Part A – Applicant

A.1 Applicant

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Part B – Scientific proposal

B.1 BASIC DETAILS

B.1.1 Title

Playful pathfinders: elucidating the direct contribution of mPFC pathways to social play behaviour in rats.

B.1.2 Abstract

Social play is thought to be fundamental for the proper development of social, motoric and cognitive skills. Therefore, deficient social play, as reported in children with autism and ADHD, could have profound consequences later in life. Therefore, studying the underlying neurobiology of social play can provide valuable knowledge to guide new therapeutic strategies for these children. Due to the highly rewarding nature of social play, most research has focused on the involvement of reward systems in social play, such as the prefrontal cortex, amygdala and striatum. Even though this has identified potential targets underlying social play, to date no single study shows the direct involvement of corticolimbic or corticostriatal pathways in social play. This study aims to investigate the direct contribution of mPFC pathways to social play and how these pathways are altered following social play deprivation. Hereto, the activity within the mPFC-NAc and mPFC-BLA projections will be recorded during social play using innovative wireless photometry and subsequently manipulated using chemogenetics to uncover whether these projections are important for the expression of and motivation for social play. This study will follow by examining how social play deprivation affects the activity within these mPFC projections later in life, specifically during social and cognitive tasks. Finally, manipulations of these mPFC pathways during social play deprivation will reveal whether its detrimental effects can be alleviated. Combined, these experiments will elucidate whether the mPFC-NAc and mPFC-BLA pathways are directly involved in social play behaviour, and how they are affected by play deprivation.

B.1.3 Layman's summary

Children with autism and ADHD often have difficulties in their social play behaviours. Social play is thought to be essential for the development of social, motoric and cognitive skills. For example social play allows children to experiment with their communication, emotions, empathy and cooperation with peers thereby advancing their social skills. A lack of social play behaviour such as in autism or ADHD can therefore have profound effects on their social and cognitive skills later in life. Therefore, efforts to discover the brain mechanisms behind social play behaviour, and which brain regions are affected by deficits in social play, could guide the development of new treatments for these children. Previous research has shown that brain regions involved in reward processing, like the prefrontal cortex (PFC), amygdala, and nucleus accumbens (NAc), are also involved in social play behaviour. Despite these studies showing which brain regions are involved in social play, it is unclear how exactly the connections between these brain regions are involved. Since a lack of social play in young rats results in less inhibitory 'stop' signals within the medial PFC (mPFC), connections between the mPFC and the basolateral amygdala (BLA) and between the mPFC and the NAc could be altered as well. Therefore, this study aims to discover how the connections between the mPFC-NAc and mPFC-BLA are involved in social play behaviour, and how the activity in these mPFC connections is changed if there is a lack of social play. To this end a new technique called wireless photometry will be used to measure brain activity within these brain connections during social play behaviour. This 'live' view will directly show if the mPFC-NAc and mPFC-BLA

connections are active during social play. Furthermore, another technique to manipulate the activity within these mPFC connections, called chemogenetics, will be used to see if these mPFC connections are necessary for social play to occur. This study will also investigate whether a lack of social play changes the activity in these brain connections later in life, and whether this contributes to the deficits in social and cognitive skills. Finally, by manipulating the activity in these mPFC connections during deficient social play, this study aims to discover how the negative effects of deficient social play can be alleviated. Combined, these experiments will reveal whether the connections between the mPFC-NAc and mPFC-BLA are directly involved in social play; and how they are affected if there is a lack of social play behaviour. Ultimately, this research could increase our understanding on how social play deficits alter the social and cognitive development of children with autism and ADHD. In turn, this knowledge could contribute to the development of new treatments for children with autism and ADHD, that focus on improving their social skills, which would be different from treatments that are currently available.

B.1.4 Keywords

Wireless photometry, chemogenetics, social play, social play deprivation, mPFC projections

B.2 SCIENTIFIC PROPOSAL

B.2.1 Research topic (What)

Social play behaviour is thought to be fundamental for proper development of social, cognitive and motoric skills of young mammals[1–6]. Evidence for the role of social play behaviour in motoric and social development comes from non-human primate studies, showing that motor and social milestones are reached at a younger age when non-human primates engaged in more social play behaviours[7]. Furthermore, it has been shown that depriving rats of social play behaviour can have long-term consequences in both cognitive and social skills. For example, play deprivation in rats can lead to increased aggressiveness[8], decreased sociability[9,10] and impaired cognitive flexibility[11]. In humans, deficits within social play behaviour are one of the key characteristics of children diagnosed with autism spectrum disorder (ASD) and attention deficit hyperactivity disorder (ADHD)[12–15]. It is suggested that these play deficits can in turn have profound effects on their social and cognitive skills later in life[13,14]. Even though play-based interventions show promising results in improving their social skills even one year after intervention, treatment still remains challenging[14,15]. **Therefore, studying the underlying neurobiology of social play behaviour and the neural circuits affected by deficits in social play behaviour, can provide valuable knowledge to guide new therapeutic strategies for these children**.

Social play is found to be highly rewarding, as shown by studies where social play is preferred over interaction with a non-playful partner[16,17]. Furthermore, rats are highly motivated to gain access to a playmate during an operant task[5]. Therefore, most research has been focused on investigating the neural reward systems in social play. Several studies have implicated the involvement of reward-associated neurotransmitters, such as opioids, endocannabinoids, dopamine and noradrenaline (for review see Achterberg 2023)[6]. Determining which brain regions these neurotransmitters act on provides a window into the possible involved neurobiology of social play behaviour. For example, the nucleus accumbens (NAc) has been identified as an important site of action for the play-enhancing effects of opioids[18] and dopamine[2], whereas the basolateral amygdala (BLA), prefrontal cortex (PFC) and habenula are important for the play-decreasing effects of norepinephrine[19]. Furthermore, the F344 rat strain, which is less playful by nature, shows altered dopamine signalling within the PFC and striatum[20]. The involvement of these corticolimbic and corticostriatal structures in social play behaviour are supported by lesion and chemical inactivation studies. For example, PFC lesions change the structure of social play[21], whereas inactivation of the PFC decreases social play behaviours without affecting its structure[22]. Lesioning striatal dopamine results in shorter play sessions[23], whereas inactivation of the NAc increases social play duration, and inactivation of the dorsomedial striatum increases the frequency of social play behaviours[22]. Lastly, lesioning the amygdala decreases social play behaviours in males, but not in females, thereby highlighting a possible role of the BLA underlying the sex differences observed in social play behaviour[24]. Combined, these studies show that corticolimbic and corticostriatal regions are clearly involved in social play behaviour, but not specifically how they are involved or what specific brain projections are underlying social play behaviour since lesioning or inactivation of a region affects all incoming and outgoing trajectories. Currently, identification of specific brain trajectories involved in social play has been limited to post-mortem c-fos correlation studies, where c-fos stainings are used as a measure of brain activity during social play. For example, after social play c-fos activity is enhanced in several PFC regions, the dorsal and ventral striatum (in which the NAc is located) and in the lateral amygdala[25]. Even though social play does not enhance c-fos within the BLA, the c-fos activity levels within the BLA have been correlated with the play-induced increases of c-fos in the PFC and ventral striatum[25]. These results are corroborated by a recent study where decreased social play behaviours are correlated with decreased levels of c-fos in the PFC and NAc, but increased levels of c-fos in the BLA[26]. **Overall, although extensive research has been conducted to identify the neurobiology of social play, to date no single study exists that shows the direct activation of specific corticolimbic or corticostriatal brain projections during social play behaviour.**

Recent advancements in technologies, such as fiber photometry to measure brain activity in freely behaving animals and chemogenetics to manipulate brain activity during behaviours, provide a toolbox to study the direct causational involvement of brain trajectories to behaviours[27,28]. Despite a multitude of studies having used these techniques for other behavioural outcomes, amongst which a few studies investigating certain brain trajectories underlying other social behaviours[1,29,30], studies using these techniques for social play behaviour are still lacking. To date, conventional fiber photometry has limited use within social play behaviours, due the optic cable restricting normal social behaviours as well as introducing noise artefacts caused by the movement of the animal[27,28]. Due to the highly energetic nature of social play behaviour[3,5,16,20], these noise artefacts provide a huge limitation in determining which brain regions are activated during social play. The recent development of wireless photometry systems overcome these limitations by eliminating the use of an optic cable, thereby reducing noise artefacts[27,28]. Therefore, wireless photometry provides a new innovative approach to study the contribution of certain brain trajectories underlying social play behaviour. Within this study, we will focus on the medial PFC (mPFC) trajectories since the mPFC is highly implicated in social play[22,25]. During the period when social play is most abundant (in rats from P21 till p42), the mPFC shows most of its development[3,11]. Recent research has shown that depriving rats of social play behaviour alters the

development of inhibitory neurons within the mPFC[3,11]. Therefore, we postulate that the mPFC projections might be sensitive to alterations by deficient social play behaviour. **This study aims to investigate the direct contribution of mPFC trajectories in social play behaviour and how these pathways are altered during social play deprivation using innovative wireless photometry systems and chemogenetics.** The abovementioned studies show the involvement of corticolimbic and corticostriatal regions in social play behaviour, however how the connections between these regions are involved is still unclear. Considering the inhibition within the mPFC decreases due to social play deprivation[3,11], it can be hypothesized that consequently activity within the projections from the mPFC to the NAc and BLA are increased. Therefore, this study will focus on characterizing the role of the mPFC-BLA and mPFC-NAc projection in social play behaviour and how these projections are affected by social play deprivation. Here, we hypothesize that the mPFC-NAc projection will underly the motivation for social play behaviour, whereas the mPFC-BLA projection will underly the expression of social play behaviour itself. **The objectives of this proposal are to 1) investigate what mPFC projections are specifically activated during social play behaviour, 2) determine whether these mPFC projections are important for the expression of and motivation for social play behaviour, 3) examine how social play deprivation affects the activity within these mPFC projections later in life, and 4) determine if the detrimental effects of social play deprivation can be alleviated by manipulation of these mPFC pathways.**

B.2.2 Approach (How)

The present study employs a combined approach of wireless photometry and chemogenetics to answer the question how the mPFC-NAc and mPFC-BLA pathways are involved in social play behaviour, and whether these mPFC projections are altered due to social play deprivation. To investigate how the activity within the mPFC-NAc and mPFC-BLA is altered during the expression of and motivation for social play behaviour, wireless photometry recordings will be made during social play sessions and during an operant task for social play behaviour. Furthermore, the activity within the mPFC-NAc and mPFC-BLA will be manipulated using chemogenetics during these behavioural tasks to determine whether these mPFC pathways are necessary for the expression of and motivation for social play behaviour. To assess whether social play deprivation in juvenile rats alters the activity within the mPFC-NAc and mPFC-BLA during social and cognitive tasks later in life, wireless photometry recordings will be acquired during social play sessions, during an operant task for social interaction and during a reversal learning task to measure cognitive flexibility. Finally, the activity of the mPFC-NAc and mPFC-BLA will be chemogenetically manipulated during the social play deprivation period, to assess whether this can alleviate the detrimental effects on social and cognitive parameters. For the generalisability of the results a pilot study will be performed, considering sex differences during social play behaviour have been reported in rodents[31,32], non-human primates[33] and human children[34]. For example, male rats engage more frequently in social play behaviours than female rats[32,35]. Even though male rats engage more in social play behaviours, this effect is abolished by lesioning the amygdala, whereas amygdala lesioning has no effect in female rats[24]. Therefore, a pilot study will investigate whether sex differences are apparent in mPFC-BLA and mPFC-NAc projection during social play behaviours and whether sex differences in these pathways are apparent following social play deprivation. The latter is especially of interest since it has been reported that preventing social play behaviour results in behavioural alterations later in life differently for male and female rats[8]. If based on this pilot we can exclude the possibility of sex differences, groups of mixed sexes will be used, whereas if sex differences are found separate sex groups will be used. Testing males and females in separate groups will require more rats, as well as more time to complete the four projects (figure 1). Sex differences are expected for the mPFC-BLA projection since it has been suggested that the BLA can underlie the sex differences during social play behaviour[16,24]. Considering these differences have not been reported for the NAc, no sex differences for the mPFC-NAc are expected.

1. **What mPFC projections are specifically activated during social play behaviour?**

To investigate whether the mPFC-NAc and mPFC-BLA are activated during the expression of and motivation for social play behaviour, wireless photometry recordings will be acquired during social play sessions and an operant task for social play behaviour (figure 2). Hereto, Wistar rats (n=16) will be injected with a Cre-dependent calcium sensor GCAMP6 (AAV-Syn-FLEX-GCaMP6f) into the mPFC. A subsequent injection of CAV2-Cre within the NAc will result the expression of GCaMP6 specifically in the NAc projecting mPFC neurons. To record from the mPFC-BLA projection, CAV2-Cre will be injected into the BLA to specifically express GCaMP6 in the mPFC-BLA neurons. After injection of these viruses, a wireless subdermal photometry device, as described by Burton and colleagues (2020)[28], will be implanted within the mPFC to be able to record the activity within the mPFC-NAc and mPFC-BLA projections. Using a subdermal wireless device avoids the introduction of large motion artifacts to which conventional fiber photometry is subjected to[27,28], especially during highly energetic social behaviours such as social play. Implantation of this system shows a comparable recovery speed with conventional fiber photometry implantations, and shows no loss of performance at least after two months of implantation[28], making it suitable to use for the duration if this study (6 weeks). Furthermore, it has been shown that recordings made with these wireless photometry systems are comparable with those made with fiber photometry[27]. Since it has been found that white furred animals have a higher

transmission of the infrared signal send out by the photometry device compared to black furred animals[28], albino Wistar rats will be used in this study to avoid the need to shave the rats before photometry recordings are acquired.

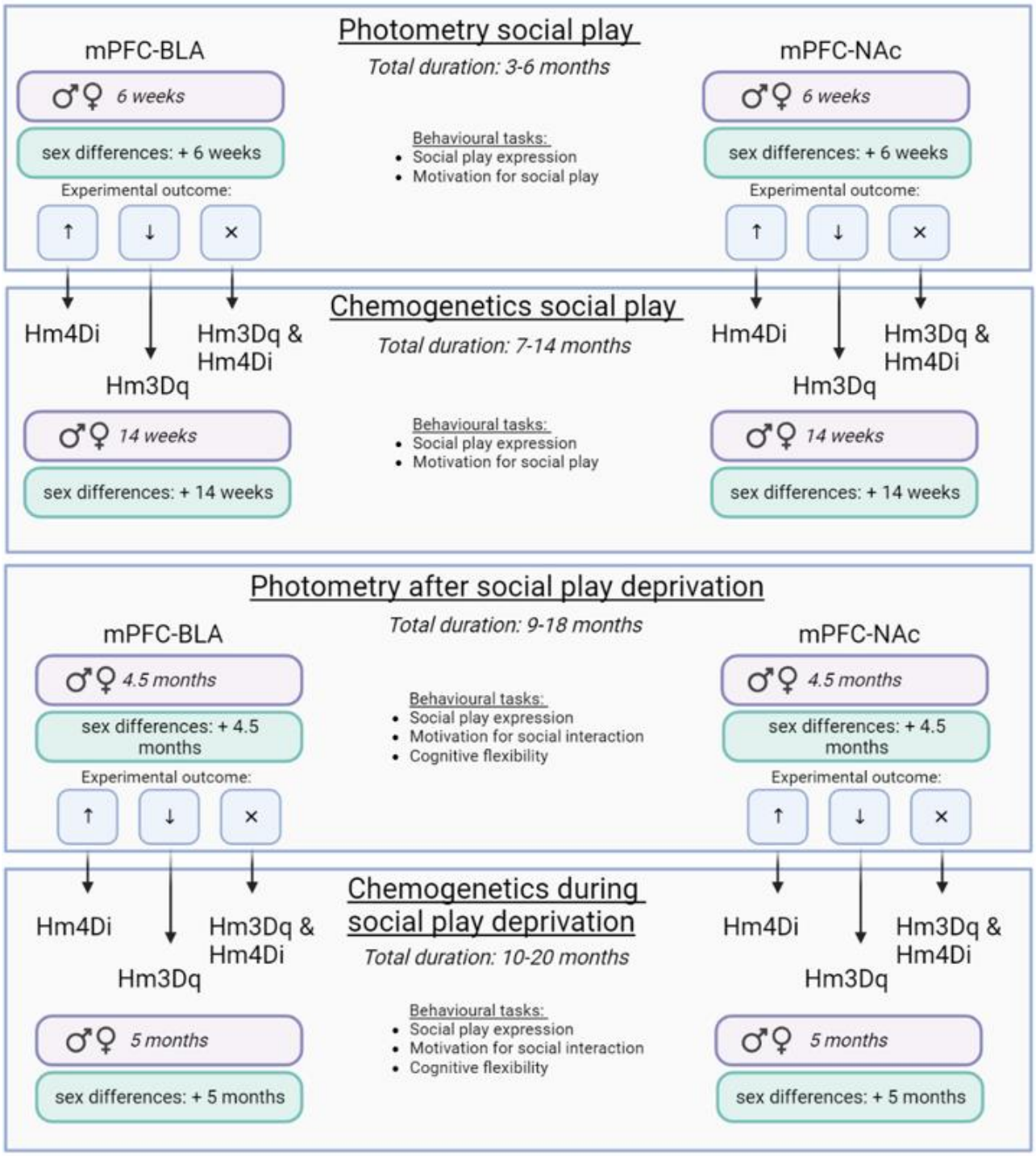

***Figure 1: Timeline of proposed research lines**. The estimated timing of each project has been indicated, as well as how long experiments for each single pathway takes while using intermixed sex groups (purple boxes). If sex differences are found during the pilot studies, additional time is required to complete the project for both sexes separately (green boxes).*

Rats will be weaned on p21, after which these stereotactic surgeries take place. Rats are allowed to recover for 2-3 days and are then pair-housed with a stimulus rat of the same sex. To allow for proper expression of the viruses, experiments do not start until 2 weeks after surgery. During this time, the rats will be trained on an operant task for social play behaviour. Prior to these training sessions rats will be isolated for 2.5 hours to enhance their engagement in the task, since it has been previously established that a short isolation period increases social play behaviour in rats[2,36]. Rats will be trained daily for 30 minutes on a fixed ratio schedule (FR1) where a single nose poke results in access to their playmate for 60 seconds. At p35, the activity within the mPFC-NAc or mPFC-BLA will be recorded during a social play session. Again, 2.5 hours prior the rats will be isolated to enhance social play behaviours during the recording session. Rats will be placed into the recording area (40 x 40 x 60 cm, length x width x height) surrounded by a coil for wireless energy delivery to the photometry system. During 10-

minute play sessions, both photometry and video recordings will be made to relate the mPFC-NAc or mPFC-BLA activity to the play behaviour expressed. Including a flashing LED light with timestamps in the photometry recording, that is also visible in the video recording allows for time-locking offline scored behaviours to the photometry recordings[37]. Pouncing (one rat attempts to nose/rub the nape of the neck of the playmate to initiate play behaviour)[5], pinning (one rat lying with its dorsal surface on the floor with the other rat standing over it)[5], total time engaged in play behaviour, and in social exploration (sniffing or grooming the playmate) will be scored to distinguish whether these mPFC projections are activated during certain play behaviours, or social behaviour in general. These social play recording sessions will be repeated on three subsequent days so that each rat has been recorded for 30 minutes in total. After completing the last recording session, rats will be performing an operant task for social play behaviour to measure whether the mPFC-NAc or mPFC-BLA are involved in the expression of social play or the motivation for social play. FR1 sessions will be repeated for two additional days, prior to which rats are isolated overnight to ensure they are properly motivated to perform the task. After these FR1 sessions, photometry recordings will be made during a progressive ratio (PR) schedule, where increasing nose pokes are required to obtain a social play reward. Here, the breakpoint, which is the maximal number of nose pokes made for a single reward, is indicative of the motivation to gain access to their playmate. PR recording sessions will be performed over three subsequent days, after which the rats will be perfused to confirm the virus and photometry placement. Exclusion will occur if either viruses or photometry device where misplaced outside the regions of interest. **Combined these experiments will elucidate whether the mPFC-NAc or mPFC-BLA are activated during the expression of social play or during the motivation for social play behaviour.**

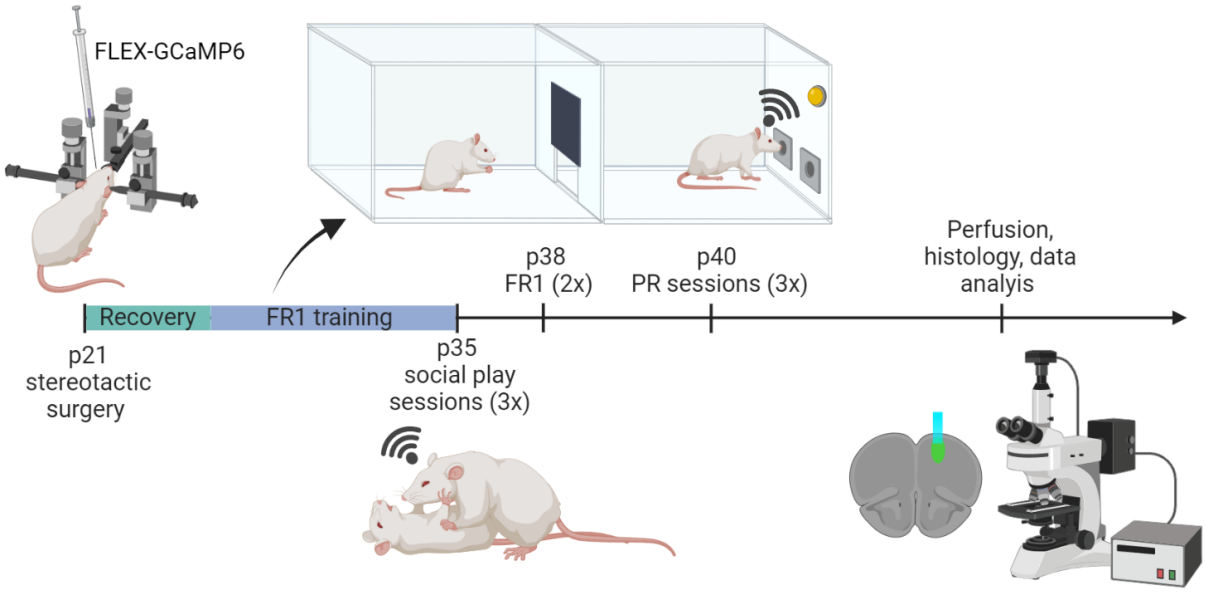

Figure 2: Experimental design for mPFC-NAc and mPFC-BLA activity during the expression of and motivation for social play behaviour*. Stereotactic surgeries will be performed at p21 after which the rats will be FR1 trained on an operant task for social play behaviour. At p35 photometry recordings will be made during three social play sessions, followed by two additional days of FR1-training. At p40 photometry recordings will be made during three progressive ratio (PR) sessions to assess their motivation for social play behaviour. Subsequently rats will be perfused to confirm the placement of the viruses and photometry device.*

2. Which mPFC projections are crucial for social play behaviour?

To investigate whether the mPFC-NAc or mPFC-BLA projection are important for the expression of social play, the activity of these pathways will be manipulated using chemogenetics during social play behaviour (figure 3). Hereto, Wistar rats will be injected with CAV2-Cre in the BLA or NAc respectively, together with an injection of a Cre-dependent activating (AAV-hSyn-DIO-Hm3D(Gq)-mCherry) or inhibiting DREADD (AAV-hSyn-DIO-Hm4D(Gi)-mCherry) into the mPFC, depending on whether this projection was activated, inhibited or unaffected during project 1 (figure 1; 2 batches of n =16, 8 DIO-mCherry controls, 8 DIO-Hm4Di or Hm3Dq). In case of activation during social play behaviour, during project 2 this projection will be inhibited; in case of inhibition during social play, the projection will here be activated; and in case no activity changes were observed during project 1, bidirectional manipulations will be performed. Rats will be weaned on p21, at which the stereotactic surgeries will occur. After recovery, rats will be pair-housed with a stimulus rat of the same sex and will be trained on an FR1 schedule as described in project 1. At p35 the effect of mPFC-NAc and mPFC-BLA manipulations will be tested during a 10-minute social play session. Similar to project 1, rats will be isolated 2.5 hours prior to the task. Furthermore, 30 minutes prior the rats will be injected either with saline (SAL) as a control condition or clozapine N-oxide (CNO) to activate the DREADD. SAL or CNO treatment will be assigned in a random and counterbalanced fashion. Pinning and pouncing frequencies, as well as total time spend on social play and social exploration will be scored during these sessions. CNO or SAL treatments will be reversed at p37, with no manipulations occurring on the day in between, so that each rat has received both SAL and CNO treatment during

social play behaviour sessions. Subsequently rats will have two additional FR1 sessions, after which rats will receive either SAL or CNO to assess the effect of mPFC-NAc or mPFC-BLA manipulations on the motivation for social play behaviour during a PR session at p40. No manipulations will occur on the following day, after which at p42 the other treatment will be evaluated during a second PR session. Rats will then be perfused to check for the placement of the viruses, and exclusion will take place if virus injections were placed outside the regions of interest. **Together, these experiments will show whether activity within the mPFC-NAc or mPFC-BLA are important for the expression of social play behaviour or for the motivation to engage is social play**.

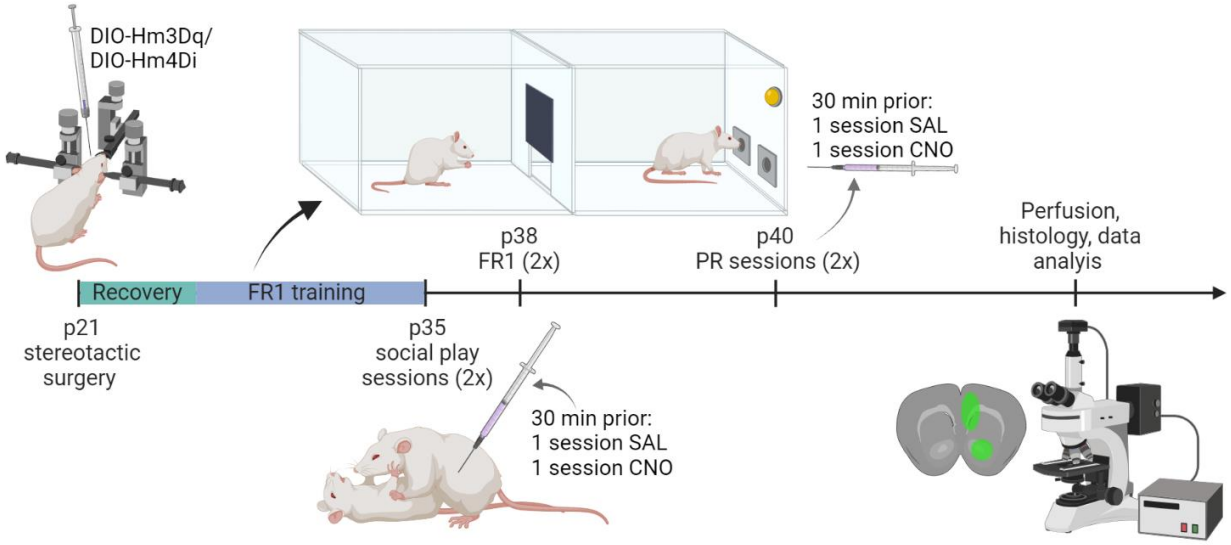

Figure 3: Experimental design for mPFC-NAc and mPFC-BLA manipulations during the expression of and motivation for social play behaviour. *Stereotactic surgeries will be performed at p21 after which rats will be FR1 trained on an operant task for social play behaviour. At p35 manipulations during two social play sessions (with one day in between) will be performed, where each rat receives either saline (SAL) or CNO in one session, and the other treatment in the second session. Rats will be trained on FR1 for two additional days, followed by manipulations during two progressive ratio sessions to assess their motivation for social play. Subsequently rats will be perfused to check the placement of the viruses.*

3. How does social play deprivation affect the activity of these mPFC projections later in life?

Recently it has been demonstrated that social play deprivation decreases the inhibitory drive within the mPFC, and that it affects the cognitive flexibility of rats in adulthood[11]. Therefore, it can be postulated that social play deprivation alters the activity within the mPFC-NAc and mPFC-BLA projections due to less inhibition on these projection neurons. To test whether social play deprivation affects the activity of mPFC-NAc and mPFC-BLA neurons during social play behaviour and during PFC-dependent tasks later in life, wireless photometry experiments will be performed (figure 4). Rats will be weaned on p21, at which they will undergo stereotactic surgery. Subsequently rats will be social play deprived until p42 (2 batches of n = 16, 8 social play deprivation and 8 pair-housed controls). Hereto, rats are pair-housed with a stimulus rat, but will be separated by a plexiglass divider containing small holes. This allows the rats to communicate, touch and smell each other, without being able to play[11]. Rats will be pair-housed for the remainder of the experiments, and the effects of social play deprivation, will be evaluated during a social play session, during an operant task for social interaction and during a reversal learning task. Wireless photometry recordings will be acquired during 10-minute play sessions as described in project 1, for three consecutive days. Subsequently, rats will be trained on an FR1 schedule during an operant task for social behaviour. Prior to these sessions rats will be isolated for 24 hours, since longer isolation periods have shown to result in higher motivation during an operant task for social play[2,5]. After 7 FR1 training sessions, wireless photometry recordings of the mPFC-NAc and mPFC-BLA will be acquired during 3 PR sessions to test if the activity for the motivation to engage in social behaviours is altered. Lastly, since it has been demonstrated that social play deprivation affects the cognitive flexibility[11], we will train the rats on a probabilistic reversal learning task to examine whether the activity in these mPFC pathways is altered during this task. Hereto, rats will be trained to nose poke to obtain a sugar pellet, after which the contingencies will be changed in three subsequent sessions. Here, only one of the nose poke hole has a high probability on the delivery of a sugar pellet (80% chance on reward), and after 8 successful nose pokes, this will change to the other previously low probability (20% chance on reward) nose poke hole. After 3 training sessions, we will record the activity in the mPFC-NAc and mPFC-BLA during three additional reversal learning sessions. Rats will be perfused to confirm the placement of the viruses and photometry system. Rats will be excluded from further analysis if either the injections or the photometry were placed outside of the regions of interest. **Combined, these experiments will elucidate whether social play deprivation alters the activity of the mPFC-NAc or mPFC-BLA during social play behaviour, during the motivation to engage in social behaviour, or during a PFC-dependent cognitive task.**

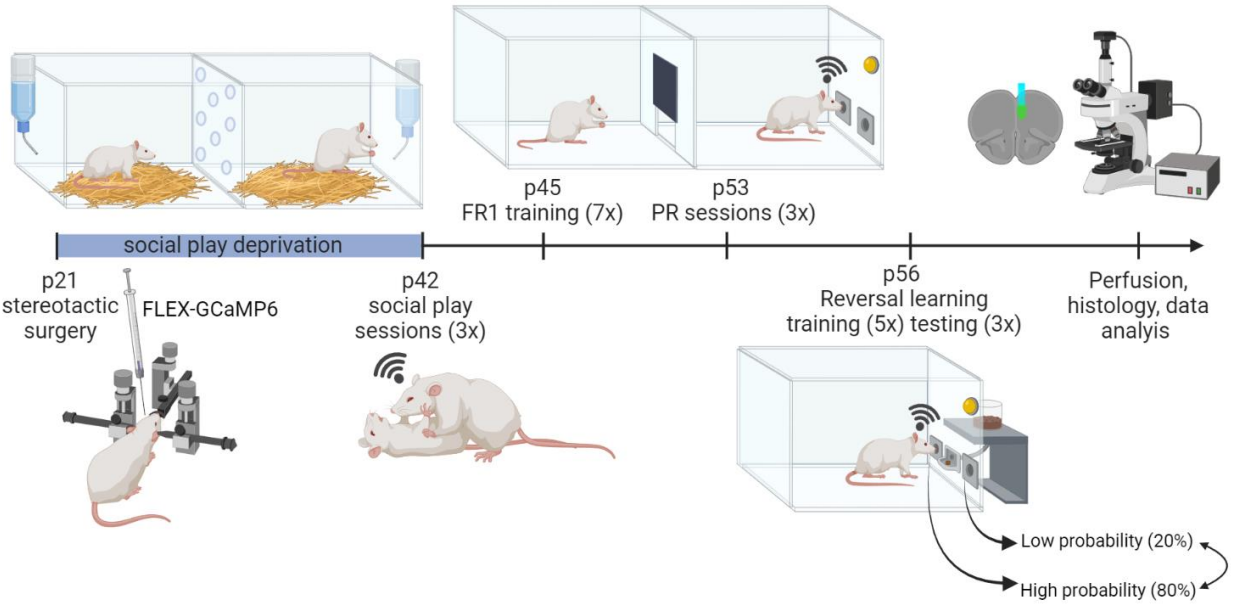

Figure 4: Experimental design for mPFC-NAc and mPFC-BLA activity after social play deprivation during the expression of social play behaviour, the motivation for social interactions and cognitive flexibility. *Stereotactic surgeries will be performed at p21 after which rats will be social play deprived. At p42 photometry recordings will be acquired during three social play sessions. Photometry recordings will be acquired during 3 PR sessions. At p56 rats will be trained and assessed on a probabilistic reversal learning task to record the mPFC-NAc and mPFC-BLA activity during cognitive flexibility. Subsequently rats will be perfused to check the placement of the viruses and photometry device.*

4. Can the detrimental effects of social play deprivation be prevented by manipulations of these mPFC pathways?

To investigate whether the detrimental effects of social play deprivation can be alleviated, chemogenetic manipulations of the mPFC-NAc or mPFC-BLA during social play deprivation will be performed to see whether it changes the outcomes on the behavioural paradigms (social play, motivation for social behaviour and cognitive flexibility; figure 5). It has been previously demonstrated that chemogenetic manipulations of the mPFC-NAc after social isolation can rescue the social recognition deficit in mice[30], therefore using chemogenetic treatments during social play deprivation might alleviate the social play deprivation deficits. Hereto, Wistar rats (2 batches of n = 16) will undergo stereotactic surgery at p14 to receive injections with either an activating (DIO-Hm3Dq) or inhibiting (DIO-Hm4Di) DREADD in the mPFC, based on the outcomes of project 3. Similar to project 2 CAV2-Cre will be injected in either the NAc or BLA to ensure projection specific expression of the DREADD. After surgery, rats will be placed back in their nest as soon as the effects of anaesthesia has worn off, and will be weaned on p21[38,39]. Careful monitoring the reaction of the mom to the pup is required to ensure the mom does not reject the pup and the pups are able to thrive (for method see Opendak (2021))[38]. Rats will be social play deprived from p21 till p42. Starting at p28, the rats will be injected twice a day with either saline as a control or CNO to activate the DREADD (SAL n=8, CNO n=8) during this social play deprivation period. At p42 the effects of these manipulations will be assessed on their social play behaviour, the motivation to engage in social behaviours and on their cognitive flexibility, similar as project 3. **Together these experiments will show whether manipulating the mPFC-BLA or mPFC-NAc activity can alleviate the detrimental effects of social play deprivation later in life.**

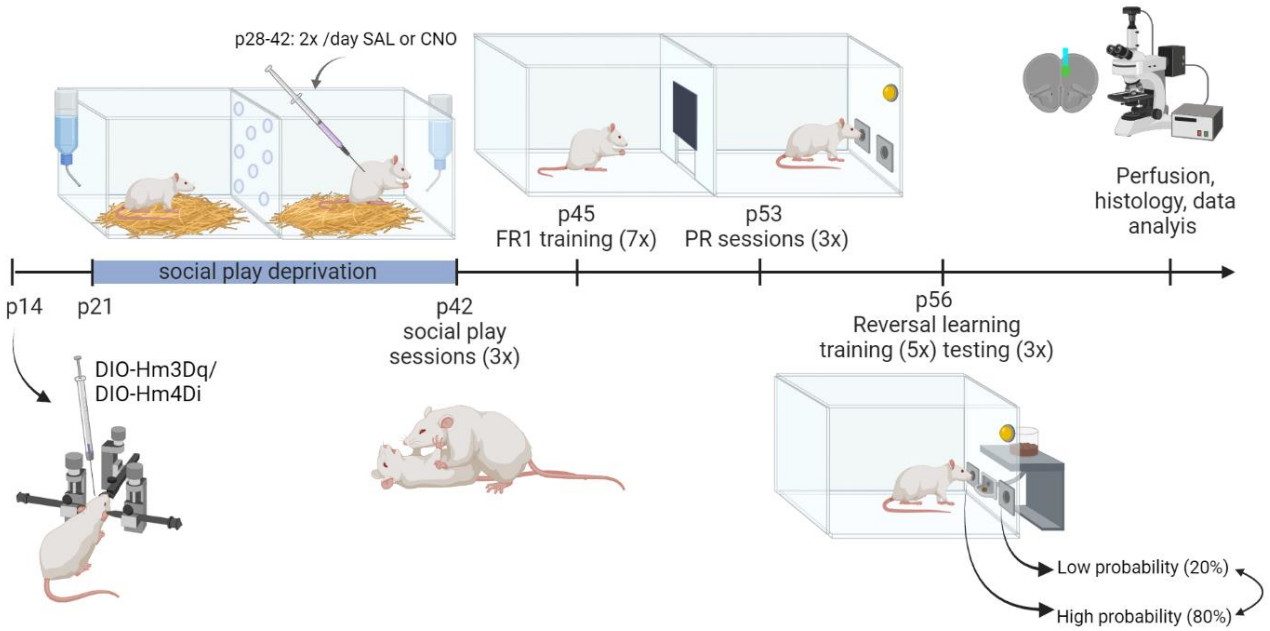

Figure 5: Experimental design for mPFC-NAc and mPFC-BLA manipulations during social play deprivation. *To assess whether these manipulations can prevent the detrimental effects of social play deprivation on sociability and cognitive flexibility, rats will be assessed during three social play sessions. Furthermore, rats will be trained on an operant task for social interactions, where the effects of these manipulations will be evaluated during three PR sessions. At p56 rats will be trained and assessed on a probabilistic reversal learning task to assess whether these manipulations prevent the detrimental effects on cognitive flexibility. Subsequently rats will be perfused to check the placement of the viruses.*

B.2.3 Feasibility / Risk assessment

This proposal is feasible within the given time-frame (figure 1), especially since the proposed techniques other than the wireless photometry system have been successfully employed in our lab[5,11]. We hypothesize that sex differences are only apparent for the mPFC-BLA projection since BLA lesions affect social play in males but not in females[24], whereas sex differences have not been reported for the NAc in social play behaviour. Using intermixed sex groups for the mPFC-NAc experiments and separate male and female groups for the mPFC-BLA experiments will take a total of 43 months. In case both projections contain sex differences, project 1, 2 and 3 can be completed as proposed, whereas both sexes cannot be addressed in project 4 due to time limitations, consequently only male rats will be addressed in project 4. If neither projection contains any sex differences the proposed research entails 28 months, thereby enabling the investigation of a third pathway. Not including the potential sex differences for an additional pathway, this research will take an additional 17 months to complete all four projects. A suitable candidate would be the projection from the BLA to mPFC since reciprocal connections between the BLA and mPFC have been implicated in social (play) behaviour[16,25,40] . Reciprocal connections between the mPFC and NAc have also been reported[41], therefore, studying the direct contribution of the NAc-mPFC projection in social play behaviour is also of interest. **Project 1 and 3:** Using a newly developed wireless photometry system requires validation of this system during social play experiments. For example, the technical properties of wireless energy delivery have only been tested in a 28 x 28 cm arena[28], which is not optimal for studying play behaviour in rats. Since 40 x 40 cm arenas have been previously used for social play behaviour[36], we will need to validate whether there is sufficient energy delivery in the centre of this arena. If this is not the case, the use of a smaller enclosure (30 x 30 cm) is required. The size of the operant chamber (25 x 30 cm)[5] for social behaviour and reversal learning does not require this validation. Even though using an innovative technique involves more risks, the benefits outweigh the risks since there are less motion artefacts, less invasive due to its subdermal design and has lower costs compared to fiber photometry systems[27,28]. Moreover, measuring brain activity during social play behaviour with fiber photometry is not feasible, therefore using wireless photometry systems is required. **Project 4:** Using chemogenetics during the social play deprivation period (p21-p42) requires surgery at p14 to allow for proper virus expression at p28. However, performing stereotactic surgeries at p14 introduces the risk of the mom rejecting the pup after returning from surgery. Previous studies have shown that implanting fiber photometry systems into rats at p13 can be done successfully, however rejection of the pup will result in the human end point[38,39]. Careful monitoring of reaction of the mom to the pups that underwent surgery is required. In case pups get rejected by the mom, the alternative is to perform surgeries at p21. Since the viruses are properly expressed after 2 weeks, chemogenetic manipulations will only be performed during the final week of social play deprivation. Since ASD and ADHD are not diagnosed directly after weaning, this still reflects whether treatments targeting these pathways in childhood could benefit the social and cognitive skills of these children later in life.

B.2.4 Scientific (a) and societal (b) impact

This proposed research is relevant for both the science community as well as for society. Firstly, previous attempts to uncover the neurobiology of social play behaviour have used crude strategies such as lesion or inactivation of certain brain regions or relate post-mortem c-fos activity to social play behaviours. Even though this has indicated some potential targets directly underlying social play behaviour, direct activation of these brain regions during social play behaviour have not been previously demonstrated. Uncovering what neurobiology directly underlies social play behaviour, and how it is affected due to social play deficits could guide the development for new therapeutic strategies for children with ASD and ADHD. It has been previously proposed that the social deficits in these children prevent them from engaging in social play behaviour[13]. Since social play behaviour is thought to be fundamental for the development of motoric, social and cognitive skills[1–5], deficient play behaviour could have profound effects later in life. Therefore, insights acquired from social play deprivation in rats, and how manipulations of certain mPFC projections could prevent deficits in sociability and cognition later in life, could be valuable for guiding the development of treatment for these children. Even though directly using chemogenetics is not feasible for human treatment due to its invasive nature, identifications of pathways that could prevent social and cognitive deficits due to a lack of social play, can help guide the development of pharmacological treatments targeting these pathways. Secondly, by using a combination of innovative wireless photometry and chemogenetics, we will be the first to assess a direct link between mPFC projections and social play behaviour. This is not only relevant to the scientific community involved in social play behaviour, since establishing the use of these wireless photometry systems could inspire other research fields to investigate which neurobiology directly underlies behaviours that are complex to measure with conventional fiber photometry, such as other social behaviours or navigation of complex 3D environments. Even though wireless photometry systems are commercially available (e.g. TeleFipho from Amuza), these still rely on external head stages with a limited battery life. If more researchers show there is a need for wireless implantable photometry systems and that these systems are easily employed in their research, this might drive the industry to further develop these systems and make them commercially available for wider use.

B.2.5 Ethical considerations

This proposed research aims to discover the direct contribution of mPFC trajectories in social play behaviour and how these pathways are affected due to social play deprivation. Since this research heavily relies on the use of an animal model, procedures will be carried out with the utmost care. We strive to minimize any potential harm, discomfort or stress experienced by these rodents throughout this study. If possible, minimally invasive techniques will be employed to collect data on which mPFC projections are active during social play behaviour. For example, the use of a wireless implanted photometry system produces less discomfort compared to wireless head-mounted photometry devices or conventional fiber photometry systems. In cases where more invasive techniques are required to gain essential insights, such as chemogenetics with frequent intraperitoneal injections, efforts are made to ensure the least possible amount of discomfort, for example by using a single play session for each injection, opposed to multiple play sessions. Furthermore, the insight acquired from these invasive procedures can have a profound societal impact. The ultimate goal of this study is to contribute to the development of new therapeutic strategies for children with ASD or ADHD. By gaining insight on which mPFC pathways can alleviate the detrimental effects of play deprivation, we hope to advance our understanding on how social play deficits impact the social and cognitive development of these children. To ensure this study has robust scientific validity and reliability, appropriate statistical methods and sample sizes will be used. Hereto, a priori power calculations will be made to use the minimum number of rats to acquire meaningful results, thereby minimizing the overall harm on these animals. The group sizes of 16 rats per condition should suffice to find large effect sizes (cohen's d 0.8) with sufficient power (80%). Furthermore, the minimum number of rats are used, by using pilot studies to exclude the possibility of sex differences. Using intermixed sex groups will require half of the rat population as opposed to including both sexes separately. Lastly, using the social play deprivation setup raises ethical concerns considering social isolation can cause stress to rats. To minimize these negative effects of a full social isolation, transparent dividers with holes will be used to allow for communication, smelling and touching with peers while preventing play behaviours. Alternatives have been considered, for example by rendering the peer non-playful with scopolamine. However, since scopolamine has shown to cause depression within rats[42], 3 weeks of play deprivation by barrier overall causes less discomfort compared to 3 weeks of scopolamine injections. We will promote transparency of the acquired data by publishing this study in an open-source environment. Furthermore, we will promote the public understanding of our research aims, methodology and outcomes to ensure this study has substantial outreach, by including a Layman's explanation to the published results.

B.2.6 Literature/references

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