61±1.2
Shock	0.35	4.48±0.6	5.85±0.5	5.49±0.5	5.64±0.3	5.75±0.4	6.20±0.6	6.70±0.7	6.56±0.5
	0.45	5.26±0.6	5.96±0.5	5.74±0.7	5.99±0.5	6.04±0.7	6.20±0.6	6.01±0.6	5.82±0.7
	0.55	5.41±0.8	6.28±0.7	6.23±0.7	6.86±0.8	5.20±0.9	6.34±0.9	5.24±0.9	6.17±1.0
Success	0.35	6.13±1.2	5.09±0.8	6.06±0.7	5.24±0.6	8.32±1.0	7.55±1.0	7.20±1.0	6.59±1.0
	0.45	5.74±0.9	4.21±0.6	4.07±0.4	4.31±0.5	6.96±0.7	5.20±0.6	5.80±0.6	5.34±0.9
	0.55	5.69±1.0	4.42±0.5	4.61±0.6	4.95±0.6	6.28±0.9	5.21±0.6	5.30±0.8	5.48±0.9

To assess anxiety-like behaviour and locomotor activity of the rats, we used the elevated plus maze (EPM, Fig. 4A) and the open field (OF). Male rats in the high-risk groups spent less time on the open arms (Fig. 4B) and showed a tendency towards more time on the closed arms of the EPM (Fig. 4C) compared to the low-risk animals. Time spent in the centre of the EPM did not differ between the groups (Fig. 4D). No differences were found between CTL and SPD groups, and locomotion on the EPM was comparable between the four groups of male rats (Fig. 4E). No differences were found between the low- and high-risk female groups but social play-deprived females spent more time on the open arms (Fig. 4F) and less time on the closed arms of the EPM (Fig. 4G) compared to control rats. No differences between groups were found in the time spent in the centre of the maze (Fig. 4H). All female groups showed comparable locomotor activity on the EPM (Fig. 4I). Behaviour of the male rats in the OF (Fig. 5A) was unaffected by play deprivation or the opportunity to take risks during play. Similar to the EPM, no differences were found in the distance travelled (Fig. 5B). Additionally, time spent in the centre, middle and periphery was comparable between all groups (Fig. 5C-E). The behaviour of the female rats in the OF was also not affected by play deprivation or the opportunity to take risks during play (Fig. 5F-I) with the exception that CTL-HR rats spent more time in the centre (Fig. 5G) and less time in the periphery (Fig. 5I) in comparison to their CTL-LR counterparts. Our observations in the EPM and OF tests showed no effect on the locomotor activity of the rats by SPD or risk-taking during play. Anxiety-like behaviour, however, was affected by risk-taking in male, but not female, rats while SPD affected anxiety-like behaviour in female, but not male, rats.

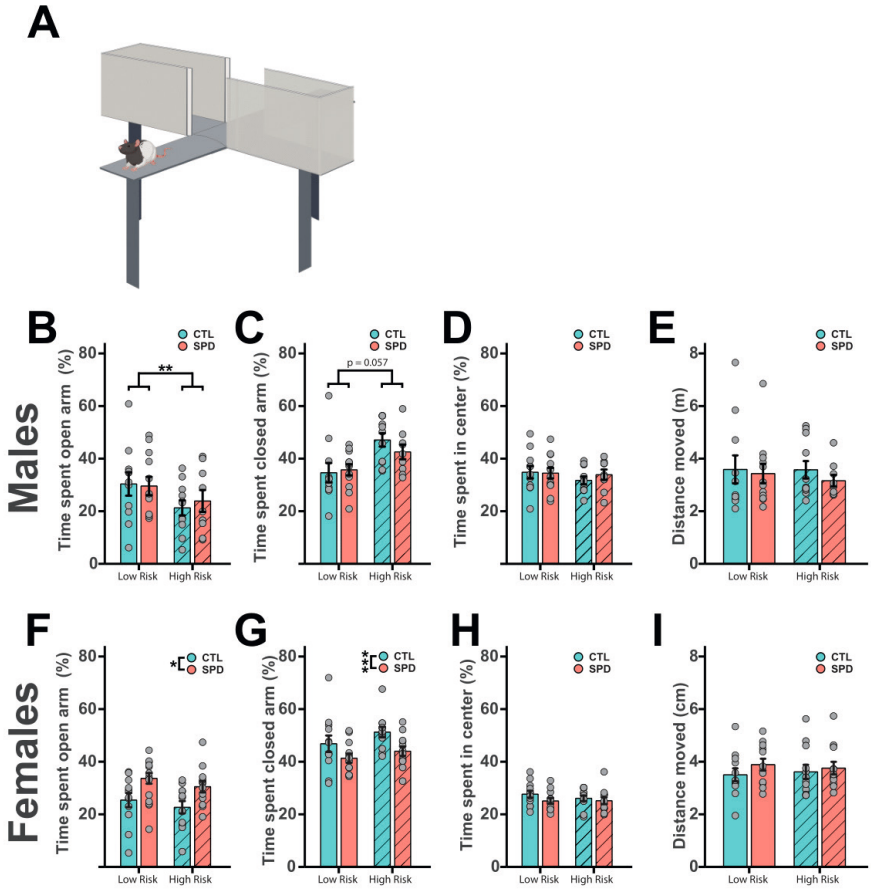

Figure 4. Behaviour on the EPM after social play deprivation with or without the opportunity for risky play

EPM results (**A**) for male (**B-E**) and female (**F-I**) rats. (**B**) Total distance moved (2W-ANOVA, Housing: p=0.50, Risk: p=0.73, Interaction: p=0.75). (**C-E**) Percentage time spent on the (**C**) open arms (2W-ANOVA, Housing: p=0.39, Risk: p=0.002, Interaction: p=0.30), (**D**) closed arms (2W-ANOVA, Housing: p=0.71, Risk: p=0.057, Interaction: p=0.74) and (**E**) in the centre zone (2W-ANOVA, Housing: p=0.68, Risk: p=0.35, Interaction: p=0.67). (**F**) Total distance moved (2W-ANOVA, Housing: p=0.29, Risk: p=0.97, Interaction: p=0.62). (**G-I**) Percentage time spent on the (**G**) open arms (2W-ANOVA, Housing: p=0.019, Risk: p=0.089, Interaction: p=0.45), (**H**) closed arms (2W-ANOVA, Housing: p<0.001, Risk: p=0.27, Interaction: p=0.78) and (**I**) in the centre zone (2W-ANOVA, Housing: p=0.27, Risk: p=0.81, Interaction: p=0.79). Statistical range (p): * p<0.05; ** p<0.01; *** p<0.001.

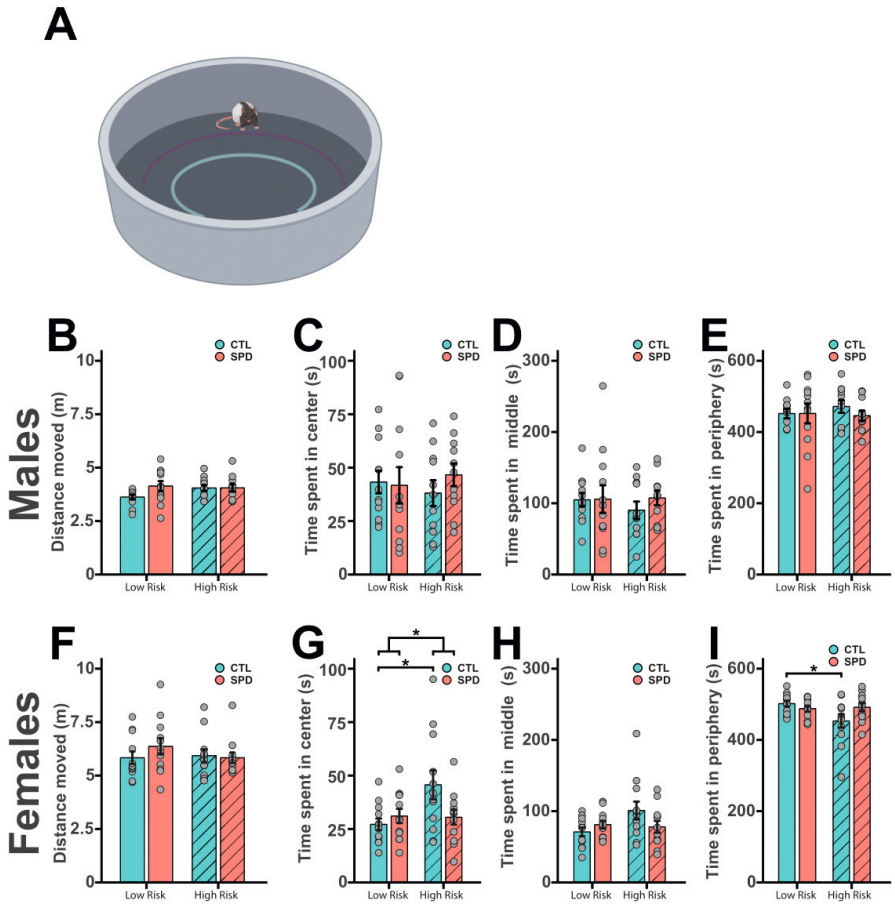

Figure 5. Behaviour in the OF after social play deprivation with or without the opportunity for risky play

OF results (**A**) for male (**B-E**) and female (**F-I**) rats. (**B**) Total distance moved (2W-ANOVA, Housing: p=0.12, Risk: p=0.33, Interaction: p=0.14). (**C-E**) Time spent in the (**C**) centre (2W-ANOVA, Housing: p=0.61, Risk: p=0.98, Interaction: p=0.44), (**D**) middle (2W-ANOVA, Housing: p=0.53, Risk: p=0.64, Interaction: p=0.55) and (**E**) periphery (2W-ANOVA, Housing: p=0.53, Risk: p=0.72, Interaction: p=0.50). (**F**) Total distance moved (2W-ANOVA, Housing: p=0.48, Risk: p=0.48, Interaction: p=0.31). (**G-I**) Time spent in the (**G**) centre (2W-ANOVA, Housing: p=0.20, Risk: p=0.046, Interaction: p=0.034; TUKEY, CTLLR-SPDLR: p=0.92, CTLLR-CTLHR: p=0.022, SPDLR-SPDHR: p=0.99, SPDHR-CTLHR: p=0.081), (**H**) middle (2W-ANOVA, Housing: p=0.46, Risk: p=0.12, Interaction: p=0.058) and (**I**) periphery (2W-ANOVA, Housing: p=0.34, Risk: p=0.078, Interaction: p=0.041; TUKEY, CTLLR-SPDLR: p=0.85, CTLLR-CTLHR: p=0.039, SPDLR-SPDHR: p=0.99, SPDHR-CTLHR: p=0.14). Statistical range (p): * p<0.05; ** p<0.01; *** p<0.001.

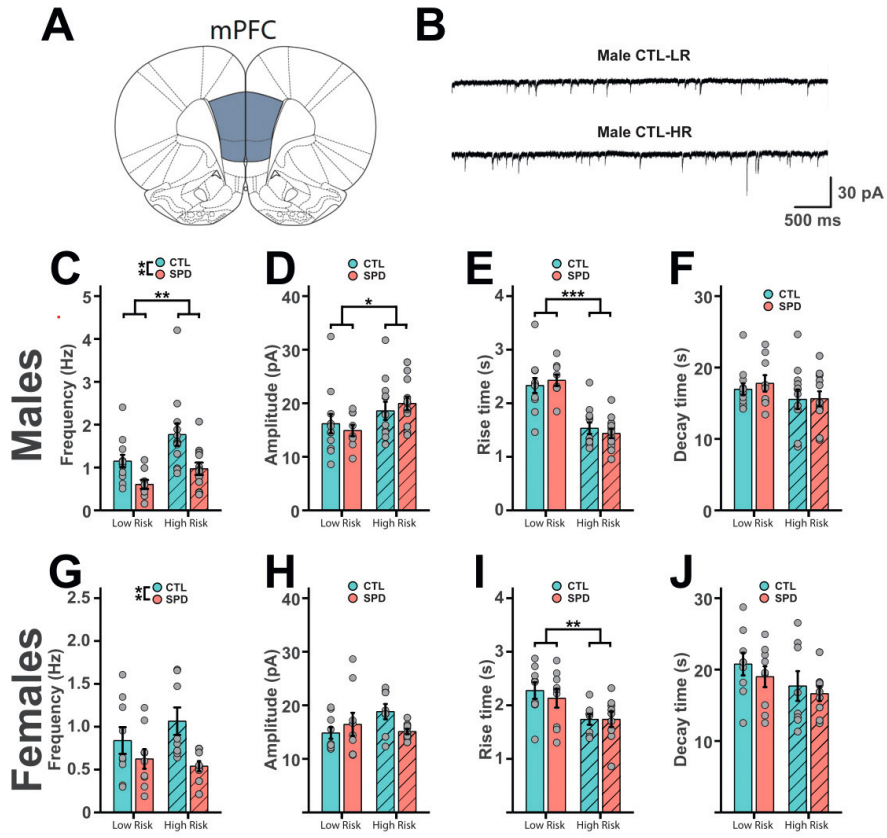

Figure 6. Prefrontal inhibition in L5 of the mPFC after social play deprivation with or without the opportunity for risky play

(A) Schematic diagram depicting the recording site in the mPFC. (B) Example traces of miniature IPSCs (mIPSCs) in L5 pyramidal cells in slices from control CTL-LR and CTL-HR male rats. Results for male (C-F) and female (G-J) rats. (C) Frequency (2W-ANOVA, Housing: p=0.001, Risk: p=0.009, Interaction: p=0.49), (D) amplitude (2W-ANOVA, Housing: p=0.79, Risk: p=0.024, Interaction: p=0.39), (E) rise time (2W-ANOVA, Housing: p=0.54, Risk: p<0.001, Interaction: p=0.40) and (F) decay time (2W-ANOVA, Housing: p=0.79, Risk: p=0.12, Interaction: p=0.74) of mIPSC events. (G) Frequency (2W-ANOVA, Housing: p=0.007, Risk: p=0.61, Interaction: p=0.23), (H) amplitude (2W-ANOVA, Housing: p=0.51, Risk: p=0.39, Interaction: p=0.069), (I) rise time (2W-ANOVA, Housing: p=0.56, Risk: p=0.004, Interaction: p=0.63) and (J) decay time (2W-ANOVA, Housing: p=0.35, Risk: p=0.089, Interaction: p=0.83) of mIPSC events. Statistical range (p): * p<0.05; ** p<0.01; *** p<0.001.

To study the impact of the opportunity to take risks during play and social deprivation on the development of PFC inhibitory circuitry, we performed voltage-clamp recordings from layer 5 pyramidal cells of the mPFC (Fig. 6A,B) of male (Fig. 6C-F) and female (Fig. 6G-J) rats. Consistent with our previous findings (Bijlsma et al., 2022, 2023), a reduction in the frequency of miniature inhibitory postsynaptic currents (mIPSCs) was found in male SPD in comparison with CTL rats (Fig. 6C). We also observed an increase in mIPSC frequency in the male high-risk groups in comparison with their low-risk counterparts (Fig. 6C). The mIPSCs in the high-risk groups also had higher amplitudes (Fig. 6D) and faster rise times (Fig. 6E). Decay kinetics (Fig. 6F) were comparable between all groups. In contrast to the males, we found no difference in mIPSC frequency between the low and high-risk groups in female rats (Fig. 6G). However, the reduction in mIPSC frequency in SPD compared to CTL was consistent with our results in males (Fig. 6G). Amplitudes of the measured events were comparable between all groups (Fig. 6H). Similar to our observation in male rats, mIPSC rise time kinetics were faster in the high-risk group (Fig. 6I) while decay kinetics remained unaffected (Fig. 6J). Consistent with our previous findings (Bijlsma et al., 2022, 2023), we show here that SPD reduces the frequency of mIPSCs in layer 5 pyramidal cells of the mPFC in both male and female rats. The opportunity to take risks increased mIPSC frequency in males, but not females, while both sexes showed faster mIPSC rise times in the high-risk groups.

We summarize the results in Table 2. Together, these data indicate that the opportunity to take risks during play alters cognitive flexibility in both males and females and improved behaviour control in a response inhibition task in male rats. Anxiety-like behaviour was modestly altered by risk. Deprivation of social play behaviour altered cognitive flexibility in both sexes but had no consequences for response inhibition. Anxiety-like behaviour was modestly reduced in female rats after deprivation of social play. Our findings also demonstrate that the reduction in inhibitory synaptic inputs in the mPFC of rats by SPD is present in both male and female rats while the opportunity to play in a high-risk environment increased mIPSC frequency only in male rats. In both sexes, mIPSCs displayed faster rise times in high risk animals.

Table 2. Schematic overview of main effects of social play deprivation and the opportunity for risky play on male and female performance and behaviour.

		Risky play		Social play deprivation	
		Males	**Females**	**Males**	**Females**
PRL	Rewards	↑	↑		↑
	Reversals		↑		
	Win-Stay	↑	↑	↑	
RI	Successes	↑			
	Shocks	↓			
	Omissions				
EPM	Open	↓			↑
	Closed				↓
	Center				
	Distance				
OF	Center		↑		
	Middle				
	Periphery				
IPSCs	Frequency	↑		↓	↓
	Amplitude	↑			
	Rise-time	↓	↓		
	Decay-time				

"↑" increased/improved/higher, "↓" decreased/impaired/lower compared to control animals

Discussion

In this study, we assessed how two important aspects of play, e.g. social play with peers and the opportunity for risk-taking during play, affect cognitive and emotional behaviour and PFC function in adulthood. Our results suggest that these two aspects of play both affect PFC circuitry and adult behaviour, but with limited overlap. We show that providing animals with the possibility to play in a high-risk environment during a peak social play period (P21-P42) altered cognitive flexibility and anxiety-like behaviour in both sexes. Behavioural inhibition was improved in males. At the neural level, we found that the frequency of mIPSCs was increased in male rats that engaged in play in a high-risk environment. The mIPSC frequency increase was not present in female rats, but mIPSCs had faster rise kinetics in high-risk animals in both sexes. SPD altered cognitive flexibility and reduced mIPSC frequency in males and females, which is in general agreement with our previous findings (Bijlsma et al., 2022, 2023). Here, we also

found that response inhibition was unaffected by SPD, while anxiety-like behaviour was affected in female, but not male, rats.

As stated in the Introduction, in this study we aimed to answer four questions. The first of these was how the opportunity for risk-taking behaviour during play alters cognitive flexibility, behavioural inhibition and anxiety-like behaviour. We found that the opportunity for risk-taking behaviour led to altered cognitive flexibility in both male and female rats in the PRL task. Similar effects have been described in other reversal learning designs after enriched or complex housing (Sampedro-Piquero et al., 2015; Zeleznikow-Johnston et al., 2017). When we assessed response inhibition, we observed that male rats that had opportunities for risk-taking during play received fewer shocks than their CTL counterparts. Performance in this behavioural inhibition task consists of multiple components like control over behaviour, appreciation of stimulus value and task engagement (Verharen et al., 2019). Our results indicate an increase in control over behaviour after risk-taking as rats are better in refraining themselves from taking the sucrose pellet. The stimulus value seems not to be affected as there are clear differences between reward collection latency during stimulus and non-stimulus trials, with no effect of risky play. Furthermore, as omissions are not affected, we think that task engagement between all groups was comparable.

Multiple studies have examined the effects of enrichment of the housing condition on anxiety related behaviour in rats. Mixed effects on behaviour in the EPM have been reported. That is, after enrichment rats have been reported to spend more time on the open arms of the EPM (Friske and Gammie, 2005; Baldini et al., 2013), less time on the open arms (Branchi and Alleva, 2006; Sparling et al., 2018) or no effect of enrichment was found at all (Brenes et al., 2009; Green et al., 2010; Li et al., 2016). In most, if not all of these studies, behavioural testing was done when the animals were still housed in their enriched environment, whereas in our study the rats had the opportunity to play in an enriched environment only when they were juveniles. In addition, our high-risk rats are well familiar with moving, climbing and playing on elevated platforms as our risky-play cage allowed them to do exactly this. The reduction in time spent on the open arms in male rats after high-risk play that we observed in the current study may therefore reflect improved risk assessment. This effect of risky play was, however, not noticeable in the OF, perhaps as a result of the fact that the OF does not include heights. Together, these data indicate that the opportunity to take risks during play alters cognitive flexibility in both males and females and improves the control over behaviour in a response inhibition task in male rats. Anxiety-like behaviour was, however, only modestly altered by risky play opportunities.

The second question was how social play experience affects the development of cognitive flexibility, behavioural inhibition and anxiety-like behaviour. We found that SPD affected the win-stay behaviour of males and the number of rewards collected in females in the PRL task, comparable to reported effects of SPD on PRL performance from our previous work (Bijlsma et al., 2022). These results also resonate well with previous studies that showed that alterations in the social domain during the post-weaning period resulted in impairments in impulsive actions and decision-making under challenging and novel circumstances (Baarendse et al., 2013; Schneider et al., 2016).

We did not observe effects of SPD on response inhibition, however. Previous social deprivation studies showed altered response to stressors (Robbins et al., 1996) and heightened fearfulness in novel environments (Einon and Morgan, 1977; Hellemans et al., 2004) in adults rats that were deprived from social play during their juvenile period, from which one would conjecture an altered performance during the RI task. The difference with our study may well be explained by the type of deprivation. We re-socialized our animals at P42 while the beforementioned studies deprived their rats from social contact (and play) until adulthood.

While no effects of SPD were found on the behaviour in the EPM in male rats, socially deprived female rats spent more time in the open arms and less time in the closed arms. This appears to be in contrast with several studies that showed that play deprivation in rats can increase anxiety-related responses (Wright et al., 1991; Da Silva et al., 1996) which would lead to a lower time spent on the open arm. However, these EPM studies were performed immediately after social isolation while in our study we tested the long-term effects of play deprivation in adulthood. Together, our data shows that deprivation of social play alters cognitive flexibility in both sexes but had no effect on the behavioural inhibition during the RI task. Anxiety-like behaviour was modestly reduced in female, but not male, rats.

The third question addressed how play manipulation affects the maturation of the mPFC. We previously showed that SPD results in a specific reduction of inhibitory currents in L5 cells in the adult mPFC, while excitatory currents remain unaffected (Bijlsma et al 2022; -2023). Here we show that this effect was apparent in both the low-risk and high-risk groups and was also present in female rats. This emphasizes the general importance of post-weaning social play, consistent with earlier studies that identified the time window between weaning and puberty (P21-P42) as a critical period for PFC maturation and behavioural development (Einon and Morgan 1977; Van Den Berg et al. 1999; Lukkes et al. 2009; Kolb et al. 2012; Baarendse et al., 2013; Whitaker et al. 2013; Bijlsma et al. 2022; 2023). In addition, we found that the frequency of mIPSCs

in L5 cells was increased in the high-risk groups in comparison to the low-risk groups, but only in male rats. In slices from both sexes, mIPSCs showed faster rise kinetics, suggesting that particularly mIPSCs with fast kinetics were enhanced. This is presumably caused by perisomatic synapses made by parvalbumin (PV)-positive interneurons, which are also affected after SPD (Bijlsma et al. 2022). Alterations in PV cells during development have been implicated in shaping cognitive capacities in adulthood (Donato et al. 2015; Canetta et al. 2021). A reduction in PFC inhibition has previously been linked to impaired cognitive flexibility (Gruber et al. 2010), and a direct link between PFC PV cell activity and social behaviour was recently demonstrated (Bicks et al. 2020; Sun et al. 2020). Our data suggests that SPD and the opportunity for risk-taking have independent, additive effects on the development of IPSCs in the PFC.

Finally our data addresses the question if the consequences of high-risk play and social play deprivation differ between males and females. Multiple enrichment studies have described sex differences in behavioural tasks after exposure to a more challenging environment (Elliott and Grunberg 2005; Simpson and Kelly 2011; Chamizo et al. 2016). However, we are the first to describe sex differences after the opportunity to take risks during social play in combination with a play deprivation paradigm. When comparing male and female rats, females tended to show more avoidance behaviour in the RI task by not approaching the reward dispenser (more omissions). A higher rate of avoidance behaviour and a lower shock threshold for females have been reported before (Svoboda et al. 2012; Yokota et al. 2017; Finnell et al. 2018). Additionally, females showed higher locomotion in the OF. Previous studies have also reported that females engage in a more active motor response compared to males in several behavioural tasks (Svoboda et al. 2012; Avcu et al. 2014; Shanazz et al. 2022).

Together, our data show that exposure to risks during juvenile play affects performance in PFC-dependent and anxiety-based tasks in adulthood and that it affects the development of inhibition in the mPFC. The deprivation of social play altered cognitive flexibility and inhibitory synaptic inputs in the mPFC in both sexes while a reduction in anxiety-like behaviour was only seen in females. Furthermore, our results indicate that interactions between risky play and SPD were limited, suggesting that social play with peers and the opportunity for risk-taking during independently affect the development of behaviour and PFC function. This implies that the effects of SPD cannot be mitigated by increasing the opportunity for risk-taking, but that both aspects of juvenile play have separate value for adult behaviour.

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CHAPTER 5

Social play deprivation in rats alters reward processing and dopaminergic innervation of the mPFC in adulthood

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ABSTRACT

Brain development is characterized by so-called critical periods during which external inputs are crucial for fine-tuning neuronal connectivity of a particular brain structure. We recently demonstrated that social play during early life is critical for the development of medial prefrontal cortex (mPFC) function and connectivity. Since social play deprivation (SPD) has been shown to affect the sensitivity to dopamine in the mPFC, and social play itself is a highly rewarding activity, we hypothesized that SPD affects reward processes. Here, we therefore investigated how SPD affects sucrose-directed behaviour. In parallel, to determine if dopaminergic modulation of the PFC is altered after SPD, we determined the density and intensity of fibres positive for tyrosine-hydroxylase (TH; the rate-limiting enzyme in the synthesis of dopamine) in the mPFC. Additionally, we recorded how inhibitory currents in the adult mPFC are modulated by dopamine D1- and D2-receptor stimulation. We report that adult rats showed an increase in sucrose intake and increased motivation to respond for sucrose after SPD. In addition, we found that the volume and intensity of TH-positive fibres was reduced in layer 5 of the mPFC after SPD. The modulation of mPFC inhibitory currents by D1- and D2-receptor agonists did not appear to be affected by SPD. These data reveal a long-lasting reduction of dopaminergic innervation of the mPFC after SPD, associated with altered reward processing in adulthood. This further emphasizes the importance of social play for shaping mPFC function and emotional capacities.

INTRODUCTION

Brain development is characterized by so-called critical or sensitive periods. During these periods, fine-tuning of brain functions takes place, which critically depends on external sensory input, to ensure optimal performance during the rest of life (Hensch, 2005). During the period when the prefrontal cortex (PFC) matures, i.e., in between weaning and early adulthood (Kolb et al., 2012), young animals display an abundance of an energetic form of social behaviour known as social play behaviour (Panksepp et al., 1984; Vanderschuren et al., 1997; Pellis and Pellis, 2009). It is widely held that social play facilitates the development of PFC-dependent skills such as flexibility, creativity, and decision-making (Špinka et al., 2001; Vanderschuren and Trezza, 2014; Pellis et al., 2023).

Social play is known to be highly rewarding (Humphreys and Einon, 1981; Ikemoto and Panksepp, 1992; Trezza and Vanderschuren, 2009; Peartree et al., 2012; Achterberg et al., 2016). Indeed, the expression of social play behaviour is modulated through neural systems, like the dopamine (DA) system, which have also been implicated in other types of reward, such as food, sex, and drugs of abuse (Trezza et al., 2010; Siviy and Panksepp, 2011; Vanderschuren et al., 2016). In addition to engaging them, it is conceivable that social play also facilitates the maturation of brain reward systems. Consistent with this idea, it has previously been shown that social play deprivation (SPD) increases the rewarding properties, motivation and consumption of alcohol, cocaine and amphetamine (Baarendse et al., 2013; Whitaker et al., 2013; Lesscher et al., 2015). With regard to natural rewards, SPD has been shown to reduce sexual exploratory behaviour, and to blunt the anticipatory response to sucrose (Van Den Berg et al., 1999a, 1999b).

DA is an important modulator of PFC function (Goldman-Rakic et al., 2000; Miller and Cohen, 2001; Seamans and Yang, 2004; Robbins and Arnsten, 2009; Floresco, 2013; Willing et al., 2017; Peters and Naneix, 2022). During early adolescence, DA fibres originating from the ventral tegmental area (VTA) continue to grow into the medial PFC (mPFC) until as late as postnatal day (P)60 (Kalsbeek et al., 1988; Berger et al., 1991; Rosenberg and Lewis, 1994; Benes et al., 1996; Naneix et al., 2012; Hoops and Flores, 2017; Willing et al., 2017; Reynolds and Flores, 2021). In parallel, basal DA levels in the mPFC increase and decreased levels of DA metabolites are found during early adolescence (Nomura et al., 1976; Naneix et al., 2012), suggesting an increase in DA availability in the PFC. In parallel, an increase in the number of PFC cells, especially GABA neurons, that interact with DA fibres can be seen (Benes et al., 1996), suggesting an increase in DA modulation of these cells. In support of an important role for social play in the development of the PFC and its DA innervation, SPD has previously been

shown to blunt the influence of DA neurotransmission on firing of mPFC pyramidal cells (Baarendse et al., 2013). More recently, we found that SPD reduces inhibitory input into pyramidal neurons in the mPFC (Bijlsma et al., 2022, 2023).

We therefore hypothesized that SPD affects reward processing and DA modulation of the mPFC. To test this, we assessed the effect of SPD on sucrose preference, consumption and the motivation to respond for sucrose. In parallel, to determine if DA modulation of mPFC is altered after SPD, we determined the density and intensity of fibres in the mPFC positive for tyrosine hydroxylase (TH), an enzyme that catalyses the rate-limiting step in the biosynthesis of DA. Additionally, we recorded how inhibitory currents in the adult mPFC, which are reduced after SPD (Bijlsma et al., 2022, 2023), are modulated by stimulation of DA D1- and D2-receptors.

MATERIALS & METHODS

Animals and housing conditions

All experiments were approved by the Animal Ethics Committee of Utrecht University and the Dutch Central Animal Testing Committee. They were conducted in agreement with Dutch (Wet op de Dierproeven, 1996; Herziene Wet op de Dierproeven, 2014) and European legislation (Guideline 86/609/EEC; Directive 2010/63/EU). Male Lister Hooded rats were obtained from Charles River (Germany) on P14 in litters with nursing mothers. All rats were subjected to a reversed 12:12h light-dark cycle with ad libitum access to water and food. Rats were weaned on P21 and were either allocated to the SPD or the control (CTL) group. All rats were housed in pairs with a littermate during the entire experiment. From P21 to P42, a transparent plexiglass divider containing small holes was placed in the middle of the home cage of SPD rats creating two separate but identical compartments. Rats in the SPD group were therefore able to receive visual, olfactory and auditive cues from one another. The holes in the plexiglass allowed the rats to have limited physical interaction but without the opportunity for social play behaviour. The divider was removed on P42 and SPD rats were housed in the same pairs for the remainder of the experiment. All experiments were conducted during the active phase of the animals (9:00 - 17:00). One week before the start of behavioural testing, i.e. around P85, the rats were subjected to food restriction. They received 4.5 grams of chow per 100 grams body weight per day, which kept them at 90% of their free-feeding body weight for the duration of behavioural testing. Rats were weighed and handled at least once a week throughout the experiment. Rats were provided with 30 sucrose

pellets (45mg, BioServ) in their home cage before operant training commenced, to reduce potential food neophobia during training.

Sucrose preference test

Two cohorts of both 12 CTL and 12 SPD rats were tested for their relative preference for sucrose over water. Before the experiments, the rats were habituated to the testing cages for 2-3 h after which they received access to two water bottles for 4 consecutive days, with one bottle containing increasing concentrations of sucrose over days (0.0%, 0.1%, 0.5% and 1%). The bottles were weighed before and after 6h of access in each session that started at 9:00 AM. Each concentration was offered once and bottle positions were switched between sessions to avoid the development of side bias. Daily consumption (ml) and the rat's body weight were used to calculate sucrose intake (ml/kg), water intake (ml/kg) and total consumption (ml/kg). Sucrose preference was calculated as the percentage of sucrose consumption of the total fluid intake ((sucrose intake/total fluid intake)*100).

Operant responding for sucrose

Responding for sucrose was tested in a separate cohort of 12 CTL and 12 SPD rats. Behavioural testing was conducted in operant conditioning chambers (Med Associates, USA) enclosed in sound-attenuating cubicles equipped with a ventilation fan. Two retractable levers were located on either side of a central food magazine into which sugar pellets could be delivered via a dispenser. A LED cue light was located above each retractable lever. A white house light was mounted in the top centre of the wall opposite the levers. Online control of the apparatus and data collection was performed using MED-PC (Med Associates) software. The rats were habituated to the operant conditioning chambers after which operant sessions started under a fixed ratio (FR) schedule of reinforcement. During the first three consecutive days, rats were tested under a FR-1 schedule, in which each active lever press resulted in the delivery of one reward. On the following days, the rats were tested under a FR-2, FR-5 and FR-10 schedule of reinforcement (during which 2, 5 or 10 lever presses were required for one reward). After the FR sessions, the rats were tested in three sessions under a progressive ratio (PR) schedule of reinforcement. Under this schedule, the animals had to meet a response requirement on the active lever that progressively increased after every earned reward (1, 2, 4, 6, 9, 12, 15, 20, 25, etc.; (Richardson and Roberts, 1996).

Immunohistochemistry

A separate cohort of 5 CTL and 4 SPD adult rats were anaesthetized around P100 with Nembutal (240 mg/kg i.p.) and transcardially perfused with 0.1 M phosphate-buffered saline (PBS, pH 7.3-7.4) followed by 4% paraformaldehyde in 0.01 M PBS. The brains were removed from the skull and post-fixed overnight in the same paraformaldehyde solution at 4°C and subsequently cryoprotected in 30% sucrose for three days at 4°C. Thereafter, the brains were rapidly frozen in isopentane on dry ice, dried and stored at −80 °C until further use. Brain sections (20 µm thick) from the mPFC between bregma levels of 4.2 - 2.2mm (Paxinos and Watson, 2007) were cut using a Leica CM 3050 S cryostat and collected in 12-well inserts filled with 1x PBS solution. Slices were blocked with 2 ml blocking buffer (10% normal goat serum, 0.25% triton-X 100 in PBS) for 1h on a shaker at room temperature. Slices were incubated overnight at 4°C in a 1.5 ml solution of blocking buffer and 2 µl of primary antibody per well (Ab TH (mouse), Sigma Aldrich, MAB318)). Sections were washed (3x15min in 1.5 ml of 1x PBS), followed by incubation in 1.5 ml solution of blocking buffer and 2 µl of secondary antibody per well (Ab goat anti-mouse(488nm), Abcam, ab150113). After another washing step (3x15min in 1.5 ml of 1x PBS), slides were mounted and stored at 4 °C until image acquisition.

Image acquisition and analysis: Images were taken with a Zeiss Confocal microscope (type LSM700). The researcher was blinded to the groups of the sections when acquiring the images and performing the quantifications. Image analysis was performed in ImageJ (National Institutes of Health USA). Image analysis was performed semi-automatically using custom-written ImageJ macros. For each image, an average intensity image was constructed from 10 z-stack slices, which was median filtered and thresholded manually. Within each cohort, the intensity data was normalized on the mean of the CTL group to prevent a difference between the staining of both cohorts affectingthe results.

Ex-Vivo electrophysiology

Slice preparation: A separate cohort of 14 CTL and 14 SPD rats were anaesthetized around P100 by induction with isoflurane and then transcardially perfused with ice-cold modified artificial cerebrospinal fluid (ACSF) containing (in mM): 92 Choline chloride, 2.5 KCl, 1.2 NaH2PO4, 30 NaHCO3, 20 HEPES, 25 glucose, 5 Na-ascorbate, 3 Na-pyruvate, 0.5 CaCl2.2H2O, and 10 MgSO4.7H2O, bubbled with 95% O2 and 5% CO2 (pH 7.3–7.4). The brain was quickly removed after decapitation and coronal slices of the mPFC (300 µm) were prepared using a vibratome (Leica VT1000S, Leica Microsystems) in ice-cold modified ACSF. Slices were initially incubated in the carbonated modified ACSF for 5 min at 35 °C and then transferred into a holding chamber containing standard

ACSF containing (in mM): 126 NaCl, 3 KCl, 1.3 MgCl2.6H2O, 2 CaCl2.2H2O, 20 glucose, 1.25 NaH2PO4 and 26 NaHCO3 bubbled with 95% O2 and 5% CO2 (pH 7.3) at room temperature for at least 30 minutes. They were subsequently transferred to the recording chamber, perfused with standard ACSF that is continuously bubbled with 95% O2 and 5% CO2 at 28–32 C.

Whole-cell recordings and analysis: Whole-cell patch-clamp recordings were performed from layer 5 (L5) pyramidal neurons in the mPFC. These neurons were visualized with an Olympus BX61W1 microscope using infrared video microscopy and differential interference contrast (DIC) optics. Patch electrodes were pulled from borosilicate glass capillaries and they had a resistance of 4-6 MΩ when filled with intracellular solutions. Inhibitory postsynaptic currents (IPSCs) were recorded in the presence of 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX) (10 µM in batch 1; 20 µM in batch 2) and D,L-2-amino-5-phosphopentanoic acid (D,L-AP5) (20 µM in batch 1; 50 µM in batch 2), with an internal solution containing (in mM): 125 CsCl, 2 MgCl2, 5 NaCl, 10 HEPES, 0.2 EGTA, 4 MgATP, 0.4 Na2GTP (pH 7.3 with CsOH; batch 1) or 70 K-gluconate, 70 KCl, 10 HEPES, 0.5 EGTA, 4 MgATP, 0.4 Na2GTP, 4 Na2phosphocreatine (pH 7.3 with KOH; batch 2). After a 2 min baseline measurement, 2 µM of the DA D1 receptor agonist SKF38393 (Tocris) or 1 µM of the DA D2 receptor agonist quinpirole (Sigma-Aldrich) was washed in and sIPSC frequency was assessed after 5 and 10 min. The membrane potential was held at -70 mV for voltage-clamp experiments. Signals were amplified, filtered at 2 kHz and digitized at 10 kHz using a MultiClamp 700B amplifier (Molecular Devices) and stored using pClamp 10 software. Series resistance was constantly monitored, and the cells were rejected from analysis if the resistance changed by >20% or it reached a value >30 MΩ. No series resistance compensation was used. Resting membrane potential was measured in bridge mode (I=0) immediately after obtaining whole-cell access. Passive and active membrane properties were analysed with Matlab (MathWorks) using a custom script and synaptic currents were analysed with Mini Analysis (Synaptosoft Inc., Decatur, GA). The detected currents were manually inspected to exclude false events.

Data processing and statistical analyses

Statistical analyses were performed with GraphPad Prism (Software Inc.) and RStudio 1_2_5019 (R version 3.6.1, R Foundation for Statistical Computing). Two-way repeated measures ANOVA (with group as between-subjects factor and sucrose concentration/session as within-subjects factor) was used followed by Tukey's post hoc test where appropriate for the sucrose preference test and operant responding

task. Electrophysiological measurements were tested with a mixed model ANOVA as the recordings on different time points did not have the same sample size. Differences between the two groups in the immunohistochemistry experiments were tested with a nonparametric Mann-Whitney-Wilcoxon test (MW), or a parametric Welch t-test (T) depending on normality that was tested with a Shapiro-Wilk test. All graphs represent the mean ± standard error of the mean (SEM) with individual data points shown in coloured (Baseline=gray, CTL=blue, SPD=red) circles. Statistical range in all figures: *p ≤ 0.05, **p < 0.01, ***p < 0.001.

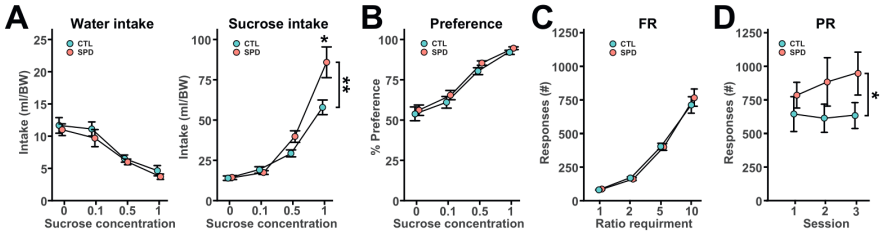

Figure 1.

(**A**) Water (Left panel; 2W-ANOVA, Housing: p=0.17, Concentration: p<0.001, Interaction: p=0.96) and sucrose (Right panel; 2W-ANOVA, Housing: p=0.0018, Concentration: p<0.001, Interaction: p=0.0016; TUKEY, CTL-SPD 0%: p=0.99, CTL-SPD 0.1%: p=0.99, CTL-SPD 0.5%: p=0.65, CTL-SPD 1%: p<0.001,) intake in the sucrose preference test. (**B**) % sucrose preference (2W-ANOVA, Housing: p=0.062, Concentration: p<0.001, Interaction: p=0.95). (**C**) The number of sucrose rewards obtained under the fixed ratio schedule of reinforcement (2W-ANOVA, Housing: p=0.65, Session: p<0.001, Interaction: p=0.81). (**D**) The number of sucrose rewards obtained under the progressive ratio schedule of reinforcement (2W-ANOVA, Housing: p=0.036, Session: p=0.85, Interaction: p=0.81). Data from 12 CTL and 12 SPD rats. Statistical range: *p ≤ 0.05, **p < 0.01.

RESULTS

Rats were deprived of social play during postnatal days (P) 21-42 after which they were re-socialized until adulthood and tested for reward-directed behaviour, using a sucrose preference task. We found that SPD rats consumed more sucrose at the highest concentrations (Fig. 1A), while their preference for sucrose (Fig. 1B) was comparable to CTL animals. To assess the motivation for sucrose, both groups of rats were tested using operant responding for sucrose, under both fixed- and progressive ratio schedules of reinforcement. SPD rats displayed higher responding rates for sucrose under a progressive ratio, but not under a fixed ratio schedule of reinforcement (Fig. 1C,D). These results indicate a higher rewarding value of sugar after SPD.

We performed immunohistochemistry to determine the density of DA fibres in the PFC. We measured the volume and intensity of TH-positive fibres within layer 5 of the mPFC (Fig. 2A). The TH intensity in slices from SPD rats was lower compared to slices from CTL rats (Fig. 2B), suggesting that the level of TH enzyme in DA axons was reduced after SPD. We further plotted the total area of the TH fibres in CTL and SPD slices, which suggested that there were also fewer TH fibres in SPD slices. (Fig. 2C). These data show that DA innervation of the mPFC is reduced after SPD.

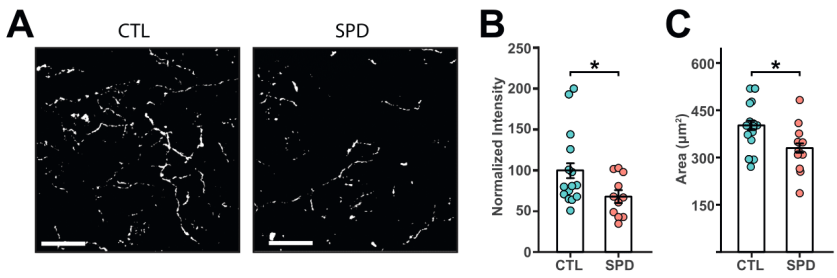

Figure 2.

(**A**) Representative confocal image of TH positive fibres in mPFC layer 5 from CTL (left panel) and SPD (right panel) slices. Scale bar is 4µm. (**B**) The normalized intensity of TH-positive fibres (T-test: p=0.049). (**C**) The area of TH-positive fibres (T-test: p=0.035). *p ≤ 0.05.

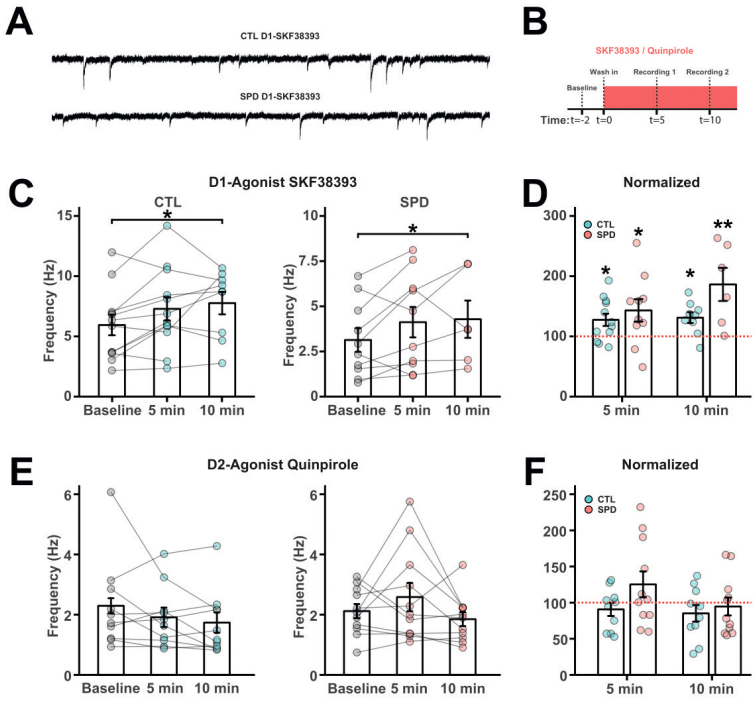

Figure 3.

(**A**) Example traces of spontaneous inhibitory postsynaptic currents (sIPSCs) in L5 pyramidal cells in slices from CTL and SPD rats. (**B**) Experimental timeline. (**C**) Frequency of sIPSCs recorded in layer 5 of the mPFC of CTL (Left panel; Mixed Model, Treatment: p=0.035; Multi comp: Base-5min p=0.052, Base-10min p=0.044) and SPD (right panel; Mixed Model, Treatment: p=0.047; Multi comp: Base-5min p=0.11, Base-10min p=0.042) rats. Baseline measurements (grey circles) were done before the wash-in of D1-agonist SKF38393. (**D**) Normalized sIPSC frequencies from (**B**). All measurements after wash-in are normalized to their within-baseline measurement (Mixed Model CTL, Treatment: p=0.011; Multi comp: 5min p=0.020. 10min p=0.015; Mixed Model SPD, Treatment: p=0.0057; Multi comp: 5min p=0.057, 10min p=0.0037). (**E**) Frequency of sIPSCs recorded in layer 5 of the mPFC of CTL (Left panel; Mixed Model, Treatment: p=0.38) and SPD (Right panel; Mixed Model, Treatment: p=0.28) rats. Baseline measurements (grey circles) were done before the wash-in of D2-agonist Quinpirole. (**F**) Normalized sIPSC frequencies from (**E**). All measurements after wash-in are normalized to their within-baseline measurement (Mixed Model CTL, Treatment: p=0.57; SPD, Treatment: p=0.15). Data from 8 CTL and 7 SPD rats for the D1 receptor stimulation measurements and from 6 CTL and 6 SPD rats for the D2 receptor stimulation measurements. Statistical range: *p ≤ 0.05, **p < 0.01.

To assess whether inhibitory synaptic currents in the mPFC are modulated by DA, we performed whole-cell patch clamp recordings in layer 5 (L5) pyramidal cells in the mPFC in slices prepared from adult CTL and SPD rats (Fig. 3A). After baseline measurement,

we washed in the DA D1 receptor agonist SKF38393 or the DA D2 receptor agonist Quinpirole and recorded sIPSC events 5 and 10 minutes after wash-in (Fig. 3B). After addition of the D1-agonist the frequency of inhibitory currents in L5 mPFC pyramidal neurons gradually increased (Fig. 3C). Baseline frequency in the SPD group was lower than in the CTL group (T-test: p=0.020), which is consistent with our previous findings (Bijlsma et al., 2022, 2023). We therefore normalized the data to their baseline values to compare the effect of D1 receptor activation on sIPSCs between CTL and SPD slices (Fig. 3D). No differences were found between CTL and SPD slices after D1 receptor stimulation (Fig. 3D: CTL vs SPD; Mixed model, Housing: p=0.16, Time: p=0.11, Interaction: p=0.25). We observed that wash-in of the D2-agonist quinpirole did not alter the frequency of sIPSCs in CTL or SPD slices (Fig. 3E). This result must be interpreted with caution however, as baseline sIPSC frequencies did not show an effect of SPD (T-test: p=0.74). Also when we normalized the data, no differences between CTL and SPD slices were found (Fig. 3F: CTL vs SPD; Mixed model, Housing: p=0.16, Time: p=0.17, Interaction: p=0.53). These results suggest that the sensitivity GABA neurotransmission to DA modulation is not altered in mPFC L5 pyramidal neurons after SPD.

DISCUSSION

In this study, we found that rats that were deprived of social play when they were young showed an increase in sucrose intake and motivation for sucrose in adulthood. We also found that the volume and intensity of TH-positive fibres was reduced in layer 5 of adult mPFC slices after SPD, while the sensitivity of inhibitory currents to dopamine D1 or D2 receptor stimulation was not altered in pyramidal neurons in the mPFC. This shows that reward processing is altered after SPD, associated with a long-term reduction in DA innervation in the mPFC.

DA has been widely implicated in social play behaviour. For example, treatment with DA receptor agonists and antagonists alters the expression of social play behaviour (Niesink and Van Ree, 1989; Vanderschuren et al., 2008; Siviy and Panksepp, 2011; Siviy et al., 2011), and DA has been implicated in the motivation for social play (Achterberg et al., 2016). Concerning the site of action of DA, it has been shown that DA release in the nucleus accumbens is increased during social play (Robinson et al., 2011), and increasing DA neurotransmission in the nucleus accumbens stimulates social play (Manduca et al., 2016). The role of mPFC dopamine in social play behaviour, however, remains to be investigated. Here we demonstrate that social play behaviour is important for the maturation of the DA system and that SPD leads to reduced DA innervation of the mPFC.

The performance of a playful action is known to be highly rewarding (Humphreys and Einon, 1981; Trezza and Vanderschuren, 2009; Achterberg et al., 2016; Vanderschuren et al., 2016). It is further thought that social play can influence the development of the neural mechanisms of positive emotions such as pleasure and motivation (Vanderschuren and Trezza, 2014; Achterberg et al., 2016; Vanderschuren et al., 2016; Tejeda et al., 2017). SPD has been shown to increase the rewarding properties, motivation and consumption of substances of abuse (Baarendse et al., 2013; Whitaker et al., 2013; Lesscher et al., 2015). When looking at sucrose specifically, we showed that SPD rats expressed a greater consumption of sucrose solution at the highest concentration, an effect that was shown also during juvenile isolation (Van Den Berg et al., 2000). These results indicate a higher rewarding value of sugar after SPD. To further explore this, we performed a reinforced learning task in which SPD rats showed a higher response rate for sucrose during a progressive ratio, but not fixed ratio, schedule of reinforcement than CTL rats, which indicates a higher motivation for sucrose. Together, these results indicate that social play influences reward system development, with SPD leading to increased sensitivity to rewarding events.

To the best of our knowledge, our study is the first to report the effects of social play deprivation on the DA innervation of the mPFC in rats. TH fibre volume gradually increases in the mPFC during the juvenile period and young adulthood (Kalsbeek et al., 1988; Naneix et al., 2012; Willing et al., 2017) and the largest increases in TH fibre volume are found around early adolescence (Willing et al., 2017), a period accompanied by a peak in social play behaviour (Baenninger, 1967; Panksepp, 1981; Spear, 2000). As SPD is also known to affect NMDA receptor-mediated glutamatergic transmission in the VTA (Whitaker et al., 2013), a subcortical area where the mesocortical dopamine pathway originates, effects here could cascade and result in reduced DA innervation of the mPFC. A direct link between the reduced mPFC DA innervation and the increased consumption of and motivation for sucrose remains to be demonstrated. Although reward processes are typically associated with dopamine projection from the VTA to the nucleus accumbens (Kelley, 2004; Wise, 2004; Berridge, 2007; Salamone and Correa, 2012), increased DA release in the mPFC during the consumption of palatable food has been repeatedly demonstrated (Bassareo and Di Chiara, 1997; Taber and Fibiger, 1997; Ahn and Phillips, 2002). DA neurotransmission in the accumbens is indirectly influenced by the PFC through its projections to the VTA (Murase et al., 1993; Taber et al., 1995; Carr and Sesack, 2000), and early studies have indicated that reduced DA function in the PFC leads to enhanced DA neurotransmission in the accumbens (Louilot et al., 1989; Mitchell and Gratton, 1992). Additionally, our previous work (Baarendse et al.,

2013) has shown that the DA-evoked reduction in the amplitude of evoked postsynaptic potentials in the mPFC was blunted after SPD. Together, these data suggest that reduced DA function in the mPFC after SPD results in increased DA neurotransmission in the accumbens, causing the animals to show an enhanced motivation for sucrose (Ikemoto and Panksepp, 1999; Phillips et al., 2003; Hamid et al., 2015). Alternatively, changes in PFC DA may alter sucrose-directed behaviour via cognitive processes such as flexibility, impulse control and decision-making (Robbins and Arnsten, 2009; Winstanley and Floresco, 2016).

Despite the difference in DA innervation, we did not detect a change in sensitivity to DA modulation in inhibitory currents in L5 pyramidal cells. D1 receptor stimulation increased the frequency of the measured sIPSCs in CTL and SPD slices. This D1 receptor stimulation-evoked increase in sIPSCs is in line with a previous study (Seamans et al., 2001) and it is thought to be caused by the depolarizing effect of D1-receptor activation on fast-spiking inhibitory interneurons (Zhou and Hablitz, 1999; Gorelova et al., 2002). Activation of D2-receptors in CTL slices did not affect sIPSC frequency, which is in line with a similar study in the mPFC (Seamans et al., 2001), but this result must be interpreted with caution. During the D2-agonist recordings, no effect of SPD was found in the baseline recordings, which contrasts with the D1 experiment and earlier studies (Bijlsma et al., 2022, 2023). We therefore cannot state with certainty whether there is an effect of SPD on the sensitivity to D2-receptor stimulation. These results show that SPD does not affect the D1-mediated modulation of sIPCSs recorded in L5 pyramidal neurons in the mPFC.

Together, our results demonstrate that social play shapes the DA innervation of the mPFC and that a lack of social play experiences enhances reward sensitivity in adulthood. This study further emphasizes the importance of juvenile play for the proper development of mPFC function and emotional capacities.

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CHAPTER 6

General Discussion

Social play behaviour is important for the development of young animals, including rats and humans (Pellegrini and Smith, 1998; Pellegrini et al., 2007). Social play allows individuals to develop and refine their social skills which are essential for building relationships and navigating social situations throughout life. Additionally, social play is a rewarding activity, which can help individuals feel more relaxed and happy. Social play often also involves physical activity, which can be beneficial for overall health and well-being. Social play behaviour is further thought to promote physical, social, cognitive, and emotional development (Špinka et al., 2001; Pellis et al., 2010; Vanderschuren and Trezza, 2014). Conversely, a lack of social play during development results in inappropriate responses to stress and fearful situations, both social and non-social (Meaney and Stewart, 1979; Potegal and Einon, 1989; Da Silva et al., 1996; Hol et al., 1999; Van Den Berg et al., 1999b, 1999c, 1999a; Von Frijtag et al., 2002; Himmler et al., 2018). Additionally, play-deprived animals tend to display higher levels of impulsivity and are less competent in solving cognitive tasks (Einon et al., 1981; Baarendse et al., 2013). In humans, disrupted social play behaviour has been associated with mental disorders such as autism spectrum disorder (ASD), attention-deficit/hyperactivity disorder (ADHD) and anxiety disorders (Alessandri, 1992; Jordan, 2003; Luman et al., 2005; Manning and Wainwright, 2010). Therefore, interventions that target social play skills can promote the development and well-being of children and may reduce the burden of mental disorders.

The overarching aim of this thesis was to test the hypothesis that social play behaviour is of major importance for the development of prefrontal cortex connectivity underlying behavioural flexibility in adulthood. For this aim, we utilised several behavioural paradigms, immunohistochemistry and ex-vivo electrophysiology.

Summary of the main findings

In this thesis, we investigated how behaviour and synaptic signalling in the adult PFC are affected when rats are deprived of social play behaviour (social play deprivation; SPD) during development. To that aim, rats were socially isolated during the three weeks in life when social play is most abundant in rats (i.e. postnatal day (P)21-P42). Our findings demonstrate that the organization of the adult GABAergic system in the medial prefrontal cortex (mPFC) of rats is robustly altered when rats are deprived of social play behaviour while the glutamatergic system appears largely unaffected (see **Chapter 2**). This was associated with a decrease in perisomatic inhibitory synapses from parvalbumin-positive GABAergic cells onto layer 5 pyramidal cells of the mPFC in adulthood. We further show that the reduction in GABAergic synapses is not limited

to the mPFC but can also be found in the orbitofrontal cortex (OFC) (see **Chapter 3**). Measuring synaptic inputs at a younger age revealed a complex interaction between social play experience and the specific developmental trajectories of the mPFC and OFC. In the mPFC, the development of inhibitory inputs was mostly affected by SPD between P21 and P42, while in the OFC inhibitory currents were mainly affected after P42. Again, excitatory inputs in both regions were unaffected.

In parallel experiments, adult SPD animals were found to use a more simplified cognitive strategy than control rats in a probabilistic reversal learning (PRL) task, which lead to SPD animals achieving more reversals (see **Chapter 2**). As early studies had shown that behavioural performance after social isolation can be partially rescued by allowing short play periods (Einon et al., 1978; Potegal and Einon, 1989), a group of SPD animals that were allowed to play for 1 hour a day was also introduced (see **Chapter 2**). The inclusion of these daily play sessions during the SPD period partially restored the cognitive performance in the PFC-dependent PRL task but did not rescue the reduction in PFC GABAergic synapses. We also found that response inhibition was unaffected by SPD, while anxiety-like behaviour was affected in female, but not male, rats (see **Chapter 4**).

We further asked how opportunities for risky play influenced the development of the brain and behaviour (see **Chapter 4**). We provided animals with the possibility to play in a high-risk environment during a peak social play period (P21-P42) after which adult male and female rats were tested for cognition, anxiety-like behaviour and mPFC function. The opportunity to take risks during play altered cognitive flexibility and anxiety-like behaviour in both sexes while behavioural inhibition was improved in males only. Interestingly, an increase in synaptic inhibition in the mPFC was observed in male, but not female rats that engaged in risky play.

We further investigated how SPD affects reward processes by measuring the motivation and preferences of rats for sucrose (see **Chapter 5**). Our data showed that social play-deprived animals ingested more sucrose, and were willing to work more for it. We found that SPD did not affect the modulation of mPFC inhibition by dopamine D1 receptors, but we observed that the density and intensity of dopaminergic fibres in the mPFC was decreased, suggesting that social play promotes the development of the dopamine projection from the ventral tegmental area to the mPFC.

Methodological considerations

Behavioural effects during and immediately after the deprivation period

We have shown in **chapter 3** that inhibitory inputs in the mPFC are mostly affected during the deprivation period, resulting in a lower frequency of spontaneous inhibitory postsynaptic currents at P42. In order to link these changes in mPFC inhibition to the PFC-dependent cognitive consequences of SPD, it is relevant to assess whether the behavioural effects found in adult rats in **chapters 2 and 4** can also be seen at this earlier time point. In this case, the PRL and RI tasks may have to be modified so that they can be performed by younger animals. Furthermore, to test the performance of P42 rats in these tasks, the animals need to be trained during the deprivation period, which entails daily handling. Although the amount of handling will be the same for control and SPD animals, we need to be aware that handling may influence the effect of SPD (Song et al., 2021). To control for this handling, new electrophysiological measurements in the mPFC are needed in which animals are handled daily during the deprivation period.

Risk-taking during the play sessions

In **chapter 4,** homemade cages were used to allow animals to play in a high-risk play environment. These cages were enriched with multiple ladders, plateaus and other objects to interact with (Chapter 4, Fig. 1C). During the two half-hour sessions per day, rats were given the possibility to climb up the sides of the cage and engage in play with objects. We observed that rats often made stretched-attend postures while approaching the ladders, plateaus and objects in the enriched cages, suggesting that the cages were perceived as challenging by the animals. However, we were unable to quantify these postures as the animals played during their active phase, i.e. under dim-light conditions, since the video recordings were of insufficient quality to be used for behavioural scoring. The enrichment provided in the cages (i.e. ladders, plateaus and other objects) also obscured the view of the camera making it hard to follow the rats and correctly identify their behaviour. We therefore cannot formally establish that genuine risk-taking took place in these rats. Accordingly, we describe enrichment as providing the possibility for risky play. For future research, the risky cages could be modified to allow recording during the sessions so a better estimation of risk-taking can be made. Furthermore, rats were only placed in the risky play cage for two blocks of 30 minutes per day between P21 and P42, while in most studies on environmental enrichment, the animals are continuously housed in this environment (Huck and Price, 1975; Schrijver et al., 2004; Simpson and Kelly, 2011; Huang et al., 2021), this was however not possible

with our risky play cages for logistical reasons. As we have shown in **chapter 2,** daily play sessions of one hour only partially rescued the effect of SPD, similarly, the effect of the risky play cage may be limited by the amount of exposure rats had to it. Modifying the risky play cages so that animals can be continuously housed in them would be important for future research.

Sex differences in behaviour and PFC function

In recent years, a growing body of research has revealed that disorders like ASD, ADHD and anxiety disorders, all of which have high incidence rates in early life, differ in their prevalence between males and females (Hoek, 2006; Vesga-López et al., 2008; Conley and Rudolph, 2009; McLean et al., 2011). Social play behaviour in rats is known to be sexually dimorphic (Auger and Olesen, 2009) with studies reporting that males initiate social play more often (Meaney and Stewart, 1981; Thor and Holloway, 1984; Pellis and Pellis, 1990; Pellis et al., 1997) and that males are more likely to pin their play partner and more frequently engage in boxing (Pellis and Pellis, 1990). Multiple studies also show differences in behaviour between socially isolated male and female rats (Weintraub et al., 2010; McCormick et al., 2013; Pisu et al., 2016). This has brought attention to the importance of taking sex differences into account when investigating brain and behaviour. While most experiments in this thesis were done in male rats, we show in **chapter 4** that the effect of social play deprivation was mostly comparable between the sexes. However, we observed important sex-specific differences in the performance in risk-based tasks after exposure to risks during play and in the development of inhibitory synaptic inputs in the mPFC. These findings support the relevance of using both male and female rats in behavioural studies. It is currently not known how social play deprivation affects the behaviour of the sexes differently, which makes it important to further examine the effects of play deprivation on molecular and behavioural levels.

Implications for brain development and possible future directions

In this thesis, we assessed how the rat's brain and behaviour is affected by the deprivation of social play. We have shown that social play and the opportunity for risk-taking during play shapes the development of inhibitory synapses in the mPFC and of cognitive and emotional function in adulthood. Moreover, we demonstrated that excitatory and inhibitory synaptic inputs in the mPFC and OFC follow distinct developmental trajectories and that lack of social play experience disrupts this development in a region-specific manner. Our results highlight the differential

vulnerability of the PFC to developmental insults, such as the lack of social play. With these findings, we answered important fundamental questions about the effect of social play deprivation on a behavioural and cellular level. This final section of this thesis will focus on new questions that have come forth from our results.

The link between PFC PV cell activity and social behaviour

Our findings demonstrate a robust alteration of specific synapses in the adult PFC after SPD. We showed that inhibition onto layer 5 pyramidal cells in the mPFC was reduced after SPD and that this was associated with by a 30% reduction in the number of perisomatic PV synapses. While a direct link between PFC PV cell activity and social behaviour was recently demonstrated (Bicks et al., 2020; Sun et al., 2020) and alterations in PV cells during development are shown to shape cognitive capacities in adulthood (Donato et al., 2015; Mukherjee et al., 2019; Canetta et al., 2022), our data does not provide a causal link between PV synapse loss and the performance in a PFC-dependent reversal learning task. It will be important to further explore the link between synaptic changes within the intricate PFC circuitry after SPD and their consequences for PFC-driven modulation of behaviour. DREADDs, a chemogenetic tool that can be used for activating specific neuronal populations, could be used to selectively silence PV synapses to see if this directly alters behaviour during a cognitive task. As we do not want all PV synapses to be affected but only around 30% of them, the right concentration of CNO, a DREADD agonist, has to be chosen.

The effect of social play deprivation in other brain regions

While we solely focused on the PFC, it is well known that social play involves a complex interplay of neural circuits in the brain and different brain regions have been implicated in the modulation of social play behaviour (Van Kerkhof et al., 2014; Siviy, 2016). It would be interesting to see if the balance between excitation and inhibition in other brain regions that have been implicated in social play are also affected, in particular those connected to the PFC. Additionally, it would be valuable to see if the effect of SPD on the E/I balance is mainly found in cortical regions, which are involved in higher-level cognitive functions or is also seen in subcortical brain regions, involved in memory, emotion and motivation. Possible regions of interest would be the insular cortex, hippocampus, amygdala, striatum, habenula and ventral midbrain (Gordon et al., 2002, 2003; Van Kerkhof et al., 2013; Vanderschuren and Trezza, 2014). It is well possible that synaptic changes in one of the regions could affect the circuit development in another region. For instance, our results suggest that the initial loss of IPSCs in the

mPFC may have caused the later effect in the OFC. It will therefore be valuable to map the development of projections between the brain regions of interest during social play deprivation. This could provide insight into the cascading effects that SPD likely cause within the brain.

Could risky play specifically affect PV perisomatic synapses?

In chapter 4 we confirmed the effect of SPD on the mIPSC inputs on L5 mPFC pyramidal neurons in play-deprived animals, but additionally, we found an increase of mIPSC inputs in high-risk animals that were allowed to take risks while playing in an enriched cage. This data suggests that there is no (or very little) interaction between the opportunity for social play and risk-taking during social. Both aspects of play have an independent effect on PFC development, and the opportunity for risk-taking does not appear to mitigate the effects of play deprivation. Interestingly, while SPD lead to an increase in the rise time of measured mIPSCs, high-risk animals showed a decrease in rise time. This raises the question if the opportunity to take risks during social play could specifically affect PV perisomatic synapses, possibly via different pathways, and increase the amount of perisomatic PV synapses, causing a higher mIPSC frequency and decreased rise time. Additionally, what does this mean for the E/I balance in high-risk animals? Are the excitatory inputs affected by high-risk play? Performing sEPSC measurements and synaptic stainings as described in chapter 2 with rats that were allowed to take risks while playing could give us answers to these questions.

Social play deprivation and dopamine signalling

Our experiments show that SPD affects the dopaminergic innervations of the PFC. However, while we see an effect on the density of TH-positive fibres we do not know if this affects the release of dopamine in the mPFC. As dopamine signalling is implicated in multiple processes within the mPFC, like working memory, stimulus discrimination, behavioural control and stress response (Horvitz, 2000; Lammel et al., 2012; Gunaydin et al., 2014), it would be valuable to measure dopamine release in the mPFC during rewarding processes in both CTL and SPD animals with a dopamine sensor, like dLight1 (Patriarchi et al., 2018). It would further be interesting to measure the same parameters, e.g. TH-positive fibre density and dopamine release, in the nucleus accumbens as the increased motivation for sucrose is typically associated with dopamine projection from the VTA to the nucleus accumbens (Ikemoto and Panksepp, 1999; Kelley, 2004; Salamone and Correa, 2012; Hamid et al., 2015).

The different aspects of social play

Our data do not reveal which aspect of play is the most important for the development of PFC circuitry and PFC-dependent cognitive processes. The effects of SPD that are described in this thesis could be caused by a loss in the social, cognitive, emotional, or physical domain of social play, or their interaction, or even by the stress caused by the impossibility to express play. To test the physical domain, for example, P21 animals could be housed with adult rats, which are known to play less than young animals (Panksepp, 1981). This way, young animals will have physical contact with another rat while social play will be limited.

Implications for humans

Access to a range of unstructured play experiences that allow children to express themselves freely in any way they desire is generally considered one of the best ways for them to maintain physical and mental health (Jarvis et al., 2014; Colliver et al., 2022). "Free" play gives children a chance to explore the world in their own way, encourages creativity and imagination (Moller, 2015), and helps to build self-control (Ginsburg et al., 2007; Yogman et al., 2018). Regrettably, there is increasing evidence that the opportunity for free play in households in post-industrialized nations is declining (Witten et al., 2013; Brussoni et al., 2015; Pynn et al., 2019). Animal studies, including those presented in this thesis, emphasize the importance of social play in general (Einon and Morgan, 1977; Hol et al., 1999; Van Den Berg et al., 1999a; Lukkes et al., 2009; Kolb et al., 2012; Whitaker et al., 2013; Vanderschuren and Trezza, 2014; Vanderschuren et al., 2016). In chapter 2, our deprived rats experienced a substantial amount of the daily social play that controls rats normally experience, but the possibility of not being able to freely express social play was missing. The results presented in Chapters 2 and 3 of this thesis suggest that the experience of unrestricted social play is important for the development of inhibitory synapses in the PFC and cognitive skills in adulthood. If we extrapolate these findings to children, this means that although the amount of time that children get the opportunity to play is important, when children are allowed to play, for example during school breaks, they should get the opportunity to freely express their play behaviour.

Along with the expression of social free play, opportunities for children to engage in risky play have steadily decreased over the years in Western societies (Valentine and McKendrick, 1997; Weir et al., 2006; Gray, 2011; Brussoni et al., 2012; Little et al., 2012). While research on the effect of risky play on behaviour is limited, Eager and Little (2011) suggested that a child that does not participate in risky activities may be more

sensitive to mental health concerns, obesity, and a decrease in learning, perception and judgment skills. In this thesis, we provide additional evidence on the importance of risk-taking during play for cognitive and emotional development, and maturation of the underlying circuitry in the PFC.

During the recent COVID-19 pandemic, far-reaching social restrictions were put in place around the world. As a result, many children were unable to play with their friends and classmates. As it is known that chronic diseases that keep children bed-bound during childhood, limiting the number of social interactions, could have adverse effects on their mental health and well-being during adulthood (Nijhof et al., 2018), concerns have been expressed about the pandemic's deleterious effects on the mental health and well-being of youth (Araújo et al., 2020; Brooks et al., 2020; Mazza et al., 2020; Robinson et al., 2020; Hawke et al., 2021; Xie et al., 2022). For example, in China, a study found that the mental health impacts caused by the pandemic may be more severe among adults under the age of 35 (Huang and Zhao, 2020). Complimentary to this, a study conducted in Canada (Hawke et al., 2020) found that youth aged 14 to 28 experienced mental health concerns in the early phases of COVID-19. As the onset of many mental disorders typically occurs in childhood or adolescence (Kessler et al., 2007) and, as shown in this thesis, the deprivation of social play interaction during this phase of life results in long-lasting, perhaps permanent, changes in brain function and connectivity, it is well conceivable that there will be long-term effects of the COVID-19 pandemic on mental health and well-being. Longitudinal studies looking into this should mainly focus on children where the lockdowns coincided with a critical period in terms of social contact and play, as while the social restrictions during the pandemic were indeed strict, they were also temporary, so you cannot rule out that the effects will eventually be better than expected because children can now play normally again.

Final remarks

The chapters of this thesis contribute to a better understanding of the importance of social play on a molecular and behavioural level and make clear that early life experiences have long-lasting effects on brain connections and function. We further emphasize the importance of play with peers in young animals, showing that different aspects of play shape different aspects of brain development and behaviour. With this thesis, we also want to increase the awareness of the importance of free, unrestricted play for children as in today's world the amount of free play time available to individuals, especially children, has been decreasing. To address this decline, it is important to strike

a balance between structured activities and free play which will be beneficial for the cognitive, social, and emotional development.

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ADDENDUM

Nederlandse samenvatting

About the author

Publications

Dankwoord

NEDERLANDSE SAMENVATTING

Sociaal gedrag is van groot belang in het leven van mens en dier en omvat een breed scala aan geavanceerde sociale interacties. Sociale gedragingen zijn cruciaal voor het overleven van het individu, de groep of de soort. Tijdens het leven ontwikkelt het gedragsrepertoire zich van eenvoudige interacties tussen baby en moeder tot complexe interacties binnen sociale netwerken. De focus van dit proefschrift zal liggen op één specifiek soort sociaal gedrag dat tot uiting komt in alle levensfasen, maar vooral wordt gezien bij jonge en vroege adolescente zoogdieren en dat uitgebreid is bestudeerd bij de rat, namelijk sociaal speelgedrag.

Sociaal speelgedrag wordt verondersteld de fysieke, sociale, cognitieve en emotionele ontwikkeling van dieren te bevorderen. Bovendien stelt het dieren die in sociale groepen leven, zoals mensen, ratten en nog vele andere dieren, in staat om hun complexe repertoire van gedragingen alvast in versimpelde vorm te oefenen. Deze periode is belangrijk, aangezien het vermogen om te reageren met passend gedrag binnen de juiste context en op het juiste moment cruciaal kan zijn om te overleven.

Sociaal spel bij ratten begint op de leeftijd van 15 tot 18 dagen, waarna de speelfrequentie sterk toeneemt totdat een piek wordt bereikt in de vroege adolescentie wanneer een rat ongeveer 35 dagen oud is. Vanaf dit moment neemt de frequentie van het speelgedrag geleidelijk af totdat het volwassen niveau is bereikt. Sociaal spel bij ratten omvat de aanval en verdediging van de nek, met als doel de nek van de speelpartner zachtjes met de snuit aan te raken. Dit resulteert er vaak in dat de verdedigende rat zichzelf op zijn rug draait, terwijl hij wordt 'vastgepind' door zijn speelpartner. Het uitvoeren van een speelse actie is een belonende activiteit. Deze activiteit wordt geassocieerd met de ervaring van positieve emotionele toestanden en vindt vaak plaats in afwezigheid van gevaren. Hierdoor is sociaal spel bij dieren een mogelijke welzijnsindicator. Tijdens het spelen wordt plezier ervaren door de activering van neurale systemen die ook betrokken zijn bij andere soorten beloning, zoals seks, smakelijk voedsel en het gebruik van verschillende drugs.

Een fundamentele eigenschap van onze hersenen is het vermogen om zich continu aan te passen aan veranderingen in de interne en externe omgeving, de zogenaamde neuroplasticiteit. Het is algemeen bekend dat veel (zo niet alle) hersengebieden een zogenaamde kritische of gevoelige periode van verhoogde plasticiteit hebben tijdens de ontwikkeling. Tijdens deze zogenaamde kritische periodes is het essentieel dat er voldoende externe prikkels zijn, zodat netwerken van hersencellen goed worden aangelegd. Klassieke voorbeelden van kritieke perioden zijn beschreven in de

olfactorische cortex en de visuele cortex. In deze laatste structuur is aangetoond dat volledige duisternis na de geboorte, om het aantal visuele ervaringen te beperken, leidt tot de ontwikkeling van een onvolgroeide visuele cortex. Bovendien resulteerde het verlies van visuele ervaringen gedurende slechts een klein, maar gevoelig tijdsbestek, in hermodellering van het volledige visuele corticale circuit en langdurige stoornissen in het visuele functioneren tijdens de volwassenheid.

In dit proefschrift testen we de hypothese dat sociaal spel essentieel is voor de ontwikkeling van het netwerk in de prefrontale cortex (PFC), een belangrijk hersengebied voor gedragsflexibiliteit op volwassen leeftijd. Om dit te testen, zijn ratten van spelgedrag gedepriveerd. Er is voor gekozen ratten in paren met een nestgenoot te huisvesten en een transparante verdeler met kleine gaatjes tussen hen te plaatsen (fig. 1 introductie). Deze gaten maakten het voor de ratten mogelijk om elkaar te zien, ruiken en horen en om beperkte fysieke interactie te hebben zonder de mogelijkheid om te spelen. Aansluitend onderzochten wij hoe de cognitieve flexibiliteit en synaptische signalering in de volwassen PFC worden beïnvloed wanneer ratten sociaal speelgedrag wordt ontnomen tijdens de ontwikkeling. Synaptische signalen werden gemeten in plakjes van de mediale PFC (mPFC) van controle (CTL) en spel gedepriveerde (SPD) ratten. In deze meting was te zien dat remmende stroompjes verminderd waren in hersenplakjes van SPD dieren, terwijl stimulerende stromen niet beïnvloed waren. Dit werd in verband gebracht met een afname in synapsen van parvalbumin-positieve cellen, een bepaalde groep cellen die voornamelijk remmende signalen afgeeft. In parallelle experimenten bereikten volwassen SPD ratten, door gebruik te maken van een meer vereenvoudigde strategie dan controle dieren, meer reversals in een PFC afhankelijke probabilistic reversal learning (PRL) taak. We introduceerden verder een tweede groep SPD-ratten die dagelijks één uur mochten spelen met hun kooigenoot tijdens de deprivatieperiode. Interessant genoeg redde een dagelijks uur spelen tijdens SPD gedeeltelijk de prestaties in de PRL, maar voorkwam dit niet de afname van remmende signalen in de PFC.

Naast onze metingen in de mPFC onderzochten we ook de invloed van sociaal spel op de ontwikkeling van stimulerende en remmende signalen in de orbitofrontale cortex (OFC), een subgebied van de PFC. We voerden opnames uit in cellen van beide regio's van jonge, adolescente en volwassen CTL en SPD ratten. We zagen dat in jonge dieren de remmende en stimulerende signalen meerdere malen hoger waren in de OFC dan in de mPFC. Bovendien had SPD geen effect op stimulerende stroompjes, maar verminderde het de remmende stromen in zowel de mPFC als de OFC. Intrigerend genoeg trad de vermindering in de mPFC op tijdens de deprivatieperiode, terwijl de vermindering in

de OFC zich pas manifesteerde na de deprivatieperiode. Onze gegevens onthulden een complexe interactie tussen sociale spelervaring en de specifieke ontwikkelingstrajecten van prefrontale subgebieden.

Verder hebben wij ook gekeken of het blootstellen aan risico's tijdens het spelen een adequate reactie op risico's en omgaan met onzekerheid bevordert. Daarbij combineerden we een gebrek aan sociaal spel met mogelijkheden om risico's te nemen. Dit werd gedaan door een kooi te verrijken met meerdere ladders, plateaus en andere objecten om mee te spelen. Op volwassen leeftijd werden de ratten getest op cognitieve flexibiliteit, gedragsremming en angstig gedrag en maakten we opnames van synaptische stromen in PFC plakjes. Wij laten in dit onderzoek zien dat blootstelling aan risico's tijdens spel de prestaties in PFC-afhankelijke taken en angstachtig gedrag op volwassen leeftijd beïnvloedt en dat het de ontwikkeling van remmende signalen in de mPFC beïnvloedt. Bovendien gaven onze resultaten aan dat de interacties tussen risicovol spel en speldeprivatie beperkt waren, wat suggereert dat sociaal spel met leeftijdsgenoten en de mogelijkheid om risico's te nemen tijdens het spelen onafhankelijk van elkaar de ontwikkeling van gedrag en PFC-functie beïnvloeden. Dit houdt in dat de effecten van speldeprivatie niet kunnen worden verzacht door de kans op het nemen van risico's te vergroten, maar dat beide aspecten van jeugdspel een afzonderlijke waarde hebben voor het gedrag van volwassenen.

Tot slot stellen wij de hypothese dat sociaal spelgedrag de ontwikkeling van remmende verbindingen beïnvloedt via dopaminerge neurotransmissie. Om dit te testen richtten we ons op het belonende aspect van sociaal spel. Hierbij zagen wij bij volwassen ratten na SPD een toename in sucrose-inname en een verhoogde motivatie om te reageren voor sucrose. In parallelle experimenten vonden we dat dopaminerge vezels in de mPFC in volume en intensiteit afnamen. Daarnaast was de modulatie van remmende stromen door dopamine, waarvan in vorige hoofdstukken werd aangetoond dat ze werden beïnvloed door SPD, vergelijkbaar tussen controle en spel gedepriveerde dieren. Samen tonen deze resultaten aan dat sociaal spel de DA-innervatie van de mPFC vormt en dat een gebrek aan sociale spelervaringen de beloningsgevoeligheid op volwassen leeftijd verhoogt. Deze studie benadrukt verder het belang van jeugdspel voor de juiste ontwikkeling van de mPFC-functie en emotionele capaciteiten.

Samengevat draagt dit proefschrift bij aan een beter begrip wat betreft het belang van sociaal spel op moleculair en gedragsniveau. Hierbij wordt duidelijk dat verschillende soorten spel invloed hebben op verschillende onderdelen van de hersenontwikkeling en gedrag. Met dit proefschrift onderschrijven we het belang van vrij en onbeperkt spel voor de cognitieve, sociale en emotionele ontwikkeling van mens en dier. Het is daarom

belangrijk om, met name, kinderen voldoende balans te bieden tussen gestructureerde dagelijkse activiteiten en de mogelijkheid tot vrij spel.

ABOUT THE AUTHOR

Ate Bijlsma was born on July 18 1994 in Duiven, the Netherlands. In 2012, he obtained his VWO Atheneum NG/NT diploma at the Candea College in Duiven. In the same year he started studying Chemistry at the Radboud University of Nijmegen. In 2013 he switched from Chemistry to Molecular Life Sciences, and he obtained his Bachelor of Science in 2016. Subsequently, he enrolled in the Master Molecular Life Sciences, with a specialization in Neurosciences, at the same university. During his first internship, he investigated how dopamine regulates voltage-gated ion channels in the somatosensory cortex of a mouse under the guidance of Dr. Wim Scheenen at the Department of Neurophysiology at the Radboud University. He performed his second internship in the same group, but now under the supervision of Dr. Fleur Zeldenrust. During this internship, he worked on developing single-neuron models on the data from his first internship to gain further insight into the effects of Dopamine on the somatosensory cortex. In 2019 he obtained his Master of Science and started as a PhD candidate under the supervision of Prof. Dr. Louk Vanderschuren and Prof. dr. Dr. Corette Wierenga at the Faculty of Veterinary Medicine and the Faculty of Science of Utrecht University. During this PhD track, he studied how the lack of social stimuli during development leads to abnormal function of the prefrontal cortex, which is important for social behaviour and complex thought processes. The results of this research are described in this thesis. Since May 2023 he has been working as a Research IT Consultant at the University Medical Center Utrecht.

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