The brain at play: neural substrates of social play behaviour in adolescent rats

Linda van Kerkhof

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Department of Neuroscience and Pharmacology, Rudolf Magnus Institute of Neuroscience, University Medical Center Utrecht, The Netherlands

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The brain at play: neural substrates of social play behaviour in adolescent rats

Het brein tijdens spel: neurale substraten voor sociaal spelgedrag in adolescente ratten

(met een samenvatting in het Nederlands)

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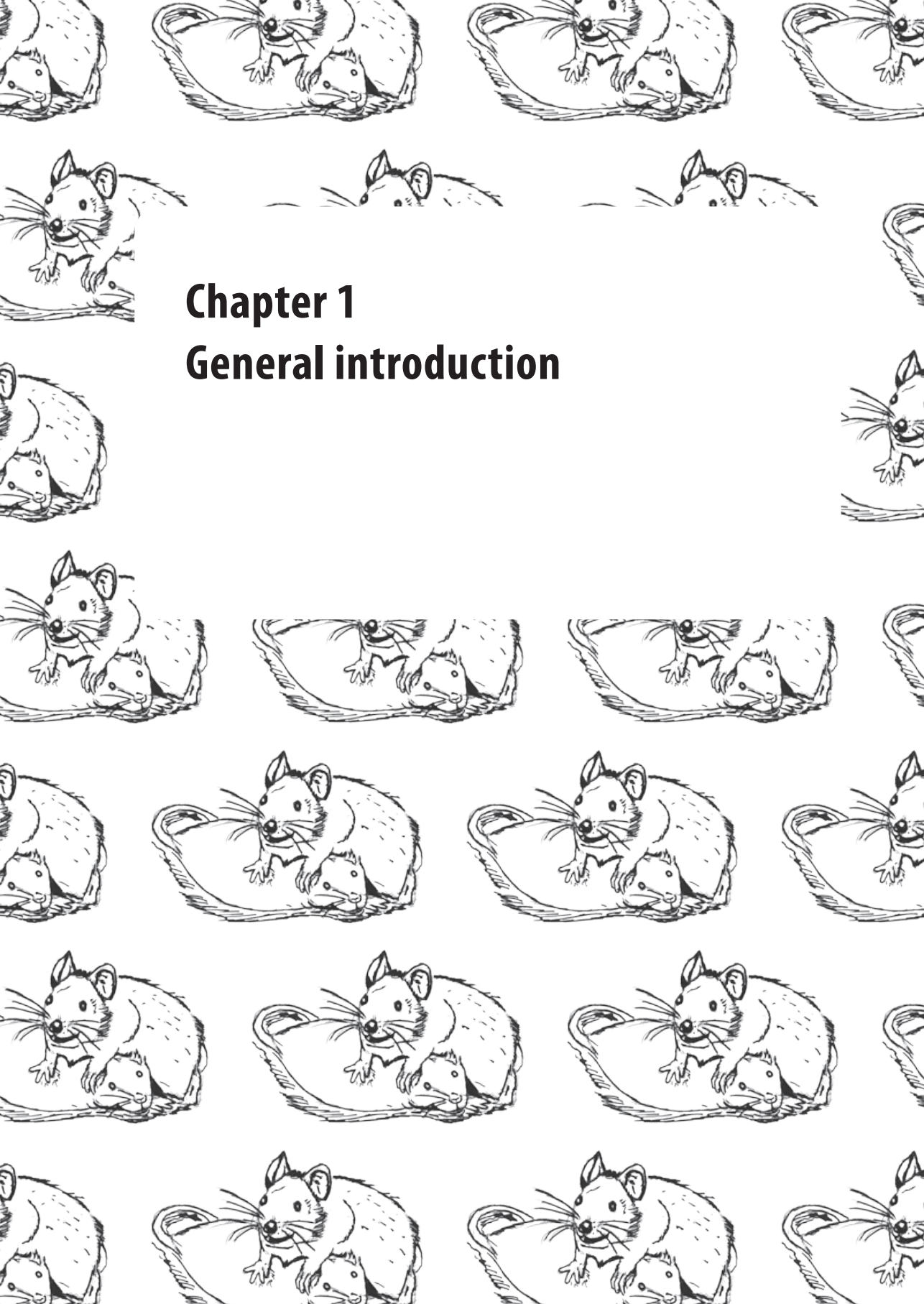


Chapter 1
General introduction

Preface

This thesis deals with the neural underpinnings of social play behaviour, a phenomenon that has intrigued scientists for many decades and is essential for proper social development (Panksepp *et al.*, 1984; Pellis and Pellis, 2009; Vanderschuren *et al.*, 1997). For this purpose, it is important to first describe and define play behaviour. The most commonly used criteria to define play are the following: animals engage in the play activity voluntarily; play is positively reinforcing; the behaviour does not seem functional in the context in which it is expressed; it is in some way structurally or temporally modified as compared to the 'functional' adult forms of the behaviour from which it seems to be derived; it is performed repeatedly, although possibly with variations; and it is mainly (if not exclusively) expressed in animals that are healthy, fed and safe (Graham and Burghardt, 2010; Panksepp *et al.*, 1984; Pellis and Pellis, 2009; Vanderschuren *et al.*, 1997). Phenomena labelled as play have been found in mammals and birds. Using the criteria mentioned above, play can even be observed in turtles, wasps, and octopuses (Pellis and Pellis, 2009). This widespread presence of play throughout the animal kingdom raises questions regarding its purpose. In this introduction section a detailed description of play behaviour, its function and the present knowledge of its neural substrates are discussed. In addition, the aim and outline of this thesis are described.

Social play behaviour

Different animal species exhibit different forms of play, such as object play which is directed at inanimate objects, predatory play which is aimed at living or dead prey, solitary play or locomotor play which refers to the appearance of spontaneous movements such as hopping or running, and social play which refers to play with conspecifics (Pellis and Pellis, 2009; Vanderschuren *et al.*, 1997). For example, play directed at inanimate objects is widely present amongst primates and carnivores, while in rodents this form of play is hardly observed (Pellis and Pellis, 2009). The most wide-spread form of play in mammals is social play (Pellis and Pellis, 1998). It is one of the earliest forms of non-mother-directed social behaviour (Vanderschuren *et al.*, 1997) and is a vigorous form of behaviour involving running, jumping, chasing, sparring and wrestling (Panksepp *et al.*, 1984; Pellis and Pellis, 1998). Social play has been defined as: "all locomotor activity directed at a conspecific, that appears to an observer to have no obvious immediate benefits for the player, in which motor patterns resembling those used in serious functional contexts may be used in modified forms" (Bekoff and Byers, 1981; Martin and Caro, 1985). Thus, social play contains elements of behavioural patterns related to adult social, sexual and aggressive behaviour. However, during play these patterns might be expressed in an exaggerated manner and/or occur out of context (Vanderschuren *et al.*, 1997).

Usually, social play involves two or more animals competing with each other to gain some advantage, with the kind of advantage differing between species. For example, in primates play usually involves biting and avoiding being bitten and in ungulates it involves head butting (Pellis and Pellis, 1998). Therefore, besides having similar characteristics, the actual movements during play are species-typical movements. The differences between species are not only apparent in the different types of movements; the frequencies and probabilities of possible initiations and responses determine the complexity of a species' play behaviour (Pellis and Pellis, 1998). Therefore, it is difficult

to point out one species as a representation of all mammals. Most studies on social play behaviour focus on the laboratory rat, *rattus norvegicus*. Rodent species are relatively easy to study in a laboratory. In addition, social play behaviour in the rat is the most complex of all rodents and easy to recognise as such (Pellis and Pellis, 2009).

Social play behaviour in the rat

In rats, the social play interaction is aimed at accessing the partner's nape area of the neck (Panksepp *et al.*, 1984; Pellis and Pellis, 2009; Trezza *et al.*, 2010; Vanderschuren *et al.*, 1997). In general, a play bout starts with one rat approaching and soliciting another rat. The soliciting rat will try to rub or nose the nape of the neck of the play partner. This solicitation is called pouncing (Fig. 1A). The partner will try to avoid giving the soliciting rat this advantage by using several tactics. Juvenile rats most often respond to a play solicitation with a complete rotation to a supine position, resulting in pinning (Fig. 1B). Pinning is one of the characteristic postures of rat play and is thought to function as a social releaser of a prolonged play bout, rather than as the endpoint of an interaction, since the rat that is in a supine position can use this position to gain access to the nape area of the partner (Vanderschuren *et al.*, 1997). Therefore, both pinning and being pinned are active phenomena during which the initiator continues to try and gain access to the nape and the partner avoiding this access (Pellis and Pellis, 1997; Vanderschuren *et al.*, 1997). Another response to a solicitation is the partial rotation. During this manoeuvre the rat rotates partially to move the nape away from the soliciting rat, while staying on the floor with its hind-paws. With this tactic the defender can push laterally against the partner or rear to an upright position; this upright position is labelled boxing (Pellis and Pellis, 1997). Another defence tactic includes evading, either by facing the partner or by moving away quickly (Pellis and Pellis, 1997; Vanderschuren *et al.*, 1997). Yet another behavioural act often present in a social interaction and that can occur after each behavioural pattern is social grooming, which is related to adult social behaviour (Vanderschuren *et al.*, 1997).
Interestingly, behavioural patterns expressed during play behaviour resemble adult behaviours although they are expressed in a modified form. For example, moving away quickly after a solicitation (approach - pounce - retreat) resembles adult sexual behaviour of the female rat (Erskine, 1989; Vanderschuren *et al.*, 1997) and rotation to a supine position resembles adult aggressive behaviour, although in serious aggressive behaviour the attacks are not directed at the nape area of the neck, but at the flanks, lower dorsum and face (Pellis and Pellis, 1998).

The probability of the different defence tactics used changes when rats mature. Social play in rats first occurs around postnatal day 18, peaks between 30-40 days, and declines when animals approach sexual maturity around day 50-60 (Pellis and Pellis, 1997). This decline in play during maturation is mainly the result of decreases in play initiation. The frequency of defence remains stable, although the pattern of defence tactics used changes during development (Pellis, 2002; Pellis and Pellis, 1990). The complete rotation is used mainly in the juvenile phase, whereas male rats at a later age more often use the partial rotation or evasion (Pellis, 2002; Pellis and Pellis, 1990). Female rats tend to increase the probability of using a complete rotation when they mature, mainly in response to the more dominant male rat (Pellis, 2002). The defence tactics used also depend on the hierarchical status of both rats. Like female rats, subordinate male rats are more likely to

adopt a supine position. The gender and status differences in defence tactics become more apparent with age (Pellis *et al.*, 2006; Pellis and Pellis, 1990).

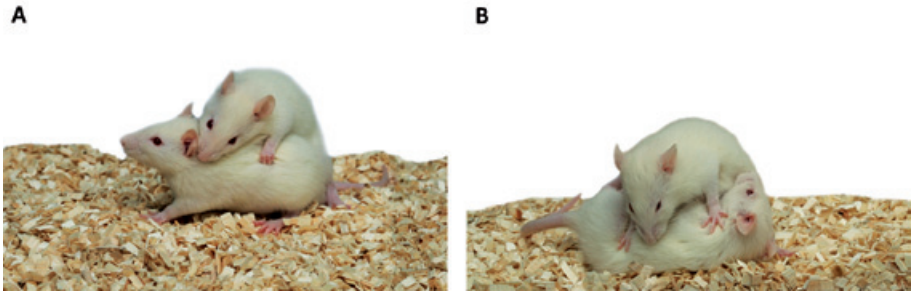


Fig. 1: The most characteristic postures of social play behaviour in the rat are pouncing (A) and pinning (B). Pictures were adapted with permission from Trezza *et al.*, 2010.

Functions of play

Social play behaviour is widespread among animal species and during the juvenile phase, animals spend a substantial amount of their time (up to 20% in mammals) on social play behaviour (Pellis and Pellis, 2009). This suggests that play must have some benefit to the animals. There have been several proposed benefits of play which will be discussed in this section, where we separately discuss the functions play may have later on in life and possible immediate benefits. Social play behaviour, like all other behaviours, has been evolving and therefore its function might have changed during evolution as well. This could mean that play serves different functions in different animal species.

Delayed functions

As mentioned before, patterns of adult social (grooming), aggressive (attack/defence), and sexual (approach-pounce-retreat) behaviour are observed during social play. Therefore, it has been suggested that play functions as practice for the possible movements and behavioural patterns needed during adulthood (Held and Špinka, 2011; Pellis *et al.*, 2010; Špinka *et al.*, 2001). However, the patterns observed during play do not only occur out of context, they are also present in modified forms. If play would serve to practice these movements, it would be expected that the movements most used during adulthood are expressed identically during play behaviour. In addition, the capacity to perform aggressive and sexual motor acts is already present in their definite form at the onset of the juvenile period, independent of social experience (Vanderschuren *et al.*, 1997).

Previous studies have shown that social play behaviour is important for learning the appropriate temporal and contextual settings of social behaviours and behavioural flexibility (Pellis and Pellis, 2009; Spinka *et al.*, 2001; Vanderschuren *et al.*, 1997). Animals deprived of play during the juvenile phase show abnormalities in the patterns of social behaviour later on in life (for review see Fone and Porkess, 2008). For example, abnormalities in aggressive (Wongwitdecha and Marsden, 1996) and sexual behaviour (Gerall *et al.*, 1967) have been observed. In addition, social isolation has been shown to cause changes in emotional and cognitive functions, such as changes in the intake of drugs of abuse (Baarendse *et al.*, 2012b; Howes *et al.*, 2000; Schenk *et al.*, 1987; Schenk *et al.*, 1990), behavioural flexibility (Jones *et al.*, 1991), impulse control (Baarendse *et*

al., 2012a) and decision-making (Baarendse *et al.*, 2012a). It is important to note that several of these studies used isolation rearing (i.e. continuous isolation from weaning), which continuously deprives animals of all post-weaning social contact, not just of play behaviour. Therefore, it is difficult to determine the specific contribution of social play behaviour to the behavioural changes seen after isolation rearing.

Several studies have used a more restricted isolation period, where animals were only isolated during postnatal week 4 and 5, the period during which social play is most abundant. These studies have indicated that isolation during this developmental period results in alterations in the social (Potegal and Einon, 1989; Van den Berg *et al.*, 1999) and cognitive domain (Baarendse *et al.*, 2012a), as well as changes in drug intake (Baarendse *et al.*, 2012b) and coping with stress (Von Frijtag *et al.*, 2002). Interestingly, 1h/day access to play decreased the abnormalities observed in open-field activity, object contact and reversal learning observed after social isolation (Einon *et al.*, 1978), suggesting that social play is indeed an essential behavioural component for proper development.

In summary, it appears that during play different behavioural acts are displayed in a variety of combinations, as if to find out which forms of behaviour fit together the best. This suggests that social play has a role in facilitating the temporal sequences and contextual organization of social behaviours. Furthermore, it has also been suggested that play facilitates the establishments of a social hierarchy, expression and understanding of intra-species communicative signals, coping with social conflicts and stress, but also improves cognitive development (Einon *et al.*, 1978; Pellis and Pellis, 2009; Vanderschuren *et al.*, 1997).

Immediate functions

Although play does not appear to have an obvious immediate function, it has been proposed that play has some immediate benefits as well (Pellis and Pellis, 2009). The most obvious immediate benefit of social play is its rewarding value (Trezza *et al.*, 2011a; Vanderschuren, 2010). Social play behaviour can be used as an incentive for maze-learning (Humphreys and Einon, 1981; Ikemoto and Panksepp, 1992; Normansell and Panksepp, 1990). Using this task it has been shown that animals prefer to interact with freely moving peers over movement-restricted peers or peers treated with amphetamine or chlorpromazine (drugs which suppress social play) (Humphreys and Einon, 1981). In addition, social play can also induce conditioned place preference (CPP) (Achterberg *et al.*, 2012; Calcagnetti and Schechter, 1992; Crowder and Hutto Jr., 1992; Douglas *et al.*, 2004; El Rawas *et al.*, 2012; Peartree *et al.*, 2012; Thiel *et al.*, 2008; Thiel *et al.*, 2009; Trezza *et al.*, 2009b). In this task rats are repeatedly placed in one compartment together with a partner. After several conditioning sessions animals will develop a preference for the social-paired compartment. Interestingly, social interaction without physical contact is also able to induce CPP, although CPP is acquired faster when physical contact is present (Peartree *et al.*, 2012). In addition, it has been shown that if the social partner is treated with methylphenidate, making it unresponsive for play, place preference was not established (Trezza *et al.*, 2009b). These results indicate that a social interaction with play is more pleasurable than social interaction without play. The pleasurable nature of social play behaviour may provide sufficient immediate benefits for the animals to keep engaging in it.

Social play can also function to reduce tension and mild stress, which might be related to its pleasurable aspects. For instance, rats that had been isolated during postnatal weeks 4 and 5 were confronted with a colony of rats that had been raised as a group. When the dominant male rat was present, the isolates failed to immobilise, which social housed rats do to diminish the number of attacks from the dominant rat. This showed, as mentioned before, that play deprivation leads to abnormalities in social behaviour later in life. However, when the dominant male rat was removed, the isolates showed increased social play behaviour. It was suggested that under mildly stressful conditions, play can serve as a release. However, in intense stress situations social play is decreased (when the dominant male is present). This suggest an immediate function for social play in reducing stress, similar to social grooming (Von Frijtag *et al.*, 2002). Additional support for this idea comes from a study using ACTH (adrenocorticotrophic hormone), which increases corticosterone release and thereby the stress level of an animal. Interestingly, administration of ACTH has the same effect as short social isolation, increasing subsequent social play levels. This shows that during minor stress, animals increase their social play behaviour (Pellis and Pellis, 2009). Together, these results indicate that immediate functions of play are mainly related to its rewarding effect and its function as a releaser of mild stress.

Clinical relevance of social play behaviour

The period of childhood and adolescence (roughly equivalent to the juvenile and adolescent stages in rodents) has received widespread attention because of its importance for behavioural development and the emergence of certain psychiatric disorders during this period (Paus *et al.*, 2008; Spear, 2000). During childhood and adolescence, substantial changes in behaviour occur, including increased complexity of the social repertoire and a remarkable increase in peer-peer interactions (Blakemore, 2008; Nelson *et al.*, 2005; Spear, 2000). A large part of these peer-peer interactions takes the form of social play behaviour (Spear, 2000). As mentioned above, social play behaviour is thought to facilitate behavioural development and provide the individual with the social and cognitive capacities required for adaptive and flexible behaviour (Baarendse *et al.*, 2012a; Gerall *et al.*, 1967; Panksepp *et al.*, 1984; Pellis and Pellis, 2009; Spinka *et al.*, 2001; Van den Berg *et al.*, 1999; Vanderschuren *et al.*, 1997; Von Frijtag *et al.*, 2002; Wongwitdecha and Marsden, 1996). Importantly, it has been described that the experience of childhood social trauma may have long-lasting repercussions, that last well into adulthood (Braun and Bock, 2011). In addition, abnormalities in play behaviour have been observed in childhood psychiatric disorders such as autism and attention deficit/hyperactivity disorder (Alessandri, 1992; Jordan, 2003; Manning and Wainwright, 2010). In view of its importance for behavioural development, and its relevance for child and adolescent psychiatry, it is essential to identify the neural substrates underlying social play behaviour, which is the aim of this thesis.

Neurobiology of social play behaviour

Our knowledge of the neurobiology of social play behaviour is mainly based on anatomical and pharmacological studies. This section provides a short overview of these

studies to present a summary of the current concepts in the field, with a special focus on the substrates and neurotransmitter systems studied in this thesis.

Anatomical studies

Cortical areas

Social play behaviour is most apparent and most complex in mammals. Mammalian species have a more elaborated cerebral cortex, which has lead to the suggestion that cortical areas might be critically involved in social play behaviour (Vanderschuren *et al.*, 1997). The role of cortical areas in play has been investigated in several studies, mainly using neonatal lesions. These studies have shown that the cortex is not required for the execution of social play behaviour; decorticated rats were still capable of playing, although there were abnormalities in the structure of social play. Decortication has been reported to reduce pinning, which was due to an altered pattern of defence (Panksepp *et al.*, 1994; Pellis *et al.*, 1992; Schneider and Koch, 2005). Animals more often responded with a partial rotation instead of a complete rotation to a supine position (Pellis *et al.*, 1992; Schneider and Koch, 2005). Several studies have indicated that decortication does not affect the number of initiations (Panksepp *et al.*, 1994; Pellis *et al.*, 1992; Schneider and Koch, 2005), but it altered the target of play initiations (Pellis *et al.*, 1992). Intact animals directed almost two-third of their play initiations at the nape of the neck and only 30% to more caudal regions like back and rump. Decorticated animals directed only 38% of their play initiations at the nape (Pellis *et al.*, 1992). Therefore, in addition to altering the pattern of response, decortication also seems to affect the performance of play initiations. It is important to note, however, that these studies used neonatal lesions, as a consequence of which compensation by other regions might have occurred during development.

The roles of the orbitofrontal cortex (OFC) and medial prefrontal cortex (mPFC) in social play behaviour have been studied in some more detail. In general, rats modulate their play behaviour in response to the partner they encounter (Pellis *et al.*, 2006; Pellis *et al.*, 2010). For example, male rats respond differently to playful attacks from dominant partners compared to subordinate partners or female rats. Rats playing with a dominant partner are more likely to respond with full rotations, whereas they are more likely to respond with partial rotations when playing with a subordinate partner or a female rat. In rats with neonatal OFC lesions this partner-related modulation of play was absent. Rats were no longer able to change their defence tactics in response to different partners (Pellis *et al.*, 2006). After neonatal lesions of the mPFC, rats failed to show the normal age-related changes in defence tactics (Bell *et al.*, 2009). During the juvenile phase they already responded as adults, which is similar to the results from studies that investigated complete decorticationes (Pellis *et al.*, 1992; Schneider and Koch, 2005).

In summary, these studies indicate that the prefrontal cortex is not essential for the expression of social play behaviour, but fine-tunes its expression in relation to social, contextual and temporal cues. This is in line with the presumed function of the prefrontal cortex in higher cognitive, so-called executive functions (Dalley *et al.*, 2004; Miller, 2000; Schoenbaum *et al.*, 2009; Wallis, 2007). Given the inherently complex and unpredictable nature of social interactions, it is likely that frontal cortical regions subserve executive functions in social contexts as well (Adolphs, 2003; Rudebeck *et al.*, 2008). Interestingly,

OFC functioning has been implicated in aggressive behaviour (de Bruin *et al.*, 1983; Rudebeck *et al.*, 2007) and the evaluation of social information (Azzi *et al.*, 2012; Bell *et al.*, 2010; Pellis *et al.*, 2006), whereas the mPFC is involved in maternal behaviour (Pereira and Morrell, 2011) and in the establishment of social hierarchy (Wang *et al.*, 2011). It remains to be determined to what extent and which of the prefrontal cortex regions are involved during the execution of play behaviour.

Striatum

The striatum is known for its involvement in controlling voluntary movement, as well as its involvement in regulating emotional and motivational aspects of behaviour (Berridge and Kringelbach, 2008; Cardinal *et al.*, 2002; Haber and Knutson, 2010; Salamone *et al.*, 2005). The size of the striatum appears to have an evolutionary relationship with the presence of social play behaviour in nonhuman primates, with a larger striatum being associated with more time spent on social play behaviour, while no correlation with the level of non-social play behaviour was observed (Graham, 2011). This suggests a specific role for the striatum in social play behaviour.

Neonatal depletion of dopamine in the striatum resulted in rats that used less complex defence strategies. In addition, animals were more likely to switch to other types of behaviour (e.g. social grooming) (Pellis *et al.*, 1993). The authors suggested that depletion of dopamine in the striatum led to impairments in maintaining the sequential organization of play fighting. Interestingly, this somewhat resembles the effect of lesions of the mPFC.

Recent studies have shown that the nucleus accumbens core and shell mediate opioid and endocannabinoid modulation of social play behaviour (Trezza *et al.*, 2011b; Trezza *et al.*, 2012). These results, together with the known function of the nucleus accumbens in hedonics and motivation (Berridge and Kringelbach, 2008; Cardinal *et al.*, 2002; Kelley, 2004; Salamone and Correa, 2012), suggest a role for the nucleus accumbens in the positive emotional properties of social play behaviour.

Amygdala

The amygdala has often been implicated in the processing of emotional information (Baxter and Murray, 2002; Cardinal *et al.*, 2002; Maren and Quirk, 2004; Morrison and Salzman, 2010; Phelps and Ledoux, 2005). One of the key features of social play behaviour is its positive emotional value (Trezza *et al.*, 2011a; Vanderschuren, 2010), suggesting involvement of the amygdala in play behaviour. Indeed, the size of the amygdala has been correlated with play behaviour in nonhuman primates, with a larger amygdala size being associated with a higher percentage of time spent on social play behaviour (Lewis and Barton, 2006). In addition, lesions of the amygdala have been reported to reduce social play behaviour, although these effects may be sex-specific (Daenen *et al.*, 2002; Meaney *et al.*, 1981). Furthermore, it has recently been shown that the amygdala is the neural site of action where endocannabinoids modulate social play (Trezza *et al.*, 2012). Enhanced levels of endocannabinoids are associated with increased social play behaviour (Trezza and Vanderschuren, 2008a; -2008b; -2009), which is mediated by cannabinoid receptors in the amygdala (Trezza *et al.*, 2012). Interestingly, the amygdala has also been associated with human social behaviour, for example in recognition of facial expressions,

and other forms of emotional processing (Phelps and Ledoux, 2005).

Together, these studies support evidence for involvement of the amygdala in social play behaviour. Previously, it has been hypothesised that the amygdala has a facilitating role in play behaviour. It may receive environmental information regarding social, temporal and contextual cues and provide associations with different emotional values so as to modulate play activities (Siviy and Panksepp, 2011).

Thalamus

The thalamus is one of the key information integration centres in the brain (Groenewegen and Witter, 2004). Lesions of the dorsomedial thalamus, posterior thalamus and the parafascicular region of the thalamus all decreased social play behaviour (Siviy and Panksepp, 1985; Siviy and Panksepp, 1987). This led to the hypothesis that the thalamic nuclei serve as an important relay station for integration of sensory information and signalling to the striatum and prefrontal cortex during social play behaviour (Siviy and Panksepp, 2011). The focus of this thesis is mainly on the midline and intralaminar thalamic nuclei, because of their strong anatomical connections with the prefrontal cortex and striatum (Groenewegen and Berendse, 1994; Groenewegen and Uylings, 2010). The midline and intralaminar nuclei have been proposed to play a role in multimodal sensory processing and motor functions as well as in awareness and cognitive functions (Van der Werf *et al.*, 2002). It is likely that similar processes are important during social play behaviour and that the thalamus is mediating these functions.

Pharmacological studies

Noradrenaline

Some of the earliest compounds identified that disrupt social play behaviour were psychomotor stimulants, such as amphetamine and methylphenidate (Beatty *et al.*, 1982; Beatty *et al.*, 1984; Vanderschuren *et al.*, 2008). Methylphenidate enhances the extracellular levels of noradrenaline and dopamine by inhibiting the noradrenaline and dopamine transporter (Ferris and Tang, 1979; Ritz *et al.*, 1987). Previously, it has been shown that the effect of methylphenidate on social play behaviour was mimicked by atomoxetine, a specific inhibitor of the noradrenaline transporter (Vanderschuren *et al.*, 2008). In contrast, a specific dopamine reuptake inhibitor, i.e. GBR-12909, did not affect social play behaviour (Vanderschuren *et al.*, 2008). Furthermore, the effect of methylphenidate on social play behaviour was blocked by administration of an α-2 adrenoceptor antagonist, RX821002, indicating that methylphenidate disrupts social play behaviour via its effect on noradrenaline neurotransmission (Vanderschuren *et al.*, 2008). In addition, administration of the α-2 adrenoceptor agonist clonidine reduced play behaviour, whereas administration of RX821002 has been reported to enhance social play behaviour (Normansell and Panksepp, 1985a; Siviy *et al.*, 1994; Siviy and Baliko, 2000), indicating that signalling through the α-2 adrenoceptor modulates the expression of social play behaviour. In addition to the α-2 adrenoceptor, a few studies have indicated a possible role for the α-1 adrenoceptor (Siviy *et al.*, 1994) and β-adrenoceptor (Beatty *et al.*, 1984).

Dopamine

Considering the highly rewarding aspects of social play behaviour (Calcagnetti and Schechter, 1992; Crowder and Hutto Jr., 1992; Humphreys and Einon, 1981; Ikemoto and Panksepp, 1992; Normansell and Panksepp, 1990; Trezza *et al.*, 2009b; Trezza *et al.*, 2010; Vanderschuren, 2010) and the known role of dopamine in mediating processes related to reward and motivation (Berridge, 2007; Ikemoto, 2007; Ikemoto and Panksepp, 1999; Robbins and Everitt, 2007; Salamone *et al.*, 2005; Salamone and Correa, 2012), it is likely that dopamine is involved in the modulation of social play behaviour as well. However, the role of dopamine is not as straightforward as expected; dopamine receptor antagonists have been reported to reduce social play behaviour, while after administration of dopamine receptor agonists both increases and decreases have been reported (Beatty *et al.*, 1984; Niesink and Van Ree, 1989; Siviy *et al.*, 1996; Vanderschuren *et al.*, 2008), and the enhancement of endogenous dopamine levels by a dopamine reuptake inhibitor had no effect on social play behaviour (Vanderschuren *et al.*, 2008). Interestingly, the increases in social play behaviour after treatment with indirect cannabinoid agonists, as well as low doses of ethanol and nicotine, depends upon dopamine signalling (Trezza *et al.*, 2009a; Trezza and Vanderschuren, 2008a; Trezza and Vanderschuren, 2009).

Serotonin

Serotonin is known to be involved in a wide array of social behaviours, such as affective behaviours (Dayan and Huys, 2009; Hariri and Holmes, 2006; Knutson *et al.*, 1997), establishing and maintaining social hierarchies (Huber *et al.*, 2001; Raleigh *et al.*, 1991), and defensive behaviour (Blanchard *et al.*, 1998). The role of serotonin has been previously investigated in social play behaviour, showing that increases in serotonin levels are not compatible with social play behaviour. Enhancement of endogenous serotonin levels by constitutive absence of the serotonin transporter or administration of a selective serotonin reuptake inhibitor, serotonin receptor agonists or a serotonin releasing agent have all been reported to reduce social play behaviour (Homberg *et al.*, 2007; Normansell and Panksepp, 1985b). However, increasing social play behaviour through a reduction in serotonin signalling has been proven difficult (Siviy *et al.*, 2011; Siviy and Panksepp, 2011), possibly due to the large diversity of serotonin receptors. In addition, it has been reported that changes in serotonin levels affects play differently in dominant and subordinate rats and in specific circumstances, such as during asymmetry in a play couple (Knutson *et al.*, 1996; Knutson and Panksepp, 1997; Siviy *et al.*, 2011). Therefore, it remains difficult to understand the exact mechanism via which serotonin modulates play behaviour.

Opioids

Opioids are important regulators of the expression of social play behaviour (Panksepp *et al.*, 1980; Siviy and Panksepp, 2011; Trezza *et al.*, 2010; Vanderschuren et al., 1997). Stimulation of μ-opioid receptors, for example, by low doses of morphine enhanced social play behaviour whereas stimulation of κ-opioid receptors reduced social play behaviour (Panksepp *et al.*, 1985; Trezza and Vanderschuren, 2008b; Vanderschuren *et al.*, 1995a; Vanderschuren *et al.*, 1995b). Stimulation of δ-opioid receptors did not affect social play behaviour (Vanderschuren *et al.*, 1995b). Recently, it has been shown that the increases in social play behaviour by stimulation of μ-opioid receptors were dependent

on cannabinoid signalling and were mediated via the nucleus accumbens (Trezza *et al.*, 2011b; Trezza and Vanderschuren, 2008a).

Cannabinoids

Several studies have reported involvement of the cannabinoid system in social play behaviour. The direction of effect depended on how the endocannabinoid system was stimulated. Stimulation of CB1 receptors by direct cannabinoid agonists has been reported to decrease social play behaviour, whereas administration of indirect agonists, which inhibit endocannabinoid degradation or reuptake, enhanced social play behaviour (Trezza and Vanderschuren, 2008a; Trezza and Vanderschuren, 2008b; Trezza and Vanderschuren, 2009). Inhibition of endocannabinoid degradation enhances their action only in synapses where they were endogenously released; therefore, administration of indirect cannabinoid agonists preserves the spatial/temporal specificity of endocannabinoid activity. Recently, it has been shown that the effect of endocannabinoids was dependent on dopamine and opioid signalling and that it was mediated via the nucleus accumbens and basolateral amygdala (Trezza *et al.*, 2012; Trezza and Vanderschuren, 2008a).

Aim and outline of this thesis

Considering the importance of social play behaviour for behavioural development and its relevance for child and adolescent psychiatry, it is of critical importance to identify the neural substrates underlying social play behaviour. The overall aim of this thesis was to further elucidate the neurobiology underlying social play behaviour. More specifically, we focussed on the role of corticostriatal system and associated limbic structures, as well as on the contribution of dopamine and noradrenaline neurotransmission to play behaviour. To that aim, we used different behavioural, pharmacological, and histological techniques.

Chapter 2 describes a detailed anatomical analysis of the neuronal activity (measured using expression of the immediate early gene c-fos) induced by social play behaviour in a wide array of regions possibly involved. Investigated brain structures include the prefrontal cortex, striatum, amygdala, thalamus, and monoamine producing regions. By correlating the levels of c-fos expression in regions with known connections, we aimed to identify a neural network engaged during social play behaviour. In the third chapter we extended the results of chapter 2 by investigating the role of the corticostriatal system in more detail. Using local administration of GABA receptor agonists or an AMPA receptor antagonist, neuronal signalling in specific subregions of the prefrontal cortex and striatum was manipulated after which social play behaviour was measured. In chapter 4 we investigated the role of the habenula in social play behaviour, since this region has been implicated in the modulation of monoamine neurotransmission and in a variety of emotional and cognitive processes. Using the immediate early gene c-fos, we identified that in this region neuronal activity was altered by social isolation and social play behaviour. Using the local pharmacological inactivation technique we further elucidated the importance of the habenula in social play behaviour.

In chapter 5 we investigate via which neural substrates methylphenidate inhibits social play behaviour. Previous studies have indicated that a noradrenergic mechanism is involved, but the site of action is unknown. Therefore, methylphenidate was locally

administered into several brain regions, including subregions of the prefrontal cortex, nucleus accumbens shell, amygdala and habenula. Subsequently, in chapter 6 we investigated which aspects (motivational and/or pleasurable) of social play behaviour are influenced by dopamine and noradrenaline neurotransmission. We established a new operant conditioning task in which rats were trained to press a lever for access to a play partner and used the previously established CPP task for social play behaviour (Trezza *et al.*, 2009b) to investigate if motivational and/or pleasurable aspects or social play behaviour were affected after treatment with the noradrenaline reuptake inhibitor atomoxetine, the dopamine reuptake inhibitor GBR12909 or the dopamine/ noradrenaline reuptake inhibitor methylphenidate.

Finally, in chapter 7 the results obtained in the present thesis are summarised and discussed in relation to the current understanding of the neural underpinnings of social play behaviour.

References

- Achterberg EJ, Trezza V, Vanderschuren LJ (2012) beta-Adrenoreceptor stimulation mediates reconsolidation of social reward-related memories. PLoS One 7:e39639.
- Adolphs R (2003) Cognitive neuroscience of human social behaviour. Nat Rev Neurosci 4:165-178.
- Alessandri SM (1992) Attention, play, and social behavior in ADHD preschoolers. J Abnorm Child Psychol 20:289-302.
- Azzi JC, Sirigu A, Duhamel JR (2012) Modulation of value representation by social context in the primate orbitofrontal cortex. Proc Natl Acad Sci U S A 109:2126-2131.
- Baarendse PJJ, Counotte DS, O'Donnel P, Vanderschuren LJMJ (2012a) Social experience during adolescence is critical for the development of cognitive control and dopamine modulation of prefrontal cortex function. Submitted.
- Baarendse PJJ, Limpens JHW, Vanderschuren L.J.M.J. (2012b) Disrupted social development enhances the motivation for cocaine. In preparation.
- Baxter MG, Murray EA (2002) The amygdala and reward. Nat Rev Neurosci 3:563-573.
- Beatty WW, Costello KB, Berry SL (1984) Suppression of play fighting by amphetamine: effects of catecholamine antagonists, agonists and synthesis inhibitors. Pharmacol Biochem Behav 20:747-755.
- Beatty WW, Dodge AM, Dodge LJ, White K, Panksepp J (1982) Psychomotor stimulants, social deprivation and play in juvenile rats. Pharmacol Biochem Behav 16:417-422.
- Bekoff M, Byers JA (1981) A critical reanalysis of the ontogeny and phylogeny of mammalian social and locomotor play: an ethological hornet's nest. In: Behavioral development (Immelmann K, Barlow GW, Petrinovich L, Main M, eds), pp 296-337. London: Cambridge University Press.
- Bell HC, McCaffrey DR, Forgie ML, Kolb B, Pellis SM (2009) The role of the medial prefrontal cortex in the play fighting of rats. Behav Neurosci 123:1158-1168.
- Bell HC, Pellis SM, Kolb B (2010) Juvenile peer play experience and the development of the orbitofrontal and medial prefrontal cortices. Behav Brain Res 207:7-13.
- Berridge KC (2007) The debate over dopamine's role in reward: the case for incentive salience. Psychopharmacology (Berl) 191:391-431.
- Berridge KC, Kringelbach ML (2008) Affective neuroscience of pleasure: reward in humans and animals. Psychopharmacology (Berl) 199:457-480.
- Blakemore SJ (2008) The social brain in adolescence. Nat Rev Neurosci 9:267-277.
- Blanchard DC, Griebel G, Rodgers RJ, Blanchard RJ (1998) Benzodiazepine and serotonergic modulation of antipredator and conspecific defense. Neurosci Biobehav Rev 22:597-612.
- Braun K, Bock J (2011) The experience-dependent maturation of prefronto-limbic circuits and the origin of developmental psychopathology: implications for the pathogenesis and therapy of behavioural disorders. Dev Med Child Neurol 53 Suppl 4:14-18.
- Calcagnetti DJ, Schechter MD (1992) Place conditioning reveals the rewarding aspect of social interaction in juvenile rats. Physiol Behav 51:667-672.
- Cardinal RN, Parkinson JA, Hall J, Everitt BJ (2002) Emotion and motivation: the role of the amygdala, ventral striatum, and prefrontal cortex. Neurosci Biobehav Rev 26:321-352.
- Crowder WF, Hutto Jr. CW (1992) Operant place conditioning measures examined using two nondrug reinforcers. Pharmacol Biochem Behav 41:817-824.
- Daenen EW, Wolterink G, Gerrits MAFM, Van Ree JM (2002) The effects of neonatal lesions in the amygdala or ventral hippocampus on social behaviour later in life. Behav Brain Res 136:571-582.

- Dalley JW, Cardinal RN, Robbins TW (2004) Prefrontal executive and cognitive functions in rodents: neural and neurochemical substrates. Neurosci Biobehav Rev 28:771-784.
- Dayan P, Huys QJ (2009) Serotonin in affective control. Annu Rev Neurosci 32:95-126.
- de Bruin JP, van Oyen HG, Van de Poll N (1983) Behavioural changes following lesions of the orbital prefrontal cortex in male rats. Behav Brain Res 10:209-232.
- Douglas LA, Varlinskaya EI, Spear LP (2004) Rewarding properties of social interactions in adolescent and adult male and female rats: impact of social versus isolate housing of subjects and partners. Dev Psychobiol 45:153-162.
- Einon DF, Morgan MJ, Kibbler CC (1978) Brief periods of socialization and later behavior in the rat. Dev Psychobiol 11:213-225.
- El Rawas R, Klement S, Salti A, Fritz M, Dechant G, Saria A, Zernig G (2012) Preventive role of social interaction for cocaine conditioned place preference: correlation with FosB/DeltaFosB and pCREB expression in rat mesocorticolimbic areas. Front Behav Neurosci 6:8.
- Erskine MS (1989) Solicitation behavior in the estrous female rat: a review. Horm Behav 23:473-502.
- Ferris RM, Tang FL (1979) Comparison of the effects of the isomers of amphetamine, methylphenidate and deoxypipradrol on the uptake of l-[3H]norepinephrine and [3H]dopamine by synaptic vesicles from rat whole brain, striatum and hypothalamus. J Pharmacol Exp Ther 210:422-428.
- Fone KC, Porkess MV (2008) Behavioural and neurochemical effects of post-weaning social isolation in rodents-relevance to developmental neuropsychiatric disorders. Neurosci Biobehav Rev 32:1087-1102.
- Gerall HD, Ward IL, Gerall AA (1967) Disruption of the male rat's sexual behaviour induced by social isolation. Anim Behav 15:54-58.
- Graham KL (2011) Coevolutionary relationship between striatum size and social play in nonhuman primates. Am J Primatol 73:314-322.
- Graham KL, Burghardt GM (2010) Current perspectives on the biological study of play: signs of progress. Q Rev Biol 85:393-418.
- Groenewegen HJ, Berendse HW (1994) The specificity of the 'nonspecific' midline and intralaminar thalamic nuclei. Trends Neurosci 17:52-57.
- Groenewegen HJ, Uylings HBM (2010) Orginization of Prefrontal-Striatal Projections. In: Handbook of Basal Ganglia Structure and Function (Steiner H, Tseng KY, eds), pp 353-365. Academic Press.
- Groenewegen HJ, Witter MP (2004) Thalamus. In: The Rat Nervous System (Paxinos G, ed), pp 407-453.
- Haber SN, Knutson B (2010) The reward circuit: linking primate anatomy and human imaging. Neuropsychopharmacology 35:4-26.
- Hariri AR, Holmes A (2006) Genetics of emotional regulation: the role of the serotonin transporter in neural function. Trends Cogn Sci 10:182-191.
- Held SDE, Špinka M (2011) Animal play and animal welfare. Anim Behav 81:891-899.
- Homberg JR, Schiepers OJG, Schoffelmeer ANM, Cuppen E, Vanderschuren LJMJ (2007) Acute and constitutive increases in central serotonin levels reduce social play behaviour in peri-adolescent rats. Psychopharmacology (Berl) 195:175-182.
- Howes SR, Dalley JW, Morrison CH, Robbins TW, Everitt BJ (2000) Leftward shift in the acquisition of cocaine self-administration in isolation-reared rats: relationship to extracellular levels of dopamine, serotonin and glutamate in the nucleus accumbens and amygdala-striatal FOS expression. Psychopharmacology (Berl) 151:55-63.

- Huber R, Panksepp JB, Yue Z, Delago A, Moore P (2001) Dynamic interactions of behavior and amine neurochemistry in acquisition and maintenance of social rank in crayfish. Brain Behav Evol 57:271-282.
- Humphreys AP, Einon DF (1981) Play as a reinforcer for maze-lerning in juvenile rats. Anim Behav 29:259-270.
- Ikemoto S (2007) Dopamine reward circuitry: two projection systems from the ventral midbrain to the nucleus accumbens-olfactory tubercle complex. Brain Res Rev 56:27-78.
- Ikemoto S, Panksepp J (1992) The effects of early social isolation on the motivation for social play in juvenile rats. Dev Psychobiol 25:261-274.
- Ikemoto S, Panksepp J (1999) The role of nucleus accumbens dopamine in motivated behavior: a unifying interpretation with special reference to reward-seeking. Brain Res Brain Res Rev 31:6-41.
- Jones GH, Marsden CA, Robbins TW (1991) Behavioural rigidity and rule-learning deficits following isolation-rearing in the rat: neurochemical correlates. Behav Brain Res 43:35-50.
- Jordan R (2003) Social play and autistic spectrum disorders: a perspective on theory, implications and educational approaches. Autism 7:347-360.
- Kelley AE (2004) Ventral striatal control of appetitive motivation: role in ingestive behavior and reward-related learning. Neurosci Biobehav Rev 27:765-776.
- Knutson B, Cole S, Wolkowitz O, Reus V, Chan T, Moore E (1997) Serotonergic intervention increases affiliative behavior in humans. Ann N Y Acad Sci 807:492-493.
- Knutson B, Panksepp J (1997) Effects of serotonin depletion on the play of juvenile rats. Ann N Y Acad Sci 807:475-477.
- Knutson B, Panksepp J, Pruitt D (1996) Effects of fluoxetine on play dominance in juvenile rats. Aggressive Behavior 22:297-307.
- Lewis KP, Barton RA (2006) Amygdala size and hypothalamus size predict social play frequency in nonhuman primates: a comparative analysis using independent contrasts. J Comp Psychol 120:31-37.
- Manning MM, Wainwright LD (2010) The role of high level play as a predictor social functioning in autism. J Autism Dev Disord 40:523-533.
- Maren S, Quirk GJ (2004) Neuronal signalling of fear memory. Nat Rev Neurosci 5:844-852.
- Martin P, Caro TM (1985) On the functions of play and its role in behavioral development. Adv Study Behav 15:59-103.
- Meaney MJ, Dodge AM, Beatty WW (1981) Sex-dependent effects of amygdaloid lesions on the social play of prepubertal rats. Physiol Behav 26:467-472.
- Miller EK (2000) The prefrontal cortex and cognitive control. Nat Rev Neurosci 1:59-65.
- Morrison SE, Salzman CD (2010) Re-valuing the amygdala. Curr Opin Neurobiol 20:221-230.
- Nelson EE, Leibenluft E, McClure EB, Pine DS (2005) The social re-orientation of adolescence: a neuroscience perspective on the process and its relation to psychopathology. Psychol Med 35:163-174.
- Niesink RJM, Van Ree JM (1989) Involvement of opioid and dopaminergic systems in isolation-induced pinning and social grooming of young rats. Neuropharmacology 28:411-418.
- Normansell L, Panksepp J (1985a) Effects of clonidine and yohimbine on the social play of juvenile rats. Pharmacol Biochem Behav 22:881-883.
- Normansell L, Panksepp J (1985b) Effects of quipazine and methysergide on play in juvenile rats. Pharmacol Biochem Behav 22:885-887.

- Normansell L, Panksepp J (1990) Effects of morphine and naloxone on play-rewarded spatial discrimination in juvenile rats. Dev Psychobiol 23:75-83.
- Panksepp J, Herman BH, Vilberg T, Bishop P, DeEskinazi FG (1980) Endogenous opioids and social behavior. Neurosci Biobehav Rev 4:473-487.
- Panksepp J, Jalowiec J, DeEskinazi FG, Bishop P (1985) Opiates and play dominance in juvenile rats. Behav Neurosci 99:441-453.
- Panksepp J, Normansell L, Cox JF, Siviy SM (1994) Effects of neonatal decortication on the social play of juvenile rats. Physiol Behav 56:429-443.
- Panksepp J, Siviy SM, Normansell L (1984) The psychobiology of play: theoretical and methodological perspectives. Neurosci Biobehav Rev 8:465-492.
- Paus T, Keshavan M, Giedd JN (2008) Why do many psychiatric disorders emerge during adolescence? Nat Rev Neurosci 9:947-957.
- Peartree NA, Hood LE, Thiel KJ, Sanabria F, Pentkowski NS, Chandler KN, Neisewander JL (2012) Limited physical contact through a mesh barrier is sufficient for social reward-conditioned place preference in adolescent male rats. Physiol Behav 105:749-756.
- Pellis SM (2002) Sex differences in play fighting revisited: traditional and nontraditional mechanisms of sexual differentiation in rats. Arch Sex Behav 31:17-26.
- Pellis SM, Castaneda E, McKenna MM, Tran-Nguyen LT, Whishaw IQ (1993) The role of the striatum in organizing sequences of play fighting in neonatally dopamine-depleted rats. Neurosci Lett 158:13-15.
- Pellis SM, Hastings E, Shimizu T, Kamitakahara H, Komorowska J, Forgie ML, Kolb B (2006) The effects of orbital frontal cortex damage on the modulation of defensive responses by rats in playful and nonplayful social contexts. Behav Neurosci 120:72-84.
- Pellis SM, Pellis VC (1990) Differential rates of attack, defense, and counterattack during the developmental decrease in play fighting by male and female rats. Dev Psychobiol 23:215-231.
- Pellis SM, Pellis VC (1997) The prejuvenile onset of play fighting in laboratory rats (*Rattus norvegicus*). Dev Psychobiol 31:193-205.
- Pellis SM, Pellis VC (1998) Play fighting of rats in comparative perspective: a schema for neurobehavioral analyses. Neurosci Biobehav Rev 23:87-101.
- Pellis SM, Pellis VC (2009) The Playful Brain. OneWorld Publications.
- Pellis SM, Pellis VC, Bell HC (2010) The function of play in the development of the social brain. American Journal of Play 2:278-296.
- Pellis SM, Pellis VC, Whishaw IQ (1992) The role of the cortex in play fighting by rats: developmental and evolutionary implications. Brain Behav Evol 39:270-284.
- Pereira M, Morrell JI (2011) Functional mapping of the neural circuitry of rat maternal motivation: effects of site-specific transient neural inactivation. J Neuroendocrinol 23:1020-1035.
- Phelps EA, Ledoux JE (2005) Contributions of the amygdala to emotion processing: from animal models to human behavior. Neuron 48:175-187.
- Potegal M, Einon D (1989) Aggressive behaviors in adult rats deprived of playfighting experience as juveniles. Dev Psychobiol 22:159-172.
- Raleigh MJ, McGuire MT, Brammer GL, Pollack DB, Yuwiler A (1991) Serotonergic mechanisms promote dominance acquisition in adult male vervet monkeys. Brain Res 559:181-190.
- Ritz MC, Lamb RJ, Goldberg SR, Kuhar MJ (1987) Cocaine receptors on dopamine transporters are related to self-administration of cocaine. Science 237:1219-1223.

- Robbins TW, Everitt BJ (2007) A role for mesencephalic dopamine in activation: commentary on Berridge (2006). Psychopharmacology (Berl) 191:433-437.
- Rudebeck PH, Bannerman DM, Rushworth MF (2008) The contribution of distinct subregions of the ventromedial frontal cortex to emotion, social behavior, and decision making. Cogn Affect Behav Neurosci 8:485-497.
- Rudebeck PH, Walton ME, Millette BH, Shirley E, Rushworth MF, Bannerman DM (2007) Distinct contributions of frontal areas to emotion and social behaviour in the rat. Eur J Neurosci 26:2315-2326.
- Salamone JD, Correa M (2012) The mysterious motivational functions of mesolimbic dopamine. Neuron 76:470-485.
- Salamone JD, Correa M, Mingote SM, Weber SM (2005) Beyond the reward hypothesis: alternative functions of nucleus accumbens dopamine. Curr Opin Pharmacol 5:34-41.
- Schenk S, Gorman K, Amit Z (1990) Age-dependent effects of isolation housing on the self-administration of ethanol in laboratory rats. Alcohol 7:321-326.
- Schenk S, Lacelle G, Gorman K, Amit Z (1987) Cocaine self-administration in rats influenced by environmental conditions: implications for the etiology of drug abuse. Neurosci Lett 81:227-231.
- Schneider M, Koch M (2005) Deficient social and play behavior in juvenile and adult rats after neonatal cortical lesion: effects of chronic pubertal cannabinoid treatment. Neuropsychopharmacology 30:944-957.
- Schoenbaum G, Roesch MR, Stalnaker TA, Takahashi YK (2009) A new perspective on the role of the orbitofrontal cortex in adaptive behaviour. Nat Rev Neurosci 10:885-892.
- Siviy SM, Baliko CN (2000) A further characterization of alpha-2 adrenoceptor involvement in the rough-and-tumble play of juvenile rats. Dev Psychobiol 37:25-34.
- Siviy SM, Deron LM, Kasten CR (2011) Serotonin, motivation, and playfulness in the juvenile rat. Dev Cogn Neurosci 1:606-616.
- Siviy SM, Fleischhauer AE, Kerrigan LA, Kuhlman SJ (1996) D2 dopamine receptor involvement in the rough-and-tumble play behavior of juvenile rats. Behav Neurosci 110:1168-1176.
- Siviy SM, Fleischhauer AE, Kuhlman SJ, Atrens DM (1994) Effects of alpha-2 adrenoceptor antagonists on rough-and-tumble play in juvenile rats: evidence for a site of action independent of non-adrenoceptor imidazoline binding sites. Psychopharmacology (Berl) 113:493-499.
- Siviy SM, Panksepp J (1985) Dorsomedial diencephalic involvement in the juvenile play of rats. Behav Neurosci 99:1103-1113.
- Siviy SM, Panksepp J (1987) Juvenile play in the rat: thalamic and brain stem involvement. Physiol Behav 41:103-114.
- Siviy SM, Panksepp J (2011) In search of the neurobiological substrates for social playfulness in mammalian brains. Neurosci Biobehav Rev 35:1821-1830.
- Spear LP (2000) The adolescent brain and age-related behavioral manifestations. Neurosci Biobehav Rev 24:417-463.
- Špinka M, Newberry RC, Bekoff M (2001) Mammalian play: training for the unexpected. Q Rev Biol 76:141-168.
- Spinka M, Newberry RC, Bekoff M (2001) Mammalian play: training for the unexpected. Q Rev Biol 76:141-168.
- Thiel KJ, Okun AC, Neisewander JL (2008) Social reward-conditioned place preference: a model revealing an interaction between cocaine and social context rewards in rats. Drug Alcohol Depend 96:202-212.

- Thiel KJ, Sanabria F, Neisewander JL (2009) Synergistic interaction between nicotine and social rewards in adolescent male rats. Psychopharmacology (Berl) 204:391-402.
- Trezza V, Baarendse PJJ, Vanderschuren LJMJ (2009a) Prosocial effects of nicotine and ethanol in adolescent rats through partially dissociable neurobehavioral mechanisms. Neuropsychopharmacology 34:2560-2573.
- Trezza V, Baarendse PJJ, Vanderschuren LJMJ (2010) The pleasures of play: pharmacological insights into social reward mechanisms. Trends Pharmacol Sci 31:463-469.
- Trezza V, Campolongo P, Vanderschuren LJMJ (2011a) Evaluating the rewarding nature of social interactions in laboratory animals. Dev Cogn Neurosci 1:444-458.
- Trezza V, Damsteegt R, Achterberg EJM, Vanderschuren LJMJ (2011b) Nucleus accumbens mu-opioid receptors mediate social reward. J Neurosci 31:6362-6370.
- Trezza V, Damsteegt R, Manduca A, Petrosino S, Van Kerkhof LWM, Pasterkamp RJ, Zhou Y, Campolongo P, Cuomo V, Di Marzo V, Vanderschuren LJMJ (2012) Endocannabinoids in amygdala and nucleus accumbens mediate social play reward in adolescent rats. J Neurosci 32:14899-14908.
- Trezza V, Damsteegt R, Vanderschuren LJMJ (2009b) Conditioned place preference induced by social play behavior: parametrics, extinction, reinstatement and disruption by methylphenidate. Eur Neuropsychopharmacol 19:659-669.
- Trezza V, Vanderschuren LJMJ (2008a) Bidirectional cannabinoid modulation of social behavior in adolescent rats. Psychopharmacology (Berl) 197:217-227.
- Trezza V, Vanderschuren LJMJ (2008b) Cannabinoid and opioid modulation of social play behavior in adolescent rats: differential behavioral mechanisms. Eur Neuropsychopharmacol 18:519-530.
- Trezza V, Vanderschuren LJMJ (2009) Divergent effects of anandamide transporter inhibitors with different target selectivity on social play behavior in adolescent rats. J Pharmacol Exp Ther 328:343-350.
- Van den Berg CL, Hol T, Van Ree JM, Spruijt BM, Everts H, Koolhaas JM (1999) Play is indispensable for an adequate development of coping with social challenges in the rat. Dev Psychobiol 34:129-138.
- Van der Werf YD, Witter MP, Groenewegen HJ (2002) The intralaminar and midline nuclei of the thalamus. Anatomical and functional evidence for participation in processes of arousal and awareness. Brain Res Brain Res Rev 39:107-140.
- Vanderschuren LJMJ (2010) How the brain makes play fun. Am J of Play 2:315-337.
- Vanderschuren LJMJ, Niesink RJM, Spruijt BM, Van Ree JM (1995a) Effects of morphine on different aspects of social play in juvenile rats. Psychopharmacology 117:225-231.
- Vanderschuren LJMJ, Niesink RJM, Spruijt BM, Van Ree JM (1995b) Mu- and kappa-opioid receptor-mediated opioid effects on social play in juvenile rats. Eur J Pharmacol 276:257-266.
- Vanderschuren LJMJ, Niesink RJM, Van Ree JM (1997) The neurobiology of social play behavior in rats. Neurosci Biobehav Rev 21:309-326.
- Vanderschuren LJMJ, Trezza V, Griffioen-Roose S, Schiepers OJG, Van Leeuwen N, De Vries TJ, Schoffelmeer ANM (2008) Methylphenidate disrupts social play behavior in adolescent rats. Neuropsychopharmacology 33:2946-2956.
- Von Frijtag JC, Schot M, Van den BR, Spruijt BM (2002) Individual housing during the play period results in changed responses to and consequences of a psychosocial stress situation in rats. Dev Psychobiol 41:58-69.
- Wallis JD (2007) Orbitofrontal cortex and its contribution to decision-making. Annu Rev Neurosci 30:31-56.

• Wang F, Zhu J, Zhu H, Zhang Q, Lin Z, Hu H (2011) Bidirectional control of social hierarchy by synaptic efficacy in medial prefrontal cortex. Science 334:693-697.
• Wongwitdecha N, Marsden CA (1996) Social isolation increases aggressive behaviour and alters the effects of diazepam in the rat social interaction test. Behav Brain Res 75:27-32.

Chapter 2
Cellular activation in limbic brain systems during social play behaviour in adolescent rats

Linda W.M. van Kerkhof[1]
Viviana Trezza[1,2]
Tessa Mulder[1]
Ping Gao[3]
Pieter Voorn[3]
Louk J.M.J. Vanderschuren[1,4]

1 Rudolf Magnus Institute of Neuroscience, Department of Neuroscience and Pharmacology, University Medical Centre Utrecht, Utrecht, The Netherlands
2 Department of Biology, University "Roma Tre", Rome, Italy
3 Department of Anatomy and Neurosciences, Neuroscience Campus Amsterdam, VU University Medical Centre, Amsterdam, The Netherlands
4 Department of Animals in Science and Society, Division of Behavioural Neuroscience, Faculty of Veterinary Medicine, Utrecht University, Utrecht, The Netherlands

In revision.

Abstract

Positive social interactions during the juvenile and adolescent phases of life are essential for proper social and cognitive development in mammals, including humans. During this developmental period, there is a marked increase in peer-peer interactions, signified by the abundance of social play behaviour. Despite its importance for behavioural development, our knowledge of the neural underpinnings of social play behaviour is limited. Therefore, the purpose of this study was to map the neural circuits involved in social play behaviour in rats. This was achieved by examining cellular activity after social play using the immediate early gene c-fos as a marker. After a session of social play behaviour, pronounced increases in c-fos expression were observed in the medial prefrontal cortex, medial and ventral orbitofrontal cortex, dorsal striatum, nucleus accumbens core and shell, lateral amygdala, several thalamic nuclei, dorsal raphe and the pedunculopontine tegmental nucleus. Importantly, the cellular activity patterns after social play were topographically organised in this network, as indicated by play-specific correlations in c-fos activity between regions with known direct connections. These correlations suggest an involvement of the projections from the medial prefrontal cortex to the striatum as well as the amygdala and monoaminergic inputs to these regions during social play behaviour. The analyses presented here outline a topographically organised neural network implicated in processes such as reward, motivation and cognitive control over behaviour, which mediates social play behaviour in adolescent rats.

Abbreviations of brain regions

AC	anterior cingulate cortex
AId	agranular insular cortex dorsal part
AIv	agranular insular cortex ventral part
BLA	basolateral amygdala
BNST	bed nucleus of the stria terminalis
CeA	central amygdala
CeM	central medial thalamic nucleus
CL	central lateral thalamic nucleus
DLO	dorsolateral orbitofrontal cortex
DRa	dorsal raphe nucleus anterior level
DRp	dorsal raphe nucleus posterior level
DS	dorsal striatum
IL	infralimbic cortex
IMDl	intermediodorsal thalamic nucleus lateral part
IMDm	intermediodorsal thalamic nucleus medial part
LA	lateral amygdala
lGP	lateral globus pallidus
LC	locus coeruleus
lCore	lateral nucleus accumbens core
LDTg	laterodorsal tegmental nucleus
LO	lateral orbitofrontal cortex
lShell	lateral nucleus accumbens shell
mCore	medial nucleus accumbens
MD	mediodorsal thalamic nucleus
MeA	medial amygdala
MO	medial orbitofrontal cortex
mPFC	medial prefrontal cortex
mShell	medial nucleus accumbens shell
NaCore	nucleus accumbens core
NAShell	nucleus accumbens shell
OFC	orbitofrontal cortex
OTu	olfactory tubercle
PC	paracentral thalamic nucleus
PPTg	pedunculopontine tegmental nucleus
PrL	prelimbic cortex
PrLd	prelimbic cortex dorsal part
PrLv	prelimbic cortex ventral part
PVl	paraventricular thalamic nucleus lateral part
PVm	paraventricular thalamic nucleus medial part
RMTg	rostromedial tegmental nucleus
SNc	substantia nigra pars compacta
SNr	substantia nigra pars reticulata
STR-	striatal region receiving input from the cortical region mentioned with it
VLO	ventrolateral orbitofrontal cortex
VO	ventral orbitofrontal cortex
VP	ventral pallidum
VS	ventral striatum
vShell	ventral nucleus accumbens shell
VTA	ventral tegmental area

Introduction

The period between weaning and sexual maturity (i.e. childhood and adolescence in humans, roughly equivalent to the juvenile and adolescent stages in rodents) has received widespread attention because of its importance for behavioural development, and conversely, because of the emergence of certain psychiatric disorders during this period (Paus *et al.*, 2008; Spear, 2000). During adolescence, substantial changes occur in brain and behaviour. In particular, there are profound changes in social behaviour, including increased complexity of the social repertoire and a remarkable increase in peer-peer interactions (Blakemore, 2008; Nelson *et al.*, 2005; Spear, 2000). For a large part, these peer-peer interactions take the form of social play behaviour. Social play behaviour is one of the earliest forms of non-mother directed social behaviour in mammals (Fagen, 1981; Panksepp *et al.*, 1984; Pellis and Pellis, 2009; Vanderschuren *et al.*, 1997), although it can also be observed in other species, e.g. reptiles, invertebrates, and avian species (Graham and Burghardt, 2010; Pellis and Pellis, 2009). Social play behaviour during adolescence is thought to be important for proper social and cognitive development (Baarendse *et al.*, 2012; Gerall *et al.*, 1967; Panksepp *et al.*, 1984; Pellis and Pellis, 2009; Špinka *et al.*, 2001; Van den Berg *et al.*, 1999; Vanderschuren *et al.*, 1997; Von Frijtag *et al.*, 2002; Wongwitdecha and Marsden, 1996). Indeed, abnormalities in play behaviour have been observed in childhood psychiatric disorders such as autism and attention deficit/hyperactivity disorder (Alessandri, 1992; Jordan, 2003; Manning and Wainwright, 2010). In addition, childhood social trauma may have long-lasting repercussions, that last well into adulthood (Braun and Bock, 2011). Therefore, in view of its importance for behavioural development, and its relevance for child and adolescent psychiatry, it is essential to identify the neural substrates underlying social play behaviour.

At present, our understanding of the neural substrates of social play behaviour is limited. Clearly, the expression of a complex behaviour such as social play involves a wide array of neural circuits. It has been hypothesised that cortical regions have a role in the facilitation of play behaviour by guiding its expression in the appropriate temporal and contextual settings. In addition, subcortical circuits may mediate the execution of the appropriate locomotor acts and the integration of sensory stimuli, as well as encoding the emotional and motivational properties of social play (Pellis and Pellis, 2007; Siviy and Panksepp, 2011; Vanderschuren *et al.*, 1997).

Neonatal lesion studies have suggested that the cortex is not essential for the execution of social play behaviour itself (Panksepp *et al.*, 1994; Pellis *et al.*, 1992; Schneider and Koch, 2005), but that frontal cortical regions are important for the fine-tuning of the expression of social play, such as adapting the play patterns to the partner's behaviour (Bell *et al.*, 2009; Bell *et al.*, 2010; Pellis *et al.*, 2006). Subcortical regions such as the thalamus and striatum have been shown to be important for expression of social play behaviour (Pellis *et al.*, 1993; Siviy and Panksepp, 1985; Siviy and Panksepp, 1987). For example, neonatal depletion of dopamine in the striatum affects the sequential ordering of play patterns (Pellis *et al.*, 1993), while lesions of certain thalamic nuclei, such as the parafascicular nucleus, have been found to reduce social play by disrupting the transmission of somatosensory stimuli related to play (Siviy and Panksepp, 1985; Siviy and Panksepp, 1987). Furthermore, previous immediate early gene expression studies have shown

enhanced cellular activity during social play behaviour in brain regions including the prefrontal cortex, amygdala and striatum (Cheng *et al.*, 2008; Gordon *et al.*, 2002).

Social play behaviour is highly rewarding (Trezza *et al.*, 2011a; Vanderschuren, 2010). It is therefore likely that brain regions involved in pleasure and motivation have an important role in this behaviour as well. These regions include the nucleus accumbens and amygdala (Lewis and Barton, 2006; Meaney *et al.*, 1981; Trezza *et al.*, 2011b; -2012). Furthermore, several studies have indicated an essential role for monoaminergic neurotransmission in social play behaviour (for review see Trezza *et al.*, 2010). Therefore, it is also likely that activity of monoamine nuclei is altered during social play behaviour.

In the present study, a broad range of the potential neural substrates involved in social play behaviour was investigated in adolescent male rats. Using expression of the immediate-early-gene c-fos as a marker, neuronal activity induced by social play behaviour was mapped in the prefrontal cortex, striatum, amygdala, thalamus, pallidum, monoamine nuclei, and tegmental regions providing input into the monoamine nuclei. In addition, it was investigated if the c-fos activity, induced by social play, would correlate between regions known to be connected. This resulted in the identification of a network of brain regions which is activated during social play behaviour in adolescent rats.

Materials and Methods

Animals

Male Wistar rats (Charles River, Sulzfeld, Germany) arrived in our animal facility at 21 days of age and were housed in groups of four in 40x26x20 cm Macrolon cages under controlled conditions (i.e. temperature 20-21 °C, 55-65 % relative humidity and 12/12 h light cycle with lights on at 7.00 a.m.). Food and water were available ad libitum. All animals used were experimentally naïve. During the first 6 days rats were handled at least twice, to familiarise them with the experimenter. All experiments were approved by the Animal Ethics Committee of Utrecht University and were conducted in accordance with Dutch regulations (Wet op de Dierproeven, 1996) and European regulations (Guideline 86/609/EEC).

Behavioural testing

Experiments were performed in a sound attenuated chamber under dim light conditions. The test arena was a Plexiglas cage (40x40x60 cm) with approximately 2 cm of wood shavings covering the floor. Animals were paired with an unfamiliar partner. Animals in a test pair did not differ more than 10 g in body weight.

Animals were repeatedly habituated to the test cage (4 consecutive days, 30 min/day) to minimise the influence of novelty of the test environment on the expression of social play behaviour (Trezza *et al.*, 2009; Trezza and Vanderschuren, 2008; Vanderschuren *et al.*, 1995a; Vanderschuren *et al.*, 1995b) and the induction of c-fos expression (Badiani *et al.*, 1998; Day *et al.*, 2001). The motivation for play was maximally enhanced by isolating the animals 24 h before the test (Niesink and Van Ree, 1989; Vanderschuren *et al.*, 1995a; -2008). On the test day, animals were placed in the test cage either in pairs ('play group', 5 pairs, n = 10) or alone ('no-play group', n = 8) for 15 min, since after social

isolation for up to 24 h, rats display most social play behaviour within the first 10-15 min of testing (Trezza *et al.*, 2009; Trezza and Vanderschuren, 2008; Vanderschuren *et al.*, 1995a; -1995b). After the test, animals were placed back into their separate cages for 30 min. This survival period was chosen because time course analysis has shown that the expression of stimulus-induced c-fos mRNA expression peaks in between 30 and 60 min after stimulation (Cullinan *et al.*, 1995; Ostrander *et al.*, 2003). Thus, the animals were sacrificed 45 min after the start of the play session. Subsequently, rats were sacrificed by decapitation, their brains were quickly removed and frozen immediately (-80 °C).

Behaviour of the animals was recorded using a camera with zoom lens, video tape recorder and television monitor. The behaviour of the play group was assessed using the Observer 5.1 software (Noldus Information Technology B.V., The Netherlands). Three behavioural elements were scored (Panksepp *et al.*, 1984; Trezza *et al.*, 2010; Vanderschuren *et al.*, 1997).

- Frequency of pinning: one animal lying with its dorsal surface on the floor with the other animal is standing over it, which is the most characteristic posture of social play in rats.
- Frequency of pouncing: one animal is attempting to nose or rub the nape of the neck of the partner, which is an index of play solicitation.
- Time spent in social exploration: one animal sniffing or grooming any part of the partner's body.

c-fos DIG *in situ* hybridization

Fresh frozen brains were cryostat sectioned (-20 °C) at 14 µm, mounted on Super-Frost Plus slides (Eric Scientific Co, Portsmouth, NH) and stored at -80 °C. Slides were warmed to room temperature before fixation with 4% PFA (4% paraformaldehyde in PBS, 154 mM NaCl, 0.896 mM KH2PO4, 4.58 mM Na2HPO4, pH = 7.5). Acetylation of the slides was performed with acetic anhydride (0.25% acetic anhydride in 1.5% triethanolamine buffer). Subsequently, slides were washed with PBS and 2x saline sodium citrate buffer (SSC buffer) before applying the hybridization mix (50% formamide, 4x SSC, 0.4% bakers yeast tRNA, 2% 50x Denhardt's reagent, 10% Dextran, 0.05% salmon sperm DNA) containing 5 ng c-fos probe per section.

The probe was generated using cDNA synthesised from total rat brain RNA and the iScript reverse transcriptase kit with random hexamers, according to manufacturer's protocol (Bio-Rad, Hercules, California). A PCR was performed with c-fos specific primers containing T3/T7 promoters. Primers (Eurogentec, Liège, Belgium) were designed using Primer3 (Rozen and Skaletsky, 2000). All primers were checked for gene specificity by BLAST searching. The primer sequences used for c-fos (Genbank NM_022197.2) were T3 antisense: AATTAACCCTCACTAAAGGGCACAGCCTGGTGAGTTTCAC and T7 sense: GTAATACGACTCA-CTATAGGGTCACCCTGCCTCTTCTCAAT. The PCR product size was checked by agarose gel electrophoresis. From these PCR products, labelled probes were generated by linear amplification using the MAXIscript Kit according to manufacturer's protocol (Applied Biosystems, Foster City, California) and probes were labelled using digoxigenin-UTP (DIG labelling mix, Roche, Penzberg, Germany). The probe size and concentration were checked using agarose gel electrophoreses. The probe was briefly heated at 95 °C before adding it to the hybridization mix

and hybridization was performed in a humid chamber at 60 °C overnight.

Post-hybridization washes were carried out with 1x SSC at 60 °C., including a wash with 2x SSC containing RNAse A (0.3 units/mL, Roche, Penzberg, Germany) at 37 °C. Before antibody incubation, slides were exposed to a blocking solution (1% blocking powder in TRIS buffer, 100 mM Tris, 150 mM NaCl, pH = 7.5) according to the DIG detection kit manual (Roche, Penzberg, Germany) for 1 h. Slides were incubated with anti-DIG-AP antibody (1:2500, DIG detection kit, Roche, Penzberg, Germany). This antibody was conjugated to alkaline phosphatase (AP), allowing the use of NBT/BCIP as a substrate to visualise the probe. The antibody incubation was performed overnight at 4 °C.

Following antibody incubation, slides were washed in TBS (100 mM TRIS, 150 mM NaCl, pH = 7.5) and a magnesium buffer (100 mM Tris, 100 mM NaCl, 50 mM MgCl, pH = 9.5). Incubation with the substrate NBT/BCIP (1:50; Roche, Penzberg, Germany) in magnesium buffer was performed in a humid chamber at room temperature for 28 h. The reaction was stopped with TBS containing EDTA (1 mM EDTA, pH = 7.5) and slides were washed twice with water to remove salt precipitate. Slides were left to dry and coverslipped using Merckoglas (Merck, New Jersey, USA).

Nissl staining

The sections adjacent to those stained for c-fos, were stained with Thionin. After fixation with PFA (4%), slides were placed in Thionin solution (0.13% in aqua bidest). Staining time was optimized for each separate experiment. Subsequently, slides were placed for 1 min in MilliQ water, 70%, 80%, 96% and 100% ethanol (3x). Thereafter, slides were placed in xylene 2 x for 2 min and coverslipped using Entellan (Merck, New Jersey, USA).

Tyrosine hydroxylase immunhistochemistry

To determine the borders of the dopaminergic regions, adjacent sections were stained for tyrosine hydroxylase (TH). After fixation with PFA (4%), slides were washed using TBS and subsequently incubated with a mouse anti-TH antibody (#22941, Incstar, Stillwater, USA) diluted 1: 2000 in TBS-T (TBS with 0.2% Triton) at 4 °C overnight. The secondary antibody was a biotinylated horse anti-mouse antibody (BA-2000, Vector, Burlingame, USA) and was diluted 1:100 in TBS-T. Slides were incubated with the secondary antibody for 2 h at room temperature. Subsequently, slides were incubated with TBS-T containing the avidine-biotin-peroxidase complex (ABC kit, Thermo Scientific, Waltham, USA) according to the manufactures' protocol and the TH protein was visualised using 3,3'-diaminobenzidine (Sigma, St. Louis, USA) as a substrate (0.005% in TBS). Afterwards, slides were dehydrated through a series of ascending concentrations of ethanol, transferred to xylene and coverslipped using Entellan (Merck, New Jersey, USA).

Quantification of c-fos immunopositive cells

Similar quantification methods have previously been described (Nordquist *et al.*, 2008). Images of all regions of interest were digitised using an objective magnification of 5x on a Leica DM/RBE photomicroscope with a Q-imaging 12 bit camera and MCID software (InterFocus Imaging, Cambridge, UK). Image acquisition was preceded by a flat field correction and a calibration routine to ensure standardised OD values. For each subject 3 (occasionally 2) sections were digitised per region of interest.

Sections were made in series of 10, therefore, sections were 140 µM apart.

An overlay was drawn on each image to define the different regions and subregions. Borders of the regions were determined using images of Nissl-stained adjacent sections (Nissl-staining as described above). Borders were drawn according to the atlas of the rat brain by Paxinos and are schematically presented in Fig. 1 (Paxinos and Watson, 2007). An algorithm was used to identify c-fos positive cells. In short, images underwent histogram equalization and smoothing (low-pass filter, kernel size 7x7). The unfiltered image was subtracted from the smoothed image, followed by a series of steps to optimise the processed image and make it a suitable measuring template for detecting objects the size and shape of c-fos immunopositive cells. The number of cells counted was corrected with a factor indicating approximate size of a cell, thus preventing two adjacent segmented objects mistakenly counted as one cell. This algorithm allows for an observer-independent measurement. Several parameters were measured: number of c-fos positive cells, optical density of each cell, total area of each cell, and the total measured surface area of the region.

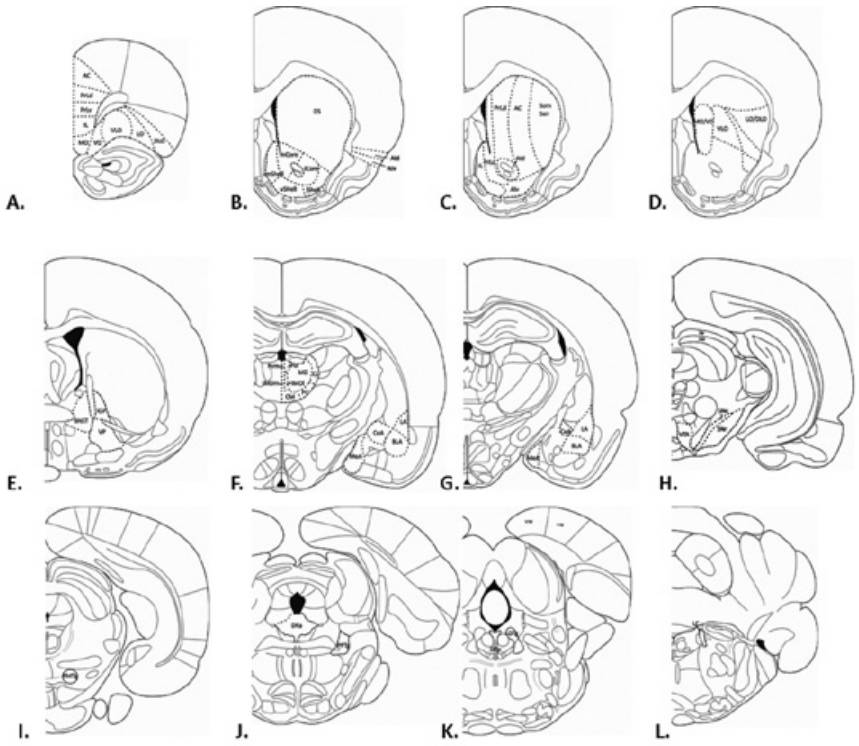


Fig. 1: Schematic representation of the regions in which the c-fos positive cells were determined. The regions measured were prefrontal cortex regions at + 3.70 mm from Bregma (A), striatal regions at +1.20 mm from Bregma (B,C,D), IGP and VP at -0.40 mm from Bregma (E), thalamus regions at -2.56 mm from Bregma (F), amygdala regions at -2.56 mm (F) and -3.30 mm (G) from Bregma, VTA and SN at -6.04 mm from Bregma (H), RMTg at -6.72 mm from Bregma (I), PPTg and the anterior part of the DR at -8.00 mm from Bregma (J), the posterior DR and the LDTg at -8.80 mm from Bregma (K), and the LC at -9.68 mm from Bregma (L). Figures adapted from Paxinos and Watson (2007)

Data analysis of c-fos expression levels

The parameters obtained from the MCID software were used to calculate the density of c-fos positive cells (number of positive cells divided by the total surface area of the region of interest). In addition, to compare the c-fos positive cell density in a manner that takes into account the labelling intensity, cells were categorised according to their labelling intensity: light, medium, and dark. Therefore, a frequency histogram of the optical densities of all cells in the no-play group was made for each brain region of interest. These histograms were used to calculate the 33rd and 67th percentile of the optical density in the no-play group. These optical density values were used to categorise the cells in all animals. The number of cells in each category was divided by the total surface area of the respective region of interest, to determine the cell density per intensity category. The cell densities of the three categories indicate shifts in the frequency histograms of the optical densities (Fig.2). An upward shift in the histogram would be reflected by an increase in the overall cell density as well as an increase in the medium category and would suggest that social play behaviour recruits a new population of cells. A rightward shift of the curve is expected when the same neurons are active, but express more c-fos as a result of play behaviour. This would be reflected by an increase in the cell density in the dark category. In a similar fashion, a downward or leftward shift of the histogram would indicate a smaller number of activated cells, or a reduced quantity of c-fos expression per activated cell.

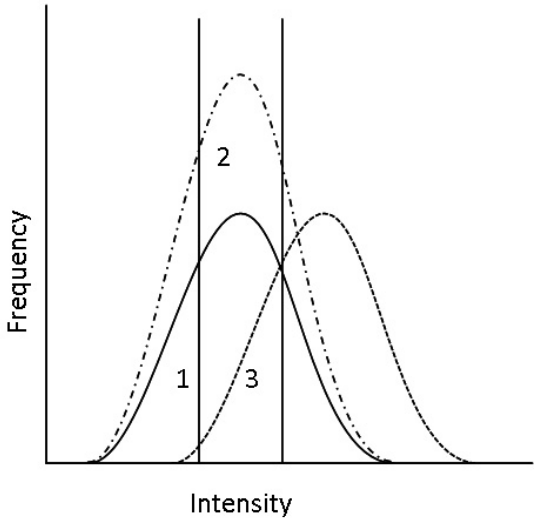


Fig. 2: Theoretical representation of potential shifts in the intensity histogram. Curve 1 indicates the frequency histogram of the control group. The two vertical lines indicate the cut-off points used to separate the cells into the light, medium and dark category (33% and 67%). The area under the curve represents the total number of c-fos positive cells per mm^2, i.e. the cell density. If social play behaviour would induce c-fos activity in a new group of neurons, an increase in the cell density is expected, which would be reflected in an upward shift of the histogram (Curve 2). This would be reflected in an enhanced cell density primarily in the medium category. Alternatively, if the same neurons are active, but express more c-fos as a result of play behaviour, a rightward shift is expected, with an increase in the cell density in the dark category (Curve 3). In a similar fashion a downward or leftward shift in the histogram could be explained. Adapted from Nordquist *et al.* 2008.

Statistical analysis

For data analysis, SPSS software 15.0 (IBM software, New York, USA) was used. To determine the effect of a play session on c-fos positive cell density, for each animal the mean of three images (taken from three subsequent sections) was calculated for all parameters. Only data from regions for which three intact sections were available were included in the analysis. To assess the effect of a play session on the density of c-fos positive cells (FpCD), data was analysed using a Student's t-test. To determine the effect of play on the FpCD per intensity category a two-way analysis of variance was used, followed by *post hoc* Student's t-test analysis if appropriate.

For the calculation of a correlation coefficient, the average total immunoreactivity for each animal was used, since this parameter takes the number of c-fos responsive cells as well as the intensity value of these cells into account. To calculate the total immunoreactivity, the OD value of a detected cell was multiplied with its cell surface, subsequently the sum of these values was calculated per region per section. The mean of three sections was calculated per animal. This average total immunoreactivity per animal was used to calculate the correlation between different regions. For analysis of the correlations a Spearman's correlation test was performed separately per group ('play' and 'no play').

Results

Expression of social play behaviour

One animal from the 'play' group was excluded from the analysis because of insufficient tissue quality, so that n=9 animals were used in the final analysis. These animals showed on average 33.56 ± 2.32 pins/15 min and the average frequency of pouncing was 52.67 ± 5.21 /15 min. The average duration of the time spent on social exploration was 41.29 ± 5.33 seconds.

Prefrontal cortex

In prefrontal cortex (Fig. 1A), social play increased the FpCD in the anterior cingulate cortex (AC: $t = 2.639$, $df = 14$, $p = 0.019$), in the dorsal and ventral prelimbic cortex (PrLd: $t = 2.715$, $df = 14$, $p = 0.017$; PrLv: $t=2.543$, $df = 14$, $p = 0.023$), in the medial orbitofrontal (MO: $t = 2.779$, $df = 12$, $p = 0.017$) and ventrolateral orbitofrontal cortex (VLO: $t = 2.560$, $df = 12$, $p = 0.025$) (Fig. 3). In contrast, social play behaviour decreased the FpCD in the dorsolateral orbitofrontal cortex (DLO: $t = 2.604$, $df = 12$, $p = 0.023$), while no significant effects were observed in the infralimbic cortex (IL: $t = 1.332$, $df = 14$, $p = 0.204$), ventral orbitofrontal cortex (VO: $t = 1.858$, $df = 12$, $p = 0.088$), lateral orbitofrontal cortex (LO: $t = -0.446$, $df = 12$, $p = 0.663$) and in the dorsal and ventral part of the agranular insular cortex (AId: $t = -0.440$, $df = 10.33$, $p = 0.669$; AIv: $t = 1.052$, $df = 8.76$, $p = 0.321$) (Fig. 3).

In addition, the c-fos expression levels were analysed taking the intensity levels into consideration (Table 1). The increase in the FpCD was specifically present in the dark intensity category in the AC, PrLd, PrLv, MO, and VLO (AC: $F_{play\ x\ category}(2,42) = 12.250$, $p < 0.001$; $t_{light} = -1.702$ $df = 14$, $p = 0.111$; $t_{medium} = 1.232$, $df = 14$, $p = 0.238$; $t_{dark} = 4.109$, $df = 14$, $p = 0.001$; PrLd: $F_{play\ x\ category}(2,42) = 12.670$, $p < 0.001$; $t_{light} = -1.630$ $df = 14$, $p = 0.125$; $t_{medium} = -0.499$, $df = 14$, $p = 0.625$; $t_{dark} = 3.646$, $df = 14$, $p = 0.003$; PrLv: $F_{play\ x\ category}(2,42) = 7.609$, p

= 0.002; t_{light} = -1.183 df = 14, p = 0.256; t_{medium} = 0.314, df = 14, p = 0.758; t_{dark} = 2.876, df = 14, p = 0.012; MO: $F_{play\ x\ category}(2,36) = 5.939$, p < 0.001; t_{light} = -0.278 df = 12, p = 0.786; t_{medium} = 0.416 df = 12, p = 0.686; t_{dark} = 6.521, df = 12, p = 0.013; VLO: $F_{play\ x\ category}(2,36) = 13.366$, p < 0.001; t_{light} = -2.121 df = 12, p = 0.055; t_{medium} = -0.667 df = 12, p = 0.517; t_{dark} = 3.571, df = 12, p = 0.004). An increase in overall FpCD together with a specific increase in the dark category may be indicative of a new group of cells that is activated and that expresses high levels of c-fos. In addition, it is possible that cells that were already expressing c-fos, express more c-fos after social play behaviour, resulting in more neurons reaching the detection limit.

The overall decrease in the FpCD observed in the DLO was mainly caused by a decrease in light cells ($F_{play\ x\ category}(2,39) = 3.561$, p = 0.038; t_{light} = -2.410 df = 13, p = 0.031; t_{medium} = -1.434 df = 13, p = 0.175; t_{dark} = 1.213, df = 13, p = 0.247) (Table 1). This may indicate that a subset of neurons expressed less c-fos, resulting in less cells reaching the detection limit.

In the IL and VO no differences were observed in the overall FpCD. However, in both areas significantly more dark cells were present (IL: $F_{play\ x\ category}(2,42) = 4.379$, p = 0.019; t_{light} = -0.863 df = 14, p=0.402; t_{medium} = -0.373, df = 14, p = 0.715; t_{dark} = 2.331, df = 14, p = 0.035; VO: $F_{play\ x\ category}(2,36) = 5.939$, p = 0.006; t_{light} = -0.912 df = 12, p=0.380; t_{medium} = 0.751, df = 12, p = 0.467; t_{dark} = 2.924, df = 12, p = 0.013) (Table 1). This suggests that the same subset of neurons expressed more c-fos after a play session in these areas.

In the AId and AIv no overall changes in the FpCD were observed, but in both areas the density of the light cells was decreased (AId: $F_{play\ x\ category}(2,42) = 4.145$, p = 0.023; t_{light} = -3.048 df = 13, p = 0.009; t_{medium} = -0.920 df = 13, p = 0.375; t_{dark} = 1.312, df = 13, p = 0.212; AIv: $F_{play\ x\ category}(2,42) = 3.995$, p = 0.026; t_{light} = -2.214 df = 13, p = 0.045; t_{medium} = 0.252 df = 13, p = 0.805; t_{dark} = 1.611, df = 13, p = 0.131) (Table 1). Possibly, the light neurons expressed more c-fos after play and were distributed over the medium and dark category, but to such a modest extent that no changes in the dark or medium category and no changes in the overall cell density could be observed.

In summary, social play induced c-fos expression in several prefrontal regions. The most pronounced effects were observed in the dorsal region of the mPFC (AC and PrL), and medial/ventral part of the OFC (including the MO and VLO).

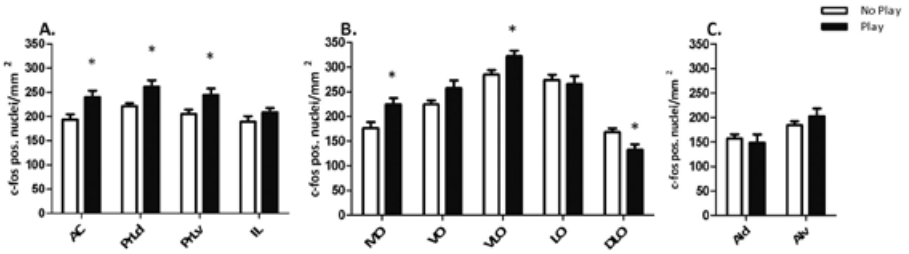


Fig. 3: Social play behaviour affected the c-fos positive cell density in prefrontal regions. The c-fos positive cell density was determined in rats after receiving a play session ('play' group) and in rats that were placed in the test cage without a partner present ('no play' group). Regions measured include medial prefrontal regions (A), orbitofrontal regions (B) and agranular insular cortical regions (C). Data are presented as mean ± SEM. * $p < 0.05$. AC = anterior cingulate cortex, PrLd = prelimbic cortex dorsal part, PrLv = prelimbic cortex ventral part, IL = infralimbic cortex, MO = medial orbitofrontal cortex, VO = ventral orbitofrontal cortex, VLO = ventrolateral orbitofrontal cortex, LO = lateral orbitofrontal cortex, DLO = dorsolateral orbitofrontal cortex, AId = agranular insular cortex dorsal part, AIv = agranular insular cortex ventral part.

Striatum

To determine involvement of the striatum in social play behaviour, the FpCD was analysed in several striatal subregions (Fig. 1B). The nucleus accumbens core and shell were measured as a whole and divided into different subregions (NaCore: medial (mCore) and lateral part (lCore); NaShell: medial (mShell), ventral (vShell), and lateral part (lShell)), because anatomical and functional differences have been reported in subdivisions of the NaCore and NaShell (van der Plasse *et al.*, 2012; Voorn *et al.*, 2004; Willuhn *et al.*, 2003). In the NaCore, an increase in the FpCD was observed (NaCore: $t = 2.707$, $df = 13$, $p = 0.018$; Fig. 4A). This increase was clearly present in the lateral part ($t = 2.831$, $df = 13$, $p = 0.014$), while in the mCore a trend towards an increase was observed ($t = 2.109$, $df = 13$, $p = 0.055$) (Fig. 4A). Social play behaviour also enhanced the FpCD in the NaShell ($t = 2.194$, $df = 13$, $p = 0.047$; Fig. 4B). This increase was found in the mShell ($t = 2.271$, $df = 13$, $p = 0.041$) and a trend towards an increase was observed in the lShell ($t = 2.107$, $df = 9.19$, $p = 0.064$) (Fig. 4B). In the vShell, no effects of social play were observed ($t = 1.287$, $df = 13$, $p = 0.220$). A large increase in FpCD after social play was observed in the dorsal striatum ($t = 6.997$, $df = 10.8$, $p < 0.001$), but not in the olfactory tubercle (OTu) ($t = 0.110$, $df = 13$, $p = 0.914$; Fig. 4C).

Dividing the c-fos positive cells into three different categories, based on intensity levels (Table 1), showed that in the NaCore enhancement of the FpCD was present in the medium and dark category ($F_{play\ x\ category}(2,39) = 3.914$, $p = 0.028$; $t_{light} = -0.588$ $df = 13$, $p = 0.567$; $t_{medium} = 3.027$, $df = 13$, $p = 0.010$; $t_{dark} = 2.581$, $df = 9.341$, $p = 0.032$). Similar changes were observed in the lCore ($F_{play\ x\ category}(2,39) = 2.991$, $p = 0.042$; $t_{light} = -0.353$ $df = 13$, $p = 0.730$; $t_{medium} = 4.240$, $df = 13$, $p = 0.001$; $t_{dark} = 3.099$, $df = 13$, $p = 0.008$). In the mCore a trend towards an interaction between play and category was found ($F_{playxcategory}(2,39) = 2.991$, $p = 0.062$). In the NaShell, an increase in the FpCD was detected in the dark category ($F_{play\ x\ category}(2,39) = 6.767$, $p = 0.003$; $t_{light} = -2.143$, $df = 13$, $p = 0.052$; $t_{medium} = 1.896$, $df = 13$, $p = 0.080$; $t_{dark} = 2.826$, $df = 8.909$, $p = 0.020$). When the medial part was analysed an increase in the FpCD was observed in the medium and dark category ($F_{play\ x\ category}(2,39) = 6.408$, p

= 0.004; t_{light} = -1.457 df = 13, p = 0.169; t_{medium} = 2.174, df = 13, p = 0.049; t_{dark} = 2.914, df = 13, p = 0.012). No significant interaction between play and category was detected in the ventral and lateral part of the NaShell (vShell: $F_{play\ x\ category}(2,39) = 2.415$, p = 0.103; lShell: $F_{play\ x\ category}(2,39) = 3.029$, p = 0.061). In the dorsal striatum social play increased the cell density of c-fos positive cells in all categories ($F_{play\ x\ category}(2,39) = 12.900$, p < 0.001; t_{light} = 2.265 df = 13, p = 0.041; t_{medium} = 7.573, df = 13, p < 0.001; t_{dark} = 5.475, df = 8.139, p = 0.001) (Table 1).

In summary, in the lCore and mShell an overall increase in FpCD and a specific increase in the medium and dark category was observed. This may be indicative of a new population of cells that is activated and/or higher levels of c-fos expression in cells that were already active. The increase in all three categories in the dorsal striatum shows an upward shift of the frequency histogram, which suggests that a new population of cells was activated after social play.

In the OTu the FpCD was reduced specifically in the light category ($F_{play\ x\ category}(2,39)$ = 3.977, p = 0.027; t_{light} = -3.306 df = 13, p=0.006; t_{medium} = -0.252, df = 13, p = 0.805; t_{dark} = 1.370, df = 13, p = 0.194). This is comparable to the pattern of effects in the AIv and AId. It may be that the light neurons expressed more c-fos after social play and were distributed over the medium an dark category. However, perhaps because of the small magnitude, this effect did not translate into changes in the dark or medium category and changes in the overall cell density.

In summary, social play behaviour induced c-fos expression in dorsal and ventral striatal regions. The c-fos activation was most evident in the dorsal striatum, where more cells were present in all three intensity categories, indicative of a new population of cells that is recruited by social play behaviour. The most pronounced changes in the ventral striatum were observed in the lCore and mShell, were social play increased the cell density of medium and dark cells.

It has been proposed that the classical division of the striatum into a dorsal part and ventral part may not be the most appropriate functional division (Voorn *et al.*, 2004). Therefore, the striatum was divided based on the inputs from the prefrontal cortex (Fig. 1C-D). This division revealed a gradient in the enhancement of the FpCD by social play. The largest increase was observed in the dorsolateral striatum, which corresponds to the part receiving input from the sensory and motor cortex (STR-SomSen = 9.463, df = 13, p < 0.001) (Fig. 5A). The magnitude of the increase in the FpCD was less prominent in the central and medial dorsal striatum. Significant increases in the FpCD were observed in the AC projection region (STR-AC: t = 5.509, df = 13, p < 0.001). In the projection area of the PrLd (STR-PrLd) a trend towards an increase was observed (t = 1.786, df = 13, p = 0.097), while in the PrLv projection area (STR-PrLv) a significant increase in the FpCD was seen (t = 2.239, df = 13, p = 0.043) (Fig. 5A). In the projection areas located in the ventral striatum no changes in the FpCD were observed (IL projection area (STR-IL): t = 0.400, df = 13, p = 0.696; AId projection area (STR-AId): t = 0.870, df = 13, p = 0.400); AIv projection area (STR-AIv): t = 0.218, df = 13, p = 0.831) (Fig. 5A).

The striatum was also divided based on the OFC projections (Groenewegen and Uylings, 2010). The striatal target regions of the OFC projections partially overlap with those from the mPFC. Indeed, a comparable gradient of c-fos activation was observed (Fig. 5B). A large increase was observed in the dorsolateral striatum region receiving inputs from

the DLO and LO (STR-DLO/LO: $t = 8.143$, $df = 13$, $p < 0.001$). The central part of the striatum receives inputs from the VLO, where an increase in the FpCD was also observed (STR-VLO: $t = 6.414$, $df = 13$, $p < 0.001$). In the medial part of the striatum, receiving input from the MO and VO, no effects of play were detected (STR-MO/VO: $t = 1.728$, $df = 13$, $p = 0.108$).

In these different divisions of the striatum, the FpCD was also analysed based on intensity of the cells (Table 1). In the dorsolateral regions, an increase in the FpCD was observed in all categories (STR-SomSen: $F_{play\ x\ category}(2,39) = 29.351$, $p < 0.001$; $t_{light} = 4.201$ $df = 13$, $p = 0.001$; $t_{medium} = 8.711$, $df = 13$, $p < 0.001$; $t_{dark} = 8.697$, $df = 7.409$, $p < 0.001$; STR-DLO/LO: ($F_{play\ x\ category}(2,39) = 27.009$, $p < 0.001$; $t_{light} = 2.393$ $df = 13$, $p = 0.033$; $t_{medium} = 7.099$, $df = 13$, $p < 0.001$; $t_{dark} = 8.698$, $df = 7.594$, $p < 0.001$). In the central striatal regions, increases in the FpCD were detected in the medium and dark category (STR-AC: ($F_{play\ x\ category}(2,39) = 10.476$, $p < 0.001$; $t_{light} = 0.843$ $df = 13$, $p = 0.415$; $t_{medium} = 6.255$, $df = 13$, $p < 0.001$; $t_{dark} = 5.248$, $df = 8.654$, $p = 0.001$; STR-VLO: ($F_{play\ x\ category}(2,39) = 7.771$, $p = 0.001$; $t_{light} = 1.614$ $df = 13$, $p = 0.130$; $t_{medium} = 5.909$, $df = 13$, $p < 0.001$; $t_{dark} = 5.193$, $df = 9.465$, $p < 0.001$). In the striatum regions receiving input from the PrLd and PrLv, social play behaviour enhanced the FpCD specifically in the dark category (STR-PrLd: ($F_{play\ x\ category}(2,39) = 3.776$, $p = 0.032$; $t_{light} = -1.251$, $df = 13$, $p = 0.233$; $t_{medium} = 1.625$, $df = 13$, $p = 0.128$; $t_{dark} = 2.239$, $df = 13$, $p = 0.043$; STR-PrLv: ($F_{play\ x\ category}(2,39) = 4.878$, $p = 0.013$; $t_{light} = -1.548$ $df = 13$, $p = 0.146$; $t_{medium} = 2.157$, $df = 13$, $p = 0.050$; $t_{dark} = 2.578$, $df = 9.252$, $p = 0.029$). In the STR-IL and STR-AIv region a significant decrease in FpCD was detected in the light category (STR-IL: ($F_{play\ x\ category}(2,39) = 4.920$, $p = 0.012$; $t_{light} = -2.605$ $df = 13$, $p = 0.022$; $t_{medium} = -0.064$, $df = 13$, $p = 0.950$; $t_{dark} = 1.965$, $df = 13$, $p = 0.071$; STR-AIv: ($F_{play\ x\ category}(2,39) = 3.820$, $p = 0.031$; $t_{light} = -3.099$ $df = 13$, $p = 0.008$; $t_{medium} = 0.300$, $df = 13$, $p = 0.769$; $t_{dark} = 1.414$, $df = 13$, $p = 0.181$). Play behaviour did not significantly change the FpCD in any category in the STR-MO/VO and STR-AId, although a few trends were found (STR-MOVO: $F_{play\ x\ category}(2,39) = 2.979$, $p = 0.063$; STR-AId: ($F_{play\ x\ category}(2,39) = 5.042$, $p = 0.011$; $t_{light} = -1.830$ $df = 13$, $p = 0.090$; $t_{medium} = 1.134$, $df = 13$, $p = 0.277$; $t_{dark} = 2.162$, $df = 13$, $p = 0.050$).

To summarise, social play behaviour increased the FpCD in different categories of the striatal regions: all categories in the dorsolateral striatum, medium and dark category in the central dorsal striatal region, dark category in the dorsomedial regions, and decreased the FpCD in the light category in the ventromedial striatal regions. This suggests that the recruitment of new cells by social play behaviour is most apparent in the dorsolateral striatum, an effect which tapers off in the medial and ventral direction. In these latter regions, it may also be that cells that were already active express more c-fos after play. Together, these results indicate that the induction of c-fos gene expression by social play behaviour is topographically organised in the striatum.

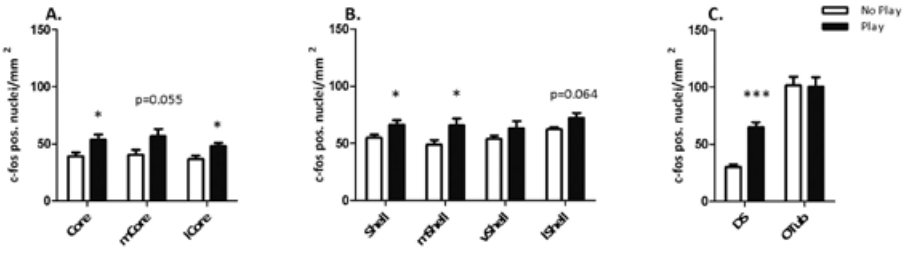


Fig. 4: Social play induced c-fos activity in dorsal and ventral striatum. The c-fos positive cell density was determined in rats after receiving a play session ('play' group) and in rats that were placed in the test cage without a partner present ('no play' group). Regions measured include the nucleus accumbens core subregions (A), nucleus accumbens shell regions (B), and the dorsal striatum and olfactory tubercle (C). Data are presented as mean ± SEM. * $p < 0.05$, *** $p < 0.001$. Core = nucleus accumbens core, mCore = medial nucleus accumbens core, lCore = lateral nucleus accumbens core, Shell = nucleus accumbens shell, mShell = medial nucleus accumbens shell, vShell = ventral nucleus accumbens shell, lShell = lateral nucleus accumbens shell, DS = dorsal striatum, OTub = olfactory tubercle.

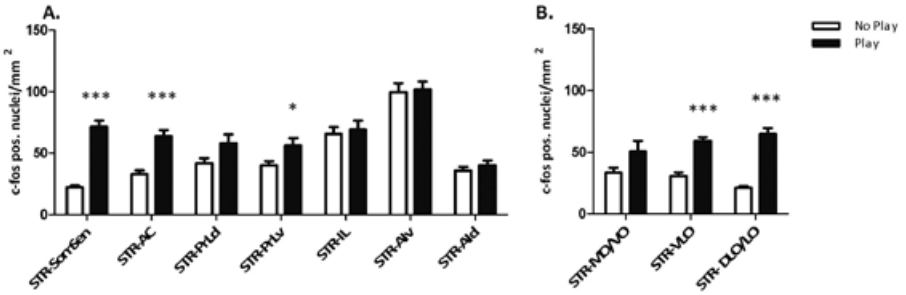


Fig. 5: The c-fos activity induced by social play behaviour in the striatum shows a dorsolateral to ventromedial gradient. The c-fos positive cell density was determined in rats after receiving a play session ('play' group) and in rats that were placed in the test cage without a partner present ('no play' group). The striatum was divided according to mPFC inputs (A) and OFC inputs (B). Data are presented as mean ± SEM. * $p < 0.05$, *** $p < 0.001$. STR- = striatal region receiving input from cortical region: SomSen = somatosensory cortex, AC = anterior cingulate cortex, PrLd = prelimbic cortex dorsal part, PrLv = prelimbic cortex ventral part, IL = infralimbic cortex, AIv = agranular insular cortex ventral part, AId = agranular insular cortex dorsal part, MO/VO = medial/ventral orbitofrontal cortex, VLO = ventrolateral orbitofrontal cortex, DLO/LO = dorsolateral/lateral orbitofrontal cortex.

Amygdala

The FpCD was determined in several amygdala regions: the lateral amygdala (LA), basolateral amygdala (BLA), central amygdala (CeA), and medial amygdala (MeA). The FpCD was measured in these regions at 2 different anterior-posterior levels (-2.56 and -3.30 mm from Bregma; Fig. 1F-G). These two levels were chosen since initial visual inspection of c-fos positive staining in both groups suggested the presence of anterior-posterior differences. In addition, the FpCD was determined in a region of the extended amygdala, the bed nucleus of the stria terminalis (BNST) (Fig. 1E).

Social play increased the FpCD in the LA at the anterior level ($t = 2.428$, $df = 14$, p

= 0.029), but not at the posterior level (t = 1.053, df = 14, p = 0.310) (Fig. 6A-B). Social play increased the FpCD in the BNST (t = 3.288, df = 14, t = 0.005 (Fig. 6C). In the other subregions of the amygdala no effect on the FpCD was observed at either level (t < 1.803, p > 0.092) (Fig. 6A-B).

Division into the three categories showed that the effect in the anterior LA was due to an increase in medium and dark cells ($F_{play\ x\ category}(2,42) = 5.77$, p = 0.006; t_{light} = -1.728 df = 14, p = 0.1061; t_{medium} = 2.581, df = 14, p = 0.022; t_{dark} = 2.432, df = 14, p = 0.029) (Table 1). In the anterior BLA an increase in FpCD was observed in the dark category ($F_{play\ x\ category}(2,42) = 4.758$, p = 0.014; t_{light} = -1.552 df = 14, p = 0.143; t_{medium} = 0.906, df = 14, p = 0.380; t_{dark} = 2.389, df = 14 p = 0.032), while no effect was observed in the posterior BLA ($F_{play\ x\ category}(2,42) = 2.158$, p = 0.149) (Table 1). In the other subregions, no alterations were detected in any of the intensity categories ($F_{play\ x\ category}$ < 2.710, p > 0.077).

In summary, social play increased the FpCD specifically in the anterior LA and BNST. In the LA, increases in the FpCD in the medium and dark category were observed, which may be the result of new cells being recruited by play, or a result of cells that were already active expressing more c-fos. In the BNST, no significant interaction was observed between the effect of play and the different categories, probably indicating that a new population of cells was recruited by social play. In the anterior BLA no overall changes in FpCD were observed, but the FpCD was enhanced in the dark category. These results suggest that in the BLA no new cells were recruited, but the same cells expressed more c-fos after a play session.

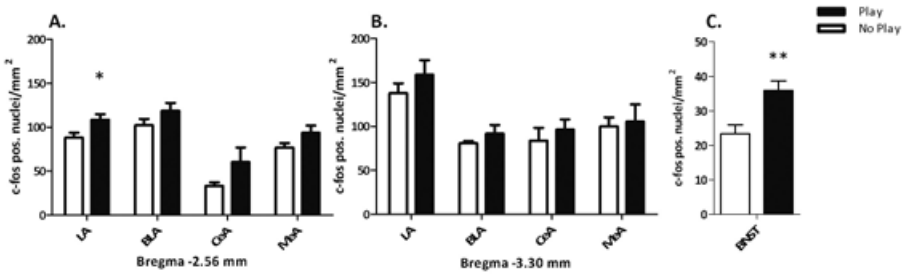


Fig. 6: Social play behaviour increased the c-fos positive cell density in the lateral amygdala and bed nucleus of the stria terminalis. The c-fos positive cell density was determined in rats after receiving a play session ('play' group) and in rats that were placed in the test cage without a partner present ('no play' group). Regions measured include the lateral amygdala (LA), basolateral amygdala (BLA), central amygdala (CeA) and medial amygdala (MeA) at two different anterior posterior levels, -2.56 mm from Bregma (A) and -3.30 mm from Bregma (B) and the bed nucleus of the stria terminalis (BNST) (C). Data are presented as mean ± SEM. * p < 0.05, ** p < 0.01.

Thalamus

Within the thalamus, we investigated regions highly connected to the striatum and prefrontal cortex; these included the mediodorsal thalamic nucleus (MD) and several of the midline and intralaminar thalamic nuclei: the central lateral thalamic nucleus (CL), the paracentral thalamic nucleus (PC), the central medial thalamic nucleus (CeM), the medial and lateral parts of the intermediodorsal thalamic nucleus (IMDm and IMDl), and the medial and lateral paraventricular thalamic nucleus (PVm and PVl) (Fig. 1F).

Social play behaviour increased the FpCD in the PC ($t = 2.642$, $df = 13$, $p = 0.020$), the IMDl ($t = 2.993$, $df = 14$, $p = 0.010$) and in the PVl ($t = 2.689$, $df = 13$, $p = 0.019$) (Fig. 7A). A trend towards an increase was observed in the CeM ($t = 2.125$, $df = 14$, $p = 0.052$), while in the other thalamic regions no differences in the FpCD were detected ($t < 1.421$, $t > 0.177$) (Fig. 7A). Division of the cells into three categories showed a trend towards an interaction between intensity category and the effect of play in the IMDl ($F_{play \times category}(2,42) = 3.026$, $p = 0.059$), while no effects were observed in any of the other regions ($F_{play \times category} < 1.830$, $p > 0.173$) (Table 1).

To summarise, these data indicate that social play induced c-fos expression in a few thalamic regions, including the PC, IMDl, and PVl. The enhancement of the FpCD in these regions was equally distributed over the different intensity categories, suggesting that a new population of cells was recruited by social play in these regions.

Pallidum

The FpCD was determined in the lateral globus pallidus (lGP) and ventral pallidum (VP) (Fig. 1E). The FpCD was decreased in the play group in the lGP ($t = -2.277$, $df = 14$, $p = 0.039$) (Fig. 7B). This was not due to a specific effect in any of the expression level categories, since there was no interaction with the effect of play ($F_{play \times category}(2,42) = 1.202$, $p = 0.311$) (Table 1). In the ventral pallidum no differences were observed on the overall FpCD, nor was there an interaction with the different expression levels categories ($t = -1.740$, $df = 14$, $p = 0.104$; $F_{play \times category}(2,42) = 0.110$, $p = 0.896$) (Fig. 7B, Table 1). These results indicate that social play behaviour decreases c-fos expression in the lGP which is equally distributed over the different intensity categories. This is indicative of a population of cells that is inhibited during play behaviour in the lGP.

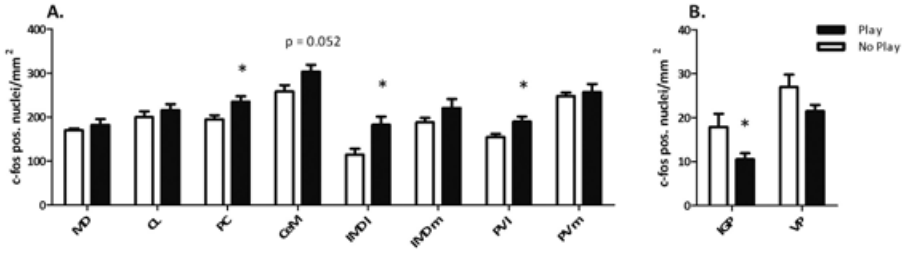


Fig. 7: Social play induced changes in the c-fos positive cell density in the thalamus and pallidum. The c-fos positive cell density was determined in rats after receiving a play session ('play' group) and in rats that were placed in the test cage without a partner present ('no play' group). Social play behaviour increased c-fos activity in several thalamic regions, while decreasing c-fos activity in the lGP. The thalamic regions measured are presented in panel A and the pallidum regions are presented in panel B. Data are presented as mean ± SEM. * $p < 0.05$. MD = mediodorsal thalamic nucleus, CL = central lateral thalamic nucleus, PC = paracentral thalamic nucleus, CeM = central medial thalamic nucleus , IMDl = intermediodorsal thalamic nucleus lateral part , IMDm = intermediodorsal thalamic nucleus medial part , PVl = paraventricular thalamic nucleus lateral part , PVm = paraventricular thalamic nucleus medial part , lGP = lateral globus pallidus, VP = ventral pallidum.

Monoamine nuclei

c-Fos expression levels after social play were also analysed in monoamine nuclei, such as the ventral tegmental area (VTA), substantia nigra pars compacta (SNc), locus coeruleus (LC), and the dorsal raphe nucleus at two different levels (DRa at -8.00 mm from Bregma, DRp at -8.72 mm from Bregma), as well as the substantia nigra pars reticulata (SNr) (Fig. 1H,J,L). No changes were observed in the VTA ($t = -0.236$, $df = 14$, $p = 0.817$), SNc ($t = -0.207$, $df = 14$, $p = 0.839$) and SNr ($t = -0.089$, $df = 14$, $p = 0.930$) (Fig. 8 A). In the dorsal raphe an increase in the FpCD was detected at the anterior level ($t = 2.637$, $df = 13$, $p = 0.021$), while no alterations were observed in the DR at the posterior level ($t = 1.446$, $df = 14$, $p = 0.170$) (Fig. 8B). No changes were observed in the LC after social play ($t = 0.994$, $df = 13$, $p = 0.338$) (Fig. 8C). No interactions were observed between play and the different categories of c-fos expression levels in any of these regions ($F_{play \times category} < 2.757$, $p > 0.075$) (Table 1). These results suggest that social play behaviour did not induce c-fos expression in the dopamine- and noradrenaline-producing regions, while it did affect c-fos expression in the serotonin-producing dorsal raphe nucleus. Since the increase in the FpCD was equally distributed over the different intensity categories, it is likely that in the anterior DR a new population of cells was recruited by social play behaviour.

Rostromedial tegmental nucleus, laterodorsal tegmental nucleus and pedunculopontine tegmental nucleus

Expression levels of c-fos, induced by play, were analysed in the rostromedial tegmental nucleus (RMTg), the laterodorsal tegmental nucleus (LDTg) and pedunculopontine tegmental nucleus (PPTg) (Fig. 1I,J,K). Social play increased the FpCD in the PPTg ($t = 2.642$, $df = 14$, $p = 0.019$), but not in RMTg or LDTg (RMTg: $t = -0.233$, $df = 14$, $p = 0.819$; LDTg: $t = 0.683$, $df = 14$, $p = 0.506$) (Fig. 8D). However, when taking into account the intensity level and comparing the FpCD per category there was an increase in the dark cells in the RMTg ($F_{play \times category}(2,40) = 4.877$, $p = 0.013$; $t_{light} = -1.935$ $df = 14$, $p=0.073$; $t_{medium} = -0.112$, $df = 14$, $p = 0.913$; $t_{dark} = 2.665$, $df = 14$, $p = 0.018$), suggesting that the same population of neurons expressed more c-fos after play. In the other regions no interaction between play and the FpCD in the three categories was observed ($F_{play \times category} < 0.263$, $p > 0.770$) (Table 1).

In summary, these results suggest that in the RMTg the same population of neurons expressed more c-fos after social play behaviour, while in the PPTg a new population of cells may be recruited.

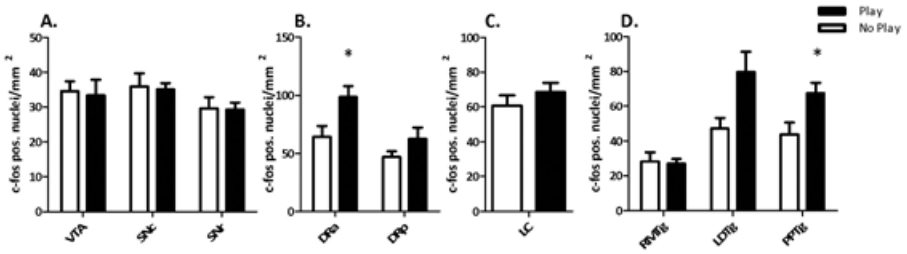


Fig. 8: Social play induced changes in monoamine nuclei and tegmental regions. The c-fos positive cell density was determined in rats after receiving a play session ('play' group) and in rats that were placed in the test cage without a partner present ('no play' group). After a social play session the level of c-fos activity was increased in the anterior dorsal raphe nucleus and the PPTg relative to the 'no play' control. Regions measured included the ventral tegmental area (VTA), substantia nigra pars compacta (SNc) and substantia nigra pars reticulata (SNr) (Fig. A), dorsal raphe nucleus anterior and posterior level (DRa and DRp; Fig. B), locus coeruleus (LC; Fig. C), rostromedial tegmental nucleus (RMTg), laterodorsal tegmental nucleus (LDTg), and pedunculopontine tegmental nucleus (PPTg) (Fig.D). Data are presented as mean ± SEM. * $p < 0.05$.

Correlations in social play-induced c-fos activity

To gain insight in the neural network activated during social play behaviour, correlations of c-fos activity were assessed between regions with known anatomical interconnections. The focus of this analysis was on connections of the prefrontal cortex and striatum with each other as well as with the amygdala, thalamus and VTA/SN complex.

Correlations of c-fos immunoreactivity between the prefrontal, striatal and thalamus regions were investigated because the three brain regions are strongly interconnected in a topographical manner (Groenewegen and Uylings, 2010; Schilman *et al.*, 2008; Van der Werf *et al.*, 2002; Voorn *et al.*, 2004). The same holds for the relationship between amygdala, prefrontal cortex and striatum. The BLA provides most inputs to the striatum (Kelley *et al.*, 1982; Voorn *et al.*, 2004), while all nuclei (measured as ROIs in the present study) provide inputs and receive outputs from the prefrontal cortex (Cassell and Wright, 1986; McDonald *et al.*, 1996; Voorn *et al.*, 2004). For this reason, correlations between BLA and striatum as well as correlations between all amygdala regions and prefrontal regions were analysed.

The VTA and SN have reciprocal projections with the striatum and prefrontal cortex, where the VTA and SNc provide a dopaminergic projection to both regions and the SNr receives GABAergic outputs from the striatum (Gerfen *et al.*, 1987; Gerfen, 2004; Ikemoto, 2007; Watabe-Uchida *et al.*, 2012). Correlations of the VTA and SNc with the prefrontal and striatal regions were analysed, as well as correlations between the SNr and striatal regions.

An overview of all correlations analysed is presented in Table 2. In addition, a summary of these correlations is visualised in a network figure, which indicates the substantial differences between the 'play' and 'no play' groups that are described below (Fig. 9).

Prefrontal cortex correlations

Significant correlations were observed between the striatal target regions and the cortical input regions in the projections of the mPFC regions PrLd, PrLv, and IL, and agranular insular regions, AId and AIv, in the play group (Table 2), whereas such correlations were absent in the no-play group. In addition, no correlations were detected in play or no play animals in the OFC projections to the striatum, or between the thalamus regions and their mPFC projection areas (Table 2). Activity in the IMDl regions of the thalamus did correlate with the AId in the play group, while not correlating in the no-play group (Table 2).

C-fos immunoreactivity levels in the BLA and MEA subnuclei of the amygdala correlated with several prefrontal cortex regions in the play group (Table 2). Correlations were observed between the anterior BLA and all mPFC regions, the ventral and lateral OFC regions, and the agranular insular cortex. These correlations were only present in the play group. At the posterior level correlations between the BLA and prefrontal regions were less pronounced, being significant in PrL, IL and AId in the play group, and in LO in the no-play group (Table 2). Activity in the anterior MeA correlated with the c-fos activity in the PrL, IL, VO, VLO, AId, and AIv and these correlations were specific for the play group. Comparable to the BLA, activity in the posterior MeA correlated with activity in fewer prefrontal regions (i.e., PrL and DLO only) relative to the anterior MeA. No correlations were observed between the prefrontal regions and the anterior LA or anterior CeA, while with the posterior LA and CeA a few correlations were observed (IL, VO and MO, VO, respectively) (Table 2).

Correlations were observed between the VTA and all mPFC regions as well as with the VLO, specifically in the play group. The c-fos activity in the SNc correlated negatively with the activity in the AC and LO, also in the play group only (Table 2).

In summary, in playing animals the most pronounced correlations in prefrontal cortex regions were observed between mPFC and striatum, between prefrontal cortex and BLA as well as MeA, and between the mPFC and VTA (Fig. 9).

Striatum correlations

For most thalamus regions, no correlations were observed in the c-fos immunoreactivity levels with those measured in their striatal projection regions, except for the PVm and its striatal target area. This correlation was specific for the play group (Table 2).

The c-fos immunoreactivity levels in the anterior BLA correlated with the levels measured in several ventral striatum regions, i.e., the mCore, mShell, lShell, and OTu, specifically in the play group. A comparable pattern of correlations was observed for the posterior BLA, except that at this level activity in the lShell did not correlate, while activity in the vShell did correlate with activity in the posterior BLA (Table 2).

The c-fos immunoreactivity levels in the VTA correlated only with the c-fos levels observed in the most ventral regions of the striatum: mShell, lShell, and OTu. These correlations were specific for the play group. In contrast, the c-fos levels in the SNc did not correlate with the levels in any of the striatal regions. C-fos immunoreactivity levels did correlate between the SNr and the mCore in the play group. In addition, the activity levels of the SNr correlated with the levels measured in the lCore in the no play animals (Table 2).

The social play-induced c-fos levels in the VP correlated with those in the mShell and lShell in the play group. In addition, c-fos immunoreactivity levels in the VP and lGP correlated negatively with the levels in the lCore in the no-play group (Table 2).

In summary, in the striatum of the playing animals the most pronounced correlations of the c-fos immunoreactivity levels were observed between BLA and ventral striatal regions, between VTA and NaShell as well as OTu and between NaShell and VP (Fig. 9).

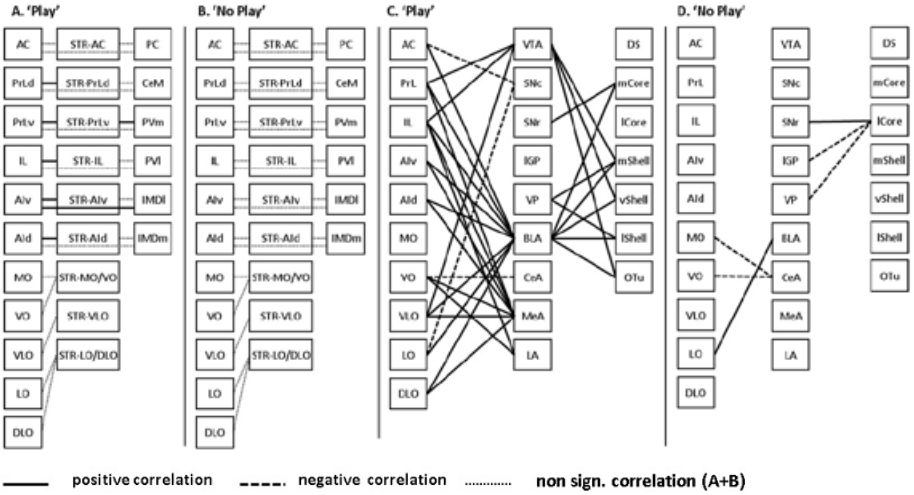


Fig. 9: Summary of correlations observed in 'play' and 'no play' animals. Data is abstracted from Table 2 and represents Spearman's rho correlation coefficient. A+B: Correlations in the thalamocorticostriatal network. Each region was only correlated with its known direct connections. Black line indicates a significant positive correlations, a dotted line indicates the absence of a significant correlation in the play (A) and no play (B) groups. C+D: Correlations of the amygdala, VTA and pallidum regions with the prefrontal cortex and striatum. Black line indicates a significant positive correlation, whereas the dotted line indicates a significant negative correlation in theplay (C) and no play (D) groups.

Discussion

In the present study, social play-induced c-fos activity was measured to investigate the neural substrates of this behaviour. The induction of c-fos expression is widely used as a neuronal activity marker (Kovacs, 2008; Morgan and Curran, 1991; Schilling *et al.*, 1991), since neurotransmitter receptor activation, depolarization, and growth factors are known to induce c-fos expression. Therefore, increases in c-fos expression levels can be interpreted as a cellular response to the given stimulus in these cells. However, it remains important to keep in mind that neuronal activation can occur in the absence of c-fos induction (Kovacs, 2008). In addition, induction of c-fos may not be detected in chronically activated neurons (Kovacs, 2008; Morgan and Curran, 1991; Schilling *et al.*, 1991).

Social play behaviour altered c-fos expression in several of the measured brain regions. These alterations were observed in regions involved in reward processing and motivation,

as well as in regions important for processing of motor and sensory information. During play behaviour, rats exhibit high levels of locomotor activity and process a large amount of sensory information (Gordon *et al.*, 2002; Pellis and Pellis, 1998; Vanderschuren *et al.*, 1997). This kind of locomotor activity and sensory processing did not occur in the control rats, since they were alone in the test cage. Behaviour expressed by the control rats existed mainly of exploration and self-grooming. Therefore, in this study all sensory, emotional and behavioural aspects of social play behaviour contributed to the observed effects. Note that, even though the animals in the play group spent a substantial amount of time on social play during testing, we can not exclude that non-playful (social or non-social) activities contributed to the changes in c-fos expression. Future studies will need to elucidate the neural underpinnings of the separate component processes that constitute social play, and whether the effect of some of these component processes (e.g., vigorous motor activity) on c-fos expression is specific for social play.

Prefrontal cortex

Several prefrontal regions were activated as a result of social play behaviour. The largest increases in the FpCD were observed in the dorsal mPFC regions and medial OFC regions, while in the lateral OFC regions a reduction in the FpCD was observed. In the dorsal mPFC regions and medial OFC regions, there was a specific enhancement in the number of dark cells, which points to either activation of a new population of cells or higher levels of c-fos expression in cells that were already active. In the IL and VO, the overall FpCD was not altered by social play behaviour, although more cells were present in the dark category. This suggests that active cells express more c-fos after social play behaviour in these regions.

Social play behaviour thus induced an inhomogeneous activity pattern in the mPFC and OFC, suggesting that the various prefrontal subregions may have a different role in modulating social play behaviour. Neonatal lesion studies have previously implicated the mPFC and OFC in social play behaviour (Bell *et al.*, 2009; Panksepp *et al.*, 1994; Pellis *et al.*, 1992; -2006; Schneider and Koch, 2005). In addition, enhanced c-fos activation after social play behaviour in rats has previously been reported in prefrontal regions (Gordon *et al.*, 2002). Separate analysis of prefrontal subregions after play has been performed in golden hamsters (Cheng *et al.*, 2008). Consistent with our study, activation was observed in the AC, PrL, and IL, whereas the OFC was not investigated (Cheng *et al.*, 2008).

Lesions of the mPFC and OFC have indicated dissociable roles of these regions in social play behaviour. Thus, neonatal lesions of the OFC did not alter the performance of play itself, but disrupted the partner-related modulation of play (Pellis *et al.*, 2006). Adolescent rats respond in a distinct manner to dominant, subordinate and female rats, but animals with neonatal lesions of the OFC responded to all different partners alike (Pellis *et al.*, 2006). Animals with neonatal lesions of the mPFC did show this partner-related change in social play behaviour. However, they expressed less complex defensive strategies compared to control rats (Bell *et al.*, 2009).

In summary, it appears that the different prefrontal regions have distinct functions in relation to social play behaviour. Previously, different roles for the mPFC and OFC have been reported in the shaping of social play behaviour in relation to social cues (Bell *et al.*, 2009; Pellis *et al.*, 2006). The present study points towards heterogeneous

functioning of the prefrontal regions in social play, which argues for future behavioural studies to include more detailed interventions in prefrontal function.

Corticostriatal systems

Similar to the prefrontal cortex, the different subregions of the striatum were not uniformly activated by social play behaviour. A large increase in the FpCD was observed in the dorsolateral striatum in all intensity categories, suggesting that a new population of cells was activated in this region. This activity may be related to the high level of motor and sensory activity in the play group, since the dorsolateral striatum receives a great deal of sensorimotor information (Voorn *et al.*, 2004). In addition, dopamine depletion in the striatum has been shown to result in alterations in the sequential order of play behaviour (Pellis *et al.*, 1993), which is in line with an important role for the striatum in the processing of sensory and motor information, and the translation into behaviour during social play.

Although to a lesser extent, social play behaviour induced c-fos activity in the ventral striatum as well, which confirms previous observations (Gordon *et al.*, 2002). The increase in c-fos activity in the ventral striatum was most pronounced in the lCore and mShell. Since these regions have been implicated in reward-related behaviours (Berridge and Kringelbach, 2008; Cardinal *et al.*, 2002; Haber and Knutson, 2010; Kelley, 2004; Nordquist *et al.*, 2008; Sesack and Grace, 2010; Voorn *et al.*, 2004; Zahm, 1999), the activity observed in the present experiments may be related to the rewarding properties of social play behaviour. Indeed, it has recently been reported that μ-opioid receptors in the NaCore and NaShell mediate the rewarding properties of social play behaviour (Trezza *et al.*, 2011b).

Interestingly, in the play group, correlations were observed between c-fos activity of the medial prefrontal/agranular insular regions and their striatal target regions. This is suggestive of a corticostriatal projection that is activated during social play behaviour. Previously, it has been reported that stimulation of cortical regions results in topographically organised induction of immediate early genes in the striatum (Miyachi *et al.*, 2005; Parthasarathy and Graybiel, 1997), which suggests that c-fos activity in the striatum after play is in part caused by activity of the prefrontal cortex. At present, our understanding of the functional role of the parallel corticostriatal connections is limited. The projection from the mPFC to the dorsomedial striatum has previously been implicated in attention and goal-directed behaviour (Balleine *et al.*, 2009; Christakou *et al.*, 2001) and the mPFC to NaCore projection has been implicated in the integration of information about consequences of actions in relation to anticipated reward (Christakou *et al.*, 2004), as well as in appetitive Pavlovian conditioning (Parkinson *et al.*, 2000) and reinstatement of drug seeking (McFarland *et al.*, 2003). Although these studies investigated the involvement of these projections in a different type of behaviour, related processes may be involved during social play behaviour. Hypothetically, dorsal prefrontal-dorsomedial striatum projections may be involved in the sequential and temporal organization of play behaviour, whereas the ventral prefrontal-ventral striatum projections underlie the rewarding aspects of social play behaviour. Interestingly, lesions of the mPFC and depletion of dopamine in the striatum both resulted in the use of less complex defence strategies during play (Bell *et al.*, 2009; Pellis *et al.*, 1993), indicating that the generation

of a playful defence strategy is indeed subserved by a corticostriatal projection.

In contrast to the mPFC and agranular insular cortex, no correlations were observed between the OFC and its striatal target regions, although social play-induced c-fos activity was observed in the OFC. It cannot be excluded that the overlap in striatal projection areas of mPFC and OFC projections masked a correlation between OFC and striatal activity. As part of the c-fos activity in the OFC projection regions may have been related to input from the mPFC, this may have overshadowed a correlation between activity in the OFC and striatal projection. Nevertheless, the correlation between mPFC and its striatal projection is apparently stronger than the possible correlation between OFC and striatum. Possibly, the OFC exerts its effects on play via different routes, with the amygdala as a potential candidate, since correlations were observed between OFC and amygdala regions (Table 2, Fig. 9). The OFC-amygdala connection is known to be involved in behavioural flexibility (Churchwell *et al.*, 2009), and the encoding of the value of expected outcomes of actions (Schoenbaum *et al.*, 2003), which may play a role in social play behaviour as well.

To conclude, cellular activity induced by social play behaviour is topographically organised in the corticostriatal system. The most pronounced alterations were observed in the dorsal mPFC, medial OFC, dorsal striatum, lCore and mShell. In addition, correlation analysis indicated a role for the mPFC-striatum and agranular insular cortex-striatum projections in social play behaviour.

Amygdala

Social play behaviour increased the expression of c-fos in the anterior LA, anterior BLA, and the BNST, which has been put forward to be part of the extended amygdala (Alheid, 2003), while in the MeA and CeA no increases in the FpCD were observed. In the anterior BLA, no overall changes in FpCD were observed, but the FpCD was enhanced in the dark cell intensity category. These results suggest that in the BLA no new cells were recruited, but that the same cells expressed more c-fos after a play session. Of the amygdala nuclei, the BLA provides most inputs to the striatum (Kelley *et al.*, 1982; Voorn *et al.*, 2004). Interestingly, the c-fos activity in the BLA correlated only in the play group with the activity in most parts of the striatum, with the exception of the most dorsolateral regions, which hardly receive amygdaloid projections (Kelley *et al.*, 1982). These results indicate a role for the amygdalo-striatal projections in social play behaviour. In addition, c-fos activity in the BLA correlated with most regions of the mPFC, several of the OFC regions, and the agranular insular cortex, suggesting an involvement of the BLA-prefrontal cortex projections in social play behaviour as well.

In line with our findings, induction of c-fos expression in the BNST has previously been reported after play in golden hamsters (Cheng *et al.*, 2008). In contrast to the present study, in golden hamsters social play-induced c-fos expression was also observed in the MeA (Cheng *et al.*, 2008). Interestingly, in our study correlations were observed between the c-fos activity in the MeA and several orbitofrontal regions, suggesting that in rats the MeA-cortical connections may indeed play a role in social play behaviour.

It is widely accepted that the amygdala is important for the processing of negative emotions (Maren and Quirk, 2004; Morrison and Salzman, 2010; Phelps and Ledoux, 2005). Over the past years, it has become clear that the amygdala is important for the

processing of positive emotions as well (Baxter and Murray, 2002; Cardinal *et al.*, 2002; Morrison and Salzman, 2010). Since one of the key features of social play behaviour is its positive emotional value (Trezza *et al.*, 2011a; Vanderschuren, 2010), it is reasonable to assume that the amygdala is involved in the pleasurable properties of play behaviour. Indeed, the size of the amygdala has been correlated with play behaviour in nonhuman primates, with a larger amygdala size being associated with a higher percentage of time spent on social play behaviour (Lewis and Barton, 2006). In addition, neonatal lesions of the amygdala have been reported to affect social play behaviour (Daenen *et al.*, 2002; Meaney *et al.*, 1981), although these effects may be sex-specific (Meaney *et al.*, 1981). Furthermore, recently it has been shown that the BLA is the site of action where endocannabinoids modulate social play reward (Trezza *et al.*, 2012). Enhanced levels of endocannabinoid activity were found to be associated with increased social play behaviour, and increased endocannabinoid neurotransmission within the BLA was necessary and sufficient for this enhancement of social play behaviour (Trezza *et al.*, 2012).

Taken together, these results indicate that the amygdala has a facilitating role in social play, receiving environmental information and modulating playful activities (Siviy and Panksepp, 2011; Trezza *et al.*, 2012). The present study shows that during play behaviour, cellular activity in the medial and OFC is correlated with activity in the amygdala, which supports the hypothesis that cortico-amygdala connections are important for social play behaviour. In addition, the correlations observed in the present study between the amygdala and ventral striatum regions as well as the known role of these structures in reward processes (Baxter and Murray, 2002; Berridge and Kringelbach, 2008; Cardinal *et al.*, 2002; Morrison and Salzman, 2010), suggest an involvement of amygdalo-striatal connections in the rewarding aspects of social play behaviour.

Thalamus

Social play increased c-fos expression in the PC, lateral IMDl, and PV thalamic nuclei. The enhancement of the FpCD in these regions was equally distributed over the different intensity categories, suggesting that a new population of cells was recruited by social play in these areas. In addition, a correlation between the PC and its striatal target region and the IMDl and its cortical target region were observed in the play group. A previous study, investigating the involvement of these regions in social play behaviour using c-fos immunohistochemistry, measured the MD and the midline and intralaminar thalamic nuclei as a whole and did not observe c-fos activation after play (Gordon *et al.*, 2002), which could be caused by a heterogeneous response of the various subnuclei.

Lesions of the dorsomedial thalamus, posterior thalamus and the parafascicular region of the thalamus have been found to decrease social play behaviour (Siviy and Panksepp, 1985; Siviy and Panksepp, 1987). This has led to the hypothesis that the thalamic nuclei serve as an important relay station in the processing of information to the striatum and prefrontal cortex during social play behaviour (Siviy and Panksepp, 2011). In addition, the intralaminar and midline thalamic nuclei are known to have a central role in arousal and attention processes (Groenewegen and Berendse, 1994). The present study indicates a specific and discrete activation in the midline and intralaminar thalamic nuclei after play, which may be related to an aroused state required for social play behaviour.

The intralaminar and midline thalamic nuclei have strong topographically organised projections towards the striatum and prefrontal cortex (Groenewegen and Witter, 2004; Voorn *et al.*, 2004), regions that were highly activated by play in the present study. However, few correlations were observed between the thalamus and its striatal and prefrontal projection regions. Apparently, the discrete projections originating from the thalamus are not decisive for the neuronal activity patterns in the prefrontal cortex and striatum. Further detailed manipulations of its subnuclei are required to better understand the role of the thalamus in social play behaviour, not least since little is known regarding the behavioural function of the individual thalamo-cortico-striatal circuits (Groenewegen and Berendse, 1994).

Striatal output structures

Besides the several striatal input structures, striatal output structures were also investigated. In the lateral globus pallidus (lGP), a significant decrease in the FpCD was observed after play behaviour. According to basal ganglia connectivity concepts (Gerfen, 2004), this is in line with the large increase in FpCD observed in the striatum, which has strong GABAergic projections towards the lGP. Activity in the lGP did not correlate with the activity in any of the striatal regions, however. This may be explained by the fact that the c-fos activity was determined in the lGP as a whole and not in subregions which are known to project to the striatum in a topographic pattern (Gerfen, 2004).

In the ventral pallidum, no effects were observed on the FpCD, although the c-fos activity in this region did correlate with several of the medial and ventral striatal regions, including the NaCore and NaShell. The fact that these correlations were positive makes it difficult to interpret the correlation in terms of functional changes restricted to the nucleus accumbens to VP projections, which are GABAergic (Gerfen, 2004). However, a seemingly paradoxical positive correlation between striatal and pallidal activity has previously been observed in studies examining the effect of dopamine depletion on striatopallidal function (Chesselet and Delfs, 1996). Instead of decreased activity in lGP upon activation of striatal input to the nucleus - as would be predicted by current basal ganglia models - an increase in deoxyglucose uptake and in metabolic activity of lGP GABAergic neurons was seen. This has been suggested to be caused by major changes in pallidal firing patterns and GABAergic neurotransmission in lGP. Interestingly, in the present experiments the correlations turned from negative to positive in non-playing vs. playing animals, respectively. This suggests a particular functional involvement of the ventral pallidum during play behaviour.

The SNr is another important output structure of the striatum. It is known that the SNr receives major GABAergic outputs from the striatum (Gerfen, 2004). However, in the present study a positive correlation was observed with the mCore in the play group and the lCore in the no-play group. By analogy to the correlation between VP and nucleus accumbens discussed above, such a positive correlation may be explained by changes in GABAergic activity in SNr that occur as a consequence of striatal activity. However, an alternative explanation may be that other than striatal inputs to pallidum or SNr contribute to the c-fos activity observed in these regions. For example, considering the lGP and VP, it may be that a sub-threshold, viz. non-significant, change in FpCD occurs in a subpopulation of neurons that is contacted by sources other than the striatum, such as

the cortex or subthalamic nucleus (Voorn, 2010).

Monoamine nuclei and related regions

Social play behaviour did not detectably enhance the levels of c-fos in the VTA and SN. Considering the major role of dopamine in motivational processes (Berridge, 2007; Robbins and Everitt, 2007; Salamone *et al.*, 2005), this was an unexpected result. However, the involvement of dopaminergic neurotransmission in social play behaviour is not straightforward. Thus, augmenting dopaminergic neurotransmission using receptor agonists or reuptake blockers does not invariably lead to increases in social play. In fact, modest increases as well as decreases have been found (Beatty *et al.*, 1984; Niesink and Van Ree, 1989; Siviy *et al.*, 1996; Vanderschuren *et al.*, 2008). Possibly, social play behaviour recruits only a small subset of dopaminergic neurons, which results in activation below the detection limit. Indeed, the anatomical organization of the VTA is highly heterogeneous (Ikemoto, 2007).

Despite the absence of a significant effect of play on c-fos activity as such, correlations were observed between the VTA/SN regions and their corticostriatal target regions. The VTA and SN provide a dopaminergic projection to the prefrontal cortex and striatum and this output is reciprocated by GABAergic and glutamatergic inputs from these regions (Gerfen *et al.*, 1987; Gerfen, 2004; Lammel *et al.*, 2008; Lammel *et al.*, 2011). Regarding the prefrontal cortex , the VTA showed correlations with all the mPFC regions and the ventrolateral orbitofrontal region, the latter being surprising considering the low number of dopamine fibres in the VLO (Van De Werd and Uylings, 2008). The VTA mainly projects to the more ventral parts of the striatum (Gerfen *et al.*, 1987; Lammel *et al.*, 2011) and correlations were observed between the VTA and NaShell and olfactory tubercle. In contrast, correlations between the SNc and striatum and prefrontal cortex were not widespread, suggesting that the increased SNc activity is not a main contributor to c-fos activity in the striatum. These results suggest that the dopaminergic modulation of the striatum and prefrontal cortex from the VTA plays a role in social play behaviour. It is likely that these projections are important for motivational and cognitive control aspects of social play behaviour, mediated by the ventral striatum and prefrontal cortex, respectively (Berridge, 2007; Dalley *et al.*, 2004; Ikemoto and Panksepp, 1999; Robbins and Arnsten, 2009; Robbins and Everitt, 2007; Salamone *et al.*, 2005; Seamans and Yang, 2004) .

Induction of c-fos activity after play was observed in the DR, which fits with the involvement of serotonin in social play behaviour (Homberg *et al.*, 2007; Siviy *et al.*, 2011; Vanderschuren *et al.*, 1997). The mechanism by which serotonin modulates social play behaviour is not completely understood, due to the high complexity and widespread distribution of serotonin receptors, but this induction of c-fos expression in the DR is supportive of a role for the serotonin system in social play.

Social play behaviour did not induce c-fos expression in the LC, despite the known involvement of noradrenaline in social play behaviour (Trezza *et al.*, 2010; Vanderschuren *et al.*, 2008). Enhancement of endogenous noradrenaline levels has been shown to disrupt social play behaviour (Vanderschuren *et al.*, 2008), suggesting that high levels of noradrenaline and social play are incompatible. It is possible that during social play behaviour, the release of noradrenaline is limited by the LC and therefore no

c-fos induction is observed in this region. Generally, basal c-fos levels are low and as a consequence reductions in c-fos levels are difficult to detect (Kovacs, 2008).

With respect to regions of the tegmentum that regulate activity in the monoamine nuclei, the RMTg did not show an overall increase in FpCD, while more cells were detected in the dark intensity category, suggesting that the same population of neurons expressed more c-fos after social play behaviour. In the PPTg, a new population of cells may be recruited by social play behaviour, since an overall increase in FpCD was observed which was not dependent on intensity levels of the cells. The RMTg has recently received a great deal of attention regarding its role in regulating the activity of VTA dopamine neurons, whereby increased activity of the RMTg results in inhibition of dopaminergic neurons (Barrot *et al.*, 2012; Hong *et al.*, 2011; Jhou *et al.*, 2009a; Jhou *et al.*, 2009b). However, since the RMTg also targets other regions (Barrot et al., 2012; Jhou *et al.*, 2009b), and the identity of the c-fos positive neurons in the RMTg is at present unknown, the implications of these changes in RMTg activity for social play remain as yet unclear.

Besides in the RMTg, social play behaviour induced c-fos expression in the PPTg, while no changes were observed in the LDTg. The PPTg and LDTg provide cholinergic and glutamatergic projections towards the dopamine producing regions, where axons from the PPTg mainly reach the SNc and those from the LDTg mainly target the VTA (Forster and Blaha, 2000; Holmstrand and Sesack, 2011). Both regions are important for regulating the activity of dopaminergic neurons, where activity in these regions is thought to enhance dopamine release (Blaha *et al.*, 1996; Blaha and Winn, 1993). The increased c-fos expression observed in the PPTg after social play behaviour, may therefore be related to its function in regulating dopamine activity.

Concluding remarks

The current study shows that social play behaviour induced cellular activity in a wide array of neural structures, which is consistent with the complex nature of this behaviour. Previous immediate early gene expression studies investigated a number of regions that were also investigated in our study. Consistent activation was observed after play behaviour in dorsal and ventral striatum (Gordon *et al.*, 2002), AC, PrL, IL and BNST (Cheng *et al.*, 2008). Contrasting results compared to the present study were reported for the MeA, where Cheng *et al.* (2008) observed an increase in activity and the AC, OFC, and dorsomedial thalamus where Gordon *et al.* (2002) observed no changes. These discrepancies may be related to experimental differences between the studies. First, the study by Cheng *et al.* (2008) investigated social play behaviour in golden hamsters, whereas Gordon *et al.* (2002) and the present study used rats. Given the subtle differences in the structure of social play behaviour between rodent species (Pellis and Pellis, 1998), it is conceivable that the neural substrates of social play behaviour in rats and hamsters somewhat differ. The absence of an increase in the AC, OFC, and thalamus after social play behaviour in the Gordon *et al.* (2002) study may be related to the fact that in that study a portion of these regions using a rectangle or circle template was measured, whereas in the present study the AC, OFC and thalamus subregions were measured separately and the borders of these regions were defined according to adjacent Nissl-stained sections. Last, both Gordon *et al.* (2002) and Cheng *et al.* (2008) detected c-fos protein levels using immunohistochemistry, whereas in the present study c-fos mRNA levels were measured

by in situ hybridisation. Thus, the differences in the results may also be related to distinct effects of social play on c-fos mRNA expression versus Fos protein levels.

In the present study, the neural substrates of social play behaviour were investigated in male rats. The structure and intensity of social play behaviour in rats somewhat differs between males and females (Pellis *et al.*, 1997). It is therefore well conceivable that social play behaviour in female rats relies on activity in a neural network, that is not identical to the network in male rats identified here, even if it is likely that there is substantial overlap between the neural substrates of social play in male and female rats.

In conclusion, the present study substantially furthers our knowledge on the neural substrates of social play behaviour by investigating a large number of brain regions that may be relevant to social play. The changes in c-fos expression induced by social play behaviour were analysed in considerable anatomical detail, allowing for the detection of subregional differences in social play-induced neuronal activity. Relationships in social play-induced cellular activity between connected regions were also taken into account, leading us to propose a potential neural network of social play behaviour as presented in Fig.9. These data indicate that activity of corticostriatal systems, together with the amygdala and monoaminergic systems, underlies social play. By demonstrating how several interconnected systems in the brain are involved in this behaviour, the present study provides an elaborate starting point for functional investigations aimed at improving our understanding of the neurobiology of social play behaviour.

Table 1

Region	Subregion	group	Light	Medium	Dark
medial prefrontal cortex	AC	No Play	64,38 (±7.99)	64,26 (±3,11)	64,84 (±8,05)
		Play	49,63 (±3.37)	72,75 (±6,14)	117,35(±9,92) **
	PrLd	No Play	71,39 (±6,40)	74,28 (±2,84)	75,43 (±8,88)
		Play	54,71 (±7,98)	72,44 (±2,36)	134,13 (±13,43) **
	PrLv	No Play	66,05 (±6,32)	69,35 (±2,81)	69,12 (±7,74)
		Play	56,72 (±4,72)	70,86 (±3,89)	116,97 (±12.69) *
	IL	No Play	62,12 (±7,74)	63,83 (±2,47)	63,60 (±9,15)
		Play	53,33 (±6,61)	62,23 (±3,51)	92,80 (±8,56) *
orbitofrontal cortex	MO	No Play	58,33 (±6,84)	60,46 (±5,25)	57,09 (±5,03)
		Play	55,57 (±7,18)	63,04 (±3,28)	106,06 (±5,57)***
	VO	No Play	73,80 (±6,88)	73,83 (±3,79)	76,76 (±7,87)
		Play	63,91(±8,38)	77,60 (±3,29)	115,61 (±10,70) *
	VLO	No Play	94,90 (±5,31)	94,78 (±4,36)	95,63 (±10,59)
		Play	68,39 (±5,31)	91,39 (±2,65)	162,23 (±15,35) **
	LO	No Play	93,15 (±5,60)	92,06 (±3,58)	88,88 (±9,99)
		Play	65,65 (±6,97) *	78,11 (±4,81) *	122,05 (±15,02)
	DLO	No Play	56,47 (±7,01)	56,47 (±4,06)	55,06 (±8,10)
		Play	35,20 (±4,95) *	44,59 (±7,57)	69,75 (±9,05)
Agranular insular cortex	AIv	No Play	61,55 (±6,78)	62,51 (±3,63)	60,81 (±7,41)
		Play	44,12 (±4,39)*	64,17 (±5,26)	82,87 (±11,02)
	AId	No Play	51,87 (±5,88)	52,77 (±3,63)	52,49 (±6,08)
		Play	31,31 (±3,68) *	46,93 (±4,98)	70,46 (±11,62)
Striatum	Core	No Play	13,00 (±1.36)	13,48 (±1,36)	13,23 (±1,60)
		Play	11,98 (±1,10)	18,88 (±1,17) *	23,65 (±3,28) *
	mCore	No Play	13,70 (±1.05)	15,18 (±1,51)	15,90 (±2,43)
		Play	11,98 (±0,62)	19,32 (±1,87)	26,90 (±5,89)
	lCore	No Play	12,74 (±1,89)	13,13 (±0,73)	14,15 (±1,17)
		Play	12,02 (±2,12)	17,45 (±0,70) **	20,50 (±3,21) **
	Shell	No Play	18,31 (±1,12)	18,93 (±1,58)	18,27 (±1,39)
		Play	14,42 (±1,38)	22,90 (±1,39)	29,46 (±3,71) *
	mShell	No Play	17,73 (±1,58)	18,94 (±2,00)	21,33 (±5,68)
		Play	14,24 (±1,47)	22,01 (±1,80) *	26,48 (±4,12) *
	vShell	No Play	18,27 (±1,27)	19,29 (±1,75)	19,39 (±2,78)
		Play	16,36 (±1,98)	23,16 (±2,45)	26,31 (±4,61)
	lShell	No Play	21,01 (±2,29)	20,89 (±1,55)	22,30 (±3,07)
		Play	19,59 (±2,76)	25,09 (±1,67)	30,26 (±3,65)
	DS	No Play	10,02 (±0,85)	10,39 (±1,09)	10,29 (±1,09)
		Play	12,58 (±0,75) *	21,67 (±1,01) **	31,86 (±3,79) ***
	Otub	No Play	33,67 (±2,61)	34,28 (±3,46)	34,34 (±4,01)
		Play	22,12 (±2,33) **	33,21 (±2,60)	45,74 (±6,92)
PFC inputs to the striatum	STR-SomSen	No Play	7,46 (±0,071)	7,41 (±0,84)	7,46 (±0,059)
		Play	11,70 (±0,69) ***	21,97 (±1,38) ***	37,95 (±3.46) ***
	STR-AC	No Play	10,79 (±1,13)	11,27 (±1,08)	10,98 (±1,13)
		Play	12,30 (±1,35)	22,73 (±1,43) *	29,02 (±3,23) *

Table 1 continued

Region	Subregion	group	Light	Medium	Dark
	STR-PrLd	No Play	13,73 (±1,07)	14,15 (±1,96)	13,92 (±1,90)
		Play	12,15 (±0,70)	18,64 (±1,94)	27,32 (±3,25) *
	STR-PrLv	No Play	13,21 (±1,15)	13,50 (±1,55)	13,32 (±1,99)
		Play	11,02 (±0.86)	19,52 (±1,70)	26,70 (±4,38) *
	STR-IL	No Play	22,02 (±2,22)	21,91 (±2,68)	21,79 (±2,18)
		Play	14,96 (±1,63) *	21,09 (±2,23)	32,70 (±4,81)
	STR-AIv	No Play	33,09 (±2,08)	32,93 (±3,45)	33,65 (±3,97)
		Play	22,96 (±2,46) *	34,15 (±2,33)	44,67 (±6,39)
	STR-AId	No Play	12,22 (±1,87)	11,95 (±1,09)	11,61 (±1,16)
		Play	7,83 (±0,59)	13,95 (±1,34)	18,38 (±2,74)
	STR-MO/VO	No Play	11,15 (±0,73)	11,57 (±1,60)	10,96 (±1,73)
		Play	11,07 (±0,78)	14,19 (±1,84)	25,42 (±6,61)
	STR-VLO	No Play	10,12 (±1,34)	10,46 (±1,25)	10,16 (±1,20)
		Play	12,98 (±1,17)	20,28 (±1,11) ***	25,91 (±2,79) ***
	STR-LO/DLO	No Play	7,22 (±0,59)	7,10 (±0,91)	7,11 (±0,63)
		Play	10,23 (±1,05) *	20,45 (±1,56) ***	34,17 (±3,05) ***
Amygdala -2.56 mm from Bregma	LA	No Play	30,35 (±2,87)	30,51 (±2,72)	27,37 (±3,80)
		Play	22,77 (±3,31)	40,47 (±2,73) *	45,20 (±6,27) *
	BLA	No Play	37,34 (±4,31)	35,77 (±3,03)	29,39 (±5,12)
		Play	28,31 (±3,91)	40,70 (±4,52)	49,68 (±6,78) *
	CeA	No Play	11,29 (±1,31)	11,63 (±1,09)	10,31 (±3,29)
		Play	11,94 (±2,59)	17,76 (±4,00)	30,52 (±12,82)
	MeA	No Play	24,46 (±2,51)	27,89 (±2,23)	24,43 (±4,46)
		Play	23,67 (±1,47)	30,74 (±2,46)	39,41 (±6,02)
Amygdala -3.30 mm from Bregma	LA	No Play	48,95 (±3,04)	47,74 (±3,85)	41,25 (±6,49
		Play	47,65 (±7,81)	53,67 (±4,93)	57,58 (±5,40)
	BLA	No Play	26,32 (±3,05)	27,89 (±1,60)	26,72 (±2,40)
		Play	26,74 (±4,08)	31,23 (±3,42)	34,01 (±3,26)
	CeA	No Play	25,36 (±3,77)	28,13 (±4,55)	30,50 (±8,10)
		Play	31,92 (±5,21)	30,42 (±5,49)	33,99 (±9,12)
	MeA	No Play	34,10 (±5,08)	33,43 (±3,47)	32,48 (±4,38)
		Play	26,89 (±5,75)	33,72 (±8,41)	44,92 (±7,28)
BNST	BNST	No Play	7,44 (±1,54)	8,19 (±0,90)	7,70 (±1,02)
		Play	8,38 (±2,22)	11,42 (±1,42)	16,05 (±1,57)
Thalamus	MD	No Play	57,89 (±7,67)	57,74 (±2,03)	54,84 (±9,31)
		Play	66,25 (±7,37)	60,57 (±7,80)	55,21 (±11.57)
	CL	No Play	66,84 (±7,18)	68,32 (±7,05)	64, 83 (±10,62)
		Play	82,98 (±9,68)	68,63 (±7,56)	64,16 (±9,74)
	PC	No Play	62,75 (±6,36)	67,10 (±4,10)	64,91 (±11,28)
		Play	75,11 (±11,20)	81,71 (±9,01)	78,39 (±11,92)
	CeM	No Play	83,49 (±7,45)	86,72 (±6,97)	87,95 (±13,66)
		Play	94,07 (±11,51)	104,79 (±8,92)	104,51 (±17.65)
	IMDI	No Play	37,12 (±7,14)	36,55 (±5,30)	41,21 (±9,53)
		Play	40,01 (±7,16)	51,02 (±5,95)	91,37 (±18,58)

Table 1 continued

Region	Subregion	group	Light	Medium	Dark
	IMDm	No Play Play	67,69 (±8,28) 60,60 (±8,97)	66,29 (±6,25) 70,37 (±9,62)	67,74 (±7,53) 89,54 (16,66)
	PVl	No Play Play	53,11 (±8,45) 51,64 (±8,56)	53,19 (±4,56) 81,53 (±9,43)	56,17 (±10.25) 56,15 (±10.51)
	PVm	No Play Play	82,93 (±10,66) 86,77 (±7,85)	84,15 (±6,54) 86,64 (±11,71)	80,69 (±10,19) 83,36 (±9,89)
Pallidum	lGP	No Play Play	5,97 (±1,47) 1,51 (±0,48)	5,59 (±1,45) 3,91 (±0,99)	6,34 (±1,54) 5,13 (±0,63)
	VP	No Play Play	8,59 (±2,18) 6,17 (±1,01)	9,20 (±1,35) 8,10 (±0.99)	9,20 (±1,54) 7,24 (±1,20)
VTA/SN	VTA	No Play Play	11,38 (±1,08) 9,17 (±1,17)	11,70 (±1,22) 11,62 (±1,62)	11,57 (±1,23) 12,64 (±2,50)
	SNc	No Play Play	11,76 (±2,06) 8,96 (±1,54)	11,92 (±2,04) 9,54 (±0,86)	12,31 (±1,83) 16,64 (±1,61)
	SNr	No Play Play	9,74 (±0,97) 8,77 (±0,80)	9,95 (±1,50) 9,76 (±1,24)	9,93 (±1,39) 10,74 (±1,50)
DR	DRa	No Play Play	19,59 (±5,96) 20,39 (±6,75)	22,15 (±3,93) 26,31 (±2,43)	21,52 (±5,05) 52,10 (±11,79)
	DRp	No Play Play	15,37 (±2,75) 20,95 (±6,75)	15,85 (±1,94) 19,91 (±2,79)	15,76 (±2,83) 21,58 (±3,65)
LC	LC	No Play Play	21,33 (±4,81) 28,87 (±8,34)	18,58 (±2,77) 19,91 (±2,79)	20,77 (±5,82) 19,88 (±6,88)
Tegmentum regions	RMTg	No Play Play	10,31 (±3,72) 2,80 (±0,71)	9,27 (±1,43) 9,06 (±1,18)	8,94 (±1,80) 15,07 (±1,43) *
	LDTg	No Play Play	16,66 (±3,50) 27,25 (±6,44)	15,62 (±2,39) 25,92 (±4,26)	14,90 (±3,36) 26,52 (±6,65)
	PPTg	No Play Play	15,48 (±4,18) 28,19 (±6,74)	13,55 (±2,43) 22,19 (±2,94)	12,43 (±1,94) 16,73 (±13,25)

Table 1: The c-fos positive cell density induced by social play behaviour divided into three categories of intensity levels (light, medium, dark). The c-fos positive cell density was determined in rats after receiving a play session ('play' group) and in rats that were placed in the test cage without a partner present ('no play' group). All regions measured are included and data are presented as the mean ± SEM. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

Table 2

		Play		No Play			Play			No Play	
Reg. 1	**Reg. 2**	**Cor.C**	**p val.**	**Cor.C**	**P val.**	**Reg. 1**	**Reg. 2**	**Cor.C**	**p val.**	**Cor.C**	**p val.**
Prefrontal - striatum						***Amygdala (-3.30 mm) - striatum (continued)***					
AC	STR-AC	0.357	0.432	0.464	0.294	BLA	lCore	0.321	0.482	-0.657	0.156
PrLd	STR-PrLd	0.738	0.037	0.214	0.645	BLA	mShell	0.964	<0.001	0.143	0.787
PrLv	STR-PrLv	0.952	<0.001	0.357	0.432	BLA	vShell	0.893	0.007	0.371	0.468
IL	STR-IL	0.857	0.014	-0.071	0.879	BLA	lShell	0.464	0.294	-0.029	0.957
MO	STR-MO/VO	0.536	0.215	-0.071	0.879	BLA	OTu	0.679	0.093	0.600	0.208
VO	STR-MO/VO	0.464	0.294	0.607	0.148	***Amygdala (-2.56 mm) - prefrontal***					
VLO	STR-VLO	0.321	0.482	-0.107	0.819	BLA	AC	0.943	0.005	0.715	0.111
LO	STR-LO/DLO	-0.214	0.645	-0.536	0.215	BLA	PrL	0.943	0.005	-0.600	0.208
DLO	STR-LO/DLO	-0.486	0.329	0.036	0.939	BLA	IL	0.829	0.042	-0.714	0.111
AId	STR-AId	0.881	0.004	0.107	0.819	BLA	MO	0.543	0.266	-0.086	0.872
AIv	STR-AIv	0.881	0.004	0.714	0.071	BLA	VO	0.600	0.208	-0.371	0.468
Thalamus - striatum						BLA	VLO	>0.999	<0.001	0.257	0.623
CL	STR-SomSen	-0.357	0.879	-0.771	0.072	BLA	LO	0.886	0.019	0.714	0.111
PC	STR-AC	-0.086	0.872	-0.086	0.872	BLA	DLO	0.943	0.005	0.143	0.787
CeM	STR-PrLd	0.607	0.148	-0.200	0.704	BLA	AId	0.893	0.007	-0.714	0.111
PVm	STR-PrLv	0.785	0.036	0.314	0.544	BLA	AIv	>0.999	<0.001	0.257	0.623
PVl	STR-IL	-0.321	0.482	0.314	0.544	LA	AC	-0.257	0.623	-0.086	0.872
IMDl	STR-AIv	0.750	0.052	-0.086	0.872	LA	PrL	-0.543	0.266	0.314	0.544
IMDm	STR-AId	0.679	0.094	-0.428	0.397	LA	IL	-0.257	0.623	-0.086	0.872
Thalamus - prefrontal						LA	MO	-0.290	0.957	-0.029	0.957
PC	AC	0.400	0.504	0.143	0.787	LA	VO	-0.486	0.329	-0.086	0.872
CeM	PrLd	0.771	0.072	0.086	0.871	LA	VLO	-0.429	0.397	0.543	0.260
PVm	PrLv	0.600	0.208	0.429	0.397	LA	LO	-0.486	0.329	0.771	0.072
PVl	IL	-0.429	0.397	0.543	0.266	LA	DLO	-0.086	0.872	0.314	0.544
IMDl	AIv	0.786	0.036	0.429	0.397	LA	AId	<0.001	>0.999	-0.314	0.544
IMDm	AId	0.642	0.119	-0.657	0.156	LA	AIv	-0.071	0.879	0.657	0.156
Amygdala (-2.56 mm) - striatum						CeA	AC	0.429	0.937	-0.771	0.072
BLA	DS	0.036	0.939	-0.543	0.266	CeA	PrL	0.200	0.704	-0.314	0.544
BLA	mCore	0.821	0.023	-0.371	0.468	CeA	IL	-0.257	0.623	-0.771	0.072
BLA	lCore	0.393	0.383	-0.771	0.072	CeA	MO	-0.314	0.544	-0.600	0.208
BLA	mShell	0.821	0.023	-0.371	0.468	CeA	VO	-0.371	0.468	-0.600	0.208
BLA	vShell	0.536	0.215	-0.143	0.787	CeA	VLO	0.257	0.623	0.371	0.468
BLA	lShell	0.821	0.023	0.486	0.329	CeA	LO	0.657	0.156	0.714	0.111
BLA	OTu	0.786	0.036	0.200	0.704	CeA	DLO	0.257	0.623	0.714	0.111
Amygdala (-3.30 mm) - striatum						CeA	AId	<0.001	>0.999	-0.257	0.623
BLA	DS	0.107	0.819	-0.371	0.468	CeA	AIv	0.214	0.645	0.429	0.397
BLA	mCore	0.821	0.023	0.143	0.787	MeA	AC	0.714	0.111	0.029	0.957

Table 2 continued

		Play		No Play				Play		No Play	
Reg. 1	**Reg. 2**	**Cor.C**	**p val.**	**Cor.C**	**P val.**	**Reg. 1**	**Reg. 2**	**Cor.C**	**p val.**	**Cor.C**	**p val.**
Amygdala (-2.56 mm) - prefrontal (continued)						***Amygdala (-3.30 mm) - prefrontal (continued)***					
MeA	PrL	0.943	0.005	-0.029	0.957	CeA	AIv	0.143	0.760	-0.143	0.787
MeA	IL	0.943	0.005	0.029	0.957	MeA	AC	0.600	0.208	0.086	0.872
MeA	MO	0.771	0.072	-0.143	0.787	MeA	PrL	0.829	0.042	-0.200	0.704
MeA	VO	0.829	0.042	0.029	0.957	MeA	IL	0.600	0.208	0.086	0.872
MeA	VLO	0.886	0.019	0.771	0.072	MeA	MO	0.314	0.544	-0.086	0.872
MeA	LO	0.600	0.208	0.543	0.266	MeA	VO	0.714	0.111	0.600	0.208
MeA	DLO	0.771	0.072	0.086	0.872	MeA	VLO	0.771	0.072	-0.200	0.704
MeA	AId	0.821	0.023	-0.086	0.872	MeA	LO	0.714	0.111	-0.657	0.156
MeA	AIv	0.929	0.003	0.543	0.266	MeA	DLO	0.829	0.042	-0.200	0.704
Amygdala (-3.30 mm) - prefrontal						MeA	AId	0.500	0.253	0.771	0.072
BLA	AC	0.771	0.072	-0.086	0.872	MeA	AIv	0.714	0.071	-0.086	0.872
BLA	PrL	0.943	0.005	-0.086	0.872	***VTA/SN - striatum***					
BLA	IL	0.943	0.005	-0.086	0.872	VTA	DS	<0.001	>0.999	0.371	0.468
BLA	MO	0.771	0.072	0.086	0.872	VTA	mCore	0.679	0.094	0.086	0.872
BLA	VO	0.600	0.208	0.086	0.872	VTA	lCore	0.143	0.760	0.429	0.397
BLA	VLO	0.886	0.019	0.771	0.072	VTA	mShell	0.786	0.036	0.086	0.872
BLA	LO	0.657	0.156	0.886	0.019	VTA	vShell	0.821	0.023	-0.086	0.872
BLA	DLO	0.714	0.111	0.086	0.872	VTA	lShell	0.286	0.535	0.486	0.329
BLA	AId	0.857	0.014	-0.426	0.397	VTA	OTu	0.821	0.023	0.029	0.957
BLA	AIv	0.714	0.071	0.771	0.072	SNc	DS	-0.500	0.253	0.371	0.468
LA	AC	0.257	0.623	0.029	0.957	SNc	mCore	-0.357	0.432	0.314	0.544
LA	PrL	0.600	0.208	0.029	0.957	SNc	lCore	-0.286	0.535	-0.086	0.872
LA	IL	0.829	0.042	0.029	0.957	SNc	mShell	-0.571	0.180	0.314	0.544
LA	MO	0.543	0.266	-0.257	0.623	SNc	vShell	-0.643	0.119	0.371	0.468
LA	VO	0.943	0.005	0.543	0.266	SNc	lShell	-0.571	0.180	0.771	0.072
LA	VLO	0.543	0.266	0.714	0.111	SNc	OTu	-0.393	0.383	0.429	0.397
LA	LO	0.143	0.787	0.257	0.623	SNr	DS	-0.464	0.294	0.600	0.208
LA	DLO	0.714	0.111	0.143	0.787	SNr	mCore	0.929	0.003	0.086	0.872
LA	AId	0.679	0.094	0.543	0.266	SNr	lCore	0.214	0.645	0.943	0.005
LA	AIv	0.714	0.071	0.771	0.072	SNr	mShell	0.750	0.052	0.086	0.872
CeA	AC	-0.086	0.872	-0.314	0.544	SNr	vShell	0.429	0.337	0.086	0.872
CeA	PrL	-0.486	0.329	-0.029	0.957	SNr	lShell	0.357	0.432	0.257	0.623
CeA	IL	-0.771	0.072	-0.314	0.544	SNr	OTu	0.643	0.119	-0.429	0.397
CeA	LO	0.086	0.872	0.029	0.957	***VTA/SN - prefrontal***					
CeA	DLO	0.371	0.468	0.771	0.072	VTA	AC	0.829	0.042	-0.543	0.266
CeA	AId	0.071	0.879	0.270	0.623	VTA	PrL	0.886	0.019	-0.600	0.208

Table 2 continued

		Play		No Play	
Reg. 1	**Reg. 2**	**Cor.C**	**p val.**	**Cor.C**	**P val.**
VTA/SN - prefrontal (continued)					
VTA	IL	0.886	0.019	-0.543	0.266
VTA	MO	0.600	0.208	-0.143	0.787
VTA	VO	0.771	0.072	0.314	0.544
VTA	VLO	0.943	0.005	-0.486	0.329
VTA	LO	0.714	0.111	-0.429	0.397
VTA	DLO	0.700	0.188	-0.029	0.957
VTA	AId	0.679	0.094	0.371	0.468
VTA	AIv	0.607	0.148	-0.143	0.787
SNc	AC	-0.829	0.042	-0.657	0.156
SNc	PrL	-0.657	0.156	-0.714	0.111
SNc	IL	-0.371	0.468	-0.657	0.156
SNc	MO	-0.029	0.957	-0.486	0.329
SNc	VO	-0.143	0.787	-0.143	0.787
SNc	LO	-0.943	0.005	0.200	0.704
SNc	DLO	-0.800	0.104	0.257	0.623
SNc	AId	-0.607	0.148	0.086	0.872
SNc	AIv	-0.714	0.071	0.200	0.704
Pallidum - striatum					
VP	DS	0.214	0.645	-0.771	0.072
VP	mCore	0.679	0.094	-0.600	0.208
VP	lCore	0.500	0.253	-0.886	0.019
VP	mShell	0.857	0.014	-0.600	0.208
VP	vShell	0.536	0.215	-0.600	0.208
VP	lShell	0.786	0.036	-0.086	0.872
VP	OTu	0.679	0.094	-0.143	0.787
lGP	DS	-0.250	0.589	-0.714	0.111
lGP	mCore	0.179	0.702	-0.429	0.397
lGP	lCore	-0.286	0.535	-0.943	0.005
lGP	mShell	0.179	0.702	-0.429	0.397
lGP	vShell	0.000	1.000	-0.429	0.397
lGP	lShell	0.357	0.432	0.029	0.957
lGP	OTu	0.429	0.337	0.143	0.787

Table 2: Overview of correlations. Correlations were determined in the 'play' and 'no play' group for relevant projections. Cor.C = Spearman's rho correlation coefficient, p val. = p value of this correlation coefficient.

References

- Alessandri SM (1992) Attention, play, and social behavior in ADHD preschoolers. J Abnorm Child Psychol 20:289-302.
- Alheid GF (2003) Extended amygdala and basal forebrain. Ann N Y Acad Sci 985:185-205.
- Baarendse PJJ, Counotte DS, O'Donnel P, Vanderschuren LJMJ (2012) Social experience during adolescence is critical for the development of cognitive control and dopamine modulation of prefrontal cortex function. Submitted.
- Badiani A, Oates MM, Day HE, Watson SJ, Akil H, Robinson TE (1998) Amphetamine-induced behavior, dopamine release, and c-fos mRNA expression: modulation by environmental novelty. J Neurosci 18:10579-10593.
- Balleine BW, Liljeholm M, Ostlund SB (2009) The integrative function of the basal ganglia in instrumental conditioning. Behav Brain Res 199:43-52.
- Barrot M, Sesack SR, Georges F, Pistis M, Hong S, Jhou TC (2012) Braking Dopamine Systems: A New GABA Master Structure for Mesolimbic and Nigrostriatal Functions. J Neurosci 32:14094-14101.
- Baxter MG, Murray EA (2002) The amygdala and reward. Nat Rev Neurosci 3:563-573.
- Beatty WW, Costello KB, Berry SL (1984) Suppression of play fighting by amphetamine: effects of catecholamine antagonists, agonists and synthesis inhibitors. Pharmacol Biochem Behav 20:747-755.
- Bell HC, McCaffrey DR, Forgie ML, Kolb B, Pellis SM (2009) The role of the medial prefrontal cortex in the play fighting of rats. Behav Neurosci 123:1158-1168.
- Bell HC, Pellis SM, Kolb B (2010) Juvenile peer play experience and the development of the orbitofrontal and medial prefrontal cortices. Behav Brain Res 207:7-13.
- Berridge KC (2007) The debate over dopamine's role in reward: the case for incentive salience. Psychopharmacology (Berl) 191:391-431.
- Berridge KC, Kringelbach ML (2008) Affective neuroscience of pleasure: reward in humans and animals. Psychopharmacology (Berl) 199:457-480.
- Blaha CD, Allen LF, Das S, Inglis WL, Latimer MP, Vincent SR, Winn P (1996) Modulation of dopamine efflux in the nucleus accumbens after cholinergic stimulation of the ventral tegmental area in intact, pedunculopontine tegmental nucleus-lesioned, and laterodorsal tegmental nucleus-lesioned rats. J Neurosci 16:714-722.
- Blaha CD, Winn P (1993) Modulation of dopamine efflux in the striatum following cholinergic stimulation of the substantia nigra in intact and pedunculopontine tegmental nucleus-lesioned rats. J Neurosci 13:1035-1044.
- Blakemore SJ (2008) The social brain in adolescence. Nat Rev Neurosci 9:267-277.
- Braun K, Bock J (2011) The experience-dependent maturation of prefronto-limbic circuits and the origin of developmental psychopathology: implications for the pathogenesis and therapy of behavioural disorders. Dev Med Child Neurol 53 Suppl 4:14-18.
- Cardinal RN, Parkinson JA, Hall J, Everitt BJ (2002) Emotion and motivation: the role of the amygdala, ventral striatum, and prefrontal cortex. Neurosci Biobehav Rev 26:321-352.
- Cassell MD, Wright DJ (1986) Topography of projections from the medial prefrontal cortex to the amygdala in the rat. Brain Res Bull 17:321-333.
- Cheng SY, Taravosh-Lahn K, Delville Y (2008) Neural circuitry of play fighting in golden hamsters. Neuroscience 156:247-256.

- Chesselet MF, Delfs JM (1996) Basal ganglia and movement disorders: an update. Trends Neurosci 19:417-422.
- Christakou A, Robbins TW, Everitt BJ (2001) Functional disconnection of a prefrontal cortical-dorsal striatal system disrupts choice reaction time performance: implications for attentional function. Behav Neurosci 115:812-825.
- Christakou A, Robbins TW, Everitt BJ (2004) Prefrontal cortical-ventral striatal interactions involved in affective modulation of attentional performance: implications for corticostriatal circuit function. J Neurosci 24:773-780.
- Churchwell JC, Morris AM, Heurtelou NM, Kesner RP (2009) Interactions between the prefrontal cortex and amygdala during delay discounting and reversal. Behav Neurosci 123:1185-1196.
- Cullinan WE, Herman JP, Battaglia DF, Akil H, Watson SJ (1995) Pattern and time course of immediate early gene expression in rat brain following acute stress. Neuroscience 64:477-505.
- Daenen EW, Wolterink G, Gerrits MAFM, Van Ree JM (2002) The effects of neonatal lesions in the amygdala or ventral hippocampus on social behaviour later in life. Behav Brain Res 136:571-582.
- Dalley JW, Cardinal RN, Robbins TW (2004) Prefrontal executive and cognitive functions in rodents: neural and neurochemical substrates. Neurosci Biobehav Rev 28:771-784.
- Day HE, Badiani A, Uslaner JM, Oates MM, Vittoz NM, Robinson TE, Watson SJ, Jr., Akil H (2001) Environmental novelty differentially affects c-fos mRNA expression induced by amphetamine or cocaine in subregions of the bed nucleus of the stria terminalis and amygdala. J Neurosci 21:732-740.
- Fagen R (1981) Animal Play Behavior. Oxford University Press, Oxford, UK.
- Forster GL, Blaha CD (2000) Laterodorsal tegmental stimulation elicits dopamine efflux in the rat nucleus accumbens by activation of acetylcholine and glutamate receptors in the ventral tegmental area. Eur J Neurosci 12:3596-3604.
- Gerall HD, Ward IL, Gerall AA (1967) Disruption of the male rat's sexual behaviour induced by social isolation. Anim Behav 15:54-58.
- Gerfen CR (2004) Basal Ganglia. In: The Rat Nervous System (Paxinos G, ed), pp 455-508.
- Gerfen CR, Herkenham M, Thibault J (1987) The neostriatal mosaic: II. Patch- and matrix-directed mesostriatal dopaminergic and non-dopaminergic systems. J Neurosci 7:3915-3934.
- Gordon NS, Kollack-Walker S, Akil H, Panksepp J (2002) Expression of c-fos gene activation during rough and tumble play in juvenile rats. Brain Res Bull 57:651-659.
- Graham KL, Burghardt GM (2010) Current perspectives on the biological study of play: signs of progress. Q Rev Biol 85:393-418.
- Groenewegen HJ, Berendse HW (1994) The specificity of the 'nonspecific' midline and intralaminar thalamic nuclei. Trends Neurosci 17:52-57.
- Groenewegen HJ, Uylings HBM (2010) Orginization of Prefrontal-Striatal Projections. In: Handbook of Basal Ganglia Structure and Function (Steiner H, Tseng KY, eds), pp 353-365. Academic Press.
- Groenewegen HJ, Witter MP (2004) Thalamus. In: The Rat Nervous System (Paxinos G, ed), pp 407-453.
- Haber SN, Knutson B (2010) The reward circuit: linking primate anatomy and human imaging. Neuropsychopharmacology 35:4-26.
- Holmstrand EC, Sesack SR (2011) Projections from the rat pedunculopontine and laterodorsal tegmental nuclei to the anterior thalamus and ventral tegmental area arise from largely separate populations of neurons. Brain Struct Funct 216:331-345.

- Homberg JR, Schiepers OJG, Schoffelmeer ANM, Cuppen E, Vanderschuren LJMJ (2007) Acute and constitutive increases in central serotonin levels reduce social play behaviour in peri-adolescent rats. Psychopharmacology (Berl) 195:175-182.
- Hong S, Jhou TC, Smith M, Saleem KS, Hikosaka O (2011) Negative reward signals from the lateral habenula to dopamine neurons are mediated by rostromedial tegmental nucleus in primates. J Neurosci 31:11457-11471.
- Ikemoto S (2007) Dopamine reward circuitry: two projection systems from the ventral midbrain to the nucleus accumbens-olfactory tubercle complex. Brain Res Rev 56:27-78.
- Ikemoto S, Panksepp J (1999) The role of nucleus accumbens dopamine in motivated behavior: a unifying interpretation with special reference to reward-seeking. Brain Res Brain Res Rev 31:6-41.
- Jhou TC, Fields HL, Baxter MG, Saper CB, Holland PC (2009a) The rostromedial tegmental nucleus (RMTg), a GABAergic afferent to midbrain dopamine neurons, encodes aversive stimuli and inhibits motor responses. Neuron 61:786-800.
- Jhou TC, Geisler S, Marinelli M, Degarmo BA, Zahm DS (2009b) The mesopontine rostromedial tegmental nucleus: A structure targeted by the lateral habenula that projects to the ventral tegmental area of Tsai and substantia nigra compacta. J Comp Neurol 513:566-596.
- Jordan R (2003) Social play and autistic spectrum disorders: a perspective on theory, implications and educational approaches. Autism 7:347-360.
- Kelley AE (2004) Ventral striatal control of appetitive motivation: role in ingestive behavior and reward-related learning. Neurosci Biobehav Rev 27:765-776.
- Kelley AE, Domesick VB, Nauta WJH (1982) The amygdalostriatal projection in the rat--an anatomical study by anterograde and retrograde tracing methods. Neuroscience 7:615-630.
- Kovacs KJ (2008) Measurement of immediate-early gene activation- c-fos and beyond. J Neuroendocrinol 20:665-672.
- Lammel S, Hetzel A, Hackel O, Jones I, Liss B, Roeper J (2008) Unique properties of mesoprefrontal neurons within a dual mesocorticolimbic dopamine system. Neuron 57:760-773.
- Lammel S, Ion DI, Roeper J, Malenka RC (2011) Projection-specific modulation of dopamine neuron synapses by aversive and rewarding stimuli. Neuron 70:855-862.
- Lewis KP, Barton RA (2006) Amygdala size and hypothalamus size predict social play frequency in nonhuman primates: a comparative analysis using independent contrasts. J Comp Psychol 120:31-37.
- Manning MM, Wainwright LD (2010) The role of high level play as a predictor social functioning in autism. J Autism Dev Disord 40:523-533.
- Maren S, Quirk GJ (2004) Neuronal signalling of fear memory. Nat Rev Neurosci 5:844-852.
- McDonald AJ, Mascagni F, Guo L (1996) Projections of the medial and lateral prefrontal cortices to the amygdala: a Phaseolus vulgaris leucoagglutinin study in the rat. Neuroscience 71:55-75.
- McFarland K, Lapish CC, Kalivas PW (2003) Prefrontal glutamate release into the core of the nucleus accumbens mediates cocaine-induced reinstatement of drug-seeking behavior. J Neurosci 23:3531-3537.
- Meaney MJ, Dodge AM, Beatty WW (1981) Sex-dependent effects of amygdaloid lesions on the social play of prepubertal rats. Physiol Behav 26:467-472.
- Miyachi S, Hasegawa YT, Gerfen CR (2005) Coincident stimulation of convergent cortical inputs enhances immediate early gene induction in the striatum. Neuroscience 134:1013-1022.
- Morgan JI, Curran T (1991) Stimulus-transcription coupling in the nervous system: involvement of the inducible proto-oncogenes fos and jun. Annu Rev Neurosci 14:421-451.

• Morrison SE, Salzman CD (2010) Re-valuing the amygdala. Curr Opin Neurobiol 20:221-230.
• Nelson EE, Leibenluft E, McClure EB, Pine DS (2005) The social re-orientation of adolescence: a neuroscience perspective on the process and its relation to psychopathology. Psychol Med 35:163-174.
• Niesink RJM, Van Ree JM (1989) Involvement of opioid and dopaminergic systems in isolation-induced pinning and social grooming of young rats. Neuropharmacology 28:411-418.
• Nordquist RE, Vanderschuren LJMJ, Jonker AJ, Bergsma M, De Vries TJ, Pennartz CMA, Voorn P (2008) Expression of amphetamine sensitization is associated with recruitment of a reactive neuronal population in the nucleus accumbens core. Psychopharmacology 198:113-126.
• Ostrander MM, Badiani A, Day HE, Norton CS, Watson SJ, Akil H, Robinson TE (2003) Environmental context and drug history modulate amphetamine-induced c-fos mRNA expression in the basal ganglia, central extended amygdala, and associated limbic forebrain. Neuroscience 120:551-571.
• Panksepp J, Normansell L, Cox JF, Siviy SM (1994) Effects of neonatal decortication on the social play of juvenile rats. Physiol Behav 56:429-443.
• Panksepp J, Siviy SM, Normansell L (1984) The psychobiology of play: theoretical and methodological perspectives. Neurosci Biobehav Rev 8:465-492.
• Parkinson JA, Willoughby PJ, Robbins TW, Everitt BJ (2000) Disconnection of the anterior cingulate cortex and nucleus accumbens core impairs Pavlovian approach behavior: further evidence for limbic cortical-ventral striatopallidal systems. Behav Neurosci 114:42-63.
• Parthasarathy HB, Graybiel AM (1997) Cortically driven immediate-early gene expression reflects modular influence of sensorimotor cortex on identified striatal neurons in the squirrel monkey. J Neurosci 17:2477-2491.
• Paus T, Keshavan M, Giedd JN (2008) Why do many psychiatric disorders emerge during adolescence? Nat Rev Neurosci 9:947-957.
• Paxinos G, Watson C (2007) The rat brain in sterotaxic coordinates. Elsevier Academic.
• Pellis SM, Castaneda E, McKenna MM, Tran-Nguyen LT, Whishaw IQ (1993) The role of the striatum in organizing sequences of play fighting in neonatally dopamine-depleted rats. Neurosci Lett 158:13-15.
• Pellis SM, Field EF, Smith LK, Pellis VC (1997) Multiple differences in the play fighting of male and female rats. Implications for the causes and functions of play. Neurosci Biobehav Rev 21:105-120.
• Pellis SM, Hastings E, Shimizu T, Kamitakahara H, Komorowska J, Forgie ML, Kolb B (2006) The effects of orbital frontal cortex damage on the modulation of defensive responses by rats in playful and nonplayful social contexts. Behav Neurosci 120:72-84.
• Pellis SM, Pellis VC (1998) Play fighting of rats in comparative perspective: a schema for neurobehavioral analyses. Neurosci Biobehav Rev 23:87-101.
• Pellis SM, Pellis VC (2007) Rough-and-tumble play and the development of the social brain. Current Directions in Psychological Science 16:95-98.
• Pellis SM, Pellis VC (2009) The Playful Brain. OneWorld Publications.
• Pellis SM, Pellis VC, Whishaw IQ (1992) The role of the cortex in play fighting by rats: developmental and evolutionary implications. Brain Behav Evol 39:270-284.
• Phelps EA, Ledoux JE (2005) Contributions of the amygdala to emotion processing: from animal models to human behavior. Neuron 48:175-187.
•Robbins TW, Arnsten AF (2009) The neuropsychopharmacology of fronto-executive function: monoaminergic modulation. Annu Rev Neurosci 32:267-287.

- Robbins TW, Everitt BJ (2007) A role for mesencephalic dopamine in activation: commentary on Berridge (2006). Psychopharmacology (Berl) 191:433-437.
- Rozen S, Skaletsky HJ (2000) Primer3 on the WWW for general users and for biologist programmers. In: Bioinformatics Methods and Protocols: Methods in Molecular Biology (Krawerts S, Misener S, eds), pp 365-386. Humana Press.
- Salamone JD, Correa M, Mingote SM, Weber SM (2005) Beyond the reward hypothesis: alternative functions of nucleus accumbens dopamine. Curr Opin Pharmacol 5:34-41.
- Schilling K, Curran T, Morgan JI (1991) Fosvergnugen. The excitement of immediate-early genes. Ann N Y Acad Sci 627:115-123.
- Schilman EA, Uylings HB, Galis-de Graaf Y, Joel D, Groenewegen HJ (2008) The orbital cortex in rats topographically projects to central parts of the caudate-putamen complex. Neurosci Lett 432:40-45.
- Schneider M, Koch M (2005) Deficient social and play behavior in juvenile and adult rats after neonatal cortical lesion: effects of chronic pubertal cannabinoid treatment. Neuropsychopharmacology 30:944-957.
- Schoenbaum G, Setlow B, Saddoris MP, Gallagher M (2003) Encoding predicted outcome and acquired value in orbitofrontal cortex during cue sampling depends upon input from basolateral amygdala. Neuron 39:855-867.
- Seamans JK, Yang CR (2004) The principal features and mechanisms of dopamine modulation in the prefrontal cortex. Prog Neurobiol 74:1-58.
- Sesack SR, Grace AA (2010) Cortico-Basal Ganglia reward network: microcircuitry. Neuropsychopharmacology 35:27-47.
- Siviy SM, Deron LM, Kasten CR (2011) Serotonin, motivation, and playfulness in the juvenile rat. Dev Cogn Neurosci 1:606-616.
- Siviy SM, Fleischhauer AE, Kerrigan LA, Kuhlman SJ (1996) D2 dopamine receptor involvement in the rough-and-tumble play behavior of juvenile rats. Behav Neurosci 110:1168-1176.
- Siviy SM, Panksepp J (1985) Dorsomedial diencephalic involvement in the juvenile play of rats. Behav Neurosci 99:1103-1113.
- Siviy SM, Panksepp J (1987) Juvenile play in the rat: thalamic and brain stem involvement. Physiol Behav 41:103-114.
- Siviy SM, Panksepp J (2011) In search of the neurobiological substrates for social playfulness in mammalian brains. Neurosci Biobehav Rev 35:1821-1830.
- Spear LP (2000) The adolescent brain and age-related behavioral manifestations. Neurosci Biobehav Rev 24:417-463.
- Špinka M, Newberry RC, Bekoff M (2001) Mammalian play: training for the unexpected. Q Rev Biol 76:141-168.
- Trezza V, Baarendse PJJ, Vanderschuren LJMJ (2009) Prosocial effects of nicotine and ethanol in adolescent rats through partially dissociable neurobehavioral mechanisms. Neuropsychopharmacology 34:2560-2573.
- Trezza V, Baarendse PJJ, Vanderschuren LJMJ (2010) The pleasures of play: pharmacological insights into social reward mechanisms. Trends Pharmacol Sci 31:463-469.
- Trezza V, Campolongo P, Vanderschuren LJMJ (2011a) Evaluating the rewarding nature of social interactions in laboratory animals. Dev Cogn Neurosci 1:444-458.
- Trezza V, Damsteegt R, Achterberg EJM, Vanderschuren LJMJ (2011b) Nucleus accumbens mu-opioid receptors mediate social reward. J Neurosci 31:6362-6370.

- Trezza V, Damsteegt R, Manduca A, Petrosino S, Van Kerkhof LWM, Pasterkamp RJ, Zhou Y, Campolongo P, Cuomo V, Di Marzo V, Vanderschuren LJMJ (2012) Endocannabinoids in amygdala and nucleus accumbens mediate social play reward in adolescent rats. J Neurosci 32:14899-14908.
- Trezza V, Vanderschuren LJMJ (2008) Cannabinoid and opioid modulation of social play behavior in adolescent rats: differential behavioral mechanisms. Eur Neuropsychopharmacol 18:519-530.
- Van De Werd HJJM, Uylings HBM (2008) The rat orbital and agranular insular prefrontal cortical areas: a cytoarchitectonic and chemoarchitectonic study. Brain Struct Funct 212:387-401.
- Van den Berg CL, Hol T, Van Ree JM, Spruijt BM, Everts H, Koolhaas JM (1999) Play is indispensable for an adequate development of coping with social challenges in the rat. Dev Psychobiol 34:129-138.
- [a] van der Plasse G, Schrama R, van Seters SP, Vanderschuren LJMJ, Westenberg HGM (2012) Deep brain stimulation reveals a dissociation of consummatory and motivated behaviour in the medial and lateral nucleus accumbens shell of the rat. PLoS One 7:e33455.
- Van der Werf YD, Witter MP, Groenewegen HJ (2002) The intralaminar and midline nuclei of the thalamus. Anatomical and functional evidence for participation in processes of arousal and awareness. Brain Res Brain Res Rev 39:107-140.
- Vanderschuren LJMJ (2010) How the brain makes play fun. Am J of Play 2:315-337.
- Vanderschuren LJMJ, Niesink RJM, Spruijt BM, Van Ree JM (1995a) Effects of morphine on different aspects of social play in juvenile rats. Psychopharmacology 117:225-231.
- Vanderschuren LJMJ, Niesink RJM, Spruijt BM, Van Ree JM (1995b) Influence of environmental factors on social play behavior of juvenile rats. Physiol Behav 58:119-123.
- Vanderschuren LJMJ, Niesink RJM, Van Ree JM (1997) The neurobiology of social play behavior in rats. Neurosci Biobehav Rev 21:309-326.
- Vanderschuren LJMJ, Trezza V, Griffioen-Roose S, Schiepers OJG, Van Leeuwen N, De Vries TJ, Schoffelmeer ANM (2008) Methylphenidate disrupts social play behavior in adolescent rats. Neuropsychopharmacology 33:2946-2956.
- Von Frijtag JC, Schot M, Van den BR, Spruijt BM (2002) Individual housing during the play period results in changed responses to and consequences of a psychosocial stress situation in rats. Dev Psychobiol 41:58-69.
- Voorn P (2010) Projections from pallidum to striatum. In: Handbook of Basal Ganglia Structure and Function (Steiner H, Tseng KY, eds), pp 249-257. Academic Press.
- Voorn P, Vanderschuren LJMJ, Groenewegen HJ, Robbins TW, Pennartz CMA (2004) Putting a spin on the dorsal-ventral divide of the striatum. Trends Neurosci 27:468-474.
- Watabe-Uchida M, Zhu L, Ogawa SK, Vamanrao A, Uchida N (2012) Whole-brain mapping of direct inputs to midbrain dopamine neurons. Neuron 74:858-873.
- Willuhn I, Sun W, Steiner H (2003) Topography of cocaine-induced gene regulation in the rat striatum: relationship to cortical inputs and role of behavioural context. Eur J Neurosci 17:1053-1066.
- Wongwitdecha N, Marsden CA (1996) Social isolation increases aggressive behaviour and alters the effects of diazepam in the rat social interaction test. Behav Brain Res 75:27-32.
- Zahm DS (1999) Functional-anatomical implications of the nucleus accumbens core and shell subterritories. Ann N Y Acad Sci 877:113-128.

Chapter 3
Social play behaviour in adolescent rats is mediated by functional activity in medial prefrontal cortex and dorsomedial striatum

Linda W.M. van Kerkhof[1]
Ruth Damsteegt[1]
Viviana Trezza[1,2]
Pieter Voorn[3]
Louk J.M.J. Vanderschuren[1,4]

1 Rudolf Magnus Institute of Neuroscience, Department of Neuroscience and Pharmacology, University Medical Centre Utrecht, Utrecht, The Netherlands
2 Department of Biology, University "Roma Tre", Rome, Italy
3 Department of Anatomy and Neurosciences, Neuroscience Campus Amsterdam, VU University Medical Centre, Amsterdam, The Netherlands
4 Department of Animals in Science and Society, Division of Behavioural Neuroscience, Faculty of Veterinary Medicine, Utrecht University, Utrecht, The Netherlands

In revision.

Abstract

Social play behaviour is a characteristic, vigorous form of social interaction in young mammals. It is highly rewarding, and thought to be of major importance for social and cognitive development. Although the neural substrates of social play remain largely unknown, there is some evidence to support a role for the prefrontal cortex and striatum in this behaviour. Using pharmacological inactivation methods, i.e. local infusions of GABA receptor agonists (baclofen and muscimol; B&M) or the AMPA/kainate receptor antagonist DNQX, we therefore investigated the involvement of several subregions of the medial prefrontal cortex and striatum in social play. Inactivation of the prelimbic cortex, infralimbic cortex and medial/ventral orbitofrontal cortex using B&M markedly reduced social play behaviour, without altering locomotor activity. Local administration of DNQX into the dorsomedial striatum increased social play, whereas infusion of B&M tended to have the same effect. However, inactivation of the nucleus accumbens core and shell did not influence social play behaviour. Thus, functional integrity of the medial prefrontal cortex is important for the proper expression of social play behaviour, whereas glutamatergic inputs into the dorsomedial striatum exert an inhibitory influence on social play. These results highlight the importance of neural circuits implicated in cognitive control, decision making and behavioural inhibition in social play behaviour.

Introduction

Substantial changes in brain and behaviour occur between weaning and early adulthood. For example, there is a marked increase in peer-peer interactions, signified by an abundance of social play behaviour, which peaks during the juvenile/early adolescent phase and declines to low levels after sexual maturation (Panksepp, 1981). Proper social interactions during this phase are thought to be important for adaptive social and cognitive development (Pellis and Pellis, 2009; Špinka *et al.*, 2001; Vanderschuren *et al.*, 1997). Indeed, abnormalities in social play behaviour have been observed in childhood psychiatric disorders such as autism and attention deficit/hyperactivity disorder (Alessandri, 1992; Manning and Wainwright, 2010). Furthermore, social traumas during childhood and adolescence are thought to increase the risk for psychopathology in later life (Braun and Bock, 2011). Therefore, identifying the neural underpinnings of social play behaviour will increase our understanding of normal social development as well as the aetiology of childhood and adolescent psychiatric disorders.

Our knowledge of the neurobiology of social play behaviour is limited (Siviy and Panksepp, 2011; Trezza *et al.*, 2010; Vanderschuren *et al.*, 1997). Interestingly, the prefrontal cortex (PFC) and striatum have previously been implicated in social play behaviour (Bell *et al.*, 2009; Panksepp *et al.*, 1994; Pellis et al., 1993; -2006; Schneider and Koch, 2005; Trezza *et al.*, 2011; -2012). Since it is in these regions that profound neural changes occur during the juvenile and adolescent period (Casey and Jones, 2010; Spear, 2000), the abundance of social play behaviour suggests that play is related to the maturation of the PFC and striatum.

Using c-fos as an activity marker, enhanced neuronal activity has been observed after social play behaviour in several medial PFC regions, i.e. anterior cingulate, prelimbic cortex (PrL) and infralimbic cortex (IL), as well as in orbitofrontal cortex (OFC) regions (Cheng *et al.*, 2008; Gordon *et al.*, 2002; Van Kerkhof *et al.*, 2012, submitted). In keeping with these findings, the structure of social play and its sensitivity to social cues was found to be altered after neonatal lesions of the PFC (Bell *et al.*, 2009; Panksepp *et al.*, 1994; Pellis *et al.*, 2006; Schneider and Koch, 2005).

With regard to the striatum, studies in nonhuman primates found that a larger size of the striatum was associated with more time spent on social play behaviour, while there was no correlation with the level of non-social play behaviour (Graham, 2011). Recent studies have also indicated a role for the ventral parts of the striatum, i.e. nucleus accumbens (NAcc), in the modulation of play behaviour by opioids and endocannabinoids (Trezza *et al.*, 2011; -2012). Furthermore, neonatal dopamine depletion in the striatum caused rats to use less complex defence strategies during a playful encounter, and to be more likely to switch to other types of behaviour (Pellis *et al.*, 1993). This indicates that striatal dopamine contributes to the maintenance of the sequential organization of play fighting. Interestingly, this somewhat resembles the effect observed after lesions of the medial PFC (Bell *et al.*, 2009), suggesting involvement of projections from medial PFC to striatum in social play behavior. Further evidence for an involvement of corticostriatal projections is provided by immediate early gene expression studies, demonstrating that social play behaviour induces c-fos expression in the striatum (Cheng *et al.*, 2008; Gordon *et al.*, 2002; Van Kerkhof *et al.*, 2012, submitted). Interestingly, the levels of c-fos induced in medial PFC regions were found to correlate with the levels of c-fos in their striatal projection

areas, suggesting that these projections might be involved in social play behaviour (Van Kerkhof *et al.*, 2012, submitted).

In the present study, the role of PFC and striatum subregions in social play behaviour was investigated using a temporary inactivation technique which allows for transient disruption of functional activity, but leaves normal brain development and function intact. We hypothesized that inactivation of PFC and striatum regions would reduce social play.

Materials and Methods

Animals

Male Wistar rats (Charles River, Sulzfeld, Germany) arrived in our animal facility at 21 days of age. They were housed in groups of four in 40x26x20 cm Macrolon cages under controlled conditions (i.e. temperature 20-21 °C, 55-65 % relative humidity and 12/12 h light cycle with lights on at 7.00 a.m.). Food and water were available *ad libitum*. All animals were experimentally naïve. During the first 6 days all rats were handled at least twice. All experiments were approved by the Animal Ethics Committee of Utrecht University and were conducted in accordance with Dutch laws (Wet op de Dierproeven, 1996) and European regulations (Guideline 86/609/EEC).

Surgical procedures

The surgical procedures were based on previous experiments (Trezza *et al.*, 2011; -2012). At 27-28 days of age, rats were anesthetised with 0.08 ml/100g (s.c.) of Hypnorm (Janssen, Belgium) and positioned in a stereotaxic apparatus (David Kopf, USA). Guide cannulae (24 gauge; Cooper's Needleworks, UK) were implanted bilaterally. The cannulae were aimed 0.5 mm above the PrL (coordinates: anterior-posterior (AP) +2.6 mm from Bregma; medial-lateral (ML) ±0.8 mm from the midline; dorsal-ventral (DV) -3.2 mm from skull surface), the IL (coordinates: AP +2.6 mm; ML ±0.8 mm; DV -4.1 mm), the medial/ventral OFC (MO/VO; coordinates: AP +3.3 mm; ML ±0.8 mm; DV -5.3 mm), the dorsomedial striatum (DMS; coordinates: AP +1.5 mm; ML ±1.9 mm; DV -5.1 mm), the NAcc core (coordinates: AP, +1.5 mm; ML ±1.9 mm; DV -6.5 mm), or 1.0 mm above the NAcc shell (coordinates: AP +1.5 mm; ML ±0.8 mm; DV -5.3 mm).

Cannulae were secured with stainless steel screws and dental acrylic. Stainless steel stylets (29 gauge) were inserted into the guide cannulae to maintain patency. After surgery, rats were individually housed for 4 days to recover, after which they were housed with their original cage mates.

Drugs and infusion procedures

The GABA-A receptor agonist muscimol and the GABA-B receptor agonist (RS)-baclofen (Tocris Bioscience, UK) were dissolved in saline. In all regions 1.0 nmol/0.3 µl baclofen and 0.1 nmol/ 0.3 µl muscimol (B&M) was administered, with the exception of the nucleus accumbens shell into which 0.1 nmol/0.3 µl baclofen/0.01 nmol/ 0.3 µl muscimol was infused. The AMPA/kainate receptor antagonist, 6,7-Dinitroquinoxaline-2,3(1H,4H)-dione (DNQX) (Sigma, USA), was dissolved in 50 % DMSO in saline, and a dose of 3.0 nmol / 0.3 µl was administered. Infusion procedures were as previously described (Trezza *et al.*,

2011; -2012). In short, bilateral infusions of drugs or equivalent volume of saline were administered using 30-gauge injection needles (Bilaney, Germany) connected to 10 µl Hamilton micro-syringes by polyethylene tubing. Over 60 s, 0.3 µl of the solution was infused using a syringe pump (Harvard Apparatus, USA). The injection needles remained within the guide cannulae for 60 s following drug infusion to facilitate diffusion and to prevent backflow of drug along the cannula track. After the infusion, the stylets were replaced and the animals were left in a holding cage for 5 min before testing.

Behavioural testing

Experiments were performed in a sound-attenuated chamber under dim light conditions. The testing arena was a Plexiglas cage (40x40x60 cm), with approximately 2 cm of wood shavings covering the floor. Animals were paired with an unfamiliar partner. Animals in a test pair did not differ more than 10 g in body weight.

One week after surgery, the rats were habituated to the experimental procedures on 2 consecutive days. On the first day, they were individually placed in the test cage for 10 min. On the second day, they were isolated for 2.5 h. Pairs of rats were then infused with saline and placed in the test cage for 15 min. On the test day, pairs of rats were isolated for 2.5 h. They were then infused, placed into a holding cage and subsequently placed into the test cage for 15 min.

Behaviour of the animals was recorded on video tape. Behaviour was assessed using the Observer software (Noldus Information Technology, The Netherlands). Three behavioural elements were scored (Panksepp *et al.*, 1984; Trezza *et al.*, 2010; Vanderschuren *et al.*, 1997).

- *Frequency of pinning:* one animal lying with its dorsal surface on the floor with the other animal standing over it. This occurs when one animal is solicited to play by its test partner (pouncing) and rotates to its dorsal surface to prolong the playful interaction.
- *Frequency of pouncing:* one animal attempts to nose or rub the nape of the neck of the partner, which is an index of play solicitation.
- *Time spent in social exploration:* one animal sniffing or grooming any part of the partner's body.

To assess whether effects of the drug treatment on social play were secondary to changes in locomotor activity, rats (at 41 days of age) were tested for horizontal locomotor activity as described previously (Trezza *et al.*, 2009; Veeneman *et al.*, 2011). Between the test for social play behaviour and locomotor activity there was at least one day without testing or infusions. The infusion protocol was as described above. After infusion, the rats were transferred to a plastic cage (l x w x h, 50 x 33 x 40 cm) and their position was tracked five times per second for 30 min using a video-tracking system (EthoVision, Noldus Information Technology, The Netherlands).

Histological confirmation of injection sites

Confirmation of the injection sites was performed as previously described (Trezza *et al.*, 2011; -2012). Only pairs of rats in which both animals had bilateral needle tracks terminating in the target area were included in the final analysis (Figs 1 and 2).

Statistical analysis

Pinning and pouncing frequencies and time spent in social exploration (s) were expressed as mean ± SEM and data was analysed using a paired samples Student's t-test. Horizontal locomotor activity was expressed as mean ± SEM of the travelled distance (cm) and analysed using a two-way repeated measures ANOVA.

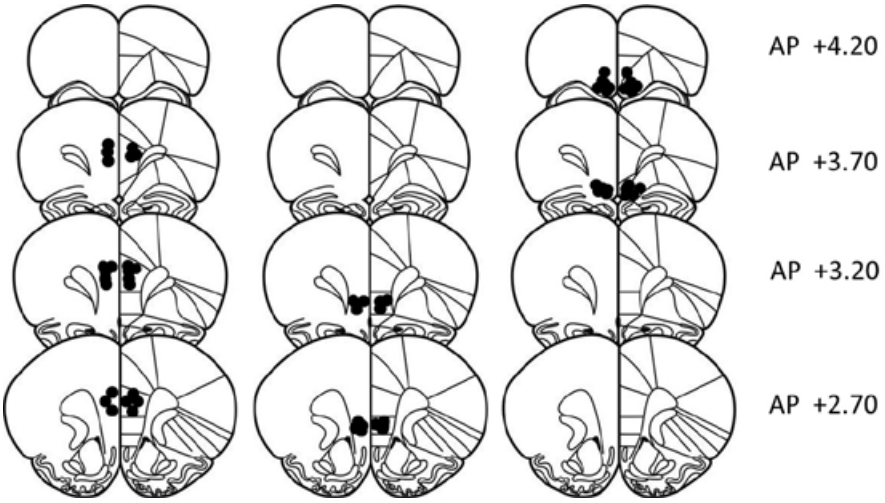


Fig. 1: Schematic representation of brain sections with microinjection placements in the prelimbic cortex (A), infralimbic cortex (B), and medial/ventral orbitofrontal cortex (C). AP = distance anterior to Bregma (in mm). Adapted from Paxinos and Watson (Paxinos and Watson, 2007).

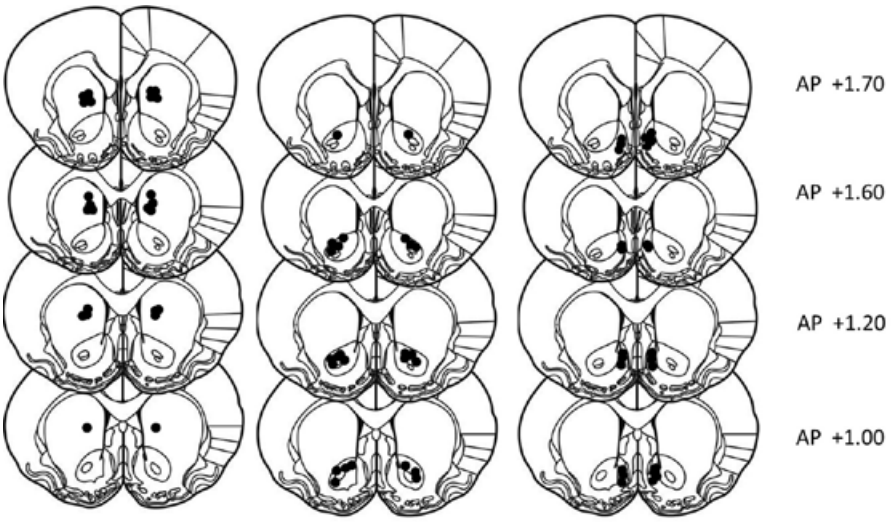


Fig. 2: Schematic representation of brain sections with microinjection placements in the dorsomedial striatum (A), nucleus accumbens core (B), and nucleus accumbens shell (C). AP = distance anterior to Bregma (in mm). Adapted from Paxinos and Watson (2007).

Results

Pharmacological inactivation of prefrontal regions

Infusion of B&M into the PrL decreased social play behaviour (Fig. 3). The frequencies of both pinning and pouncing were reduced (pinning: t = 3.145, df = 8, p =0.014; pouncing: t = 3.509, df = 8, p = 0.008), while there was an increase in the duration of social exploration (t = -2.322, df = 8, p = 0.049). Administration of B&M into the PrL did not affect locomotor activity ($F_{treatment}$ (1,23) = 0.801, p = 0.380; F_{time}(5,115) = 66.445, p < 0.001; $F_{treatment\ x\ time}$ (5,115) = 0.177, p = 0.971) (Fig.3D).

After administration of B&M into the IL a decrease in pinning and pouncing was observed (pinning: t = 4.616, df = 7, p = 0.002; pouncing: t = 5.285, df = 7, p = 0.001) (Fig. 3E-F). Infusion of B&M into the IL had no effect on social exploration (t = -2.105, df = 7, p = 0.073) or locomotor activity ($F_{treatment}$ (1,18) = 0.817, p = 0.378; F_{time}(5,90) =57.835, p < 0.001; $F_{treatment\ x\ time}$ (5,90) = 0.223, p = 0.952) (Fig. 3G-H).

Administration of B&M into the MO/VO resulted in a decrease in both play parameters (pinning: t = 4.408, df = 5, p = 0.007; pouncing: t = 4.146, df = 5, p = 0.009) (Fig. 3I-J), while no effects were observed on social exploration (t = -0.384, df = 5, p = 0.717) or locomotor activity ($F_{treatment}$ (1,15) = 0.160, p = 0.695; F_{time} (5,75) = 36.445, p < 0.001; $F_{treatment\ x\ time}$ (5,75) = 0.354, p = 0.878) (Fig.3K-L).

For the play parameters the effect size (i.e. percentage decrease compared to saline) was comparable in the three regions (pinning: PrL: -41.14 ± 10.45; IL: -57.47 ± 12.01; MO/VO: 50.80 ± 8.65; pouncing: PrL: -32.51 ± 8.46; IL: -55.29 ± 8.23; MO/VO: 40.33 ± 8.26. No difference was observed when the reduction in pinning and pouncing was compared between the three regions (pinning: F_{region} (2,20) = 0.633, p = 0.541; pouncing: F_{region} (2,20) = 0.2.338, p = 0.122).

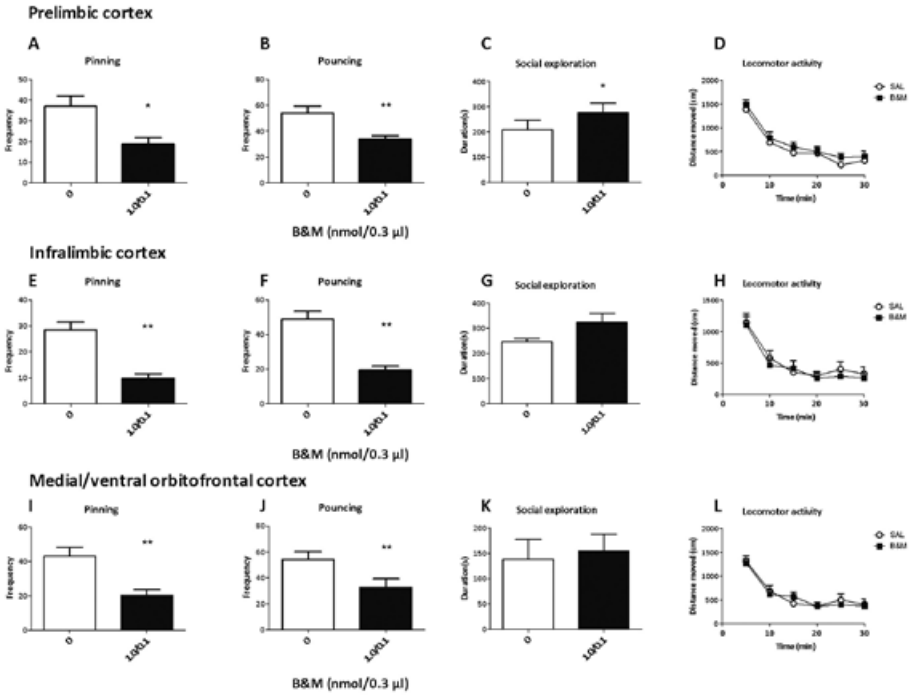


Fig. 3: The effect of B&M administration (1.0 nmol/ 0.3 µl & 0.1 nmol/ 0.3 µl) into prefrontal regions on social play behaviour. Data are presented as mean ± SEM. B&M administration into the prelimbic cortex (n= 9) reduced the levels of pinning (A) and pouncing (B), increased the time spent on social exploration (C), and had no effect on locomotor activity (D). Infusion of B&M into the infralimbic cortex (n = 8) reduced the levels of pinning (E) and pouncing (F), while no effects were observed on social exploration (G) or locomotor activity (H). After administration of B&M into the medial/ventral orbitofrontal cortex (n = 6), a reduction in pinning (I) and pouncing (J), but no effect on social exploration (K) or locomotor activity (L) was found. *p< 0.05, **p <0.01 compared to saline (0 B&M; paired samples Student's t-test).

Pharmacological inactivation of striatal regions

Administration of B&M into the DMS did not affect pinning (t = -1.726, df = 12, p = 0.110), but a trend towards an increase in the frequency of pouncing was observed (t = -2.168, df = 12, p = 0.051; Fig. 4A-B). Treatment with B&M also tended to decrease social exploration (t = 2.126, df = 12, p = 0.055; Fig. 4C), but it did not affect locomotor activity ($F_{treatment}$ (1,17) = 0.512, p = 0.484; F_{time} (5,85) = 24.767, p < 0.001; $F_{treatment\ x\ time}$ (5,85) = 0.684, p = 0.637; Fig. 4D).

Infusion of B&M into the NAcc core did not influence pinning (t = -1.138, df = 10, p = 0.281) or pouncing (t = -0.278, df = 10, p = 0.787) (Fig. 4E-F). In addition, administration of B&M into the NAcc core had no effect on social exploration (t = -0.916, df = 10, p = 0.381) or locomotor activity ($F_{treatment}$ (1,13) = 0.462, p = 0.509; F_{time} (5,65) = 47.795, p < 0.001; $F_{treatment\ x\ time}$ (5,65) = 0.220, p = 0.953) (Fig. 4G-H).

Administration of B&M at the dose used in the other regions (1.0 nmol baclofen and 0.1 nmol muscimol/ 0.3 µl) into the NAcc shell resulted in a complete blockade of play (data not shown), which was secondary to striking effects on eating. Animals would spend

all their time on non-specific eating (eating e.g. sawdust and faeces) (see Stratford and Kelley, 1997). Therefore, a lower dose of B&M (0.1 nmol baclofen and 0.01 nmol muscimol/ 0.3 µl), which had no general disruptive effects on behaviour was administered into the NAcc shell. This dose had no effect on any of the parameters measured (pinning: t = 0.353, df = 4, p = 0.742; pouncing: t = 0.105, df = 4, p = 0.922; social exploration: t = -0.683, df = 4, p = 0.532; locomotor activity: $F_{treatment}$ (1,13) = 0.113, p = 0.742; F_{time} (5,65) = 31.444, p < 0.001; $F_{treatment\ x\ time}$ (5,65) = 2.342, p = 0.051) (Fig. 4I-L).

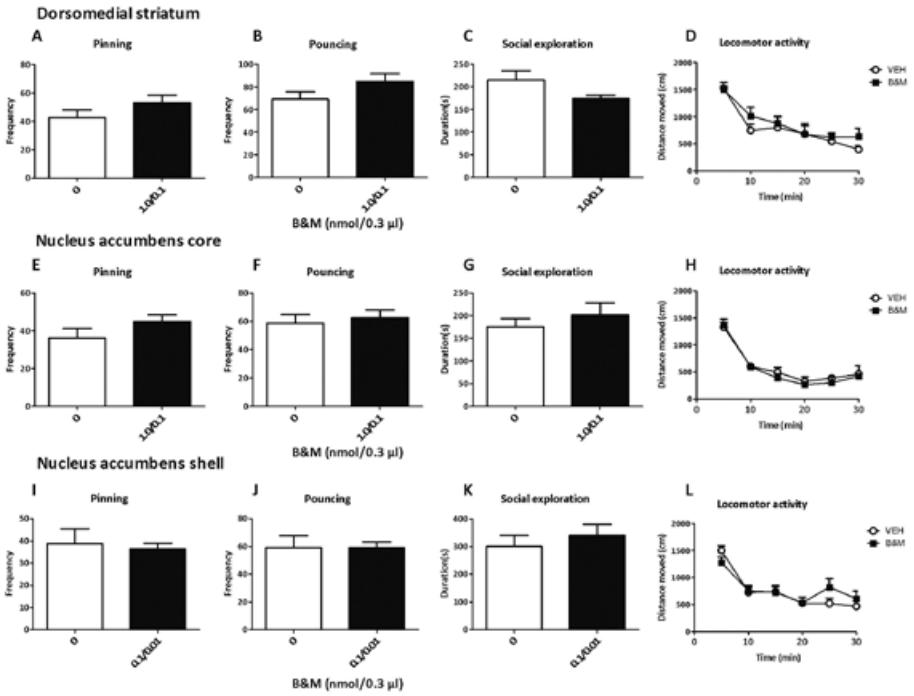


Fig. 4: The effect of B&M administration into striatal regions on social play behaviour. Data are presented as mean ± SEM. B&M was administered into the dorsomedial striatum (A-D; n = 13) and nucleus accumbens core (E-H; n = 10) in a dose of 1.0 nmol/ 0.3 µl & 0.1 nmol/ 0.3 µl and into the nucleus accumbens shell (I-L; n = 5)) in a dose of 0.1 nmol/ 0.3 µl & 0.01 nmol/ 0.3 µl. Administration of B&M did not affect any of the measured parameters: pinning (A,E,I), pouncing (B,F,J), social exploration (C,G,K), and locomotor activity (D,H,L), although in the dorsomedial striatum a trend towards an increase in pouncing (p = 0.051; B) as well as a trend towards a reduction in the time spent on social exploration (p = 0.055; C) was found.

Involvement of glutamatergic input to the striatum

In view of the large effects observed after pharmacological inactivation of the prefrontal regions, their known glutamatergic projections towards the striatum (Groenewegen and Uylings, 2010; Voorn *et al.*, 2004), and the trend towards an effect of B&M in the DMS, the role of glutamatergic input into the striatum in social play was investigated using local administration of the AMPA receptor antagonist DNQX. Administration of DNQX into the DMS increased the frequency of pinning (t = -2.552, df = 8, p = 0.034) and pouncing (t = 1.403, df = 8, p = 0.032), bit it did not affect social exploration (t = 1.403, df = 8, p =

0.198) or locomotor activity ($F_{treatment}$ (1,18) = 0.059, p = 0.811; $F_{treatment\ x\ time}$ (5,90) = 1.395, p = 0.234) (Fig. 5A-D).

Infusion of DNQX into the NAcc core did not affect social play behaviour (pinning: t = -1.769, df = 7 p =0.120; pouncing: t = -1.254, df = 7, p =0.250), social exploration (t = 1.382, df = 7, p = 0.210), or locomotor activity ($F_{treatment}$ (1,17) = 0.877, p = 0.362; $F_{treatment\ x\ time}$ (5,85) = 0.182, p = 0.969 (Fig. 5E-H). DNQX was not administered into the NAcc shell, since this is known to enhance feeding behaviour in a comparable fashion as administration of B&M into this region (Maldonado-Irizarry *et al.*, 1995).

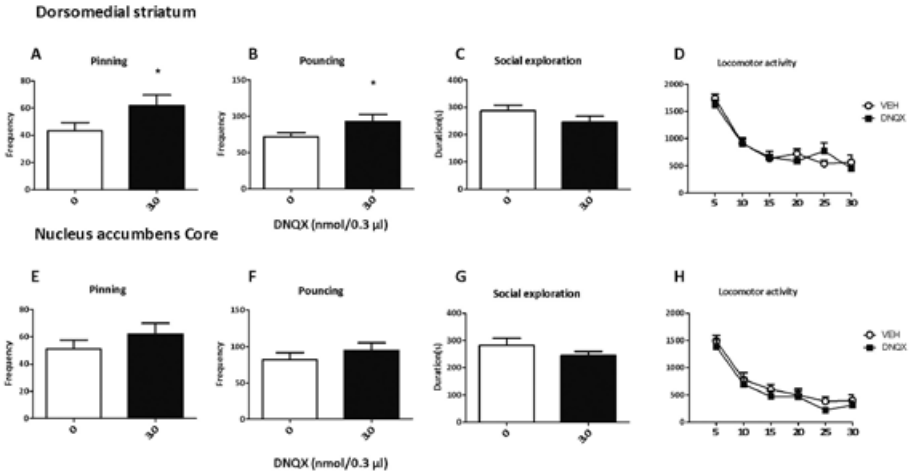


Fig. 5: The effect of DNQX administration (3.0 nmol/ 0.3 µl) into the dorsomedial striatum and nucleus accumbens core on social play behaviour. Data are presented as mean ± SEM. DNQX administration into the dorsomedial striatum (n = 9) increased the frequency of pinning (A) and pouncing (B), while it did not affect the time spent on social exploration (C) or locomotor activity (D). Infusion of B&M into the nucleus accumbens core (n = 8) did not change the level of pinning (E), pouncing (F), social exploration (G), or locomotor activity (H). *p< 0.05 compared to saline (0 DNQX; paired samples Student's t-test).

Discussion

Importance of the PFC for social play behaviour

Temporary inactivation of the medial PFC regions reduced pinning and pouncing, while locomotor activity was not altered. Inactivation of the PrL, but not the IL or MO/VO increased social exploration. These results indicate that temporary inactivation of medial PFC regions specifically decreases social play behaviour, whereby both play initiation as well as the response to play initiations are affected.

Altered responses to play initiations have previously been reported after neonatal PFC lesions (Bell *et al.*, 2009; Panksepp *et al.*, 1994; Pellis *et al.*, 2006; Schneider and Koch, 2005). Lesions of the lateral and ventral OFC have been found to disrupt the partner-related modulation of play (Pellis *et al.*, 2006). Thus, control but not lesioned rats respond differently to dominant, subordinate and female rats. Animals with medial PFC lesions still show partner-related changes in social play behaviour, but they use less complex

defensive strategies (Bell *et al.*, 2009). These, and other, studies indicate that after PFC lesions, play behaviour can still be observed, but that its structure and sensitivity to social cues is altered (Bell *et al.*, 2009; Panksepp *et al.*, 1994; Pellis *et al.*, 2006; Schneider and Koch, 2005). In the present study, however, the effect of temporary PFC inactivation was more substantial and, in contrast to the lesion studies, play initiations were affected as well.

The methodological approach of the aforementioned lesion studies differs in several respects from the present study. Relatively large lesions were made at an early age (postnatal day 3-7) which may have impaired brain development and damaged fibres of passage. In contrast, local administration of GABA receptor agonists, as used here, temporarily reduces regional output without affecting brain development and connectivity (Martin, 1991; van Duuren *et al.*, 2007). After neonatal lesions, other regions may functionally compensate for the loss. However, our data show that disruption of PFC signalling markedly interferes with the execution of social play behaviour, indicating that functional integrity of the PFC is required during social play behaviour.

In the present study, comparable effects of inactivation were observed in the three PFC regions tested. Muscimol administration into a certain cortical region may influence neuronal activity in adjacent regions through local cortico-cortical circuits (Martin, 1991). However, if in the present study the effects of B&M in some regions were mediated via cortico-cortical interactions, the behavioural effects would have to be distance-dependent. Since no difference was observed in the effect size between the PFC regions we conclude that each of the three PFC regions has an important role in social play behaviour.

The PFC and OFC have been widely implicated in executive functions, such as attention, planning, cognitive flexibility and decision making (Dalley *et al.*, 2004; Wallis, 2007). Given the complex and unpredictable nature of social interactions, it is likely that frontal cortical regions subserve executive functions in social contexts as well (Adolphs, 2003). Indeed, OFC lesions have been reported to enhance aggressive behaviour (de Bruin *et al.*, 1983; Rudebeck *et al.*, 2007) and the OFC is also involved in the evaluation of social information (Azzi *et al.*, 2012; Pellis *et al.*, 2006). The medial PFC has been implicated in maternal behaviour (Pereira and Morrell, 2011), social hierarchy (Wang *et al.*, 2011) and social comparisons in decision-making (Bault *et al.*, 2011). Inactivation of the PrL, IL and MO/VO may, therefore, disrupt social play behaviour by interfering with cognitive flexibility and decision making processes necessary for a proper social interaction. Given the anatomical (Groenewegen and Uylings, 2010; Vertes, 2004) and functional (e.g. Chudasama *et al.*, 2003) differences within the PFC, it is likely that different components of social play are affected by inactivation of the three different regions.

Inactivation of the NAcc does not affect social play behaviour

A complete blockade of play was observed after administration of B&M (1.0 nmol/ 0.3 µl and 0.1 nmol/ 0.3 µl) into the NAcc shell. However, this effect was probably secondary to a large increase in non-specific eating, leaving no opportunity for the animals to engage in social interaction (see Stratford and Kelley, 1997). A lower dose of B&M, which did not induce eating, did not alter social play. Since the effects of NAcc shell infusions of GABA receptor agonists and AMPA receptor antagonists on eating behaviour are highly

comparable (Maldonado-Irizarry *et al.*, 1995; Stratford and Kelley, 1997), DNQX was not administered into the shell. Thus, understanding the effect of inactivation of the NAcc shell on social play behaviour remains difficult.

Administration of B&M or DNQX into the NAcc core did not affect social play. Therefore, functional activity of the core is apparently not required for the execution of social play behaviour. These results appear at odds with previous findings, implicating the NAcc core and shell in social play behaviour (Gordon *et al.*, 2002; Trezza *et al.*, 2011; -2012; Van Kerkhof *et al.*, 2012, submitted). Thus, after social play behaviour, c-fos activity was enhanced in the NAcc (Gordon *et al.*, 2002; Van Kerkhof *et al.*, 2012, submitted). In addition, the NAcc core and shell were found to mediate opioid and cannabinoid effects on social play (Trezza *et al.*, 2011; -2012). In view of the involvement of the NAcc in hedonics and incentive motivation (Berridge and Kringelbach, 2008; Kelley, 2004; Salamone and Correa, 2012), these findings suggest a role for the NAcc in the positive emotional properties of social play behaviour. The lack of effect of NAcc core inactivation suggests that if output from the core is inhibited, social play is mediated by other regions, such as the NAcc shell. If, however, activity of a critical neurotransmitter system, such as opioids or cannabinoids, is altered, then NAcc output is changed in such a way that social play behaviour is affected (Trezza *et al.*, 2011; -2012).

Glutamatergic DMS inputs inhibit social play behaviour

Infusion of B&M into the DMS tended to increase play initiation. In addition, administration of DNQX into the DMS enhanced pinning and pouncing. This suggests that glutamatergic input into the DMS exerts an inhibitory influence on social play behaviour. Possibly, inactivation of the DMS with B&M locally affects opposing processes resulting in the absence of a clear effect as observed after AMPA/kainate receptor blockade. In other words, an optimal balance of different inputs into the DMS may be necessary for normal expression of social play behaviour, whereas B&M infusion affects different inputs in a comparable fashion resulting in a smaller effect of inactivation.

We previously observed a correlation between social play-induced c-fos activity in the DMS and its inputs from the PrL (Van Kerkhof *et al.*, 2012, submitted), suggesting that glutamatergic inputs from the PrL to the DMS are involved in social play behaviour. However, inhibition of PrL output reduced social play behaviour, whereas blocking glutamatergic inputs into the DMS increased it. Thus, involvement of a glutamatergic PrL-to-DMS projection in social play does not appear likely. However, since inactivation of the PrL will inhibit all its outputs, the specific involvement of the PrL-DMS projection may be overshadowed by the effect of PrL B&M on other PrL outputs. In addition, it is possible that other glutamatergic inputs are responsible for the effects of DMS manipulation, such as those from the amygdala and thalamus. Indeed, these regions have been implicated in social play behaviour (Siviy and Panksepp, 2011; Trezza *et al.*, 2010; -2012)).

Previous lesion (Devan *et al.*, 1999; Eagle and Robbins, 2003) and inactivation studies (Corbit and Janak, 2007) have shown involvement of the DMS in response selection and response inhibition. Thus, animals lacking a functional DMS have been found to display various forms of disinhibited behaviour. Of particular interest are the findings that DMS lesions impair (Eagle and Robbins, 2003), whereas with methylphenidate improves stop signal reaction time task performance (Eagle *et al.*, 2007). These findings echo the effects

of methylphenidate (Vanderschuren *et al.*, 2008), which reduces social play, and DMS DNQX, which enhances it. This hints at the possibility that expression of vigorous social play behaviour can be kept in control through inhibitory mechanisms mediated by the DMS.

Conclusion

The present study indicates a more direct involvement of the medial PFC and OFC in social play behaviour than previously thought. Besides facilitating the performance of appropriate responses to play initiations from the partner, as previously reported (Bell *et al.*, 2009; Pellis *et al.*, 2006), functional integrity of the PFC is important for the animal to initiate a playful interaction itself. Furthermore, glutamatergic inputs into the DMS, but not the NAcc core, exert inhibitory control over social play behaviour. These results provide important opportunities for further investigations as to how the PFC and striatum are involved in social play behaviour.

References

- Adolphs R (2003) Cognitive neuroscience of human social behaviour. Nat Rev Neurosci 4:165-178.
- Alessandri SM (1992) Attention, play, and social behavior in ADHD preschoolers. J Abnorm Child Psychol 20:289-302.
- Azzi JC, Sirigu A, Duhamel JR (2012) Modulation of value representation by social context in the primate orbitofrontal cortex. Proc Natl Acad Sci U S A 109:2126-2131.
- Bault N, Joffily M, Rustichini A, Coricelli G (2011) Medial prefrontal cortex and striatum mediate the influence of social comparison on the decision process. Proc Natl Acad Sci U S A 108:16044-16049.
- Bell HC, McCaffrey DR, Forgie ML, Kolb B, Pellis SM (2009) The role of the medial prefrontal cortex in the play fighting of rats. Behav Neurosci 123:1158-1168.
- Berridge KC, Kringelbach ML (2008) Affective neuroscience of pleasure: reward in humans and animals. Psychopharmacology (Berl) 199:457-480.
- Braun K, Bock J (2011) The experience-dependent maturation of prefronto-limbic circuits and the origin of developmental psychopathology: implications for the pathogenesis and therapy of behavioural disorders. Dev Med Child Neurol 53 Suppl 4:14-18.
- Casey BJ, Jones RM (2010) Neurobiology of the adolescent brain and behavior: implications for substance use disorders. J Am Acad Child Adolesc Psychiatry 49:1189-1201.
- Cheng SY, Taravosh-Lahn K, Delville Y (2008) Neural circuitry of play fighting in golden hamsters. Neuroscience 156:247-256.
- Chudasama Y, Passetti F, Rhodes SE, Lopian D, Desai A, Robbins TW (2003) Dissociable aspects of performance on the 5-choice serial reaction time task following lesions of the dorsal anterior cingulate, infralimbic and orbitofrontal cortex in the rat: differential effects on selectivity, impulsivity and compulsivity. Behav Brain Res 146:105-119.
- Corbit LH, Janak PH (2007) Inactivation of the lateral but not medial dorsal striatum eliminates the excitatory impact of Pavlovian stimuli on instrumental responding. J Neurosci 27:13977-13981.
- Dalley JW, Cardinal RN, Robbins TW (2004) Prefrontal executive and cognitive functions in rodents: neural and neurochemical substrates. Neurosci Biobehav Rev 28:771-784.
- de Bruin JP, van Oyen HG, Van de Poll N (1983) Behavioural changes following lesions of the orbital prefrontal cortex in male rats. Behav Brain Res 10:209-232.
- Devan BD, McDonald RJ, White NM (1999) Effects of medial and lateral caudate-putamen lesions on place- and cue-guided behaviors in the water maze: relation to thigmotaxis. Behav Brain Res 100:5-14.
- Eagle DM, Robbins TW (2003) Inhibitory control in rats performing a stop-signal reaction-time task: effects of lesions of the medial striatum and d-amphetamine. Behav Neurosci 117:1302-1317.
- Eagle DM, Tufft MR, Goodchild HL, Robbins TW (2007) Differential effects of modafinil and methylphenidate on stop-signal reaction time task performance in the rat, and interactions with the dopamine receptor antagonist cis-flupenthixol. Psychopharmacology (Berl) 192:193-206.
- Gordon NS, Kollack-Walker S, Akil H, Panksepp J (2002) Expression of c-fos gene activation during rough and tumble play in juvenile rats. Brain Res Bull 57:651-659.
- Graham KL (2011) Coevolutionary relationship between striatum size and social play in nonhuman primates. Am J Primatol 73:314-322.
- Groenewegen HJ, Uylings HBM (2010) Orginization of Prefrontal-Striatal Projections. In: Handbook of Basal Ganglia Structure and Function (Steiner H, Tseng KY, eds), pp 353-365. Academic Press.
- Kelley AE (2004) Ventral striatal control of appetitive motivation: role in ingestive behavior and reward-related learning. Neurosci Biobehav Rev 27:765-776.

- Maldonado-Irizarry CS, Swanson CJ, Kelley AE (1995) Glutamate receptors in the nucleus accumbens shell control feeding behavior via the lateral hypothalamus. J Neurosci 15:6779-6788.
- Manning MM, Wainwright LD (2010) The role of high level play as a predictor social functioning in autism. J Autism Dev Disord 40:523-533.
- Martin JH (1991) Autoradiographic estimation of the extent of reversible inactivation produced by microinjection of lidocaine and muscimol in the rat. Neurosci Lett 127:160-164.
- Panksepp J (1981) The ontogeny of play in rats. Dev Psychobiol 14:327-332.
- Panksepp J, Normansell L, Cox JF, Siviy SM (1994) Effects of neonatal decortication on the social play of juvenile rats. Physiol Behav 56:429-443.
- Panksepp J, Siviy SM, Normansell L (1984) The psychobiology of play: theoretical and methodological perspectives. Neurosci Biobehav Rev 8:465-492.
- Paxinos G, Watson C (2007) The rat brain in sterotaxic coordinates. Elsevier Academic.
- Pellis SM, Castaneda E, McKenna MM, Tran-Nguyen LT, Whishaw IQ (1993) The role of the striatum in organizing sequences of play fighting in neonatally dopamine-depleted rats. Neurosci Lett 158:13-15.
- Pellis SM, Hastings E, Shimizu T, Kamitakahara H, Komorowska J, Forgie ML, Kolb B (2006) The effects of orbital frontal cortex damage on the modulation of defensive responses by rats in playful and nonplayful social contexts. Behav Neurosci 120:72-84.
- Pellis SM, Pellis VC (2009) The Playful Brain. OneWorld Publications.
- Pereira M, Morrell JI (2011) Functional mapping of the neural circuitry of rat maternal motivation: effects of site-specific transient neural inactivation. J Neuroendocrinol 23:1020-1035.
- Rudebeck PH, Walton ME, Millette BH, Shirley E, Rushworth MF, Bannerman DM (2007) Distinct contributions of frontal areas to emotion and social behaviour in the rat. Eur J Neurosci 26:2315-2326.
- Salamone JD, Correa M (2012) The mysterious motivational functions of mesolimbic dopamine. Neuron 76:470-485.
- Schneider M, Koch M (2005) Deficient social and play behavior in juvenile and adult rats after neonatal cortical lesion: effects of chronic pubertal cannabinoid treatment. Neuropsychopharmacology 30:944-957.
- Siviy SM, Panksepp J (2011) In search of the neurobiological substrates for social playfulness in mammalian brains. Neurosci Biobehav Rev 35:1821-1830.
- Spear LP (2000) The adolescent brain and age-related behavioral manifestations. Neurosci Biobehav Rev 24:417-463.
- Špinka M, Newberry RC, Bekoff M (2001) Mammalian play: training for the unexpected. Q Rev Biol 76:141-168.
- Stratford TR, Kelley AE (1997) GABA in the nucleus accumbens shell participates in the central regulation of feeding behavior. J Neurosci 17:4434-4440.
- Trezza V, Baarendse PJJ, Vanderschuren LJMJ (2009) Prosocial effects of nicotine and ethanol in adolescent rats through partially dissociable neurobehavioral mechanisms. Neuropsychopharmacology 34:2560-2573.
- Trezza V, Baarendse PJJ, Vanderschuren LJMJ (2010) The pleasures of play: pharmacological insights into social reward mechanisms. Trends Pharmacol Sci 31:463-469.
- Trezza V, Damsteegt R, Achterberg EJM, Vanderschuren LJMJ (2011) Nucleus accumbens mu-opioid receptors mediate social reward. J Neurosci 31:6362-6370.

- Trezza V, Damsteegt R, Manduca A, Petrosino S, Van Kerkhof LWM, Pasterkamp RJ, Zhou Y, Campolongo P, Cuomo V, Di Marzo V, Vanderschuren LJMJ (2012) Endocannabinoids in amygdala and nucleus accumbens mediate social play reward in adolescent rats. J Neurosci 32:14899-14908.
- van Duuren E, van der Plasse G, van der Blom R, Joosten RN, Mulder AB, Pennartz CM, Feenstra MG (2007) Pharmacological manipulation of neuronal ensemble activity by reverse microdialysis in freely moving rats: a comparative study of the effects of tetrodotoxin, lidocaine, and muscimol. J Pharmacol Exp Ther 323:61-69.
- Van Kerkhof LWM, Trezza V, Mulder T, Gao P, Voorn P, Vanderschuren LJMJ (2012) Cellular activation in limbic brain systems during social play behaviour in adolescent rats. Submitted.
- Vanderschuren LJMJ, Niesink RJM, Van Ree JM (1997) The neurobiology of social play behavior in rats. Neurosci Biobehav Rev 21:309-326.
- Vanderschuren LJMJ, Trezza V, Griffioen-Roose S, Schiepers OJG, Van Leeuwen N, De Vries TJ, Schoffelmeer ANM (2008) Methylphenidate disrupts social play behavior in adolescent rats. Neuropsychopharmacology 33:2946-2956.
- Veeneman MMJ, Boleij H, Broekhoven MH, Snoeren EMS, Guitart MM, Cousijn J, Spooren W, Vanderschuren LJMJ (2011) Dissociable roles of mGlu5 and dopamine receptors in the rewarding and sensitizing properties of morphine and cocaine. Psychopharmacology 214:863-876.
- Vertes RP (2004) Differential projections of the infralimbic and prelimbic cortex in the rat. Synapse 51:32-58.
- Voorn P, Vanderschuren LJMJ, Groenewegen HJ, Robbins TW, Pennartz CMA (2004) Putting a spin on the dorsal-ventral divide of the striatum. Trends Neurosci 27:468-474.
- Wallis JD (2007) Orbitofrontal cortex and its contribution to decision-making. Annu Rev Neurosci 30:31-56.
- Wang F, Zhu J, Zhu H, Zhang Q, Lin Z, Hu H (2011) Bidirectional control of social hierarchy by synaptic efficacy in medial prefrontal cortex. Science 334:693-697.

Chapter 4
Functional integrity of the habenula is necessary for social play behaviour in adolescent rats

Linda W.M. van Kerkhof[1]
Ruth Damsteegt[1]
Viviana Trezza[1,2]
Pieter Voorn[3]
Louk J.M.J. Vanderschuren[1,4]

1 Rudolf Magnus Institute of Neuroscience, Department of Neuroscience and Pharmacology, University Medical Centre Utrecht, Utrecht, The Netherlands
2 Department of Biology, University "Roma Tre", Rome, Italy
3 Department of Anatomy and Neurosciences, Neuroscience Campus Amsterdam, VU University Medical Centre, Amsterdam, The Netherlands
4 Department of Animals in Science and Society, Division of Behavioural Neuroscience, Faculty of Veterinary Medicine, Utrecht University, Utrecht, The Netherlands

Submitted.

Abstract

During adolescence, a marked increase in peer-peer interactions is observed in all mammals, including humans, which is signified by the abundance of social play behaviour. Social play is highly rewarding, and known to be modulated through monoaminergic neurotransmission. Recently, the habenula has received widespread attention because of its role in the regulation of monoaminergic neurotransmission as well as in a variety of emotional and cognitive functions. Therefore, in the present study, we investigated the involvement of the habenula in social play behaviour. Using the neuronal activity maker c-fos, we show that the habenula is activated after 24 h of social isolation in adolescent rats, and that a subsequent social play interaction reduces c-fos activity in the medial part of the lateral habenula. This suggests that habenula activity modulates the aversive properties of social isolation, which is alleviated by the positive effects of social play. Furthermore, after functional inactivation of the habenula, using a mixture of the GABA receptor agonists baclofen and muscimol, social play behaviour is markedly reduced, whereby responsiveness to play solicitation is more sensitive to habenula inactivation than play solicitation itself. Together, our data indicate an important role for the habenula in the processing of positive (social play behaviour) and negative (social isolation) social information in adolescent rats. Altered habenula function might be related to the social impairments in childhood and adolescent psychiatric disorders such as autism, attention deficit/hyperactivity disorder and early-onset schizophrenia.

Introduction

Social play behaviour is a vigorous, rewarding form of social interaction in young mammals (Panksepp *et al.*, 1984; Pellis and Pellis, 1997; Spear, 2000; Vanderschuren *et al.*, 1997). Social play behaviour is thought to be important for the acquisition of communication skills, the formation and maintenance of social bonds, and for social and cognitive development (Palagi, 2006; Pellis and Pellis, 2009; Potegal and Einon, 1989; Špinka *et al.*, 2001; Van den Berg *et al.*, 1999). Conversely, abnormalities in social play behaviour have been observed in several childhood and adolescent psychiatric disorders (Alessandri, 1992; Jordan, 2003; Manning and Wainwright, 2010; Moller and Husby, 2000). Therefore, identifying the neural substrates of social play behaviour will advance our understanding of normal social development and the aetiology of childhood and adolescent psychiatric disorders.

The neural substrates of social play behaviour remain incompletely understood. Lesion and intracranial drug administration studies have implicated the frontal cortex (Bell *et al.*, 2009; Panksepp *et al.*, 1994; Pellis *et al.*, 2006; Schneider and Koch, 2005), as well as the thalamus (Siviy and Panksepp, 1985; -1987), amygdala (Daenen *et al.*, 2002; Meaney *et al.*, 1981; Trezza *et al.*, 2012), striatum(Pellis *et al.*, 1993) and nucleus accumbens (Trezza *et al.*, 2011b; Trezza *et al.*, 2012) in social play. Furthermore, monoaminergic (i.e., dopaminergic, serotoninergic and noradrenergic) neurotransmission plays a prominent role in the modulation of social play (Homberg *et al.*, 2007; Siviy *et al.*, 1996; -2011; Trezza *et al.*, 2010; Vanderschuren *et al.*, 1997; -2008).

In recent years, the habenula has received a great deal of attention because of its key role in regulating monoaminergic neurotransmission (Hikosaka, 2010; Lecourtier and Kelly, 2007). The habenula can be subdivided into a medial (mHB) and a lateral part (lHb), whereby the latter can be further divided into the lateral habenula medial part (lHbm) and lateral habenula lateral part (lHbl). These regions provide regionally distributed input to monoamine nuclei, including the ventral tegmental area (VTA), dorsal raphe nucleus, and locus coeruleus (Goncalves *et al.*, 2012; Kim, 2009; Lecourtier and Kelly, 2007). Considering its interconnections with monoaminergic pathways, it is not surprising that the habenula has been implicated in functions that depend on monoaminergic signalling, such as reward, punishment, decision-making, learning, attention and stress (for review see Hikosaka, 2010; Lecourtier and Kelly, 2007). Furthermore, the habenula has been suggested to be involved in several psychiatric disorders that involve altered monoaminergic neurotransmission, including depression, ADHD, and schizophrenia (Hikosaka, 2010; Lee and Goto, 2011; Yang *et al.*, 2008).

Given its involvement in a variety of cognitive and emotional behavioural processes, it is reasonable to expect that the habenula is involved in social behaviour as well. However, except for one study that has implicated the habenula in maternal behaviour (Matthews-Felton *et al.*, 1995), its role in social behaviour is unknown. The present study fills the hiatus by mapping habenular neuronal activity during social play behaviour and determining the effects of inhibition of habenula activity on the quality and intensity of social play behaviour.

Materials and Methods

Animals

Male Wistar rats (Charles River, Sulzfeld, Germany) arrived in our animal facility at 21 days of age. They were housed in groups of four in 40x26x20 cm Macrolon cages under controlled conditions (i.e. temperature 20-21 °C, 55-65 % relative humidity and 12/12 h light cycle with lights on at 7.00 a.m.). Food and water were available ad libitum. All animals used were experimentally naïve. During the first 6 days rats were handled at least twice. All experiments were approved by the Animal Ethics Committee of Utrecht University and were conducted in accordance with Dutch regulations (Wet op de Dierproeven, 1996) and European regulations (Guideline 86/609/EEC).

Surgical procedures

The surgical procedures were based on previous experiments (Trezza *et al.*, 2011b; -2012). At 27-28 days of age, rats were anesthetised with 0.08 ml/100g (s.c.) Hypnorm (fentanylcitrate 0.315 mg/ml and fluanison 10 mg/ml, Janssen, Belgium) and positioned into a stereotactic frame (David Kopf Instruments, USA). Guide cannulae (24 gauge microblasted thinwalled stainless steel, Cooper's Needleworks, UK) were implanted bilaterally. The cannulae were aimed 0.5 mm above the habenula (coordinates: anterior-posterior (AP) -3.0 mm from Bregma; medial-lateral (ML) ±0.8 mm from the midline; dorsal-ventral (DV) -4.2 mm from skull surface). Coordinates were determined by pilot placements in rats 28 days of age.

Cannulae were secured with stainless steel screws and dental acrylic. Stainless steel stylets (29 gauge) were inserted into the guide cannulae to maintain patency. After surgery, rats were individually housed for 4 days to recover, after which they were housed with their original cage mates.

Drugs and infusion procedures

The GABA-A receptor agonist muscimol (Tocris Bioscience, UK) and the GABA-B receptor agonist (RS)-baclofen (Tocris Bioscience, UK) were dissolved in saline. Infusion procedures were as previously described (Trezza *et al.*, 2011b; Trezza *et al.*, 2012). In short, bilateral infusions of drugs or an equivalent volume of saline were administered using 30-gauge injectors (Bilaney, Germany) that were connected to 10 µl Hamilton micro-syringes by polyethylene (PE-20) tubing. Over 60 s, 0.3 µl of the drugs or saline was infused using a syringe pump (model 975A; Harvard Apparatus, USA), and the injectors were left in place for another 60 s to allow for diffusion. Two different doses were used: a mixture of 1.0 nmol/ 0.3 µl baclofen and 0.1 nmol/ 0.3 µl muscimol or a mixture of 0.3 nmol/ 0.3 µl baclofen and 0.03 nmol/ 0.3 µl muscimol. After the procedure, stylets were replaced and animals were left in a holding cage for 5 min before testing.

Behavioural testing

Experiments were performed, as previously described (Trezza and Vanderschuren, 2008), in a sound attenuated chamber under red light conditions. The testing arena was a Plexiglas cage (40x40x60 cm) with approximately 2 cm of wood shavings covering

the floor. Animals were randomly paired with an unfamiliar partner. Animals in a test pair did not differ more than 10 g in body weight.

The experiment investigating the effect of functional inactivation of the habenula on social play behaviour was conducted as follows (Trezza *et al.*, 2011b; Trezza *et al.*, 2012). One week post-surgery, the rats were habituated to the experimental procedures on 2 consecutive days. On the first habituation day, rats were individually placed into the test cage for 10 min. On the second habituation day, the animals were socially isolated for 2.5 h. Pairs of rats were then infused with vehicle solutions and placed into the test cage for 15 min, to habituate them to the infusion and testing procedures. On the test day, pairs of rats (isolated for 3.5 or 24 h) received either a drug or a saline infusion and were placed into the test cage for 15 min.

The experiment aimed to determine the c-fos expression in the habenula after social play behaviour was performed as previously described (Van Kerkhof *et al.*, 2012, submitted). Animals were separately habituated to the test cage for 30 min on 4 consecutive days, to minimise the influence of novelty of the test environment on c-fos expression. The motivation for play was enhanced to half-maximal and maximal levels, respectively, by isolating the animals for 3.5 h or 24 h before the test (Niesink and Van Ree, 1989; Vanderschuren *et al.*, 1995; -2008). On the test day, animals were placed into the test cage either in pairs ('play group') or alone ('no play group') for 15 min. After the test, animals were placed back into their separate cages for 30 min. Subsequently, rats were sacrificed by decapitation, their brains were quickly removed and frozen immediately (-80 °C).

Behaviour of the animals was recorded using a camera with zoom lens, video tape recorder and television monitor. The behaviour of the playing rats was assessed using the Observer 5.1 software (Noldus Information Technology B.V., The Netherlands). Three behavioural elements were scored (Panksepp *et al.*, 1984; Trezza *et al.*, 2010; Vanderschuren *et al.*, 1997).

- *Frequency of pinning:* one animal lying with its dorsal surface on the floor with the other animal is standing over it, which is the most characteristic posture of social play in rats.
- *Frequency of pouncing:* One animal attempts to nose or rub the nape of the neck of the partner, which is an index of play solicitation.
- *Time spent in social exploration:* one animal sniffing or grooming any part of the partner's body.

To assess whether effects of the drug treatment on social play were secondary to changes in locomotor activity, the rats were subsequently tested for horizontal locomotor activity as previously described (Trezza *et al.*, 2009; Veeneman *et al.*, 2011). There was at least one day without infusions or testing between the tests for social play behaviour and locomotor activity. The infusion protocol was similar to the one described above. After the infusion procedure, rats were transferred to a plastic cage (l x w x h, 50 x 33 x 40 cm) and their position was tracked five times per second for 30 min using a video-tracking system (EthoVision, Noldus Information Technology, The Netherlands).

Histological confirmation of injection sites

Animals were sacrificed using carbon dioxide inhalation and microinjected with 0.3 µl of black ink (Parker) over 1 min through the guide cannulae, comparable to the drug infusion procedure. After the infusion, animals were immediately decapitated, their brains removed and immediately frozen. Cryostat sections (20 µm) were collected and stained using Nissl-staining. In short, slides were placed in Thionin solution (0.13% in MilliQ) for 1-5 min. Subsequently, slides were placed for 1 min in MilliQ water, 70%, 80%, 96% and 100% ethanol (3x). Next, slides were placed in xylene (2x) for 2 min and coverslipped using Entellan (Merck). Placement of the microinjection sides was determined using a light microscope according to the atlas of Paxinos and Watson (2007). Only pairs in which both animals had bilateral needle tracks terminating into the target area were included in the final analysis (see Fig. 1A).

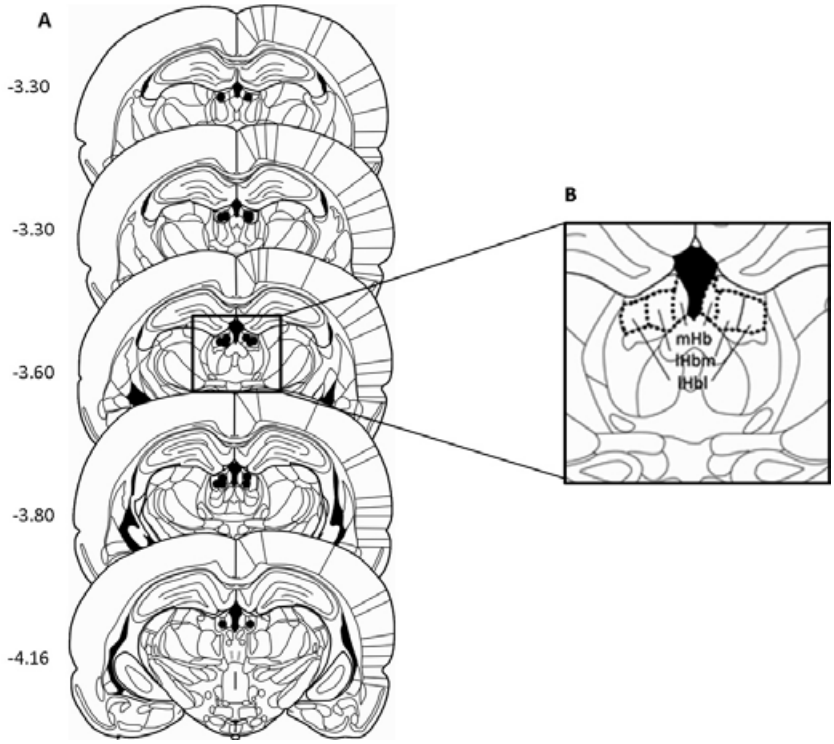


Fig. 1: A. Schematic representation of brain sections with the spread of microinjection placements (black dots) in the habenula. B. Schematic representation of a brain section outlining the borders of the different habenula subnuclei analysed for c-fos expression. Figures are adapted from Paxinos and Watson (2007).

c-fos DIG *in situ* hybridization

Fresh frozen brains were cryostat sectioned (-20 °C) at 14 µm, mounted on Super-Frost Plus slides (Eric Scientific Co, Portsmouth, NH) and stored at -80 °C. Slides were warmed to room temperature before fixation with 4% PFA (4% paraformaldehyde in PBS, 154 mM NaCl, 0.896 mM KH2PO4, 4.58 mM Na2HPO4, pH = 7.5). Acetylation of the slides was performed with acetic anhydride (0.25% acetic anhydride in 1.5% triethanolamine

buffer). Subsequently, slides were washed with PBS and 2x saline sodium citrate buffer (SSC buffer) before applying the hybridization mix (50% formamide, 4x SSC, 0.4% bakers yeast tRNA, 2% 50x Denhardt's reagent, 10% Dextran, 0.05% salmon sperm DNA) containing 5 ng c-fos probe per section.

The probe was generated using cDNA synthesised from total rat brain RNA and the iScript reverse transcriptase kit with random hexamers, according to manufacturer's protocol (Bio-Rad, Hercules, California). A PCR was performed with c-fos specific primers containing T3/T7 promoters. Primers (Eurogentec, Liège, Belgium) were designed using Primer3 (Rozen and Skaletsky, 2000). All primers were checked for gene specificity by BLAST searching. The primer sequences used for c-fos (Genbank NM_022197.2) were T3 antisense: AATTAACCCTCACTAAAGGG-CACAGCCTGGTGAGTTTCAC and T7 sense: GTAATACGACTCACTATAGGG-TCACCCTGCCTCTTCTCAAT. The PCR product size was checked by agarose gel electrophoresis. From these PCR products, labelled probes were generated by linear amplification using the MAXIscript Kit according to manufacturer's protocol (Applied Biosystems, Foster City, California) and probes were labelled using digoxigenin-UTP (DIG labelling mix, Roche, Penzberg, Germany). The probe size and concentration were checked using agarose gel electrophoreses. The probe was briefly heated at 95 °C before adding it to the hybridization mix and hybridization was performed in a humid chamber at 60 °C overnight.

Post-hybridization washes were carried out with 1x SSC at 60 °C, including a wash with 2x SSC containing RNAse A (0.3 units/ml, Roche, Penzberg, Germany) at 37 °C. Before antibody incubation, slides were exposed to a blocking solution (1% blocking powder in TRIS buffer, 100 mM Tris, 150 mM NaCl, pH = 7.5) according to the DIG detection kit manual (Roche, Penzberg, Germany) for 1 h. Slides were incubated with anti-DIG-AP antibody (1:2500, DIG detection kit, Roche, Penzberg, Germany). This antibody was conjugated to alkaline phosphatase (AP), allowing the use of NBT/BCIP as a substrate to visualise the probe. The antibody incubation was performed overnight at 4 °C.

Following antibody incubation, slides were washed in TBS (100 mM TRIS, 150 mM NaCl, pH = 7.5) and a magnesium buffer (100 mM Tris, 100 mM NaCl, 50 mM MgCl, pH = 9.5). Incubation with the substrate NBT/BCIP (1:50; Roche, Penzberg, Germany) in magnesium buffer was performed in a humid chamber at room temperature for 28 h. The reaction was stopped with TBS containing EDTA (1 mM EDTA, pH = 7.5) and slides were washed twice with water to remove salt precipitate. Slides were left to dry and coverslipped using Merckoglas (Merck, New Jersey, USA).

Quantification of c-fos immunopositive cells

The quantification methods have previously been described (Nordquist *et al.*, 2008). Images of all regions of interest were digitised using an objective magnification of x5 on a Leica DM/RBE photomicroscope with a Q-imaging 12 bit camera and MCID software (InterFocus Imaging, Cambridge, UK). Image acquisition was preceded by a flat field correction and a calibration routine to ensure standardised OD values. For each subject 3 (occasionally 2) sections were digitised per region of interest. Sections were made in series of ten, therefore, sections were 140 µM apart.

Regions of interest were determined using images of Nissl-stained adjacent sections (Nissl-staining as described above) according to the atlas of the rat brain by Paxinos

and Watson (2007), as schematically presented in Fig. 1B (Paxinos and Watson, 2007). An algorithm was used to identify c-fos positive cells. In short, images underwent histogram equalization and smoothing (low-pass filter, kernel size 7x7). The unfiltered image was subtracted from the smoothed image, followed by a series of steps to optimise the processed image and make it a suitable measuring template for detecting objects the size and shape of c-fos immunopositive cells. The number of cells counted was corrected with a factor indicating approximate size of a cell, thus preventing two adjacent segmented objects mistakenly counted as one cell. This algorithm allows for an observer-independent measurement. Several parameters were measured: number of c-fos positive cells, optical density of each cell, and the total measured surface area of the region.

Data analysis of c-fos expression levels

The parameters obtained from the MCID software were used to calculate the density of c-fos positive cells (number of positive cells divided by the total surface area of the region). In addition, to compare the c-fos positive cell density in a manner that takes the labelling intensity into account, cells were categorised in three conditions: light, medium, and dark. Therefore, a frequency histogram of the optical densities of all cells in the non-playing animals was made for each brain region. These histograms were used to calculate the 33rd and 67th percentile of the optical density in the non-playing animals. These optical density values were used to categorise the cells in all animals. The number of cells in each category was divided by the total surface area of the respective region of interest, to determine the cell density per intensity category. The cell densities of the three categories indicate shifts in the frequency histograms of the optical densities (Fig.2). An upward shift in the histogram would be reflected by an increase in the overall cell density as well as an increase in the medium category and would suggest recruitment of a new population of cells. A rightward shift of the curve is expected when the same neurons are active, but express more c-fos. This would be reflected by an increase in the cell density in the dark category. In a similar fashion, a downward or leftward shift of the histogram would indicate a smaller number of activated cells, or a reduced quantity of c-fos expression per activated cell, respectively.

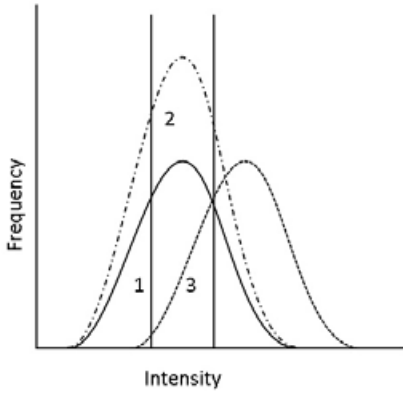


Fig. 2: Theoretical representation of potential shifts in the intensity histogram. Curve 1 indicates the frequency histogram of the control group. The two vertical lines indicate the cut-off points used to separate the cells into the light, medium and dark category (33% and 67%). The area under the curve represents the total number of c-fos positive cells per mm2, i.e. the cell density. If social play behaviour induces c-fos activity in a new group of neurons, an increase in the cell density is expected, which would be reflected in an upward shift of the histogram (curve 2). This should be apparent as an enhanced cell density primarily in the medium category. Alternatively, if the same neurons are active, but express more c-fos as a result of play behaviour, a rightward shift is expected, with an increase in the cell density in the dark category (curve 3). A downward or leftward shift in the histogram could be explained in a similar fashion. Adapted from Nordquist et al., 2008.

Statistical analysis

For data analysis, SPSS software 15.0 (IBM software, New York, USA) was used. For each animal the mean c-fos positive cell density (FpCD) of three images (taken from three subsequent sections) was calculated. To assess the effect of isolation time and play experience on the FpCD, data was analysed using a two-way analysis of variance with social isolation and play experience as between subject factors. To determine the effect of play on the FpCD per intensity category, a two-way analysis of variance was used as well, with play experience and social isolation time as between subject factors, followed by *post hoc* Student's t-test analysis if appropriate. To assess the effect of baclofen/muscimol infusions on social play behaviour, data was analysed using a paired samples Student's t-test or an independent Student's t-test, depending on the experimental setup. The effect of baclofen/muscimol administration on locomotor activity was analysed using a one-way analysis of variance.

Results

Social isolation induces c-fos expression in the habenula

The mean c-fos positive cell density (FpCD) was determined in rats (n=8/group) that were socially isolated for 3.5 h or 24 h. After these isolation periods, half of the animals was placed in the test cage with a partner for 15 min ('play' group), while the other group was placed in the same test cage alone ('no play' group). In all habenula regions, social isolation for 24 h induced a significant increase in the FpCD compared to social

isolation for 3.5 h (mHb: $F_{isolation}(1,28) = 2.522$, $p = 0.026$; lHbm: $F_{isolation}(1,28) = 33.524$, $p < 0.001$; lHbl: $F_{isolation}(1,28) = 22.556$, $p < 0.001$) (Fig.3). These results indicate that after social isolation for 24 h, c-fos activity is increased in the entire habenula. No effect of play on the FpCD was observed in any of the habenula regions (mHb: $F_{play}(1,28) = 0.013$ $p = 0.910$; lHbm: $F_{play}(1,28) = 1.337$, $p = 0.257$; lHbl: $F_{play}(1,28) = 1.167$, $p = 0.289$), nor was there an interaction observed between isolation and play (mHb: $F_{play \times isolation}(1,28) = 2.780$ $p = 0.107$; lHbm: $F_{play \times isolation}(1,28) = 1.912$, $p = 0.178$; lHbl: $F_{play \times isolation}(1,28) = 1.101$, $p = 0.303$).

In addition, the c-fos expression levels were analysed taking the intensity levels into account (Fig. 4). This entailed the differentiation of the c-fos positive cell density per category of intensity: light, medium and dark cells (see Fig. 2). Results indicate that in all regions, 24 h of social isolation decreased the FpCD in the light category (mHb: $F_{isolation}(1,27) = 71.539$, $p < 0.001$; lHbm: $F_{isolation}(1,28) = 28.479$, $p < 0.001$; lHbl: $F_{isolation}(1,28) = 32.231$, $p < 0.001$; Fig.4A-C), while it increased the FpCD in the medium (Fig. 4D-F) and dark categories (Fig. 4G-I) (medium: mHb: $F_{isolation}(1,27) = 63.848$, $p < 0.001$; lHbm: $F_{isolation}(1,28) = 38.411$, $p < 0.001$; lHbl: $F_{isolation}(1,28) = 29.267$, $p < 0.001$; dark: mHb: $F_{isolation}(1,28) = 49.644$, $p < 0.001$; lHbm: $F_{isolation}(1,28) = 104.981$, $p < 0.001$; lHbl: $F_{isolation}(1,28) = 38.210$, $p < 0.001$) (Fig. 4). These results suggest that social isolation for 24 h enhances the levels of c-fos in cells that already express c-fos as well as recruiting previously inactive cells.

Interestingly, in the lHbm, a significant effect of play was observed in the dark category ($F_{play}(1,28) = 4.348$, $p = 0.046$), as well as an interaction between the effect of play and the effect of isolation ($F_{isolation \times play}(1,28) = 4.943$, $p = 0.034$) (Fig. 4F). These results indicate that play has a differential effect on the dark c-fos positive cells after 3.5 h and 24 h of isolation in the lHbm. Post hoc independent t-tests showed that there was no effect of play after 3.5 h isolation ($t = 0.381$, $df = 14$, $p = 0.709$), while after 24 h of isolation, play reduced the FpCD in the dark category ($t = -2.191$, $df = 14$, $p = 0.046$). In none of the other regions, effects of play were observed in the different categories (light category: mHb: $F_{play}(1,27) = 1.859$, $p = 0.184$; lHbm: $F_{play}(1,28) = 0.789$, $p = 0.382$; lHbl: $F_{play}(1,28) = 0.377$, $p = 0.544$; medium category: mHb: $F_{play}(1,27) = 0.758$, $p = 0.392$; lHbm: $F_{play}(1,28) = 0.791$, $p = 0.381$; lHbl: $F_{play}(1,28) = 1.343$, $p = 0.256$; dark category: mHb: $F_{play}(1,27) = 0.186$, $p = 0.670$; lHbl: $F_{play}(1,28) = 0.595$, $p = 0.447$) (Fig. 4).

In summary, social isolation enhances c-fos expression throughout the habenula. Social play behaviour reduces the number of dark c-fos expressing cells after 24 h of social isolation in the lHbm.

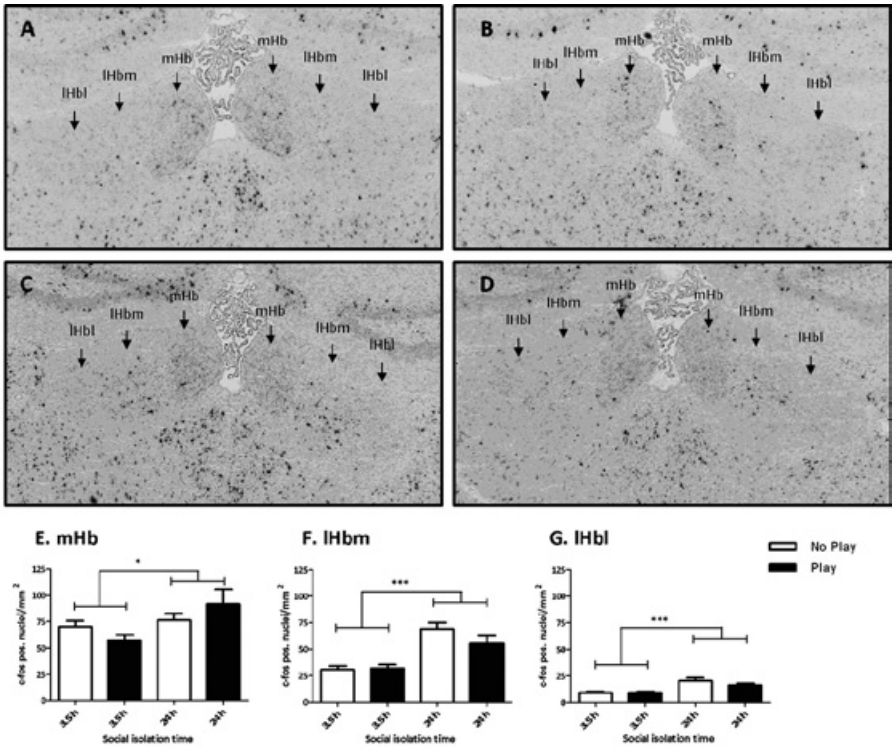


Fig. 3: Social isolation for 24 h enhances the cell density of c-fos positive cells. The c-fos positive cell density was determined in rats after 3.5 h or 24 h of social isolation and subsequently receiving a play session and in rats that were placed in the test cage without a partner present. Panel A-D shows representative pictures of c-fos labelling after 3.5 h no play (A), 3.5 h play (B), 24 h no play (C), and 24 h play (D). For delineation of the different habenula subregions see Fig. 1B. Panel E-F show quantification of c-fos positive cells in the four groups in the mHb (E), lHbm (F), lHbl (G). Data are presented as mean ± SEM. *$p < 0.05$, ***$p < 0.001$.

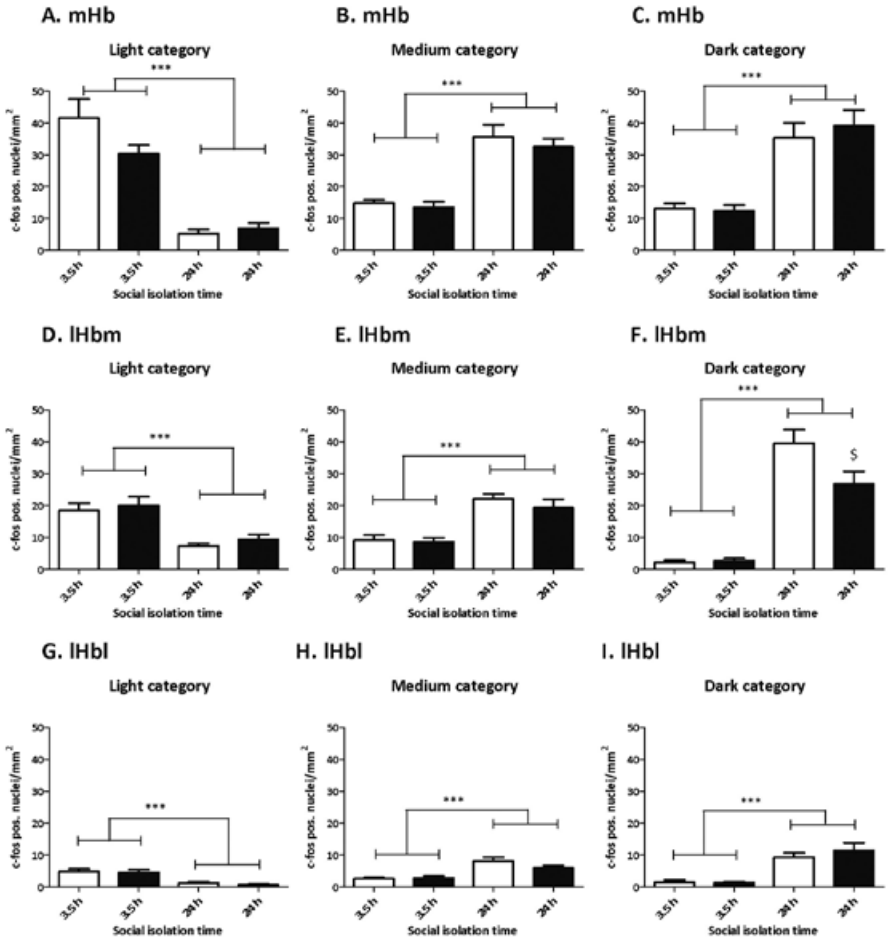


Fig. 4: Social isolation for 24 h reduces the cell density of light c-fos positive cells, while it increases the cell density of light and medium positive cells in the mHb (A-C), lHbm (D-F), and lHbm (G-I). Social play behaviour reduces the density of dark c-fos positive cells after 24 h of social isolation in lHbm (F). Data are presented as mean ± SEM. White bars = 'no play group', black bars – 'play group'. ***p <0.001 3.5 h vs. 24 h of social isolation, $p <0.05 play vs. no play group.

Temporary inactivation of the habenula reduced social play behaviour

To further investigate the role of the habenula in social play behaviour, this region was temporarily inactivated by local administration of a baclofen and muscimol mixture (B&M). The effect of B&M administration was investigated after both 3.5 h and 24 h of social isolation (Fig. 5). The dose of 1.0 nmol/ 0.3 µl baclofen and 0.1 nmol/ 0.3 µl muscimol almost completely abolished the levels of pinning after 24 h of isolation ($t = 4.808$, $df = 5$, $p = 0.005$) (Fig. 5A). Pouncing was decreased as well ($t = 4.074$, $df = 5$, $p = 0.010$), while social exploratory behaviour was unaffected ($t = -0.849$, $df = 5$, $p = 0.435$). These results indicate a specific effect of habenula inactivation on social play behaviour, without affecting non-playful social interactions.

Since this dose of B&M profoundly reduced social play behaviour, a lower dose of B&M

(0.3 nmol/ 0.3 µl baclofen and 0.03 nmol/ 0.3 µl muscimol) was also tested after 24 h of social isolation. This dose also reduced pinning (t = 3.177, df = 10, p = 0.010), while it did not affect pouncing (t = 1.662, df = 10, p = 0.128). These results suggest that at a lower dose, inactivation of the habenula specifically affects the responsiveness to social play initiations. Similar to the higher dose, this dose did not influence social exploration (t = -1.616, df = 10, p = 0.137).

Since social isolation affected FpCD in the habenula (Fig. 3-4), the effect of temporary inactivation of the habenula using the lower dose of B&M was also investigated after 3.5 h of social isolation (Fig. 5B). Comparable to what happened after 24 h of social isolation, B&M reduced pinning (t = 5.106, df = 10, p < 0.001), but not pouncing (t = 1.664, df = 10, p = 0.127) or social exploration (-1.837, df = 10, p = 0.096). None of the doses tested influenced locomotor activity ($F_{treatment}$ (2.28) = 1.390, p = 0.266) (Fig. 5C).

In summary, these results indicate that the inactivation of the habenula specifically affects social play behaviour, leaving locomotor activity and non-playful social investigation intact. Responsiveness to play solicitation seems more sensitive to habenula inactivation than the initiation of play. Furthermore, the disruption of play by inactivation of the habenula is independent of the duration of social isolation.

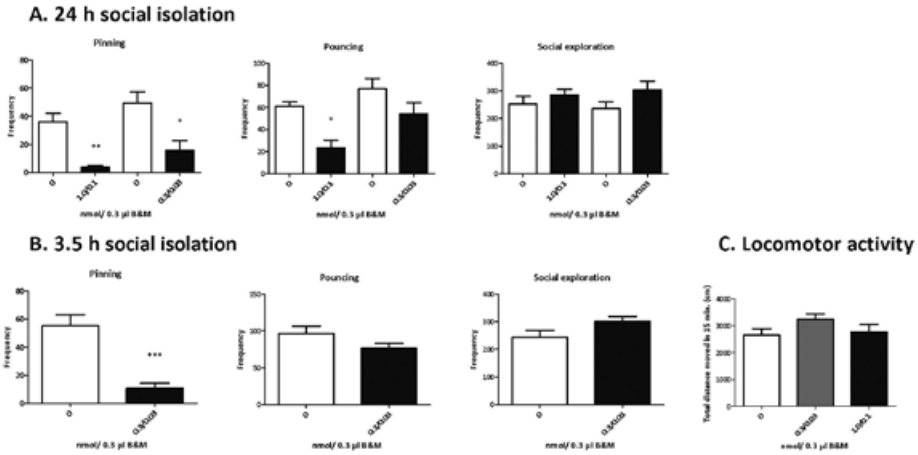


Fig. 5: Pharmacological inactivation with B&M reduces social play after 24 h (A) and 3.5 h (B) of isolation, but does not affect social exploration (A-B) or locomotor activity (C). Data are presented as mean ± SEM. *p< 0.05, **p <0.01, ***p <0.001.

Discussion

The present study is the first to report involvement of the habenula in social behaviour in adolescent rats. Our data show that the habenula becomes activated after social isolation. This social isolation-induced activation is reduced in the lHbm after social play. In addition, functional inactivation of the habenula suppressed social play behaviour.

The habenula responds to social isolation

In the present study, c-fos activity in the habenula was investigated after two periods of social isolation that have been shown to induce half-maximal and maximal levels of social play behaviour (Niesink and Van Ree, 1989; Vanderschuren *et al.*, 1995; -2008). Social isolation for 24 h enhanced the number of cells expressing c-fos relative to 3.5 h of social isolation, throughout the entire habenula. Previous studies, as well as our own observations, using c-fos as a neuronal activity marker have shown that the basal c-fos level in the habenula is low in naïve animals (Wirtshafter *et al.*, 1994; Zhang *et al.*, 2005). It is therefore possible that after 3.5 h of isolation the levels of c-fos are enhanced compared to baseline, but to a lesser extent than after 24 h of isolation. This suggests that cellular activity in the habenula is a function of the duration of social isolation. However, this conclusion should be drawn with caution because in the present study the c-fos levels were not determined after 0 h of isolation. A 0 h isolation group was not included, because eliminating the isolation period before testing results in a highly variable level of play behaviour (Vanderschuren *et al.*, 2008). These levels of play likely depend on the amount of social interaction in the home cage immediately before testing, which makes the interpretation of cellular activity results of a 0 h isolation group difficult.

Activation of the habenula after social isolation may be related to its role in the processing of aversive information (Hikosaka, 2010). Indeed, stressful stimuli have been shown to enhance c-fos expression in the lateral habenula (Wirtshafter *et al.*, 1994) and to induce immune responses in the medial habenula (Cirulli *et al.*, 1998; Sugama *et al.*, 2002). In addition, neuronal activity in the medial and lateral habenula has been found to be enhanced in rat models of depression (Caldecott-Hazard *et al.*, 1988; Shumake *et al.*, 2003). It is therefore likely that in the present study the habenula was activated as a result of the negative emotional effects of social isolation.

The habenula is positioned as a key regulator of monoaminergic neurotransmission. Activation of the habenula results in decreased activity of dopaminergic and serotonergic neurons, and/or increased noradrenergic activity, via direct and indirect projections to the VTA, dorsal raphe nucleus and locus coeruleus, respectively (Kalen *et al.*, 1989a; -1989b; Lecourtier *et al.*, 2008; Lecourtier and Kelly, 2007; Stern *et al.*, 1979). Recently, it has been shown that the projection of the lateral habenula to the rostromedial tegmental nucleus (RMTg), which in turn regulates dopamine activity via its inhibitory projections to the VTA and substantia nigra, signals aversive information (Hong *et al.*, 2011; Stamatakis and Stuber, 2012). Therefore, activity in the habenula after social isolation may result in altered monoamine levels, which signal the affective states associated with social isolation and the modulation of subsequent social interaction.

Social play reduces c-fos expression in the habenula after social isolation

The opportunity to play reduced the number of darkly labelled c-fos positive cells in the lHbm after 24 h of social isolation. The reduction in FpCD after play in animals isolated for 24 h may be related to the increase in c-fos expression as a result of social isolation. Social play behaviour is known to be highly rewarding (Trezza *et al.*, 2011a; Vanderschuren, 2010). The experience of a positive social event may therefore reduce the activity evoked by the aversive properties of social isolation in this part of the habenula. It is then reasonable to expect that the experience of social play behaviour has a larger effect on habenula activity after longer isolation periods, which is in keeping with our observations.

Interestingly, social play specifically reduced the FpCD in the lHbm, while it did not affect c-fos levels in the mHb or lHbl. These different habenula subregions are known to have distinct efferent and afferent connections, whereby the lateral habenula has dense projections to monoamine-producing nuclei (Aghajanian and Wang, 1977; Goncalves *et al.*, 2012; Jhou *et al.*, 2009; Lecourtier and Kelly, 2007). More specifically, the lHbl mainly projects to the RMTg, which provides a GABAergic input to the VTA and dorsal raphe nucleus, whereas the lHbm mainly projects directly to the VTA and dorsal raphe nucleus and has only sparse projections to the RMTg (Goncalves *et al.*, 2012; Kim, 2009). It has been hypothesised that the lHbl is involved in signalling aversive stimuli via the RMTg-VTA pathway to inhibit dopamine release (Hong *et al.*, 2011; Stamatakis and Stuber, 2012), whereas the lHbm is part of a habenula-VTA feedback loop, because the lHbm directly projects to, and also receives direct inputs from the VTA (Goncalves *et al.*, 2012; Skagerberg *et al.*, 1984). The experience of social play behaviour after 24 h of social isolation therefore alters the activity in the habenula-VTA loop, reflected by changes in lHbm activity.

The lateral habenula is also implicated in regulating serotonin and noradrenaline neurotransmission. This may be related to the effects of play on habenula c-fos levels as well, since these neurotransmitters are known to modulate social play behaviour (Homberg *et al.*, 2007; Siviy *et al.*, 2011; Siviy and Panksepp, 2011; Trezza *et al.*, 2010; Vanderschuren *et al.*, 1997; -2008). It has recently been shown that signalling from the basal ganglia to the habenula in response to aversive stimuli is reduced by serotonin (Shabel *et al.*, 2012), and that antidepressants reduce the c-fos response to stress in the medial habenula (Silva *et al.*, 2012). Thus, the play-induced suppression of habenula activity may be mediated by serotonin.

In summary, the positive experience of social play behaviour may reduce habenula activity when the animal is in a negative emotional state as a result of social isolation, through altered dopamine or serotonin neurotransmission.

Inactivation of the habenula reduces social play behaviour

In the present study, we used local administration of a mixture of two GABA receptor agonists to temporarily inactivate the habenula (Majchrzak and Di Scala, 2000; Martin and Ghez, 1999; McFarland and Kalivas, 2001; van Duuren *et al.*, 2007). Pharmacological inactivation of the habenula decreased the frequency of pinning, whereas a higher dose of the agonists reduced pouncing as well. The effect of habenula inactivation was specific for playful social behaviour, since neither social exploration nor locomotor activity was

affected. The absence of an effect on general social interest is in keeping with the effects of lesions of the habenula, which were found not to impair social interaction in adult rats (Lecourtier *et al.*, 2004). These results therefore suggest that the habenula is specifically involved in playful, rewarding social interactions.

The current study shows that the responsiveness to a playful solicitation is more sensitive to habenula inactivation than initiation of a playful interaction. Inactivation of the habenula reduced pinning, which is the response to a playful solicitation (Panksepp *et al.*, 1984; Pellis and Pellis, 1987; Trezza *et al.*, 2010; Vanderschuren *et al.*, 1997), and at a high dose reduced pouncing (i.e., play solicitation) as well. This reduction in pouncing may be an indirect effect of the reduction in responsiveness of the test partner. Alternatively, it could be that distinct subregions of the habenula, that are differentially sensitive to GABA receptor agonism, modulate different aspects of play. Either way, these data indicate that the habenula is an important modulator of social play behaviour in adolescent rats.

The habenula complex has direct efferent and afferent connections with regions that have previously been reported to be involved in social play behaviour, such as the thalamus and septum (Beatty *et al.*, 1982; Lecourtier and Kelly, 2007; Siviy and Panksepp, 1985; -1987). In addition, the habenula is a key regulator of monoaminergic neurotransmission, and inactivation of the habenula is likely to interfere with this process. As mentioned previously, manipulations of habenula signalling have been reported to alter dopamine (Lecourtier *et al.*, 2008), serotonin (Kalen *et al.*, 1989b; Stern *et al.*, 1979; Yang *et al.*, 2008) and noradrenaline neurotransmission (Kalen *et al.*, 1989a). Numerous previous studies have indicated that a correct balance in monoaminergic activity is essential for the expression of social play behaviour (for reviews see Siviy and Panksepp, 2011; Trezza *et al.*, 2010; Vanderschuren *et al.*, 1997). Therefore, it is likely that inactivation of the habenula disrupts social play behaviour via alteration of monoaminergic signalling and/or via its projection to other regions, such as the thalamus.

In conclusion, the present study indicates an important role for the habenula in the processing of positive (social play behaviour) and negative (social isolation) social information in adolescent rats. Elucidating the mechanisms by which the habenula is involved in social events may improve our understanding of the pathology of disorders in which social interactions are affected.

References

- Aghajanian GK, Wang RY (1977) Habenular and other midbrain raphe afferents demonstrated by a modified retrograde tracing technique. Brain Res 122:229-242.
- Alessandri SM (1992) Attention, play, and social behavior in ADHD preschoolers. J Abnorm Child Psychol 20:289-302.
- Beatty WW, Dodge AM, Traylor KL, Donegan JC, Godding PR (1982) Septal lesions increase play fighting in juvenile rats. Physiol Behav 28:649-652.
- Bell HC, McCaffrey DR, Forgie ML, Kolb B, Pellis SM (2009) The role of the medial prefrontal cortex in the play fighting of rats. Behav Neurosci 123:1158-1168.
- Caldecott-Hazard S, Mazziotta J, Phelps M (1988) Cerebral correlates of depressed behavior in rats, visualized using 14C-2-deoxyglucose autoradiography. J Neurosci 8:1951-1961.
- Cirulli F, Pistillo L, De Acetis L, Alleva E, Aloe L (1998) Increased number of mast cells in the central nervous system of adult male mice following chronic subordination stress. Brain Behav Immun 12:123-133.
- Daenen EW, Wolterink G, Gerrits MAFM, Van Ree JM (2002) The effects of neonatal lesions in the amygdala or ventral hippocampus on social behaviour later in life. Behav Brain Res 136:571-582.
- Goncalves L, Sego C, Metzger M (2012) Differential projections from the lateral habenula to the rostromedial tegmental nucleus and ventral tegmental area in the rat. J Comp Neurol 520:1278-1300.
- Hikosaka O (2010) The habenula: from stress evasion to value-based decision-making. Nat Rev Neurosci 11:503-513.
- Homberg JR, Schiepers OJG, Schoffelmeer ANM, Cuppen E, Vanderschuren LJMJ (2007) Acute and constitutive increases in central serotonin levels reduce social play behaviour in peri-adolescent rats. Psychopharmacology (Berl) 195:175-182.
- Hong S, Jhou TC, Smith M, Saleem KS, Hikosaka O (2011) Negative reward signals from the lateral habenula to dopamine neurons are mediated by rostromedial tegmental nucleus in primates. J Neurosci 31:11457-11471.
- Jhou TC, Geisler S, Marinelli M, Degarmo BA, Zahm DS (2009) The mesopontine rostromedial tegmental nucleus: A structure targeted by the lateral habenula that projects to the ventral tegmental area of Tsai and substantia nigra compacta. J Comp Neurol 513:566-596.
- Jordan R (2003) Social play and autistic spectrum disorders: a perspective on theory, implications and educational approaches. Autism 7:347-360.
- Kalen P, Lindvall O, Bjorklund A (1989a) Electrical stimulation of the lateral habenula increases hippocampal noradrenaline release as monitored by in vivo microdialysis. Exp Brain Res 76:239-245.
- Kalen P, Strecker RE, Rosengren E, Bjorklund A (1989b) Regulation of striatal serotonin release by the lateral habenula-dorsal raphe pathway in the rat as demonstrated by in vivo microdialysis: role of excitatory amino acids and GABA. Brain Res 492:187-202.
- Kim U (2009) Topographic commissural and descending projections of the habenula in the rat. J Comp Neurol 513:173-187.
- Lecourtier L, Defrancesco A, Moghaddam B (2008) Differential tonic influence of lateral habenula on prefrontal cortex and nucleus accumbens dopamine release. Eur J Neurosci 27:1755-1762.
- Lecourtier L, Kelly PH (2007) A conductor hidden in the orchestra? Role of the habenular complex in monoamine transmission and cognition. Neurosci Biobehav Rev 31:658-672.

- Lecourtier L, Neijt HC, Kelly PH (2004) Habenula lesions cause impaired cognitive performance in rats: implications for schizophrenia. Eur J Neurosci 19:2551-2560.
- Lee YA, Goto Y (2011) Neurodevelopmental disruption of cortico-striatal function caused by degeneration of habenula neurons. PLoS One 6:e19450.
- Majchrzak M, Di Scala G (2000) GABA and muscimol as reversible inactivation tools in learning and memory. Neural Plast 7:19-29.
- Manning MM, Wainwright LD (2010) The role of high level play as a predictor social functioning in autism. J Autism Dev Disord 40:523-533.
- Martin JH, Ghez C (1999) Pharmacological inactivation in the analysis of the central control of movement. J Neurosci Methods 86:145-159.
- Matthews-Felton T, Corodimas KP, Rosenblatt JS, Morrell JI (1995) Lateral habenula neurons are necessary for the hormonal onset of maternal behavior and for the display of postpartum estrus in naturally parturient female rats. Behav Neurosci 109:1172-1188.
- McFarland K, Kalivas PW (2001) The circuitry mediating cocaine-induced reinstatement of drug-seeking behavior. J Neurosci 21:8655-8663.
- Meaney MJ, Dodge AM, Beatty WW (1981) Sex-dependent effects of amygdaloid lesions on the social play of prepubertal rats. Physiol Behav 26:467-472.
- Moller P, Husby R (2000) The initial prodrome in schizophrenia: searching for naturalistic core dimensions of experience and behavior. Schizophr Bull 26:217-232.
- Niesink RJM, Van Ree JM (1989) Involvement of opioid and dopaminergic systems in isolation-induced pinning and social grooming of young rats. Neuropharmacology 28:411-418.
- Nordquist RE, Vanderschuren LJMJ, Jonker AJ, Bergsma M, De Vries TJ, Pennartz CMA, Voorn P (2008) Expression of amphetamine sensitization is associated with recruitment of a reactive neuronal population in the nucleus accumbens core. Psychopharmacology 198:113-126.
- Palagi E (2006) Social play in bonobos (Pan paniscus) and chimpanzees (Pan troglodytes): Implications for natural social systems and interindividual relationships. Am J Phys Anthropol 129:418-426.
- Panksepp J, Normansell L, Cox JF, Siviy SM (1994) Effects of neonatal decortication on the social play of juvenile rats. Physiol Behav 56:429-443.
- Panksepp J, Siviy SM, Normansell L (1984) The psychobiology of play: theoretical and methodological perspectives. Neurosci Biobehav Rev 8:465-492.
- Paxinos G, Watson C (2007) The rat brain in sterotaxic coordinates. Elsevier Academic.
- Pellis SM, Castaneda E, McKenna MM, Tran-Nguyen LT, Whishaw IQ (1993) The role of the striatum in organizing sequences of play fighting in neonatally dopamine-depleted rats. Neurosci Lett 158:13-15.
- Pellis SM, Hastings E, Shimizu T, Kamitakahara H, Komorowska J, Forgie ML, Kolb B (2006) The effects of orbital frontal cortex damage on the modulation of defensive responses by rats in playful and nonplayful social contexts. Behav Neurosci 120:72-84.
- Pellis SM, Pellis VC (1987) Play-fighting differs from serious fighting in both target of attack and tactics of fighting in the laboratory rat Rattus norvegicus. Aggressive Behavior 13:227-242.
- Pellis SM, Pellis VC (1997) The prejuvenile onset of play fighting in laboratory rats (Rattus norvegicus). Dev Psychobiol 31:193-205.
- Pellis SM, Pellis VC (2009) The Playful Brain. OneWorld Publications.
- Potegal M, Einon D (1989) Aggressive behaviors in adult rats deprived of playfighting experience as juveniles. Dev Psychobiol 22:159-172.

- Rozen S, Skaletsky HJ (2000) Primer3 on the WWW for general users and for biologist programmers. In: Bioinformatics Methods and Protocols: Methods in Molecular Biology (Krawerts S, Misener S, eds), pp 365-386. Humana Press.
- Schneider M, Koch M (2005) Deficient social and play behavior in juvenile and adult rats after neonatal cortical lesion: effects of chronic pubertal cannabinoid treatment. Neuropsychopharmacology 30:944-957.
- Shabel SJ, Proulx CD, Trias A, Murphy RT, Malinow R (2012) Input to the lateral habenula from the basal ganglia is excitatory, aversive, and suppressed by serotonin. Neuron 74:475-481.
- Shumake J, Edwards E, Gonzalez-Lima F (2003) Opposite metabolic changes in the habenula and ventral tegmental area of a genetic model of helpless behavior. Brain Res 963:274-281.
- Silva M, Aguiar DC, Diniz CR, Guimaraes FS, Joca SR (2012) Neuronal NOS inhibitor and conventional antidepressant drugs attenuate stress-induced fos expression in overlapping brain regions. Cell Mol Neurobiol 32:443-453.
- Siviy SM, Deron LM, Kasten CR (2011) Serotonin, motivation, and playfulness in the juvenile rat. Dev Cogn Neurosci 1:606-616.
- Siviy SM, Fleischhauer AE, Kerrigan LA, Kuhlman SJ (1996) D2 dopamine receptor involvement in the rough-and-tumble play behavior of juvenile rats. Behav Neurosci 110:1168-1176.
- Siviy SM, Panksepp J (1985) Dorsomedial diencephalic involvement in the juvenile play of rats. Behav Neurosci 99:1103-1113.
- Siviy SM, Panksepp J (1987) Juvenile play in the rat: thalamic and brain stem involvement. Physiol Behav 41:103-114.
- Siviy SM, Panksepp J (2011) In search of the neurobiological substrates for social playfulness in mammalian brains. Neurosci Biobehav Rev 35:1821-1830.
- Skagerberg G, Lindvall O, Bjorklund A (1984) Origin, course and termination of the mesohabenular dopamine pathway in the rat. Brain Res 307:99-108.
- Spear LP (2000) The adolescent brain and age-related behavioral manifestations. Neurosci Biobehav Rev 24:417-463.
- Špinka M, Newberry RC, Bekoff M (2001) Mammalian play: training for the unexpected. Q Rev Biol 76:141-168.
- Stamatakis AM, Stuber GD (2012) Activation of lateral habenula inputs to the ventral midbrain promotes behavioral avoidance. Nat Neurosci.
- Stern WC, Johnson A, Bronzino JD, Morgane PJ (1979) Effects of electrical stimulation of the lateral habenula on single-unit activity of raphe neurons. Exp Neurol 65:326-342.
- Sugama S, Cho BP, Baker H, Joh TH, Lucero J, Conti B (2002) Neurons of the superior nucleus of the medial habenula and ependymal cells express IL-18 in rat CNS. Brain Res 958:1-9.
- Trezza V, Baarendse PJJ, Vanderschuren LJMJ (2009) Prosocial effects of nicotine and ethanol in adolescent rats through partially dissociable neurobehavioral mechanisms. Neuropsychopharmacology 34:2560-2573.
- Trezza V, Baarendse PJJ, Vanderschuren LJMJ (2010) The pleasures of play: pharmacological insights into social reward mechanisms. Trends Pharmacol Sci 31:463-469.
- Trezza V, Campolongo P, Vanderschuren LJMJ (2011a) Evaluating the rewarding nature of social interactions in laboratory animals. Dev Cogn Neurosci 1:444-458.
- Trezza V, Damsteegt R, Achterberg EJM, Vanderschuren LJMJ (2011b) Nucleus accumbens mu-opioid receptors mediate social reward. J Neurosci 31:6362-6370.

- Trezza V, Damsteegt R, Manduca A, Petrosino S, Van Kerkhof LWM, Pasterkamp RJ, Zhou Y, Campolongo P, Cuomo V, Di Marzo V, Vanderschuren LJMJ (2012) Endocannabinoids in amygdala and nucleus accumbens mediate social play reward in adolescent rats. J Neurosci 32:14899-14908.
- Trezza V, Vanderschuren LJMJ (2008) Cannabinoid and opioid modulation of social play behavior in adolescent rats: differential behavioral mechanisms. Eur Neuropsychopharmacol 18:519-530.
- Van den Berg CL, Hol T, Van Ree JM, Spruijt BM, Everts H, Koolhaas JM (1999) Play is indispensable for an adequate development of coping with social challenges in the rat. Dev Psychobiol 34:129-138.
- van Duuren E, van der Plasse G, van der Blom R, Joosten RN, Mulder AB, Pennartz CM, Feenstra MG (2007) Pharmacological manipulation of neuronal ensemble activity by reverse microdialysis in freely moving rats: a comparative study of the effects of tetrodotoxin, lidocaine, and muscimol. J Pharmacol Exp Ther 323:61-69.
- Van Kerkhof LWM, Trezza V, Mulder T, Gao P, Voorn P, Vanderschuren LJMJ (2012) Cellular activation in limbic brain systems during social play behaviour in adolescent rats. Submitted.
- Vanderschuren LJMJ (2010) How the brain makes play fun. Am J of Play 2:315-337.
- Vanderschuren LJMJ, Niesink RJM, Spruijt BM, Van Ree JM (1995) Effects of morphine on different aspects of social play in juvenile rats. Psychopharmacology 117:225-231.
- Vanderschuren LJMJ, Niesink RJM, Van Ree JM (1997) The neurobiology of social play behavior in rats. Neurosci Biobehav Rev 21:309-326.
- Vanderschuren LJMJ, Trezza V, Griffioen-Roose S, Schiepers OJG, Van Leeuwen N, De Vries TJ, Schoffelmeer ANM (2008) Methylphenidate disrupts social play behavior in adolescent rats. Neuropsychopharmacology 33:2946-2956.
- Veeneman MMJ, Boleij H, Broekhoven MH, Snoeren EMS, Guitart MM, Cousijn J, Spooren W, Vanderschuren LJMJ (2011) Dissociable roles of mGlu5 and dopamine receptors in the rewarding and sensitizing properties of morphine and cocaine. Psychopharmacology 214:863-876.
- Wirtshafter D, Asin KE, Pitzer MR (1994) Dopamine agonists and stress produce different patterns of Fos-like immunoreactivity in the lateral habenula. Brain Res 633:21-26.
- Yang LM, Hu B, Xia YH, Zhang BL, Zhao H (2008) Lateral habenula lesions improve the behavioral response in depressed rats via increasing the serotonin level in dorsal raphe nucleus. Behav Brain Res 188:84-90.
- Zhang F, Zhou W, Liu H, Zhu H, Tang S, Lai M, Yang G (2005) Increased c-Fos expression in the medial part of the lateral habenula during cue-evoked heroin-seeking in rats. Neurosci Lett 386:133-137.

Chapter 5
Methylphenidate inhibits social play behaviour via the amygdala and habenula

L.W.M. van Kerkhof[1]
R. Damsteegt[1]
E.J.M. Achterberg[1]
V. Trezza[1,2]
L.J.M.J. Vanderschuren[1,3]

1 Rudolf Magnus Institute of Neuroscience, Department of Neuroscience and Pharmacology, University Medical Centre Utrecht, Utrecht, The Netherlands
2 Department of Biology, University "Roma Tre", Rome, Italy
3 Department of Animals in Science and Society, Division of Behavioural Neuroscience, Faculty of Veterinary Medicine, Utrecht University, Utrecht, The Netherlands

In preparation.

Abstract

Positive social interactions during the juvenile and adolescent phases of life are essential for proper social and cognitive development. Like human children, most young mammals show a marked increase in peer-peer interactions, signified by the abundance of social play behaviour. Previously, it has been reported that treatment with methylphenidate markedly reduced the expression of social play behaviour in adolescent rats, by enhancement of endogenous noradrenaline levels. However, the neural substrates via which methylphenidate influences social play behaviour are unknown. Since methylphenidate is widely used for the treatment of attention-deficit/hyperactivity disorder, determining its site of action in social play behaviour may be informative for studies on its clinical mechanism of action. Therefore, the aim of the present study was to identify which neural substrates mediate the effect of methylphenidate on social play behaviour. Since methylphenidate is thought to act on cognition and behaviour via corticostriatal systems, methylphenidate (5.0 µg/0.3 µL) was administered into the prelimbic cortex, medial/ventral orbitofrontal cortex, ventrolateral orbitofrontal cortex and nucleus accumbens shell. In addition, methylphenidate was administered into the amygdala and habenula, regions known to be important for the expression of social play behaviour. Surprisingly, methylphenidate did not influence social play behaviour after administration into the cortical or striatal regions. However, social play was reduced after administration of methylphenidate into amygdala or habenula, while no effects were observed on social exploration behaviour or locomotor activity. These results indicate that the effect of systemic methylphenidate on social play is mediated via the amygdala and habenula. This study provides two sites of action for methylphenidate in the disruption of social play and stresses the importance of subcortical structures during methylphenidate treatment.

Introduction

Social play behaviour is a highly vigorous form of social interaction, abundantly expressed during childhood and adolescence (Panksepp *et al.*, 1984; Vanderschuren *et al.*, 1997). The experience of social play behaviour during childhood and adolescence is critical for normal social and cognitive development (Baarendse *et al.*, 2012; Potegal and Einon, 1989; Van den Berg *et al.*, 1999). Over the past years several studies have investigated the neural substrates of social play behaviour (for reviews see Pellis and Pellis, 2009; Siviy and Panksepp, 2011; Trezza *et al.*, 2010; Vanderschuren *et al.*, 1997). Some of the earliest compounds identified to disrupt social play behaviour were psychomotor stimulants, such as amphetamine and methylphenidate (Beatty *et al.*, 1982; Beatty *et al.*, 1984; Vanderschuren *et al.*, 2008). Interestingly, methylphenidate (Ritalin®, Concerta®) is widely used for the treatment of attention-deficit/hyperactivity disorder (ADHD) in children and adolescents (Kutcher *et al.*, 2004). Despite the widely recognised efficacy of methylphenidate in the treatment of ADHD, its underlying mechanism of action is incompletely understood. Elucidating the mechanism by which methylphenidate disrupts social play behaviour may increase our understanding of the therapeutic effects, and/or side effects of this drug.

Methylphenidate enhances the extracellular levels of noradrenaline and dopamine by inhibiting the noradrenaline and dopamine transporter (Ferris and Tang, 1979; Ritz *et al.*, 1987). Previously, it has been shown that the effect of methylphenidate on social play behaviour was mimicked by the noradrenaline reuptake inhibitor atomoxetine and blocked by the α-2 adrenoceptor antagonist RX821002 (Vanderschuren *et al.*, 2008). These results showed that methylphenidate disrupts social play behaviour via noradrenergic neurotransmission. It was hypothesised that the effect of methylphenidate on social play behaviour was related to enhanced behavioural inhibition (Vanderschuren *et al.*, 2008), since methylphenidate also enhances behavioural inhibition in a stop-signal reaction time task, through a noradrenergic mechanism in rats (Eagle *et al.*, 2007) and humans (Aron *et al.*, 2003a; Tannock *et al.*, 1989).

Both animal and human studies have implicated the frontal cortex in behavioural inhibition (Aron *et al.*, 2003b; Eagle *et al.*, 2008; for review see Humby and Wilkinson, 2011). Indeed, local administration of atomoxetine in the dorsal prelimbic cortex and orbitofrontal cortex improves stop-signal task performance in the rat (Bari *et al.*, 2011), indicating that noradrenergic mechanisms in these regions influence behavioural inhibition. Prefrontal and orbitofrontal regions have been implicated in social play behaviour as well (Bell *et al.*, 2009; Panksepp *et al.*, 1994; Pellis *et al.*, 2006; Schneider and Koch, 2005; Van Kerkhof *et al.*, 2012c, submitted; -2012d, submitted).

Previous studies have also indicated that striatum is a key neural substrate for social play behaviour (Graham, 2011; Pellis *et al.*, 1993; Trezza *et al.*, 2011; -2012; Van Kerkhof *et al.*, 2012d, submitted). Interestingly, it has been shown that the striatum is also involved in behavioural inhibition in the five-choice serial reaction time task and delayed reward task (Dalley *et al.*, 2008). Noradrenergic projections to the striatum mainly target the nucleus accumbens shell (Berridge *et al.*, 1997; Delfs *et al.*, 1998; McKittrick and Abercrombie, 2007).

The amygdala and habenula are two other candidate regions for mediating the effect of methylphenidate on social play. The amygdala is known to have an important role in

social play behaviour (Daenen *et al.*, 2002; Meaney *et al.*, 1981; Trezza *et al.*, 2012) and most amygdala nuclei express α-2 adrenoceptors (Unnerstall *et al.*, 1984). The habenula has recently received widespread interest regarding its role in reward-related behaviours (Bromberg-Martin and Hikosaka, 2011; Hikosaka, 2010; Hong *et al.*, 2011). Furthermore, the habenula is a key regulator of monoaminergic signalling (Lecourtier and Kelly, 2007) and receives noradrenergic inputs (Lecourtier and Kelly, 2007). In addition, it has recently been shown that the habenula is an essential structure for the expression of social play behaviour (Van Kerkhof et al., 2012b, submitted).

In the present study, the effect of local methylphenidate administration on social play behaviour was therefore investigated after infusion into the prelimbic cortex, medial/ventral orbitofrontal cortex, ventrolateral orbitofrontal cortex, nucleus accumbens shell, amygdala, and habenula.

Materials and Methods

Animals

Male Wistar rats (Charles River, Sulzfeld, Germany) arrived in our animal facility at 21 days of age. They were housed in groups of four in 40x26x20 cm Macrolon cages under controlled conditions (i.e. temperature 20-21 °C, 55-65 % relative humidity and 12/12 h light cycle with lights on at 7.00 a.m.). Food and water were available *ad libitum*. All animals used were experimentally naïve. During the first 6 days rats were handled at least twice. All experiments were approved by the Animal Ethics Committee of Utrecht University and were conducted in accordance with Dutch regulations (Wet op de Dierproeven, 1996) and European regulations (Guideline 86/609/EEC).

Surgical procedures

The surgical procedures were based on previous experiments (Trezza *et al.*, 2011; -2012). At 27-28 days of age, rats were anesthetised with 0.08 ml/100g (s.c.) Hypnorm (fentanylcitrate 0.315 mg/ml and fluanison 10 mg/ml, Janssen, Belgium) and positioned into a stereotactic frame (David Kopf Instruments, USA). Guide cannulae (24 gauge microblasted thinwalled stainless steel, Cooper's Needleworks, UK) were implanted bilaterally. The cannulae were aimed 0.5 mm above prelimbic cortex (coordinates: anterior-posterior (AP) +2.6 mm from Bregma; medial-lateral (ML) ± 0.8 mm from the midline; dorsal-ventral (DV) -3.2 mm from skull surface), the medial/ventral orbitofrontal cortex (coordinates: AP +3.3 mm; ML ± 0.8 mm; DV -5.3 mm), the ventrolateral orbitofrontal cortex (coordinates: AP +3.3 mm; ML ± 1.9 mm; DV -4.2 mm), the habenula (coordinates: AP -3.0 mm; ML ± 0.8 mm; DV -4.7 mm), 1.0 mm above the nucleus accumbens shell (coordinates: AP +1.5 mm; ML ±0.8 mm; DV -5.3 mm), or amygdala (coordinates: AP -1.9 mm; ML ± 4.4 mm; DV -7.8 mm). Coordinates were based on previous experiments (Trezza et al., 2011; -2012; Van Kerkhof *et al.*, 2012b, submitted; -2012c, submitted) or determined by pilot placements in rats 28 days of age. Cannulae were secured with stainless steel screws and dental acrylic. Stainless steel stylets (29 gauge) were inserted into the guide cannulae to maintain patency. After surgery, rats were individually housed for 4 days to recover, after which they were housed with their original cage mates.

Drugs and infusion procedures

Methylphenidate-HCL (Sigma, St. Louis, USA) was dissolved in saline. In all regions 5.0 µg/0.3 µL was administered, based on previous studies (Tye *et al.*, 2010; Zheng et al., 2008). Infusion procedures were as previously described by Trezza *et al.* (Trezza *et al.*, 2011; -2012). In short, bilateral infusions of methylphenidate or equivalent volume of saline were administered using 30-gauge injection needles (Bilaney, Germany) that were connected to 10 µL Hamilton microsyringes by polyethylene (PE-20) tubing. Over 60 s, 0.3 µL of methylphenidate or saline was infused using a syringe pump (model 975A; Harvard Apparatus, USA), and the injectors were left in place for another 60 s to allow for diffusion. After the procedure, stylets were replaced and animals were left in a holding cage for 5 min before testing.

Behavioural testing

Experiments were performed, as previously described (Trezza and Vanderschuren, 2008), in a sound attenuated chamber under red light conditions. The testing arena was a Plexiglas cage (40x40x60 cm) with approximately 2 cm of wood shavings covering the floor. Animals were paired with an unfamiliar partner. Animals in a test pair did not differ more than 10 g in body weight. One week post-surgery, the rats were habituated to the experimental procedures on 2 consecutive days. On the first habituation day, rats were individually placed into the test cage for 10 min. On the second habituation day, the animals were socially isolated for 2.5 h. Pairs of rats were then infused with vehicle solutions and placed into the test cage for 15 min, to habituate them to the infusion and testing procedures. In all experiments a within-subject design was used, except for the amygdala experiment. On the test day, pairs of rats (isolated for 2.5 h) received either a methylphenidate or a saline infusion and were placed into the test cage for 15 min. On the second test day treatment was reversed, thus, each animal received a saline and methylphenidate treatment. The first and second test day were separated by a wash-out day on which the animals received no treatment and were not tested. In all experiments this within-subject design was used, except for the amygdala experiment, in which two independent groups of animals were used. In this experiment half of the pairs received saline and half of the pairs received methylphenidate and the animals were only tested once.

Behaviour of the animals was recorded using a camera with zoom lens, video tape recorder and television monitor. The behaviour of the rats was assessed using the Observer 5.1 software (Noldus Information Technology B.V., The Netherlands). Three behavioural elements were scored (Panksepp *et al.*, 1984; Trezza *et al.*, 2010; Vanderschuren *et al.*, 1997).

- *Frequency of pinning:* one animal lying with its dorsal surface on the floor with the other animal is standing over it, which is the most characteristic posture of social play in rats.
- *Frequency of pouncing:* one animal attempts to nose or rub the nape of the neck of the partner, which is an index of play solicitation.
- T*ime spent in social exploration:* one animal sniffing or grooming any part of the partner's body.

To assess whether effects of the drug treatment on social play were secondary to changes in locomotor activity, the rats were subsequently tested for horizontal locomotor activity as previously described (Trezza *et al.*, 2009; Veeneman *et al.*, 2011). There was at least one day without infusions or testing between the tests for social play behaviour and locomotor activity. The infusion protocol was similar to the one described above. After the infusion procedure, rats were transferred to a plastic cage (l x w x h, 50 x 33 x 40 cm) and their position was tracked five times per second for 30 min using a video-tracking system (EthoVision, Noldus Information Technology, The Netherlands).

Histological confirmation of injection sites

Animals were sacrificed using carbon dioxide inhalation and microinjected with 0.3 µl of black ink (Parker) over 1 min through the guide cannulae, comparable to the drug infusion procedure. After the infusion, animals were immediately decapitated, their brains removed and immediately frozen. Cryostat sections (20 µm) were collected and a cresyl violet staining was performed. Placement of the microinjection sides was determined using a light microscope according to the atlas of Paxinos and Watson (Paxinos and Watson, 2007). Only pairs in which both animals had bilateral needle tracks terminating into the target area were included in the final analysis (see Fig. 1 and 2).

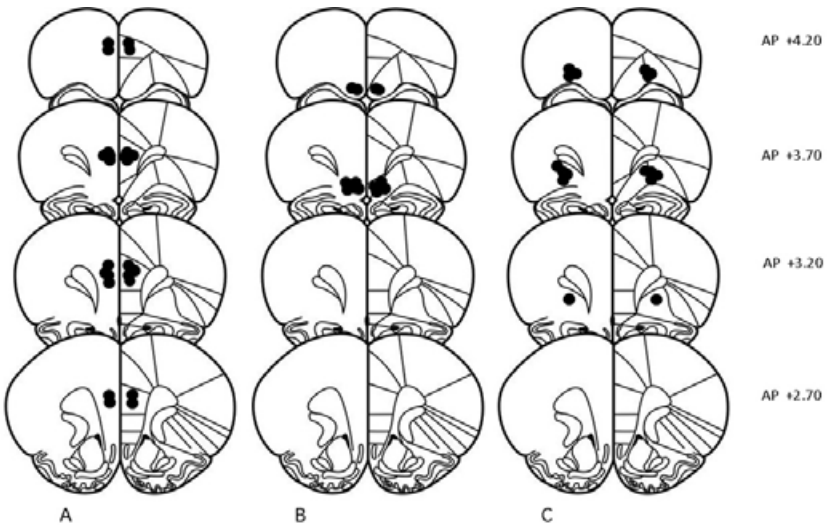


Fig. 1: Schematic representation of brain sections with microinjection placements in the prelimbic cortex (A), medial/ventral orbitofrontal cortex (B), and ventrolateral orbitofrontal cortex (C). AP = anterior-posterior level in mm from Bregma. Adapted from Paxinos and Watson (2007).

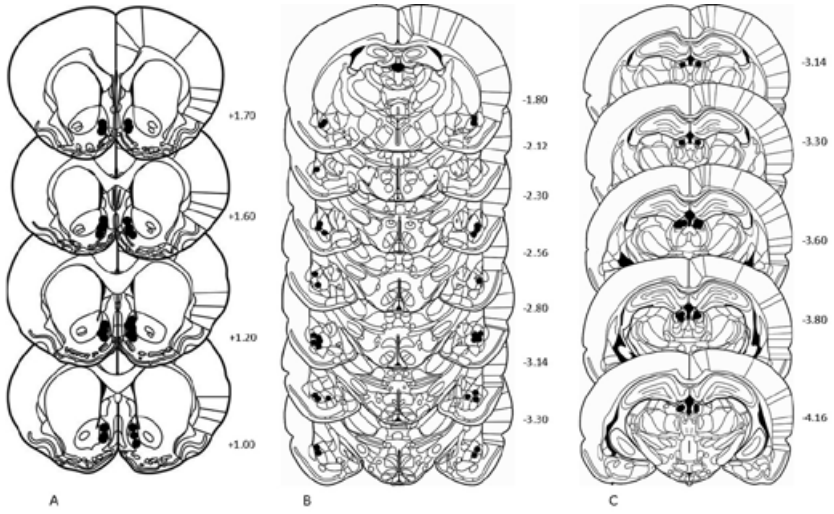


Fig. 2: Schematic representation of brain sections with microinjection placements in the nucleus accumbens shell (A), amygdala (B), and habenula (C). AP = anterior-posterior level in mm from Bregma. Adapted from Paxinos and Watson (2007).

Statistical analysis

Pinning and pouncing frequencies and time spent on social exploration (s) are expressed as mean ± SEM. To assess the effect of methylphenidate administration on social play behaviour, data were analysed using a paired samples Student's t-test. In the experiment where methylphenidate was administered into the amygdala a separate test and control group were used; therefore, data were analysed using an unpaired Student's t-test. Horizontal locomotor activity was expressed as mean ± SEM travelled distance (cm). The effects of methylphenidate administration on locomotor activity were analysed using a two-way repeated measures ANOVA.

Results

Prefrontal cortex and nucleus accumbens shell

Infusion of methylphenidate into the prelimbic cortex did not affect pinning ($t = -0.798$, $df = 8$, $p = 0.448$) or pouncing ($t = -0.190$, $df = 8$, $p = 0.785$; $n = 9$) (Fig. 3A-B). In addition, no effect was observed on social exploratory behaviour ($t = 0.282$, $df = 8$, $p = 0.785$) (Fig. 3C). Social play behaviour was also not changed after infusion of methylphenidate into the medial/ventral orbitofrontal cortex (pinning: $t = 0.447$, $df = 6$, $p = 0.671$; pouncing: $t = 0.777$, $df = 6$, $p = 0.467$; $n = 7$; Fig. 3D-E). In addition, no changes were observed in the time spent on social exploratory behaviour ($t = -1.528$, $df = 6$, $p = 0.177$; Fig.3F). Furthermore, after administration of methylphenidate into the ventrolateral orbitofrontal cortex (n=7), no changes were observed in pinning ($t = 0.736$, $df = 6$, $p = 0.490$; Fig. 3G), pouncing ($t = 0.059$, $df = 6$, $p = 0.955$; Fig. 3H), or social exploratory behaviour ($t = 0.485$, $df = 6$, $p = 0.645$; Fig. 3I).

Administration of methylphenidate (5.0 µg/0.3 µL) into the nucleus accumbens shell

did not affect social play behaviour, since the frequency of pinning and pouncing was not altered (pinning: t = 0.511, df = 9, p = 0.622; pouncing: 0.149, df = 9, p = 0.885; n = 10) (Fig. 4A-B). In addition, no effects were observed on the time spent on social exploration (t = -0.238, df = 9, p = 0.247) or locomotor activity ($F_{treatment}(1,16) = 1.082$, p = 0.314; $F_{time}(5,80) = 14.675$, $p < 0.001$; $F_{time \times treatment}(5,80) = 0.852$, p = 0.517) (Fig. 4C-D).

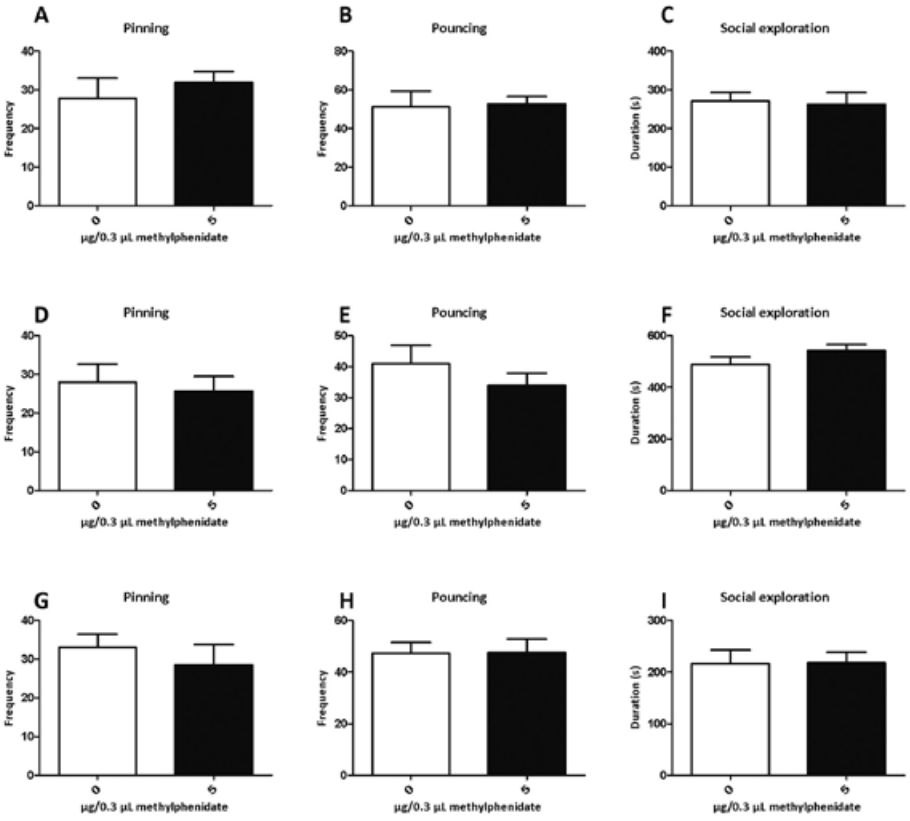


Fig. 3: The effect of methylphenidate administration (5.0 µg/0.3 µL) in the prelimbic cortex (A-C; n = 9), medial/ventral orbitofrontal cortex (D-F; n = 7), and ventrolateral orbitofrontal cortex (G-I; n = 7) on social play behaviour. Data are presented as mean ± SEM. Methylphenidate infusion did not affect the level of pinning (A,D,G), pouncing (B,E,H) or social exploration (C,F,I).

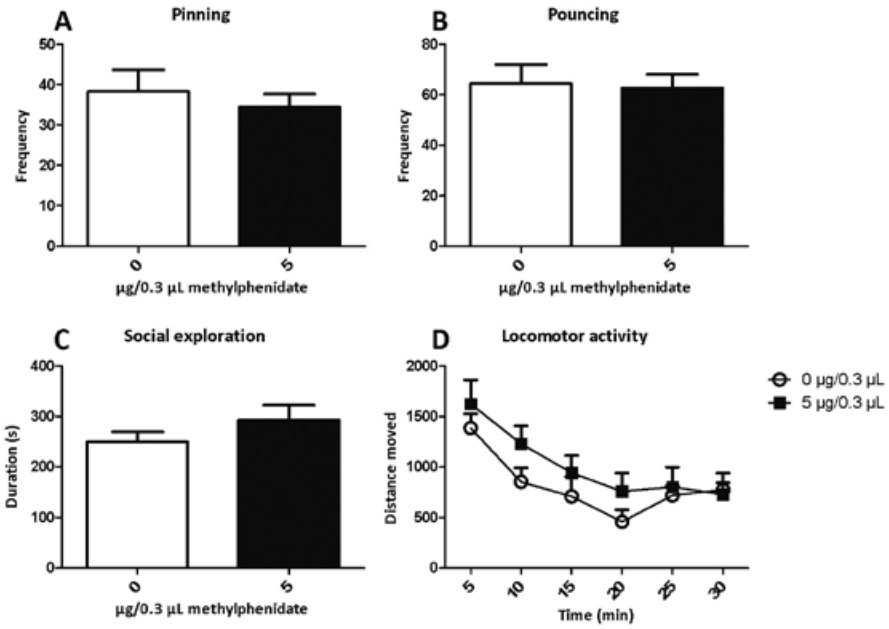


Fig. 4: The effect of methylphenidate administration (5.0 µg/0.3 µL) in the nucleus accumbens shell on social play behaviour (n = 10). Data are presented as mean ± SEM. Methylphenidate infusion did not affect the level of pinning (A), pouncing (B), social exploration (C), or locomotor activity (D; saline n = 9, methylphenidate n = 10).

Amygdala and habenula

Infusion of methylphenidate into the amygdala (n = 6 per group) reduced the frequency of pinning (t = 2.732, df = 10, p = 0.021) and pouncing (t = 2.822, df = 10, p = 0.018), without affecting the level of social exploration (t = 0.858, df = 10, p = 0.411) or locomotor activity ($F_{treatment}(1,17) = 0.215$, p = 0.649; $F_{time}(5,85) = 26.475$, p < 0.001; $F_{time \times treatment}(5,85) = 0.749$, p = 0.589) (Fig. 5).

A reduction in the frequency of both play parameters was also observed after administration of methylphenidate into the habenula (pinning: t = 4.874, df = 8, p = 0.001; pouncing : t = 5.577, df = 8, p = 0.001; n = 9) (Fig. 6A-B). This effect was specific for social play behaviour, since no changes were detected in the time spent on social exploration (t = -0.192, df = 8, p = 0.853) or locomotor activity ($F_{treatment}(1,18) = 0.147$, p = 0.706; $F_{time}(5,90) = 49.335$, p < 0.001; $F_{time \times treatment}(5,90) = 0.218$, p = 0.954) (Fig. 6C-D).

In summary, these results indicate that the amygdala and habenula are involved in the effect of methylphenidate on social play behaviour.

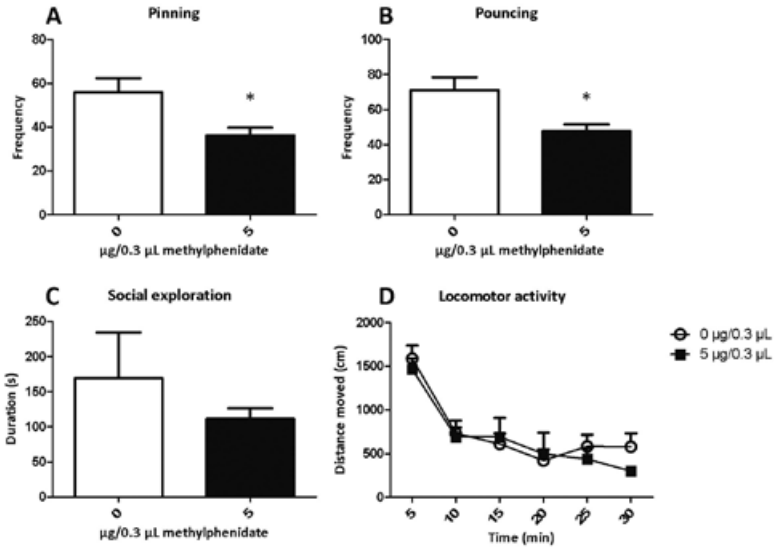


Fig. 5: The effect of methylphenidate administration (5.0 µg/0.3 µL) in the amygdala on social play behaviour (n = 6). Data are presented as mean ± SEM. Methylphenidate infusion into the amygdala reduced the level of pinning (A) and pouncing (B), while it did not affect social exploration (C) or locomotor activity (D; saline n = 10, methylphenidate n = 9). * $p < 0.05$

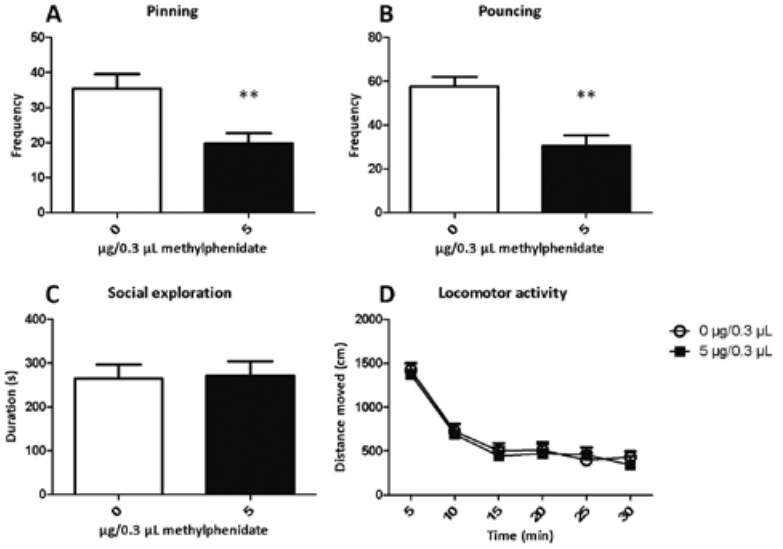


Fig. 6: The effect of methylphenidate administration (5.0 µg/0.3 µL) in the habenula on social play behaviour (n = 9). Data are presented as mean ± SEM. Methylphenidate infusion into the habenula decreased the frequency of pinning (A) and pouncing (B), while it did not affect the time spend on social exploration (C) or locomotor activity (D; saline n = 12, methylphenidate n = 8). ** $p < 0.01$

Discussion

The aim of the present study was to investigate which brain regions mediate the effects of methylphenidate on social play behaviour in adolescent rats. Administration of methylphenidate into the amygdala and habenula decreased the expression of social play behaviour, whereas administration into the prelimbic cortex, medial/ventral orbitofrontal cortex, ventrolateral orbitofrontal cortex, and nucleus accumbens shell did not affect social play.

Prefrontal cortex and nucleus accumbens shell are not involved in the disruption of social play by methylphenidate

The behavioural effects of methylphenidate have previously been ascribed to prefrontal and striatal regions in humans (Rubia *et al.*, 2009; -2011; Tomasi *et al.*, 2011) and rodents (Claussen *et al.*, 2012; Lee *et al.*, 2008; Spencer *et al.*, 2012). Methylphenidate is known to enhance behavioural inhibition in rats (Eagle *et al.*, 2007) and humans (Aron *et al.*, 2003a; Tannock *et al.*, 1989) and previous studies have indicated that mainly the prelimbic cortex, orbitofrontal cortex and nucleus accumbens shell are important for behavioural inhibition (Bari *et al.*, 2011; Dalley *et al.*, 2008; Eagle and Baunez, 2010; Economidou *et al.*, 2012). Therefore, in the present study methylphenidate was administered into the prelimbic cortex, medial/ventral orbitofrontal cortex, ventrolateral orbitofrontal cortex, and nucleus accumbens shell. In contrast to our expectations, none of these regions mediated the effect of methylphenidate on social play behaviour, since after infusion of methylphenidate in these regions, no changes in any of the parameters were observed. It is important to note that a few prefrontal regions were not investigated in the present study (e.g. anterior cingulate cortex and infralimbic cortex). Therefore, it is possible that other prefrontal regions are involved, although these regions have not been explicitly implicated in behavioural inhibition (Bari *et al.*, 2011; Eagle *et al.*, 2008). The effect of methylphenidate on social play behaviour was shown to be dependent on noradrenergic neurotransmission (Vanderschuren *et al.*, 2008) and considering the noradrenergic inputs into the striatum (Berridge *et al.*, 1997; Delfs et al., 1998; McKittrick and Abercrombie, 2007), it appears unlikely that striatal regions not investigated in this study (i.e. other than the nucleus accumbens shell) are involved in the effect of methylphenidate on social play behaviour. However, it cannot be excluded that the dose of methylphenidate used in the present study was not optimal to cause an effect in the prefrontal regions or nucleus accumbens shell. However, given that this dose of methylphenidate was effective when administered into the amygdala and habenula, this seems unlikely.

The disruption of social play behaviour by methylphenidate is mediated via the amygdala and habenula

A reduction in social play behaviour was observed, after administration of methylphenidate into the amygdala and the habenula. This reduction was behaviourally specific, since no changes were observed in the time spent on social exploration or in locomotor activity. After systemic administration of methylphenidate a comparable specific reduction in play behaviour has been observed (Vanderschuren *et al.*, 2008). Therefore, it is likely that the systemic effect of methylphenidate is mediated by the amygdala and habenula.

Both the amygdala and habenula have previously been implicated in the modulation and

expression of social play behaviour (Cheng *et al.*, 2008; Daenen *et al.*, 2002; Meaney *et al.*, 1981; Trezza *et al.*, 2012; Van Kerkhof *et al.*, 2012d) and both regions receive noradrenergic inputs (Gottesfeld, 1983; Lecourtier and Kelly, 2007; Unnerstall *et al.*, 1984). Systemic treatment with methylphenidate has been reported to decrease glucose metabolism in the habenula (Porrino and Lucignani, 1987), which suggests that the enhancement of noradrenaline levels by methylphenidate results in decreased habenula activity. This is consistent with a previous study showing that inactivation of the habenula by local administration of GABA receptor agonists resulted in decreased expression of social play behaviour (Van Kerkhof *et al.*, 2012b, submitted). Noradrenaline has also been reported to reduce neuronal activity in the lateral and basolateral amygdala via α2-adrenoceptors using *in vitro* and *in vivo* studies (Buffalari and Grace, 2007; Ferry *et al.*, 1997; Johnson *et al.*, 2011). The α2-adrenoceptors are involved in the reduction of social play behaviour by methylphenidate (Vanderschuren *et al.*, 2008), suggesting that the reduction in social play is associated with reduced neuronal activity in the amygdala. The hypothesis that a reduction in amygdala function causes decreases in social play behaviour is consistent with previous studies where lesions of the amygdala were reported to reduce social play behaviour in male rats (Daenen *et al.*, 2002; Meaney *et al.*, 1981).

We have recently shown that methylphenidate increases the motivation for social play behaviour, but this effect was dopamine-dependent (Van Kerkhof *et al.*, 2012a, in preparation). Interestingly, administration of the specific noradrenaline reuptake inhibitor, atomoxetine, decreased responding in the operant conditioning task for social play behaviour, suggesting that enhancement of endogenous noradrenaline levels (which also reduces the expression of social play) decreases the motivation to play (Van Kerkhof *et al.*, 2012a, in preparation). Both the amygdala and habenula are known to be involved in several aspects of reward signalling (Baxter and Murray, 2002; Bromberg-Martin and Hikosaka, 2011; Cardinal *et al.*, 2002; Everitt *et al.*, 2003; Hikosaka, 2010; Lecourtier and Kelly, 2007; Morrison and Salzman, 2010). It may therefore be that the disruption of amygdala and habenula signalling by methylphenidate influences the reinforcing properties of social play behaviour. Interestingly, methylphenidate reduced both the initiation to play and responsiveness to play initiations (Van Kerkhof *et al.*, 2012a, in preparation; Vanderschuren *et al.*, 2008), which may reflect reduced motivation to play.

An additional aspect of social play that might be influenced by methylphenidate during play behaviour are cognitive aspects of the social interaction. Methylphenidate may enhance the animal's attention for its surroundings and as a consequence it will engage less in a play interaction, since during a play interaction an animal is mainly focussed on its partner. Interestingly, local administration of methylphenidate into the amygdala has been reported to enhance cue-reward learning (Tye *et al.*, 2010), suggesting that methylphenidate within the amygdala enhances sensitivity to environmental cues. Moreover, in children with ADHD, methylphenidate is known to reduce distractibility (Swanson and Petrovich, 1998; Urban and Gao, 2012). On the other hand, a certain amount of behavioural flexibility is also required for play behaviour, since animals constantly need to adjust their behavioural strategy in response to the partner's action. Methylphenidate has been reported to improve flexibility in a discrimination task, through a noradrenergic mechanism of action (Seu *et al.*, 2009). This suggests that either

methylphenidate-induced improvements in flexibility are not related to its effects on play, or that they lead to exaggerated flexibility, which causes to animals to cut short the complex chains of behaviour in a social interaction (Lyon and Robbins, 1975). Indeed, the amygdala has been associated with emotional attention in humans and rodents (Henckens *et al.*, 2012; Meck and Macdonald, 2007; Troiani *et al.*, 2012) and some of the behavioural abnormalities observed after habenula lesions might be related to attention processes (Hikosaka, 2010; Lecourtier and Kelly, 2005; Lee and Huang, 1988). Moreover, studies in rats have implicated the amygdala in behavioural flexibility (Churchwell *et al.*, 2009; Schoenbaum *et al.*, 2003). Further studies are required to investigate if the effect of methylphenidate on social play mediated via the amygdala and habenula is related to attention or flexibility.

The habenula and amygdala are both known to be involved in stress and anxiety related behaviours (Hikosaka, 2010; Roozendaal *et al.*, 2009; Shin and Liberzon, 2010). Indeed, increased noradrenaline levels in the amygdala have been associated with stress and anxiety (for review see Tanaka *et al.*, 2000). Therefore, it could be that the enhancement of noradrenaline in the amygdala and habenula by methylphenidate augments stress or anxiety to reduce social play behaviour. However, since no alterations were observed on social exploration behaviour and locomotor activity, it is unlikely that changes in stress and/or anxiety explain the effects of methylphenidate on social play behaviour.

Considering the involvement of the habenula and amygdala, a common functional pathway might be involved in the regulation of social play behaviour. One of the regions that is targeted by both structures is the thalamus (De Olmos *et al.*, 2004; Lecourtier and Kelly, 2007), which has been implicated in social play behaviour (Siviy and Panksepp, 1985; Siviy and Panksepp, 1987; Van Kerkhof *et al.*, 2012d, submitted). Another common target is the rostromedial tegmental nucleus (RMTg), which receives direct projections from the habenula and indirect projections from the amygdala. This structure has been proposed to integrate information from both structures to regulate locomotor activity in response to aversive and positive events (Jhou *et al.*, 2009). The amygdala to RMTg projection via the periaqueductal grey is thought to regulate behavioural inhibition required for freezing responses (Jhou *et al.*, 2009). Lesions of the habenula or RMTg both result in hyperactivity and increased impulsivity (Jhou *et al.*, 2009; Lecourtier and Kelly, 2005; Murphy *et al.*, 1996). However, the effects of methylphenidate on social play behaviour are not related to general changes in social or locomotor activity (Vanderschuren *et al.*, 2008 ; present study). Therefore, involvement of a amygdala/habenula-RMTg projection in the effects of methylphenidate on social play may indicate that this pathway also mediates more subtle changes in behaviour. Another common target of the habenula and the amygdala is the ventral tegmental area (VTA) (Kaufling *et al.*, 2009; Lecourtier and Kelly, 2007), and dopaminergic projections from the VTA have an important role in social play behaviour (Siviy and Panksepp, 2011; Trezza *et al.*, 2010).

In summary, there are multiple routes via which the amygdala and habenula might be communicating and modulating the expression of social play behaviour. This study provides new insights into the mechanism of action of methylphenidate and indicates subcortical structures such as the amygdala and habenula may play a

more important role in the effects of methylphenidate on behaviour than often thought. Further studies into the mechanism by which methylphenidate acts in the amygdala and habenula may advance our understanding of the properties of this drug in the treatment of ADHD.

References

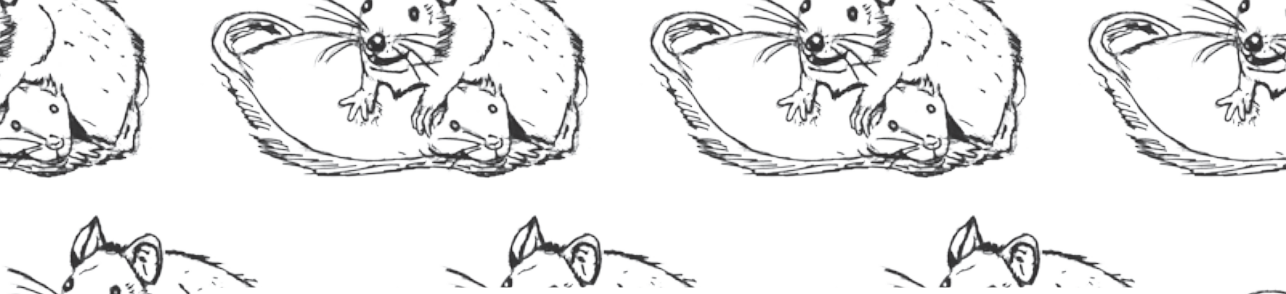
- Aron AR, Dowson JH, Sahakian BJ, Robbins TW (2003a) Methylphenidate improves response inhibition in adults with attention-deficit/hyperactivity disorder. Biol Psychiatry 54:1465-1468.
- Aron AR, Fletcher PC, Bullmore ET, Sahakian BJ, Robbins TW (2003b) Stop-signal inhibition disrupted by damage to right inferior frontal gyrus in humans. Nat Neurosci 6:115-116.
- Baarendse PJJ, Counotte DS, O'Donnel P, Vanderschuren LJMJ (2012) Social experience during adolescence is critical for the development of cognitive control and dopamine modulation of prefrontal cortex function. Submitted.
- Bari A, Mar AC, Theobald DE, Elands SA, Oganya KC, Eagle DM, Robbins TW (2011) Prefrontal and monoaminergic contributions to stop-signal task performance in rats. J Neurosci 31:9254-9263.
- Baxter MG, Murray EA (2002) The amygdala and reward. Nat Rev Neurosci 3:563-573.
- Beatty WW, Costello KB, Berry SL (1984) Suppression of play fighting by amphetamine: effects of catecholamine antagonists, agonists and synthesis inhibitors. Pharmacol Biochem Behav 20:747-755.
- Beatty WW, Dodge AM, Dodge LJ, White K, Panksepp J (1982) Psychomotor stimulants, social deprivation and play in juvenile rats. Pharmacol Biochem Behav 16:417-422.
- Bell HC, McCaffrey DR, Forgie ML, Kolb B, Pellis SM (2009) The role of the medial prefrontal cortex in the play fighting of rats. Behav Neurosci 123:1158-1168.
- Berridge CW, Stratford TL, Foote SL, Kelley AE (1997) Distribution of dopamine beta-hydroxylase-like immunoreactive fibers within the shell subregion of the nucleus accumbens. Synapse 27:230-241.
- Bromberg-Martin ES, Hikosaka O (2011) Lateral habenula neurons signal errors in the prediction of reward information. Nat Neurosci 14:1209-1216.
- Buffalari DM, Grace AA (2007) Noradrenergic modulation of basolateral amygdala neuronal activity: opposing influences of alpha-2 and beta receptor activation. J Neurosci 27:12358-12366.
- Cardinal RN, Parkinson JA, Hall J, Everitt BJ (2002) Emotion and motivation: the role of the amygdala, ventral striatum, and prefrontal cortex. Neurosci Biobehav Rev 26:321-352.
- Cheng SY, Taravosh-Lahn K, Delville Y (2008) Neural circuitry of play fighting in golden hamsters. Neuroscience 156:247-256.
- Churchwell JC, Morris AM, Heurtelou NM, Kesner RP (2009) Interactions between the prefrontal cortex and amygdala during delay discounting and reversal. Behav Neurosci 123:1185-1196.
- Claussen CM, Chong SL, Dafny N (2012) Selective bilateral lesion to caudate nucleus modulates the acute and chronic methylphenidate effects. Pharmacol Biochem Behav 101:208-216.
- Daenen EW, Wolterink G, Gerrits MAFM, Van Ree JM (2002) The effects of neonatal lesions in the amygdala or ventral hippocampus on social behaviour later in life. Behav Brain Res 136:571-582.
- Dalley JW, Mar AC, Economidou D, Robbins TW (2008) Neurobehavioral mechanisms of impulsivity: fronto-striatal systems and functional neurochemistry. Pharmacol Biochem Behav 90:250-260.
- De Olmos JS, Beltramico CA, Alheid G (2004) Amygdala and Extended Amygdala of the Rat: A Cytoarchitectonical, Fibroarchitectonical, and Chemoarchitectonical Survey. In: The Rat Nervous System (Paxinos G, ed), pp 509-603.
- Delfs JM, Zhu Y, Druhan JP, Aston-Jones GS (1998) Origin of noradrenergic afferents to the shell subregion of the nucleus accumbens: anterograde and retrograde tract-tracing studies in the rat. Brain Res 806:127-140.
- Eagle DM, Baunez C (2010) Is there an inhibitory-response-control system in the rat? Evidence from anatomical and pharmacological studies of behavioral inhibition. Neurosci Biobehav Rev 34:50-72.

- Eagle DM, Baunez C, Hutcheson DM, Lehmann O, Shah AP, Robbins TW (2008) Stop-signal reaction-time task performance: role of prefrontal cortex and subthalamic nucleus. Cereb Cortex 18:178-188.
- Eagle DM, Tufft MR, Goodchild HL, Robbins TW (2007) Differential effects of modafinil and methylphenidate on stop-signal reaction time task performance in the rat, and interactions with the dopamine receptor antagonist cis-flupenthixol. Psychopharmacology (Berl) 192:193-206.
- Economidou D, Theobald DE, Robbins TW, Everitt BJ, Dalley JW (2012) Norepinephrine and dopamine modulate impulsivity on the five-choice serial reaction time task through opponent actions in the shell and core sub-regions of the nucleus accumbens. Neuropsychopharmacology 37:2057-2066.
- Everitt BJ, Cardinal RN, Parkinson JA, Robbins TW (2003) Appetitive behavior: impact of amygdala-dependent mechanisms of emotional learning. Ann N Y Acad Sci 985:233-250.
- Ferris RM, Tang FL (1979) Comparison of the effects of the isomers of amphetamine, methylphenidate and deoxypipradrol on the uptake of l-[3H]norepinephrine and [3H]dopamine by synaptic vesicles from rat whole brain, striatum and hypothalamus. J Pharmacol Exp Ther 210:422-428.
- Ferry B, Magistretti PJ, Pralong E (1997) Noradrenaline modulates glutamate-mediated neurotransmission in the rat basolateral amygdala in vitro. Eur J Neurosci 9:1356-1364.
- Gottesfeld Z (1983) Origin and distribution of noradrenergic innervation in the habenula: a neurochemical study. Brain Res 275:299-304.
- Graham KL (2011) Coevolutionary relationship between striatum size and social play in nonhuman primates. Am J Primatol 73:314-322.
- Henckens MJ, van Wingen GA, Joels M, Fernandez G (2012) Time-dependent effects of cortisol on selective attention and emotional interference: a functional MRI study. Front Integr Neurosci 6:66.
- Hikosaka O (2010) The habenula: from stress evasion to value-based decision-making. Nat Rev Neurosci 11:503-513.
- Hong S, Jhou TC, Smith M, Saleem KS, Hikosaka O (2011) Negative reward signals from the lateral habenula to dopamine neurons are mediated by rostromedial tegmental nucleus in primates. J Neurosci 31:11457-11471.
- Humby T, Wilkinson LS (2011) Assaying dissociable elements of behavioural inhibition and impulsivity: translational utility of animal models. Curr Opin Pharmacol 11:534-539.
- Jhou TC, Fields HL, Baxter MG, Saper CB, Holland PC (2009) The rostromedial tegmental nucleus (RMTg), a GABAergic afferent to midbrain dopamine neurons, encodes aversive stimuli and inhibits motor responses. Neuron 61:786-800.
- Johnson LR, Hou M, Prager EM, Ledoux JE (2011) Regulation of the Fear Network by Mediators of Stress: Norepinephrine Alters the Balance between Cortical and Subcortical Afferent Excitation of the Lateral Amygdala. Front Behav Neurosci 5:23.
- Kaufling J, Veinante P, Pawlowski SA, Freund-Mercier MJ, Barrot M (2009) Afferents to the GABAergic tail of the ventral tegmental area in the rat. J Comp Neurol 513:597-621.
- Kutcher S, Aman M, Brooks SJ, Buitelaar J, van Daalen E, Fegert J, Findling RL, Fisman S, Greenhill LL, Huss M, Kusumakar V, Pine D, Taylor E, Tyano S (2004) International consensus statement on attention-deficit/hyperactivity disorder (ADHD) and disruptive behaviour disorders (DBDs): clinical implications and treatment practice suggestions. Eur Neuropsychopharmacol 14:11-28.
- Lecourtier L, Kelly PH (2005) Bilateral lesions of the habenula induce attentional disturbances in rats. Neuropsychopharmacology 30:484-496.
- Lecourtier L, Kelly PH (2007) A conductor hidden in the orchestra? Role of the habenular complex in monoamine transmission and cognition. Neurosci Biobehav Rev 31:658-672.

•Lee EH, Huang SL (1988) Role of lateral habenula in the regulation of exploratory behavior and its relationship to stress in rats. Behav Brain Res 30:265-271.
• Lee MJ, Swann AC, Dafny N (2008) Methylphenidate sensitization is prevented by prefrontal cortex lesion. Brain Res Bull 76:131-140.
• Lyon M, Robbins TW (1975) The action of central nervous system stimulant drugs: a general theory concerning amphetamine effects. In: Current Developments in Psychopharmacology (Essman W, Valzelli L, eds), pp 79-163. Spectrum: New York.
• McKittrick CR, Abercrombie ED (2007) Catecholamine mapping within nucleus accumbens: differences in basal and amphetamine-stimulated efflux of norepinephrine and dopamine in shell and core. J Neurochem 100:1247-1256.
• Meaney MJ, Dodge AM, Beatty WW (1981) Sex-dependent effects of amygdaloid lesions on the social play of prepubertal rats. Physiol Behav 26:467-472.
• Meck WH, Macdonald CJ (2007) Amygdala inactivation reverses fear's ability to impair divided attention and make time stand still. Behav Neurosci 121:707-720.
• Morrison SE, Salzman CD (2010) Re-valuing the amygdala. Curr Opin Neurobiol 20:221-230.
• Murphy CA, DiCamillo AM, Haun F, Murray M (1996) Lesion of the habenular efferent pathway produces anxiety and locomotor hyperactivity in rats: a comparison of the effects of neonatal and adult lesions. Behav Brain Res 81:43-52.
• Panksepp J, Normansell L, Cox JF, Siviy SM (1994) Effects of neonatal decortication on the social play of juvenile rats. Physiol Behav 56:429-443.
• Panksepp J, Siviy SM, Normansell L (1984) The psychobiology of play: theoretical and methodological perspectives. Neurosci Biobehav Rev 8:465-492.
• Paxinos G, Watson C (2007) The rat brain in sterotaxic coordinates. Elsevier Academic.
• Pellis SM, Castaneda E, McKenna MM, Tran-Nguyen LT, Whishaw IQ (1993) The role of the striatum in organizing sequences of play fighting in neonatally dopamine-depleted rats. Neurosci Lett 158:13-15.
• Pellis SM, Hastings E, Shimizu T, Kamitakahara H, Komorowska J, Forgie ML, Kolb B (2006) The effects of orbital frontal cortex damage on the modulation of defensive responses by rats in playful and nonplayful social contexts. Behav Neurosci 120:72-84.
• Pellis SM, Pellis VC (2009) The Playful Brain. OneWorld Publications.
• Porrino LJ, Lucignani G (1987) Different patterns of local brain energy metabolism associated with high and low doses of methylphenidate. Relevance to its action in hyperactive children. Biol Psychiatry 22:126-138.
• Potegal M, Einon D (1989) Aggressive behaviors in adult rats deprived of playfighting experience as juveniles. Dev Psychobiol 22:159-172.
• Ritz MC, Lamb RJ, Goldberg SR, Kuhar MJ (1987) Cocaine receptors on dopamine transporters are related to self-administration of cocaine. Science 237:1219-1223.
• Roozendaal B, McEwen BS, Chattarji S (2009) Stress, memory and the amygdala. Nat Rev Neurosci 10:423-433.
• Rubia K, Halari R, Cubillo A, Mohammad AM, Brammer M, Taylor E (2009) Methylphenidate normalises activation and functional connectivity deficits in attention and motivation networks in medication-naive children with ADHD during a rewarded continuous performance task. Neuropharmacology 57:640-652.

- Rubia K, Halari R, Mohammad AM, Taylor E, Brammer M (2011) Methylphenidate normalizes frontocingulate underactivation during error processing in attention-deficit/hyperactivity disorder. Biol Psychiatry 70:255-262.
- Schneider M, Koch M (2005) Deficient social and play behavior in juvenile and adult rats after neonatal cortical lesion: effects of chronic pubertal cannabinoid treatment. Neuropsychopharmacology 30:944-957.
- Schoenbaum G, Setlow B, Nugent SL, Saddoris MP, Gallagher M (2003) Lesions of orbitofrontal cortex and basolateral amygdala complex disrupt acquisition of odor-guided discriminations and reversals. Learn Mem 10:129-140.
- Seu E, Lang A, Rivera RJ, Jentsch JD (2009) Inhibition of the norepinephrine transporter improves behavioral flexibility in rats and monkeys. Psychopharmacology (Berl) 202:505-519.
- Shin LM, Liberzon I (2010) The neurocircuitry of fear, stress, and anxiety disorders. Neuropsychopharmacology 35:169-191.
- Siviy SM, Panksepp J (1985) Dorsomedial diencephalic involvement in the juvenile play of rats. Behav Neurosci 99:1103-1113.
- Siviy SM, Panksepp J (1987) Juvenile play in the rat: thalamic and brain stem involvement. Physiol Behav 41:103-114.
- Siviy SM, Panksepp J (2011) In search of the neurobiological substrates for social playfulness in mammalian brains. Neurosci Biobehav Rev 35:1821-1830.
- Spencer RC, Klein RM, Berridge CW (2012) Psychostimulants act within the prefrontal cortex to improve cognitive function. Biol Psychiatry 72:221-227.
- Swanson LW, Petrovich GD (1998) What is the amygdala? Trends Neurosci 21:323-331.
- Tanaka M, Yoshida M, Emoto H, Ishii H (2000) Noradrenaline systems in the hypothalamus, amygdala and locus coeruleus are involved in the provocation of anxiety: basic studies. Eur J Pharmacol 405:397-406.
- Tannock R, Schachar RJ, Carr RP, Chajczyk D, Logan GD (1989) Effects of methylphenidate on inhibitory control in hyperactive children. J Abnorm Child Psychol 17:473-491.
- Tomasi D, Volkow ND, Wang GJ, Wang R, Telang F, Caparelli EC, Wong C, Jayne M, Fowler JS (2011) Methylphenidate enhances brain activation and deactivation responses to visual attention and working memory tasks in healthy controls. Neuroimage 54:3101-3110.
- Trezza V, Baarendse PJJ, Vanderschuren LJMJ (2009) Prosocial effects of nicotine and ethanol in adolescent rats through partially dissociable neurobehavioral mechanisms. Neuropsychopharmacology 34:2560-2573.
- Trezza V, Baarendse PJJ, Vanderschuren LJMJ (2010) The pleasures of play: pharmacological insights into social reward mechanisms. Trends Pharmacol Sci 31:463-469.
- Trezza V, Damsteegt R, Achterberg EJM, Vanderschuren LJMJ (2011) Nucleus accumbens mu-opioid receptors mediate social reward. J Neurosci 31:6362-6370.
- Trezza V, Damsteegt R, Manduca A, Petrosino S, Van Kerkhof LWM, Pasterkamp RJ, Zhou Y, Campolongo P, Cuomo V, Di Marzo V, Vanderschuren LJMJ (2012) Endocannabinoids in amygdala and nucleus accumbens mediate social play reward in adolescent rats. J Neurosci 32:14899-14908.
- Troiani V, Price ET, Schultz RT (2012) Unseen fearful faces promote amygdala guidance of attention. Soc Cogn Affect Neurosci.
- Tye KM, Tye LD, Cone JJ, Hekkelman EF, Janak PH, Bonci A (2010) Methylphenidate facilitates learning-induced amygdala plasticity. Nat Neurosci 13:475-481.

- Unnerstall JR, Kopajtic TA, Kuhar MJ (1984) Distribution of alpha 2 agonist binding sites in the rat and human central nervous system: analysis of some functional, anatomic correlates of the pharmacologic effects of clonidine and related adrenergic agents. Brain Res Rev 319:69-101.
- Urban KR, Gao WJ (2012) Evolution of the Study of Methylphenidate and Its Actions on the Adult Versus Juvenile Brain. J Atten Disord.
- Van den Berg CL, Hol T, Van Ree JM, Spruijt BM, Everts H, Koolhaas JM (1999) Play is indispensable for an adequate development of coping with social challenges in the rat. Dev Psychobiol 34:129-138.
- Van Kerkhof LWM, Achterberg EJM, Servadio M, Trezza V, Vanderschuren LJMJ (2012a) Fractionating play: dissociable roles of dopamine and noradrenaline in social play performance, reward and motivation. In preparation.
- Van Kerkhof LWM, Damsteegt R, Trezza V, Gao P, Voorn P, Vanderschuren L.J.M.J. (2012b) Functional integritiy of the habenula is necessary for social play behaviour in adolescent rats. Submitted.
- Van Kerkhof LWM, Damsteegt R, Trezza V, Voorn P, Vanderschuren L.J.M.J. (2012c) Social play behaviour in adolescent rats is mediated by functional activity in medial prefrontal cortex and dorsomedial striatum. Submitted.
- Van Kerkhof LWM, Trezza V, Mulder T, Gao P, Voorn P, Vanderschuren LJMJ (2012d) Cellular activation in limbic brain systems during social play behaviour in adolescent rats. Submitted.
- Vanderschuren LJMJ, Niesink RJM, Van Ree JM (1997) The neurobiology of social play behavior in rats. Neurosci Biobehav Rev 21:309-326.
- Vanderschuren LJMJ, Trezza V, Griffioen-Roose S, Schiepers OJG, Van Leeuwen N, De Vries TJ, Schoffelmeer ANM (2008) Methylphenidate disrupts social play behavior in adolescent rats. Neuropsychopharmacology 33:2946-2956.
- Veeneman MMJ, Boleij H, Broekhoven MH, Snoeren EMS, Guitart MM, Cousijn J, Spooren W, Vanderschuren LJMJ (2011) Dissociable roles of mGlu5 and dopamine receptors in the rewarding and sensitizing properties of morphine and cocaine. Psychopharmacology 214:863-876.
- Zheng X, Liu F, Wu X, Li B (2008) Infusion of methylphenidate into the basolateral nucleus of amygdala or anterior cingulate cortex enhances fear memory consolidation in rats. Sci China C Life Sci 51:808-813.



Chapter 6
Dissecting play: contrasting roles of dopamine and noradrenaline in social play expression, reward and motivation

L.W.M. van Kerkhof[1]
E.J.M. Achterberg[2]
M. Servadio[1]
V. Trezza[3]
P. Voorn[4]
L.J.M.J. Vanderschuren[1,2]

1 Rudolf Magnus Institute of Neuroscience, Department of Neuroscience and Pharmacology, University Medical Centre Utrecht, Utrecht, The Netherlands.
2 Department of Animals in Science and Society, Division of Behavioural Neuroscience, Faculty of Veterinary Medicine, Utrecht University, Utrecht, The Netherlands.
3 Department of Biology, University 'Roma Tre', Rome, Italy.
4 Department of Anatomy and Neurosciences, Neuroscience Campus Amsterdam, VU University Medical Centre, Amsterdam, The Netherlands.

In preparation.

Abstract

Social play behaviour is a vigorous form of social interaction abundantly expressed during the juvenile and adolescent phases of life. The experience of positive social interactions during these developmental stages is essential for proper social and cognitive development in mammals, including humans. Social play is highly rewarding and as such its expression depends on its motivational and pleasurable properties. Previously, it has been shown that the expression of social play behaviour is differentially influenced by dopamine and noradrenaline neurotransmission. In the present study we investigated whether dopamine and noradrenaline neurotransmission are differently involved in the motivational and pleasurable aspects of play, using the dopamine/noradrenaline reuptake inhibitor methylphenidate, the dopamine reuptake inhibitor GBR12909, the dopamine receptor antagonist alpha-flupenthixol and the noradrenaline reuptake inhibitor atomoxetine. To investigate the pleasurable aspects of play, we used the previously established social play-induced conditioned place preference (CPP) task. To investigate its motivational properties, we developed an operant conditioning task for social play reward. Treatment with GBR12909 increased operant responding and disrupted the acquisition of CPP, but did not affect the expression of social play behaviour. Atomoxetine treatment reduced operant responding and the expression of social play behaviour, but did not affect the acquisition of social play-induced CPP. After treatment with methylphenidate, operant conditioning was enhanced, expression of social play was reduced and acquisition of CPP was blocked. The latter effect was prevented by pretreatment with alpha-flupenthixol. These results demonstrate the involvement of dopaminergic neurotransmission in the pleasurable and motivational properties of social play, and noradrenergic involvement in the expression and motivational properties of play. In conclusion, the expression, pleasurable and motivational properties of social play behaviour rely on dissociable neural substrates.

Introduction

The experience of social interactions during childhood and adolescence is critical for social and cognitive development (Baarendse *et al.*, 2012a; Potegal and Einon, 1989; Van den Berg *et al.*, 1999). Throughout this developmental period, a specific form of social interaction, i.e. social play behaviour, is abundantly expressed (Panksepp *et al.*, 1984; Pellis and Pellis, 1998; Spear, 2000). Social play behaviour is a highly vigorous form of social interaction, in which components of other social behaviours are present, albeit expressed in an adapted and/or out-of-context manner (Pellis and Pellis, 2009; Vanderschuren *et al.*, 1997).

Previous studies have indicated that the expression of social play behaviour can be modulated by dopaminergic and noradrenergic neurotransmission. For example, treatment with dopamine receptor agonists and antagonists alters the expression of social play behaviour (Niesink and Van Ree, 1989; Siviy *et al.*, 1996; Siviy and Panksepp, 2011; Trezza *et al.*, 2010). In addition, modulation of play behaviour by cannabinoids, ethanol and nicotine depends upon dopamine signalling (Trezza *et al.*, 2009a; Trezza and Vanderschuren, 2008). Administration of the α-2 adrenoceptor agonist clonidine reduced social play behaviour, whereas administration of RX821002, an α-2 adrenoceptor antagonist, has been reported to enhance social play behaviour (Normansell and Panksepp, 1985; Siviy *et al.*, 1994; Siviy and Baliko, 2000). In addition, administration of methylphenidate, which blocks the reuptake of dopamine and noradrenaline (Ferris and Tang, 1979; Ritz *et al.*, 1987), inhibited the expression of social play behaviour (Beatty *et al.*, 1982; Vanderschuren *et al.*, 2008). The effect of methylphenidate could be mimicked by administration of atomoxetine, a specific inhibitor of the noradrenaline transporter, but not by the dopamine reuptake inhibitor GBR12909 (Vanderschuren *et al.*, 2008). Furthermore, the effect of methylphenidate on social play behaviour was prevented by pretreatment with the α-2 adrenoceptor antagonist, RX821002, indicating that α-2 adrenoceptors have an important role in social play behaviour (Vanderschuren *et al.*, 2008).

Social play behaviour is highly rewarding (Trezza *et al.*, 2011a; Vanderschuren, 2010) and it is modulated through neural systems involved in other rewards such as food, sex, and drugs of abuse (Trezza *et al.*, 2010). It has been shown that several components of reward can be dissociated: its pleasurable ('hedonic') properties, incentive motivational properties, and effects on learning (Berridge *et al.*, 2009). These components are mediated via different neural systems (Berridge *et al.*, 2009). For example, opioids and endocannabinoids are thought to influence the pleasurable properties of a reward, whereas dopamine is thought to be mainly involved in its motivational aspects (Barbano and Cador, 2007; Berridge *et al.*, 2009; Kelley, 2004; Salamone and Correa, 2012).

In the present study, we investigated whether dopamine and noradrenaline are involved in the pleasurable and/or motivational aspects of social play behaviour. Since methylphenidate has a marked inhibitory effect on the expression of social play, we also investigated how methylphenidate influenced these different aspects of social play behaviour. To measure the motivational aspects of social play behaviour, we developed an operant conditioning task, in which rats were trained to press a lever for access to a playful partner under a progressive ratio schedule of reinforcement. In addition, we investigated whether changes in dopamine and noradrenaline neurotransmission

affected the acquisition of social play-induced conditioned place preference (CPP). In this task, rats learn to associate a set of environmental cues with social play. Rats will only develop a preference for the play-associated compartment if the play encounter is perceived as pleasurable and if they are able to encode the context-reward association (Trezza *et al.*, 2009b). The combination of these two tasks provides new information on the involvement of dopamine and noradrenaline neurotransmission in social play behaviour.

Materials and Methods

Animals

Male Wistar rats (Charles River, Sulzfeld, Germany) arrived in our animal facility at 21 days of age and were housed in groups of four in 40 × 26 × 20 cm (l × w × h) Macrolon cages under controlled conditions (ambient temperature 20-21°C, 60-65 % relative humidity, and 12/12 h light cycle with lights on at 7.00 a.m.). Food and water were available *ad libitum*. All animals used were experimentally naïve. All experiments were approved by the Animal Ethics Committee of Utrecht University and were conducted in accordance with Dutch laws (Wet op de Dierproeven, 1996) and European regulations (Guideline 86/609/EEC).

Drugs

Methylphenidate hydrochloride (BUFA, Castricum, The Netherlands), atomoxetine hydrochloride (Tocris Bioscience, Bristol, UK) and α-flupenthixol dihydrochloride (Sigma-Aldrich, Schnelldorf, Germany) were dissolved in saline. GBR-12909 dihydrochloride (Sigma-Aldrich, Schnelldorf, Germany) was dissolved in MilliQ water. Methylphenidate and GBR-12909 were administered subcutaneously (s.c.). Atomoxetine and α-flupenthixol were administered intra-peritoneally (i.p.). Drug doses and pre-treatment intervals were based on previous studies (Baarendse *et al.*, 2012b; Baarendse and Vanderschuren, 2012; Vanderschuren *et al.*, 2008). Drug doses were calculated as salt. Drugs were administered 30 min before testing, except for when methylphenidate treatment was combined with α-flupenthixol treatment, in which case α-flupenthixol was administered 45 min before test, i.e. 15 min prior to methylphenidate administration. In view of the importance of the neck area in the expression of social play behaviour, s.c. injections were administered in the flank.

Operant conditioning paradigm

Apparatus

Behavioural testing was conducted in an operant conditioning chamber (Med Associates, Vermont, USA) divided into two equally sized compartments (25 x 30 x 25 cm , *l x w x h*). The compartments were separated by a Plexiglas wall with 42 small holes (Ø 0.5 cm) and an automated metal door in the middle. Both compartments had a metal grid floor and a Plexiglas lid which contained a house-light (2 W). One compartment (the 'lever pressing compartment') was equipped with two 4.8 cm-wide retractable levers, located on opposite sides of the compartment. Above each lever was a cue light (2.5 W). One lever was designated as the active lever and the other as the inactive lever; allocation

of the left or right lever as active was counterbalanced between animals. Experimental events and data recording were controlled using Med PC software (Med Associates, Vermont, USA).

Experimental procedure

All experiments were performed under red light conditions. Animals were randomly paired with a test partner from another home cage. Animals in a test pair did not differ more than 10 grams in body weight at the start of the experiment. A test pair consisted of one 'lever-pressing' animal and one play 'stimulus' partner. At 24 days of age, test pairs were habituated to the test cage for 10 min. Animals were isolated for 24 h/day for 5 days/week after the habituation session. In the experiment to determine the effect of isolation time on responding for play, one group of animals was isolated for 2 h/day for 5 days/week. Following the habituation day, animals received two shaping sessions on two consecutive days. During these shaping sessions, approaching the active lever resulted in presentation of the cue light, retraction of the lever and opening of the door by the experimenter. Rats were allowed to interact for two minutes after which the door was closed. This procedure was repeated 7 times in each shaping session. In addition, if an animal did not perform any active lever presses during acquisition sessions, it received a shaping session on the next day. On the third day, the lever pressing sessions commenced under a fixed ratio (FR) 1 schedule of reinforcement. Under this FR 1 schedule of reinforcement, each active lever press resulted in presentation of the cue light, retraction of both levers, and opening of the door, after which animals were allowed to freely interact for 2 min. After 2 min, the door automatically closed and the house-light was illuminated during a 25 s intra-trial interval. During this interval, the experimenter placed each rat back into its starting compartment. Animals received one 20 min session per day, for 5 days/week. During the other 2 days/week animals were socially housed with their original cage-mates. As a result of time limit of 20 min, under this schedule a maximum of eight rewards could be obtained. After acquisition of the task under the FR 1 schedule (i.e., when an animal obtained at least six out of eight possible rewards on two consecutive days), a progressive ratio (PR) schedule of reinforcement was introduced. 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, 25, etc; Richardson and Roberts, 1996). When rats met the response requirement on the active lever, the cue light was illuminated, both levers retracted and the door opened for 1 min, during which the animals could freely interact. A PR session continued until an animal failed to obtain a reward within 10 min. Animals were trained under this schedule until responding had stabilized, defined as obtaining at least six rewards on three consecutive days with a variation of no more than two rewards. After responding had stabilized, drug treatment started according to a Latin Square design. GBR-12909 and atomoxetine were tested in one cohort of animals, and all other treatments were tested in separate groups of animals. Inactive lever presses were recorded, but had no programmed consequences.

Analysis of social play behaviour

During social interactions (i.e., when the door between the two compartments was open), behaviour of the playing rats was assessed on-line using the Observer 5.1 software (Noldus Information Technology B.V., The Netherlands). In addition to the on-line analysis, behaviour of the animals was recorded using a camera with zoom lens, video tape recorder and television monitor. Three behavioural elements were scored (Panksepp *et al.*, 1984; Trezza *et al.*, 2010; Vanderschuren *et al.*, 1997).

- *Frequency of pinning:* one animal lying with its dorsal surface on the floor with the other animal is standing over it, which is the most characteristic posture of social play in rats.
- *Frequency of pouncing:* one animal is attempting to nose or rub the nape of the neck of the partner, which is an index of play solicitation.
- *Time spent in social exploration:* one animal sniffing or grooming any part of the partner's body.

Place conditioning paradigm

Apparatus

The place conditioning setup (TSE System, Bad Homburg, Germany) comprised eight boxes, each consisting of three compartments with removable Plexiglas lids. The two conditioning compartments were equally sized (30 cm x 25 cm x 30 cm; *l x w x h*) and separated by the third, neutral, compartment (10 cm x 25 cm x 30 cm; *l x w x h*). The two conditioning compartments had different visual and tactile cues: one had black-and-white striped walls and a floor with wide metal mesh, and the other had black walls and a floor with fine metal mesh. The compartment with black walls had a white light (2 W) mounted on the Plexiglas lid, to achieve a comparable light intensity in both conditioning compartments. The middle compartment had white walls, a smooth floor, and a white light (2 W) on the lid. The position of the animal in the apparatus was monitored by an array of photo-beam sensors located 2.5 cm above the floor. The time spent in each compartment (msec) was recorded by a computer. All experiments were performed in a dimly lit room.

Experimental procedure

Place conditioning was performed as previously described (Achterberg *et al.*, 2012; Trezza *et al.*, 2009b; Trezza *et al*., 2011b). At 26 days of age (day 1), each rat was placed in the middle compartment of the apparatus and pre-conditioning side preference was determined by allowing the rats to move freely in the three compartments for 15 min. On the basis of their preference scores, rats were assigned to a compartment in which they would be allowed social interaction during conditioning. A counterbalanced place conditioning design was used (Tzschentke, 2007; Veeneman *et al.*, 2011), meaning that the pre-conditioning preference in each experimental group for rats to be social-paired or non-social paired approximated 50%. Thus, based on their pre-conditioning performance, half of the rats were conditioned in their preferred compartment, while the other half were conditioned in their non-preferred compartment. After the pre-conditioning test, rats were individually housed to increase their motivation for social interaction and to

facilitate the development of social play-induced CPP (Achterberg *et al.*, 2012; Niesink and Van Ree, 1989; Trezza *et al.*, 2009b; Trezza *et al.*, 2011b; Vanderschuren *et al.*, 2008). Place conditioning began on day 2. On days 2, 4, 6, and 8 rats were placed for 30 min in one compartment with an initially unfamiliar partner (social session) in the morning and were placed alone in the other compartment (non-social session) in the afternoon. On day 3, 5, 7, and 9 the order of the sessions was reversed. Social and non-social sessions were separated by at least one hour. Drugs were administered 30 min before the start of each social session. On day 10, rats were placed in the middle compartment and were allowed to explore the entire apparatus for 15 min. The time spent in each compartment during this test was recorded to determine place preference.

Statistical analysis

Data were analysed using SPSS software 15.0 for Windows and expressed as mean ± SEM. The frequency of pinning and pouncing during operant conditioning was calculated per minute of interaction time. The duration of social exploration was calculated as a percentage of the interaction time. In experiments where a single drug dose was tested, data were analysed using paired Student's t-test. If multiple doses were tested, data were analysed using a repeated measures ANOVA followed by a paired Student's t-test when appropriate. Operant responding was analysed with lever and treatment as a within-subjects factor. The breakpoints under the PR schedule of reinforcement are derived from an escalating curve, which violates the homogeneity of variance. Therefore, breakpoints were analysed using the non-parametric Friedman test, followed by a post-hoc Wilcoxon signed ranks test if appropriate. Place conditioning data were expressed as mean time spent in the social paired and non-social paired compartment. Place conditioning data were analysed using a two-way ANOVA analysis, with compartment and treatment as factors, followed by paired Student's t-test when appropriate.

Results

Establishment of the operant conditioning task

The results indicated that the rats were able to acquire the task, i.e. press a lever for the opportunity for a social interaction (Fig. 1A). However, only after 24 h of isolation did all tested animals (6/6) reach criterion under the FR 1 schedule of reinforcement within 8 days of training (after which the PR schedule of reinforcement was introduced), whereas only one third (2/6) of the animals isolated for 2 h reached the FR 1 criterion (i.e., at least six out of eight possible rewards on two consecutive days) within 8 days (Fig. 1A). In view of the limited age window during which social play behaviour is abundant (Baenninger, 1967; Meaney and Stewart, 1981; Panksepp, 1981; Pellis and Pellis, 1990), fast acquisition of the task is necessary to allow for a sufficient age window to remain for further (pharmacological) testing. This experiment shows that a duration of 24 h of isolation during conditioning and testing is most suited for these experiments. After acquisition of the task under the FR1 schedule, the rats were trained to respond under a PR schedule of reinforcement. Results show that rats respond differently on the active and inactive lever ($F_{lever}(1,5) = 22.605$, $p = 0.005$), indicating that they are capable of discriminating between the active and inactive lever. After 24 h of isolation, rats perform more active and inactive

responses ($F_{isolation}(1,5) = 35.657$, $p = 0.002$; $F_{lever\ x\ isolation}(1,5) = 27.470$, $p = 0.003$) (Fig. 1B). Post hoc analysis confirmed that animals isolated for 24 h made more active and inactive responses (active: $t=5.880$, $df = 5$, $p = 0.002$; inactive: $t = 2.812$, $df = 5$, $p = 0.037$) (Fig. 1B). In addition, after 24 h isolation, rats obtained more rewards ($t = 12.872$, $df = 5$, $p < 0.001$) (Fig. 1C) and reached a higher breakpoint ($Z = -2.201$, $p = 0.028$) than after 2 h of isolation (Fig. 1D).

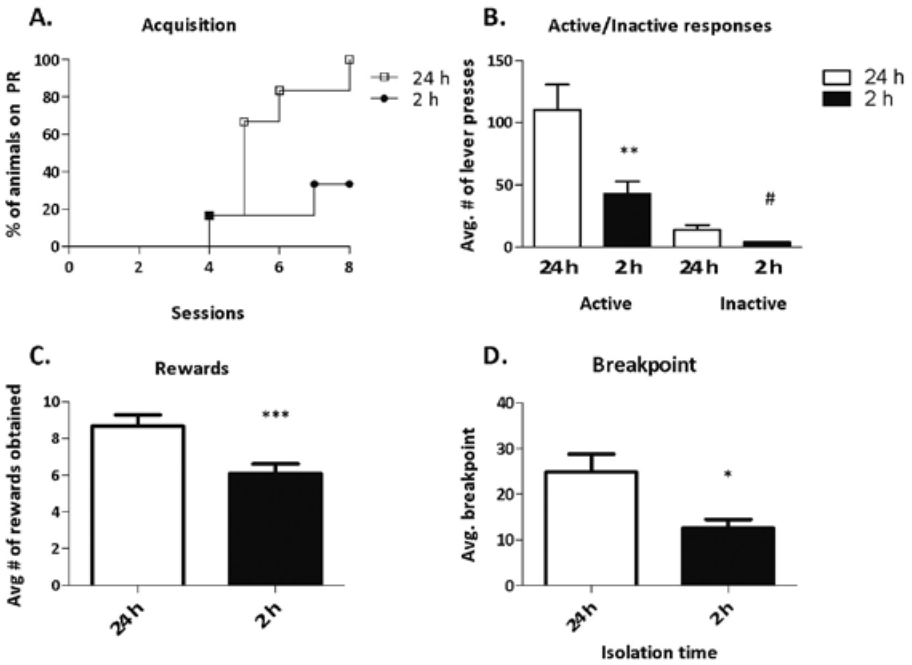


Fig. 1: Duration of social isolation influenced the acquisition and performance in the operant task for social play behaviour. Shorter social isolation (2 h) resulted in a slower acquisition of the task (A; n = 6/group). In addition, shorter isolation (2 h) reduced operant responding (n=6) as reflected in the number of responses (B), the number of rewards obtained (C), and the breakpoint (D). In panel A data is presented as the percentage of animals that reached the criteria of responding under an FR-1 schedule of reinforcement (i.e., at least 6 out of 8 rewards obtained on two consecutive days), in panels B-D, data are presented as mean ± SEM

The effect of selective dopamine and noradrenaline reuptake inhibitors on operant responding for social play behaviour

The animals discriminated between the active and inactive lever ($F_{lever}(1.6) = 37.836$, $p = 0.001$), and GBR-12909 selectively increased responding on the active lever ($F_{treatment}(1.6) = 5.547$, $p = 0.057$; $F_{lever\ x\ treatment}(1,6) = 7.435$, $p = 0.034$; active: $t = -2.551$, $df = 6$, $p = 0.043$; inactive: $t = 1.671$, $df = 6$, $p = 0.146$) (Fig. 2A). In addition, GBR-12909 increased the number of rewards obtained ($t= -2.931$, $df = 6$, $p = 0.026$) (Fig. 2B) and the breakpoint ($Z = -2.207$, $p = 0.027$) (Fig. 2C). GBR-12909 treatment did not affect the expression of social play behaviour. The frequency of pinning ($t = 0.885$, $df = 6$, $p = 0.420$), pouncing ($t = 0.204$, $df = 6$, $p = 0.845$) and the duration of social exploratory

behaviour (t = 1.140, df = 6, p = 0.298) were not altered by 10 mg/kg of GBR-12909 (Fig. 2D-F).

Administration of atomoxetine reduced operant responding for play. Treatment with atomoxetine decreased the number of responses ($F_{treatment}(2.14) = 28.843$, $p < 0.001$). Animals were capable of discriminating between the active and inactive lever ($F_{lever}(1.7) = 112.150$, $p<0.001$) and atomoxetine differently affected responding on the active and inactive lever ($F_{lever\ x\ treatment}(2.14) = 27.423$, $p < 0.001$). *Post hoc* analysis revealed that responding was decreased on both levers. However, atomoxetine was more potent in reducing responding on the active than on the inactive lever, since active lever presses were significantly decreased by both doses of atomoxetine, whereas only the highest dose reduced inactive presses (active: sal vs. 1: t = 4.061, df = 7, p = 0.005; sal. vs. 3: t = 8.014, df = 7, p < 0.001; inactive: sal. vs. 1: t = 2.081, df = 7, p = 0.076; sal. vs. 3: 3.412, df = 7, p = 0.011) (Fig. 3A). In addition, atomoxetine decreased the number of rewards obtained ($F_{treatment}(2,14) = 48.309$, $p < 0.001$; sal. vs. 1: t = 5.338, df = 7, p = 0.001; sal. vs. 3: t = 9.000, df = 7, p < 0.001) (Fig. 3B) and the breakpoint reached ($X^2 = 15.000$, df = 2, p < 0.001; sal. vs. 1: Z = -2.392, p = 0.01; sal. vs. 3: Z = -2.524, p = 0.012) (Fig. 3C). Atomoxetine reduced the expression of social play behaviour as well. Treatment with both doses of atomoxetine decreased the frequency of pinning ($F_{treatment}(2,14) = 9.645$, $p = 0.002$; sal. vs. 1: t = 2.438, df = 7, p = 0.045; sal. vs. 3: t = 3.870, df = 7, p = 0.006) (Fig. 3D). The frequency of pouncing was decreased after administration of 3 mg/kg atomoxetine ($F_{treatment}(2,14) = 6.625$, $p = 0.009$; sal. vs. 1: t = 2.077, df = 7, p = 0.076; sal. vs. 3: t = 3.281, df = 7, p = 0.013) (Fig. 3E). The time spent on social exploration was not affected by atomoxetine treatment ($F_{treatment}(2,14) = 2.013$, $p = 0.170$) (Fig. 3F).

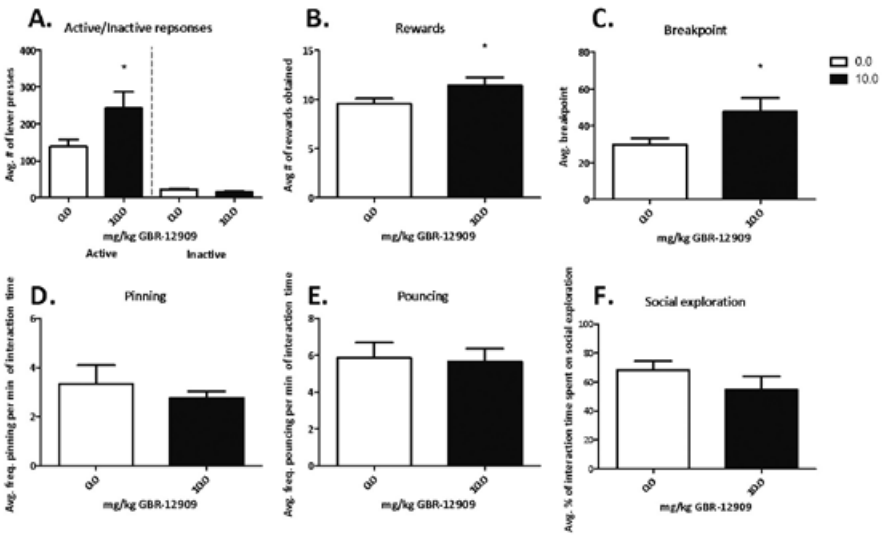


Fig. 2: Treatment with GBR-12909 enhanced operant responding for social play behaviour (n = 7). GBR-12909 increased the number of active responses (A), the number of rewards obtained (B) and the breakpoint (C). Administration of GBR-12909 did not affect the frequency of pinning (D), the frequency of pouncing (E), or the time spent on social exploration behaviour (F). Data are presented as mean ± SEM. * $p < 0.05$.

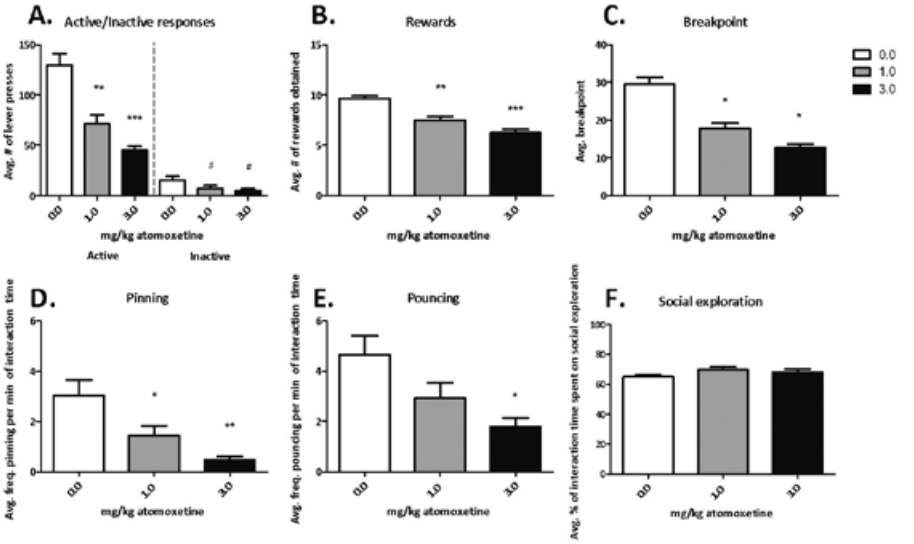


Fig. 3: Administration of atomoxetine reduced operant responding for social play behaviour (n = 8). After treatment with atomoxetine the number of active and inactive responses was reduced (A), fewer rewards were obtained (B) and the breakpoint was lower (C). In addition, the frequency of pinning (D) and pouncing (E) was reduced. The time spent on social exploration was unaffected (F). Data are presented as mean ± SEM. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, relative to saline treatment. # $p < 0.05$ inactive lever presses relative to saline treatment.

Methylphenidate enhances operant responding for social play behaviour

Treatment with methylphenidate (1-3 mg/kg) enhanced the number of lever presses in rats responding for social play under a PR schedule of reinforcement ($F_{treatment}(2,10) = 20.606$, $p < 0.001$). After treatment with methylphenidate, there was a significant increase in the number of active lever presses and a trend towards an increase of responses on the inactive lever ($F_{lever\ x\ treatment}(2,10) = 17.404$, $p = 0.001$; active: sal. vs. 1 mg/kg: $t = -0.873$, $df = 5$, $p = 0.422$; sal. vs. 3 mg/kg: $t = -4.314$, $df = 5$, $p = 0.008$; inactive: sal. vs. 1 mg/kg: $t = 0.060$, $df = 5$, $p = 0.955$; sal. vs. 3 mg/kg: $t = -2.490$, $df = 5$, $p = 0.055$) (Fig. 4A). In addition, the number of rewards obtained ($F_{treatment}(2,10) = 19.935$, $p < 0.001$; sal. vs. 1 mg/kg: $t = -0.415$, $df = 5$, $p = 0.695$; sal. vs. 3 mg/kg: $t = -4.392$, $df = 5$, $p = 0.007$) (Fig. 4B) as well as the breakpoint was increased after treatment with 3 mg/kg of methylphenidate ($X^2 = 8.273$, $df = 2$, $p = 0.016$; sal. vs. 1 mg/kg: $Z = -0.552$, $p = 0.581$; sal. vs. 3 mg/kg: $Z = -2.060$, $p = 0.039$) (Fig. 4C).

In contrast to the enhancement of operant responding, treatment with methylphenidate decreased the frequency of pinning and pouncing at both doses (pinning: $F_{treatment}(2,10) = 65.972$, $p < 0.001$; sal. vs. 1 mg/kg: $t = 7.255$, $df = 5$, $p = 0.001$; sal. vs. 3 mg/kg: $t = 9.219$, $df = 5$, $p < 0.001$; pouncing: $F_{treatment}(2,10) = 49.035$, $p < 0.001$; sal. vs. 1 mg/kg: $t = 5.756$, $df = 5$, $p = 0.002$; sal. vs. 3 mg/kg: $t = 8.329$, $df = 6$, $p < 0.001$) (Fig. 4D-E). In addition, methylphenidate increased the duration of social exploration ($F_{treatment}(2,10) = 8.726$, $p = 0.006$; sal. vs. 1 mg/kg: $t = -2.401$, $df = 5$, $p = 0.064$; sal. vs. 3 mg/kg: $t = -3.920$, $df = 5$, $p = 0.011$) (Fig. 4F). These results suggest that despite decreasing the expression of social play, methylphenidate enhances operant responding for play.

Subsequently, we investigated whether the enhancement in operant responding after methylphenidate was the result of an extinction overshoot. Thus, the rats may have increased responding because they received less play reward. Therefore, the rats were treated with 3 mg/kg methylphenidate or saline for five consecutive days. The results show that methylphenidate increased the number of rewards ($F_{treatment}(1,6) = 27.836$, $p = 0.002$), but that this effect did not change with repeated treatment ($F_{time}(4,24) = 0.754$, $p = 0.565$; $F_{treatment \times time}(4,24) = 2.192$, $p = 0.100$)) (Fig.4G). These results indicate that the enhancement by methylphenidate of operant conditioning was not caused by an extinction overshoot.

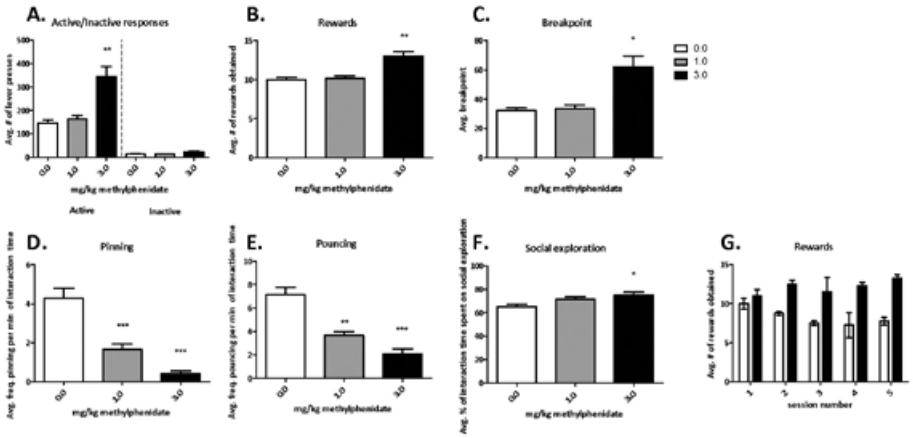


Fig. 4: Methylphenidate influenced operant responding for social play behaviour. Treatment of methylphenidate enhanced the number of active lever presses (A), the number of rewards obtained (B), the breakpoint reached (C), and the time spent on social interaction not related to play (F), while it reduced the frequency of pinning (D) and pouncing (E)(n = 6). The effect of methylphenidate on the number of rewards obtained was similar over 5 consecutive days of treatment (G) (n = 4/group). Data are presented as mean ± SEM. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, relative to saline treatment.

The effect of selective dopamine and noradrenaline reuptake inhibitors on the acquisition of social play-induced CPP

Methylphenidate has been reported to disrupt the acquisition of CPP induced by social play (Trezza *et al.*, 2009b). However, the role of dopamine and noradrenaline neurotransmission in this effect remains unknown. Therefore, in the present study, the effect of the specific dopamine reuptake inhibitor GBR-12909 and the specific noradrenaline reuptake inhibitor atomoxetine on social play-induced CPP was investigated. These drugs were administered prior to each social conditioning session. After eight conditioning sessions, preference for each compartment was tested in a drug-free state.

Treatment with GBR-12909 altered the acquisition of social play-induced CPP ($F_{compartment (comp)}(1,40) = 12.652$, $p = 0.001$; $F_{treatment}(1,40) < 0.001$, $p = 0.992$; $F_{comp \times treatment}(1,40) = 4.630$, $p = 0.038$). Post hoc analysis revealed that the control group had a significant preference for the social compartment ($t = 2.881$, $df = 11$, $p = 0.015$), whereas the animals

treated with GBR-12909 showed no preference ($t = 0.774$, $df = 9$, $p = 0.459$) (Fig. 5A). GBR-12909 treatment did not affect the total number of entries into both compartments (mean number of entries: saline 69.50 ± 4.25; GBR-12909 66.60 ± 8.76; $t = 0.070$, $df = 20$, $p = 0.945$) suggesting that the absence of CPP in the GBR-12909 treated animals is not related to alterations in locomotor activity. These results indicate that an increase in endogenous dopamine levels interferes with the acquisition of CPP induced by social play behaviour.

Administration of atomoxetine did not affect the acquisition of social play-induced CPP. A significant effect of compartment was observed, but no effect of treatment or an interaction between treatment and compartment was detected ($F_{comp}(1,70) = 28.051$, $p < 0.001$; $F_{treatment}(2,70) = 0.195$, $p = 0.824$; $F_{comp \times treatment}(2,70) = 0.725$, $p = 0.487$) (Fig. 5B). These results indicate that increased endogenous noradrenaline levels do not interfere with the acquisition of social play induced CPP.

We next examined whether the disruption of social play-induced CPP by methylphenidate, as observed in our previous study (Trezza *et al.*, 2009b), was related to the effect of methylphenidate on dopamine levels, using the dopamine receptor antagonist α-flupenthixol. First, we observed that administration of α-flupenthixol prior to the social conditioning sessions did not influence the acquisition of CPP (Fig. 5C). A significant effect of compartment was found, but neither an effect of treatment, nor a treatment with compartment interaction was detected ($F_{comp}(1,104) = 42.886$, $p < 0.001$; $F_{treatment}(3,104) = 0.236$, $p = 0.852$; $F_{comp \times treatment}(3,104) = 0.969$, $p = 0.410$). These results indicate that α-flupenthixol did not influence the acquisition of social play behaviour. In a subsequent experiment, the lowest dose of α-flupenthixol, 0.125 mg/kg, was used to combine with methylphenidate treatment. Acquisition of CPP was altered in this experiment, since besides a compartment effect, an interaction between treatment and compartment was detected ($F_{comp}(1,50) = 94.063$, $p < 0.001$; $F_{treatment}(2,50) = 0.652$, $p = 0.525$; $F_{comp \times treatment}(2,50) = 4.731$, $p = 0.013$). Post hoc analysis revealed a significant preference for the social compartment in the control group ($t = 7.669$, $df = 8$, $p < 0.001$) as well as in the animals receiving the combined treatment of methylphenidate and α-flupenthixol ($t = 6.351$, $df = 9$, $p < 0.001$) (Fig. 5D). In contrast, animals treated with methylphenidate did not show a preference for any of the compartment ($t = 1.880$, $df = 8$, $p = 0.097$), similar to a previous report (Trezza *et al.*, 2009b). Treatment did not affect the total number of entries into both compartments (mean number of entries: saline 98.00 ± 2.61; methylphenidate 102.11 ± 4.50; methylphenidate + α-flupenthixol 108.20 ± 5.91; $F_{treatment}(2.27) = 1.160$, $p = 0.330$), suggesting that locomotion was unaltered during the test. These results indicate that α-flupenthixol prevents the disruption of CPP acquisition by methylphenidate, which suggests that the effect of methylphenidate on CPP is mediated via a dopaminergic mechanism.

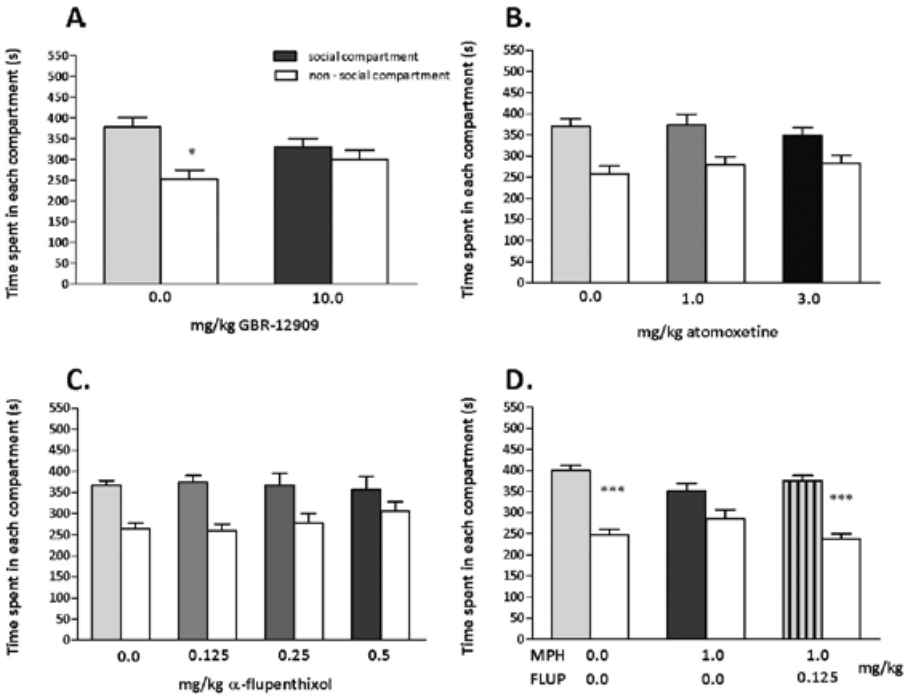


Fig. 5: Effect of GBR-12909, atomoxetine, α-flupenthixol, and methylphenidate treatment on the acquisition of social play induced CPP. Treatment with GBR-12909 disrupts the acquisition of social play-induced CPP (A; n = 12/10), while treatment with atomoxetine (B; n = 18/10/10) and α-flupenthixol (C; n = 24/12/10/10) had no effect on the acquisition of CPP. Methylphenidate treatment disrupts the acquisition of social play induced CPP, which is blocked by pre-treatment with α-flupenthixol (D; n = 9 per group). * $p < 0.05$, *** $p < 0.001$, relative to time spent in social compartment.

Discussion

Establishment of an operant conditioning task for social interaction

The aim of the present study was to investigate the motivational and pleasurable aspects of social play behaviour. Responding under a progressive ratio schedule of reinforcement is a widely used method to measure the motivational value of rewards (Richardson and Roberts, 1996). In the present study, we developed a novel setup in which rats were trained to lever-press for social play behaviour. To the best of our knowledge, ours is the first study to show that rats are willing to perform an operant task to obtain access to a playful social interaction. Previous studies have shown that there is a close relationship between the length of social isolation and the amount of play behaviour expressed subsequently (Niesink and Van Ree, 1989; Vanderschuren *et al.*, 1995; Vanderschuren *et al.*, 2008). Therefore, longer social isolation probably enhances the motivation for social play behaviour. This is in line with the results observed in the present study, where animals acquire the operant task faster and reach a higher breakpoint after a longer period of social isolation (24 h vs. 2 h). These results indicate that it is possible to measure

differences in social motivation with this operant conditioning task. In addition, after 24 h of isolation, the majority of rats acquired the task within 5-8 sessions, and already reach a near maximal number of rewards during task acquisition. This fast acquisition is required considering the limited age window during which social play behaviour is abundantly expressed (Baenninger, 1967; Meaney and Stewart, 1981; Panksepp, 1981; Pellis and Pellis, 1990).

Dissociable roles of dopamine and noradrenaline in the motivation for social play

Administration of methylphenidate enhanced responding for social play behaviour. In contrast to this enhancement in the motivation for play, the expression of social play behaviour itself was reduced. This reduction in social play behaviour is similar to previous reports (Beatty *et al.*, 1982; Vanderschuren *et al.*, 2008). In addition, the enhancement of responding remained present during repeated treatment with methylphenidate, which excludes the possibility that the increase in responding is the result of an extinction overshoot. Administration of the specific dopamine reuptake inhibitor GBR-12909 did not affect the expression of social play behaviour, as previously reported (Vanderschuren *et al.*, 2008). However, similar to methylphenidate treatment, GBR-12909 enhanced operant responding for social play behaviour, suggesting that the enhancement of operant responding by methylphenidate is mediated via a dopaminergic mechanism. The present study shows that treatment with the specific noradrenaline reuptake inhibitor atomoxetine reduced the expression of social play behaviour as well as operant responding for play. Previously, it was reported that the reduction in the expression of social play behaviour by methylphenidate was mimicked by atomoxetine and mediated via α2-adrenoceptors (Vanderschuren *et al.*, 2008). These results suggest that enhanced endogenous noradrenaline signalling reduces the motivation for and expression of social play behaviour.

Combining the effects of methylphenidate, GBR-12909, and atomoxetine treatment, it appears that methylphenidate has dissociable actions on social play behaviour, via dopaminergic and noradrenergic signalling. Enhancement of endogenous noradrenaline signalling reduces the motivation to play and the expression of social play behaviour, whereas enhancement of endogenous dopamine signalling has no influence on the expression of social play behaviour, but enhances operant responding. After treatment with methylphenidate, the noradrenergic effect on the expression of social play is apparent, whereas the effect on dopamine neurotransmission on operant responding has the upper hand. Interestingly, methylphenidate reduces the expression of social play behaviour at both 1 and 3 mg/kg, while the effect on operant responding was only apparent at the dose of 3 mg/kg, which supports the idea that different neural systems underlie the effects of methylphenidate on these two aspects of social play behaviour.

The dissociation between enhanced responding and reduced reward consumption after methylphenidate treatment is in line with previous studies. Changes in dopamine levels affect the motivation for a reward, without markedly changing reward consumption (for reviews see: Baldo and Kelley, 2007; Barbano and Cador, 2007; Berridge, 2007; Salamone and Correa, 2012). For example, administration of amphetamine into the nucleus accumbens enhanced operant responding for food (e.g. Zhang *et al.*, 2003), while it did not increase food consumption (e.g. Hanlon *et al.*, 2004). Our observations are

therefore consistent with the view that dopaminergic neurotransmission plays a critical role in activating aspects of motivation, that is, in the invigoration of appetitive approach towards a goal (Robbins and Everitt, 2007; Salamone and Correa, 2012), but not in reward consumption.

Dissociable effects of dopamine and noradrenaline on acquisition of social play induced CPP

The present study indicates that dopamine and noradrenaline have distinct effects on the acquisition of CPP induced by social play behaviour. Administration of the selective dopamine reuptake inhibitor GBR-12909 prior to social conditioning sessions disrupted the establishment of CPP, whereas treatment with atomoxetine did not affect the acquisition of CPP. Consistent with a previous study, we also show that treatment with methylphenidate disrupts the acquisition of CPP (Trezza *et al.*, 2009b). In addition, pre-treatment with the dopamine receptor antagonist α-flupenthixol blocked the disruptive effect of methylphenidate on CPP acquisition. The dose of α-flupenthixol used in this experiment had no effect on play behaviour by itself. These results indicate that methylphenidate inhibits the acquisition of social play-induced CPP via enhancement of endogenous dopamine levels.

It is unlikely that the enhancement in endogenous dopamine levels by methylphenidate or GBR-12909 interfered with learning and memory processes during conditioning. For example, treatment with methylphenidate itself induces CPP, which shows that adolescent rats treated with methylphenidate are capable of acquiring a place-reward association (Trezza *et al.*, 2009b). The most parsimonious explanation for our data is therefore that enhancement of endogenous dopamine levels reduced the hedonic value of play behaviour, which resulted in the absence of CPP. It has repeatedly been shown that manipulating dopaminergic neurotransmission does not change the hedonic value of food (Dickinson *et al.*, 2000; Wassum *et al.*, 2011; Wyvell and Berridge, 2000). To the best of our knowledge, however, our findings are the first to suggest that increasing dopaminergic neurotransmission can actually reduce the hedonic value of a reward, although more work is needed before this conclusion can be firmly drawn. Our data further showed that increased endogenous dopamine levels resulted in enhanced operant responding for social play behaviour and that the expression of social play behaviour was not changed. Possibly, increases in dopamine resulted in a reduction of the pleasurable properties but an enhanced motivation to play and as a consequence, the level of social play behaviour was unaltered. These three different aspects of social play behaviour are closely related, and enhancement of endogenous dopamine levels affects all aspects differently. This may explain why treatment with dopaminergic drugs has been reported to have variable effects on social play (Trezza *et al.*, 2010).

Considering noradrenergic involvement in social play behaviour, it is clear that enhanced noradrenaline levels result in a reduction in social play (Vanderschuren *et al.*, 2008; present study). The present study indicates that these effects are most likely related to a reduced motivation for social play behaviour, since operant responding for social play behaviour is reduced as well. In addition, increased noradrenaline levels do not interfere with the acquisition of social play induced CPP, suggesting that play is still perceived as pleasurable. Visual inspection of the data in figure 5B suggest that at the higher dose of

atomoxetine the magnitude of CPP is slightly reduced, which may be related to the large reduction in social play behaviour with this dose of atomoxetine (Vanderschuren *et al.*, 2008). These data suggest that increased endogenous noradrenaline levels mainly affect the motivation for social play behaviour, while leaving its hedonic aspects unaffected.

Concluding remarks

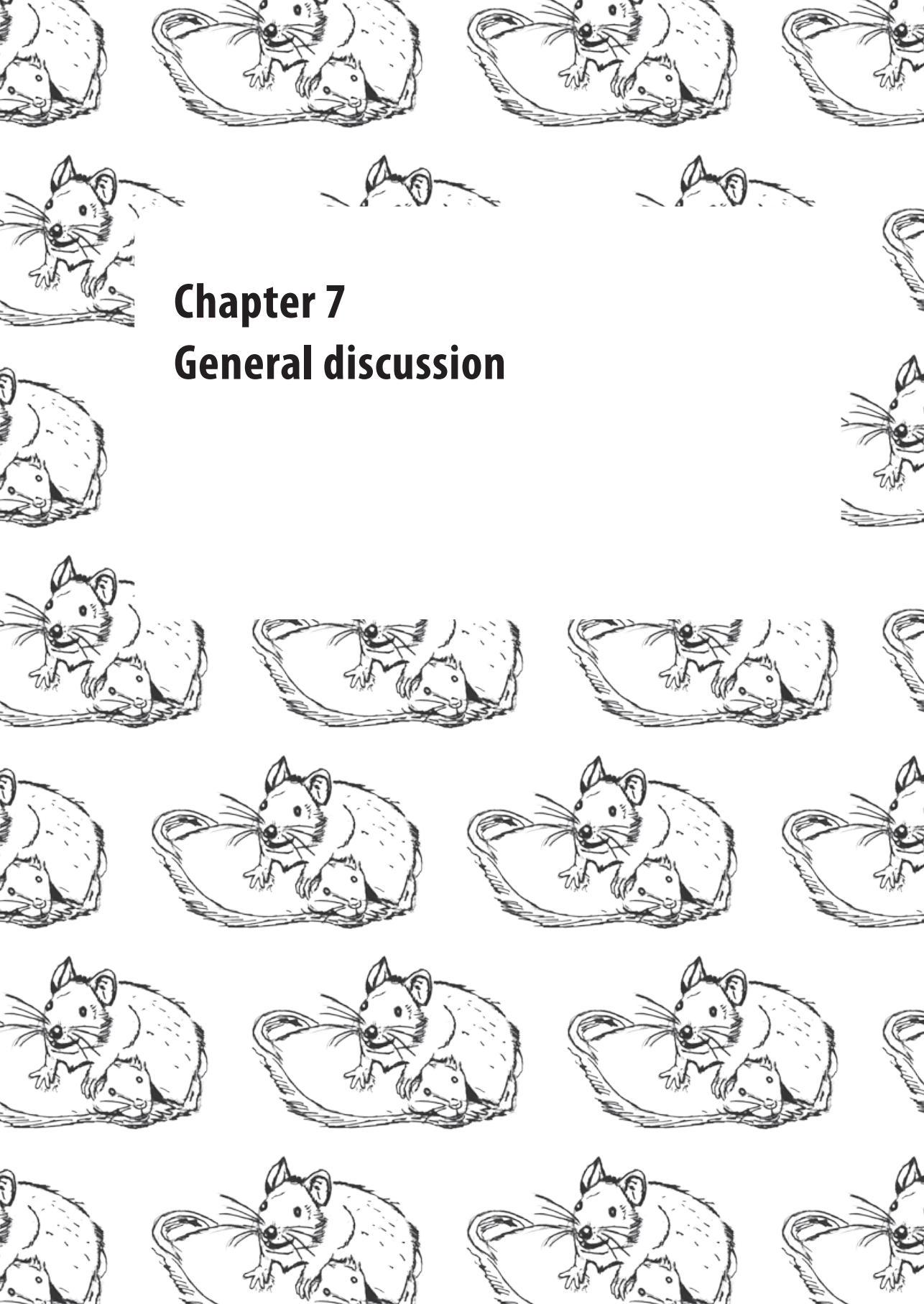
The present study shows that dopaminergic and noradrenergic signalling affect different aspects of social play behaviour. Increased operant responding for play and disruption of the acquisition of social play-induced CPP by are mediated via a dopaminergic mechanism. Increases in noradrenergic neurotransmission reduce the motivation for as well as the expression of social play behaviour, without affecting the acquisition of social play-induced CPP. These data provide new insights into the intricate mechanisms by which catecholamines modulate social play behaviour in rats. Elucidating the neural underpinnings of social behaviour in the young may increase our understanding of normal, adaptive social development, as well as in the pathophysiology of childhood and adolescent psychiatric disorders characterized by aberrant social behaviour.

References

- Achterberg EJ, Trezza V, Vanderschuren LJ (2012) beta-Adrenoreceptor stimulation mediates reconsolidation of social reward-related memories. PLoS One 7:e39639.
- Baarendse PJJ, Counotte DS, O'Donnel P, Vanderschuren LJMJ (2012a) Social experience during adolescence is critical for the development of cognitive control and dopamine modulation of prefrontal cortex function. Submitted.
- Baarendse PJJ, Vanderschuren LJMJ (2012) Dissociable effects of monoamine reuptake inhibitors on distinct forms of impulsive behavior in rats. Psychopharmacology (Berl) 219:313-326.
- Baarendse PJJ, Winstanley CA, Vanderschuren LJMJ (2012b) Simultaneous blockade of dopamine and noradrenaline reuptake promotes disadvantageous decision making in a rat gambling task. Psychopharmacology (Berl).
- Baenninger LP (1967) Comparison of behavioural development in socially isolated and grouped rats. Anim Behav 15:312-323.
- Baldo BA, Kelley AE (2007) Discrete neurochemical coding of distinguishable motivational processes: insights from nucleus accumbens control of feeding. Psychopharmacology (Berl) 191:439-459.
- Barbano MF, Cador M (2007) Opioids for hedonic experience and dopamine to get ready for it. Psychopharmacology (Berl) 191:497-506.
- Beatty WW, Dodge AM, Dodge LJ, White K, Panksepp J (1982) Psychomotor stimulants, social deprivation and play in juvenile rats. Pharmacol Biochem Behav 16:417-422.
- Berridge KC (2007) The debate over dopamine's role in reward: the case for incentive salience. Psychopharmacology (Berl) 191:391-431.
- Berridge KC, Robinson TE, Aldridge JW (2009) Dissecting components of reward: 'liking', 'wanting', and learning. Curr Opin Pharmacol 9:65-73.
- Dickinson A, Smith J, Mirenowicz J (2000) Dissociation of Pavlovian and instrumental incentive learning under dopamine antagonists. Behav Neurosci 114:468-483.
- Ferris RM, Tang FL (1979) Comparison of the effects of the isomers of amphetamine, methylphenidate and deoxypipradrol on the uptake of l-[3H]norepinephrine and [3H]dopamine by synaptic vesicles from rat whole brain, striatum and hypothalamus. J Pharmacol Exp Ther 210:422-428.
- Hanlon EC, Baldo BA, Sadeghian K, Kelley AE (2004) Increases in food intake or food-seeking behavior induced by GABAergic, opioid, or dopaminergic stimulation of the nucleus accumbens: is it hunger? Psychopharmacology (Berl) 172:241-247.
- Kelley AE (2004) Ventral striatal control of appetitive motivation: role in ingestive behavior and reward-related learning. Neurosci Biobehav Rev 27:765-776.
- Meaney MJ, Stewart J (1981) A descriptive study of social development in the rat (Rattus norvegicus). Anim Behav 29:34-35.
- Niesink RJM, Van Ree JM (1989) Involvement of opioid and dopaminergic systems in isolation-induced pinning and social grooming of young rats. Neuropharmacology 28:411-418.
- Normansell L, Panksepp J (1985) Effects of clonidine and yohimbine on the social play of juvenile rats. Pharmacol Biochem Behav 22:881-883.
- Panksepp J (1981) The ontogeny of play in rats. Dev Psychobiol 14:327-332.
- Panksepp J, Siviy SM, Normansell L (1984) The psychobiology of play: theoretical and methodological perspectives. Neurosci Biobehav Rev 8:465-492.
- Pellis SM, Pellis VC (1990) Differential rates of attack, defense, and counterattack during the developmental decrease in play fighting by male and female rats. Dev Psychobiol 23:215-231.

- Pellis SM, Pellis VC (1998) Play fighting of rats in comparative perspective: a schema for neurobehavioral analyses. Neurosci Biobehav Rev 23:87-101.
- Pellis SM, Pellis VC (2009) The Playful Brain. OneWorld Publications.
- Potegal M, Einon D (1989) Aggressive behaviors in adult rats deprived of playfighting experience as juveniles. Dev Psychobiol 22:159-172.
- Richardson NR, Roberts DC (1996) Progressive ratio schedules in drug self-administration studies in rats: a method to evaluate reinforcing efficacy. J Neurosci Methods 66:1-11.
- Ritz MC, Lamb RJ, Goldberg SR, Kuhar MJ (1987) Cocaine receptors on dopamine transporters are related to self-administration of cocaine. Science 237:1219-1223.
- Robbins TW, Everitt BJ (2007) A role for mesencephalic dopamine in activation: commentary on Berridge (2006). Psychopharmacology (Berl) 191:433-437.
- Salamone JD, Correa M (2012) The mysterious motivational functions of mesolimbic dopamine. Neuron 76:470-485.
- Siviy SM, Baliko CN (2000) A further characterization of alpha-2 adrenoceptor involvement in the rough-and-tumble play of juvenile rats. Dev Psychobiol 37:25-34.
- Siviy SM, Fleischhauer AE, Kerrigan LA, Kuhlman SJ (1996) D2 dopamine receptor involvement in the rough-and-tumble play behavior of juvenile rats. Behav Neurosci 110:1168-1176.
- Siviy SM, Fleischhauer AE, Kuhlman SJ, Atrens DM (1994) Effects of alpha-2 adrenoceptor antagonists on rough-and-tumble play in juvenile rats: evidence for a site of action independent of non-adrenoceptor imidazoline binding sites. Psychopharmacology (Berl) 113:493-499.
- Siviy SM, Panksepp J (2011) In search of the neurobiological substrates for social playfulness in mammalian brains. Neurosci Biobehav Rev 35:1821-1830.
- Spear LP (2000) The adolescent brain and age-related behavioral manifestations. Neurosci Biobehav Rev 24:417-463.
- Trezza V, Baarendse PJJ, Vanderschuren LJMJ (2009a) Prosocial effects of nicotine and ethanol in adolescent rats through partially dissociable neurobehavioral mechanisms. Neuropsychopharmacology 34:2560-2573.
- Trezza V, Baarendse PJJ, Vanderschuren LJMJ (2010) The pleasures of play: pharmacological insights into social reward mechanisms. Trends Pharmacol Sci 31:463-469.
- Trezza V, Campolongo P, Vanderschuren LJMJ (2011a) Evaluating the rewarding nature of social interactions in laboratory animals. Dev Cogn Neurosci 1:444-458.
- Trezza V, Damsteegt R, Achterberg EJM, Vanderschuren LJMJ (2011b) Nucleus accumbens mu-opioid receptors mediate social reward. J Neurosci 31:6362-6370.
- Trezza V, Damsteegt R, Vanderschuren LJMJ (2009b) Conditioned place preference induced by social play behavior: parametrics, extinction, reinstatement and disruption by methylphenidate. Eur Neuropsychopharmacol 19:659-669.
- Trezza V, Vanderschuren LJMJ (2008) Bidirectional cannabinoid modulation of social behavior in adolescent rats. Psychopharmacology (Berl) 197:217-227.
- Tzschentke TM (2007) Measuring reward with the conditioned place preference (CPP) paradigm: update of the last decade. Addict Biol 12:227-462.
- Van den Berg CL, Hol T, Van Ree JM, Spruijt BM, Everts H, Koolhaas JM (1999) Play is indispensable for an adequate development of coping with social challenges in the rat. Dev Psychobiol 34:129-138.
- Vanderschuren LJMJ (2010) How the brain makes play fun. Am J of Play 2:315-337.

• Vanderschuren LJMJ, Niesink RJM, Spruijt BM, Van Ree JM (1995) Mu- and kappa-opioid receptor-mediated opioid effects on social play in juvenile rats. Eur J Pharmacol 276:257-266.
• Vanderschuren LJMJ, Niesink RJM, Van Ree JM (1997) The neurobiology of social play behavior in rats. Neurosci Biobehav Rev 21:309-326.
• Vanderschuren LJMJ, Trezza V, Griffioen-Roose S, Schiepers OJG, Van Leeuwen N, De Vries TJ, Schoffelmeer ANM (2008) Methylphenidate disrupts social play behavior in adolescent rats. Neuropsychopharmacology 33:2946-2956.
• Veeneman MMJ, Boleij H, Broekhoven MH, Snoeren EMS, Guitart MM, Cousijn J, Spooren W, Vanderschuren LJMJ (2011) Dissociable roles of mGlu5 and dopamine receptors in the rewarding and sensitizing properties of morphine and cocaine. Psychopharmacology 214:863-876.
• Wassum KM, Ostlund SB, Balleine BW, Maidment NT (2011) Differential dependence of Pavlovian incentive motivation and instrumental incentive learning processes on dopamine signaling. Learn Mem 18:475-483.
• Wyvell CL, Berridge KC (2000) Intra-accumbens amphetamine increases the conditioned incentive salience of sucrose reward: enhancement of reward "wanting" without enhanced "liking" or response reinforcement. J Neurosci 20:8122-8130.
• Zhang M, Balmadrid C, Kelley AE (2003) Nucleus accumbens opioid, GABaergic, and dopaminergic modulation of palatable food motivation: contrasting effects revealed by a progressive ratio study in the rat. Behav Neurosci 117:202-211.



Chapter 7
General discussion

Positive social interactions during the juvenile and adolescent phases of life are essential for proper social and cognitive development in mammals, including humans. Social play behaviour is thought to facilitate behavioural development and provide the individual with the social and cognitive capacities required for adaptive and flexible behaviour (Baarendse *et al.*, 2012; Gerall *et al.*, 1967; Panksepp *et al.*, 1984; Pellis and Pellis, 2009; Spinka *et al.*, 2001; Van den Berg *et al.*, 1999; Vanderschuren *et al.*, 1997; Von Frijtag *et al.*, 2002; Wongwitdecha and Marsden, 1996). Abnormalities in play behaviour have been observed in childhood psychiatric disorders such as autism and attention deficit/hyperactivity disorder (Alessandri, 1992; Jordan, 2003; Manning and Wainwright, 2010). In view of its importance for behavioural development, and its relevance for child and adolescent psychiatry, it is essential to identify the neural substrates underlying social play behaviour. In this thesis, we aimed to further elucidate the neurobiology of social play behaviour.

Clearly, the expression of a complex behaviour such as social play involves a wide array of neural circuits. These include circuits involved in processing of sensory information, emotional and cognitive processes and the modulation of voluntary movement. To determine the key brain regions involved during social play behaviour, neuronal activity was mapped using the immediate early gene c-fos as a marker (chapter 2). A detailed anatomical analysis method was used to determine the involvement of a substantial number of brain regions and subregions. In line with previous immediate early gene expression studies (Cheng *et al.*, 2008; Gordon *et al.*, 2002), neuronal activity was observed in cortical regions, striatum, and amygdala. In addition, we demonstrate subregional differences in activation patterns within these structures. Furthermore, we found social play-induced increases in neuronal activity in brain regions that were not detected in previous immediate early gene expression studies, such as the thalamus, pedunculopontine tegmental nucleus, dorsal raphe nucleus, and habenula. Based on correlations in c-fos activity between regions with known anatomical connections, we identified a potential 'play network' of communicating neural structures. Since these correlations in c-fos expression were highly specific for social play, these data indicate that during play behaviour a network of brain regions is activated.

In the subsequent chapters we further specified the involvement of corticostriatal systems and the habenula in social play behaviour (chapter 3 and 4). Furthermore, we investigated the site of action through which methylphenidate influences social play behaviour (chapter 5), and investigated the role of dopaminergic and noradrenergic neurotransmission in the rewarding and motivational properties of social play (chapter 6). These studies provide new insights into the neurobiology of social play behaviour that will be discussed in the next sections.

Cortical areas

The results described in chapter 2 of this thesis demonstrate that neuronal activity is enhanced in dorsal medial prefrontal regions and medial orbitofrontal regions during social play behaviour. These data suggest that prefrontal subregions are differently involved in social play behaviour. The follow-up study described in chapter 3 showed that temporary inactivation of the prelimbic cortex, infralimbic cortex, and medial/ventral orbitofrontal cortex, using local administration of GABA receptor agonists, resulted in

marked decreases in social play behaviour. Inactivation of these three regions affected both play initiations and responses to play initiations (i.e. pouncing and pinning).

No previous study has used a temporary inactivation technique to probe the role of cortical regions in social play, but several studies have investigated the effect of neonatal prefrontal cortex lesions (Bell *et al.*, 2009; Panksepp *et al.*, 1994; Pellis *et al.*, 1992; Pellis *et al.*, 2006; Schneider and Koch, 2005) . In contrast to our study, these lesion studies reported only minor alterations in social play behaviour: lesions of several cortical regions mainly affected the frequency of pinning, while the number of play initiations was not altered (Bell *et al.*, 2009; Panksepp *et al.*, 1994; Pellis *et al.*, 1992; Pellis *et al.*, 2006; Schneider and Koch, 2005). The discrepancies between the earlier studies and those described in this thesis suggest that after neonatal cortical lesions, other regions compensate for the loss of function. The results described in this thesis clearly show that disruption of normal prefrontal signalling markedly interferes with the execution of social play behaviour.

Previously, Bell *et al.* (2010) have observed differences in the development of the medial prefrontal cortex and the orbitofrontal cortex as a consequence of social experience (Bell *et al.*, 2010). The neuronal morphology of the orbitofrontal cortex was much more complex in rats raised with multiple peers compared to rats raised with a single peer. In contrast, neuronal morphology of the orbitofrontal cortex was not changed in rats that were raised with multiple adult rats compared to rats raised with multiple peers, suggesting that the orbitofrontal cortex is mainly sensitive to the number of social partners. In contrast, neuronal morphology of the medial prefrontal cortex was a function of the presence of play. The presence of a single or multiple peers resulted in less complex morphology of the medial prefrontal cortex compared to rats raised with multiple adults. It was hypothesised that social play improves the maturation of the medial prefrontal cortex by enhancing pruning (Bell *et al.*, 2010). Consistent with this notion, recent work from our laboratory has shown that social isolation during the juvenile/early adolescent period leads to a long-lasting change in the physiological properties of medial prefrontal pyramidal neurons, as well as impairments in the performance of cognitive tasks (i.e., the 5-choice serial reaction time task and the rat gambling task) that depend on prefrontal mechanisms (Baarendse *et al.*, 2012).

Both human and animal studies have implicated the medial and orbitofrontal cortex in a variety of complex behaviours, including social behaviour, learning, and decision-making (Rudebeck *et al.*, 2008). Manipulations of the orbitofrontal cortex have been reported to enhance aggressive behaviour (de Bruin *et al.*, 1983; Rudebeck *et al.*, 2007), alter outcome expectancies (Schoenbaum *et al.*, 2011), and the orbitofrontal cortex appears to be involved in the evaluation of social information (Azzi *et al.*, 2012; Bell *et al.*, 2010; Pellis *et al.*, 2006). On the basis of these findings, it can be hypothesized that during social play behaviour the orbitofrontal cortex is required for processing information related to the partner and its behaviour, such as the value of the social interaction, and selection of the appropriate behavioural responses in response to the partner.

The medial prefrontal cortex has been reported to be involved in maternal behaviour (Pereira and Morrell, 2011), social hierarchy (Wang *et al.*, 2011) and social comparisons in decision-making (Bault *et al.*, 2011). Possibly, during social play behaviour the medial prefrontal cortex serves to process information on how well the play acts are performed in relation to behaviour of the partner. In this way, the animal's social position in relation

to its partner can be evaluated. It is known that altered medial prefrontal functioning re-establishes social hierarchies (Wang *et al.*, 2011) and lesions of the medial prefrontal cortex disrupt the age-related changes in play response, which are related to the establishment of a social hierarchy (Bell *et al.*, 2009).

In conclusion, the results presented in this thesis, together with previous studies, provide evidence for a functional differentiation within the prefrontal cortex in relation to social play behaviour. In addition, the results in this thesis indicate that the prefrontal cortex regions have an essential role during the execution of social play behaviour, which stands in apparent contrast to the results from previous neonatal lesion studies. Interference with on-going neuronal signalling, by temporary pharmacological inactivation, disrupts the execution of social play behaviour, indicating that during social play behaviour signalling processes in these regions are related to the expression of meaningful behavioural components of social play.

Striatum

The results described in chapter 2 of this thesis show that during social play behaviour neuronal activity is enhanced in dorsal and ventral subregions of the striatum. Activation of the striatum during social play behaviour is in line with a previous immediate early gene expression study (Gordon *et al.*, 2002). Similar to the prefrontal cortex, induction of c-fos activity was heterogeneous within the striatum. The most profound increase was observed in the dorsolateral striatum and this increase gradually declined towards the ventromedial direction. Furthermore, pharmacological manipulations indicated that glutamatergic inputs into the dorsomedial striatum inhibit social play behaviour, whereas inactivation of the nucleus accumbens core did not affect social play behaviour (chapter 3).

The c-fos activity pattern observed within the striatum correlated with the c-fos activity in the medial prefrontal regions providing input to the striatum, suggesting an involvement of corticostriatal projections in social play (chapter 2). Therefore, involvement of glutamatergic inputs into the dorsomedial striatum and the nucleus accumbens core, which receive projections from the prelimbic cortex, was investigated by local administration of the AMPA receptor antagonist DNQX (chapter 3). Administration of DNQX into the dorsomedial striatum, but not the nucleus accumbens core, facilitated social play behaviour. Intriguingly, as mentioned above, inhibition of prelimbic output by local administration of GABA receptor agonists markedly reduced the expression of social play behaviour, which is in contrast with the effects of DNQX infusions into the dorsomedial striatum. This does not necessarily imply that glutamatergic prelimbic-dorsomedial projections are not involved in social play. For example, the output regions of the prelimbic cortex mediating the reduction in play after pharmacological inactivation might be non-striatal regions. Thus, an involvement of prelimbic-dorsomedial projections might be overshadowed by the inhibiting effect of other prelimbic output regions.

Previous studies have established the involvement of the dorsomedial striatum in response selection and inhibition, by showing that animals lacking a functional dorsomedial striatum display various forms of disinhibited behaviour (Corbit and Janak, 2007; Devan *et al.*, 1999; Eagle and Robbins, 2003; Rogers *et al.*, 2001). Interestingly, the projection from the mPFC to the dorsomedial striatum has previously been implicated in

attention processes (Christakou *et al.*, 2001). This could mean that reducing certain forms of selective attention facilitates play behaviour, since during a social interaction animals need to continuously switch between behavioural patterns in relation to the partner's action and other environmental cues. Possibly, the expression of vigorous social play behaviour can be kept in control through dorsomedial striatal behavioural inhibition mechanisms that are at least partially regulated by medial prefrontal inputs.

Enhancement of c-fos activity by social play behaviour was also observed in the ventral striatum, with the most profound increases in the lateral nucleus accumbens core and medial nucleus accumbens shell (chapter 2). Pharmacological inactivation of these regions did not confirm a critical involvement of the nucleus accumbens core, while the role of the nucleus accumbens shell in social play behaviour could not be established because of non-specific pharmacological effects on eating behaviour (chapter 3). The nucleus accumbens has been previously implicated in social play behaviour (Gordon *et al.*, 2002; Trezza *et al.*, 2011; Trezza *et al.*, 2012), which is most likely related to the role of the nucleus accumbens in reward-related behaviours (Berridge and Kringelbach, 2008; Cardinal *et al.*, 2002; Zahm, 1999). For example, the nucleus accumbens is a critical region for the positive modulation of social play behaviour by μ-opioid receptors and it also has a role in the play-facilitating effects of endocannabinoids (Trezza *et al.*, 2011; Trezza *et al.*, 2012). Together, these results indicate that mechanisms within the nucleus accumbens modulate the expression of social play behaviour, but that functional activity within accumbens subregions is not critical for its execution. This indicates that if functional activity from the nucleus accumbens core is inhibited, social play is likely mediated by other regions, such as the accumbens shell. If, however, activity of a critical neurotransmitter system, such as opioids or cannabinoids, is altered, then nucleus accumbens output is changed in such a way that social play behaviour is affected (Trezza *et al.*, 2011; -2012).

In conclusion, striatal subregions are differently involved in social play behaviour. The dorsolateral striatum is most likely involved in processing sensory and motor information, the dorsomedial striatum exerts an inhibitory influence over social play behaviour, whereas the nucleus accumbens core is a modulator of the rewarding properties of social play behaviour. However, there is likely to be functional redundancy between nucleus accumbens subregions in the execution of social play.

Habenula

The results described in this thesis identify the habenula as a key player during social play behaviour (chapter 4). Our results indicate that c-fos gene expression is induced in the habenula after social isolation for 24 h and this increase in activity can be reduced by social play behaviour. Furthermore, we demonstrate that proper habenula signalling is needed for social play, since pharmacological inactivation of the habenula markedly reduced the expression of social play behaviour.

Previous studies have shown that the habenula is a key regulator of monoamine neurotransmission via direct and indirect projections towards the VTA, dorsal raphe nucleus, and locus coeruleus (Hikosaka, 2010; Hong *et al.*, 2011; Kalen *et al.*, 1989a; Kalen *et al.*, 1989b; Lecourtier *et al.*, 2008; Lecourtier and Kelly, 2007; Stern *et al.*, 1979). The monoamines are known modulators of social play behaviour (Beatty *et al.*, 1982;

Pellis *et al.*, 1993; Siviy *et al.*, 1996; -2011; Siviy and Panksepp, 2011; Trezza *et al.*, 2010; Vanderschuren *et al.*, 1997; -2008). Therefore, we postulated that the disruption of social play behaviour by manipulations of the habenula is mediated via alterations in monoaminergic signalling (chapter 4).

Temporary inactivation of the habenula, by administration of an AMPA receptor antagonist, increased dopamine release in the prefrontal cortex, dorsal striatum, and nucleus accumbens (Lecourtier *et al.*, 2008). Dopamine agonists and antagonists have both been reported to reduce social play behaviour, whereas enhancing endogenous dopamine levels by administration of a selective dopamine reuptake inhibitor did not alter social play behaviour (Siviy *et al.*, 1996; Trezza *et al.*, 2010; Vanderschuren *et al.*, 2008). Possibly, inactivation of the habenula interferes with the dopamine levels normally present during social play behaviour and thereby disrupts social play behaviour.

Lesions of the habenula have also been reported to enhance serotonin turnover in the dorsal raphe nucleus (Yang *et al.*, 2008). Interestingly, increases in serotonin levels have been reported to be incompatible with social play behaviour. Enhancement of endogenous serotonin levels by constitutive absence of the serotonin transporter, administration of a selective serotonin reuptake inhibitor, as well as administration of a serotonin releasing agent have been reported to reduce social play behaviour (Homberg *et al.*, 2007). Therefore, inactivation of the habenula might have decreased social play behaviour as a result of enhanced serotonin levels.

Since stimulation of the habenula has been shown to increase noradrenaline levels in the hippocampus, prefrontal cortex, striatum, and nucleus accumbens (Cenci *et al.*, 1992; Kalen *et al.*, 1989a), the levels of noradrenaline might be decreased after inactivation. However, this has so far not been pertinently tested. Administration of a selective noradrenaline reuptake inhibitor, which increases endogenous noradrenaline levels, has been reported to reduce social play behaviour (Vanderschuren *et al.*, 2008). In the present study, inactivation of the habenula, possibly resulting in decreased noradrenaline levels, reduced social play behaviour. At first glance, this suggests that the reduction in play after habenula inactivation is not related to altered noradrenaline levels, although it is important to keep in mind that reductions in play have been observed with some types of noradrenaline receptor antagonists (Beatty *et al.*, 1984; Trezza *et al.*, 2010).

In conclusion, the habenula appears to have an important role in social play behaviour and social isolation. The observed effects most likely involve one or several of the monoamines. Interestingly, the results described in chapter 5 indicate that the reduction of social play behaviour by methylphenidate is at least partially mediated through the habenula. These data indicate that dopamine and/ or noradrenaline are able to modulate habenula function, which in turn affects social play behaviour. Possibly, the habenula acts as an integrator of several information processes and provides feedback via modulation of monoamine neurotransmission. If proper signalling in this region is disrupted, the expression of a complex behaviour such as social play behaviour is compromised.

Methylphenidate

In chapter 5 we investigated the site of action through which methylphenidate influenced social play behaviour. Methylphenidate is widely used for the treatment of attention deficit/hyperactivity disorder (Kutcher *et al.*, 2004), a disorder highly prevalent

during childhood and adolescence. Understanding the mechanism through which methylphenidate influences social play behaviour may therefore be of great clinical relevance.

Methylphenidate's site of action was investigated by local administration into several candidate regions: prelimbic cortex, medial/ventral orbitofrontal cortex, ventrolateral orbitofrontal cortex, nucleus accumbens shell, habenula, and amygdala. These regions were selected based on their function in play behaviour and known noradrenergic innervations (for detailed description per region see chapter 5) The results of this study indicate that the habenula and amygdala both mediate the effect of methylphenidate, since local administration of methylphenidate into these regions reduced the expression of social play behaviour, whereas administration into the other regions had no effect (chapter 5). These results indicate that enhanced dopamine and/or noradrenaline levels in the habenula and amygdala cause a reduction in social play behaviour. Previously, it has been demonstrated that the effect of methylphenidate on social play behaviour is mediated via the α2-adrenoceptor (Vanderschuren *et al.*, 2008). Therefore, it is likely that enhancement of noradrenaline levels in the habenula and amygdala is causing a reduction in social play behaviour.

The behavioural effects of methylphenidate have previously been ascribed to prefrontal and striatal regions in humans (Rubia *et al.*, 2009; -2011; Tomasi *et al.*, 2011) and rodents (Claussen *et al.*, 2012; Lee *et al.*, 2008; Spencer *et al.*, 2012). In addition, methylphenidate is known to enhance behavioural inhibition in rats (Eagle *et al.*, 2007) and humans (Aron *et al.*, 2003; Tannock *et al.*, 1989) and previous studies have indicated that mainly the prelimbic cortex, orbitofrontal cortex and nucleus accumbens shell are important for behavioural inhibition (Bari *et al.*, 2011; Dalley *et al.*, 2008; Eagle and Baunez, 2010; Economidou *et al.*, 2012). Thus, it was surprising that in the present study these regions appeared not to be involved in the effect of methylphenidate on social play behaviour.

Given the involvement of both the habenula and amygdala, a common functional pathway may be involved in the regulation of social play behaviour by methylphenidate. The habenula and amygdala share several target regions, which are interesting candidates in relation to play behaviour. For example, common targets include the thalamus, rostromedial tegmental nucleus, and VTA. These regions have all been associated with social play behaviour (Siviy and Panksepp, 1985; Siviy and Panksepp, 1987; Van Kerkhof *et al.*, 2012, submitted (chapter 2). Interestingly, lesions of the habenula or RMTg both result in hyperactivity and increased impulsivity (Jhou *et al.*, 2009; Lecourtier and Kelly, 2005; Murphy *et al.*, 1996), suggesting that activity in the habenula via the RMTg results in inhibiting locomotor responses as well. In addition, the habenula-amygdala-VTA network has previously been shown to be a functional network associated with error monitoring in humans (Ide and Li, 2011). Investigating the mechanism via which methylphenidate influences habenula and amygdala function to inhibit social play behaviour is an interesting topic for further studies.

Dopamine and noradrenaline

In chapter 6 we investigated how pleasurable and motivational aspects of social play behaviour are modulated by dopamine and noradrenaline, using methylphenidate, a selective dopamine reuptake inhibitor (GBR-12909), and a selective noradrenaline

reuptake inhibitor (atomoxetine), in two pertinent setups. These were the social play-induced conditioned place preference (CPP) task and an operant conditioning task for social interaction. The results described in this chapter indicate that enhancement of endogenous dopamine levels disrupted the acquisition of CPP and increased operant responding for social play behaviour, while the expression of social play behaviour was not altered. In contrast, enhancement of endogenous noradrenaline levels reduced operant responding for social play behaviour and reduced the expression of social play behaviour, while it did not affect the acquisition of social play-induced CPP. Interestingly, administration of methylphenidate (enhancing both dopamine and noradrenaline levels), disrupted the acquisition of CPP, enhanced operant responding and reduced the expression of social play behaviour. Thus, methylphenidate influences the acquisition of CPP and operant responding via a dopaminergic mechanism, while it affects the expression of social play behaviour via a noradrenergic mechanism. This study indicates that dopamine and noradrenaline affect different aspects of social play behaviour in a distinct manner, possibly via different neural substrates.

Enhancement of endogenous dopamine levels has previously been reported to enhance the motivation for rewards, without affecting reward consumption (for reviews see: Baldo and Kelley, 2007; Barbano and Cador, 2007; Berridge, 2007; Salamone and Correa, 2012). Motivation can be dissociated into directional (i.e., behaviour being directed towards or away from certain stimuli) and activational (i.e. the invigoration of behaviour directed at rewards, in terms of speed, persistence, and work output) components (see Salamone and Correa, 2012). Dopamine has been particularly implicated in the latter, although the data in chapter 6 do not allow for a strict distinction between the two. The disruption of CPP acquisition by a selective dopamine reuptake inhibitor suggests that enhancement of endogenous dopamine levels alters the hedonic value of social play behaviour, similar to methylphenidate treatment. However, enhancement of endogenous dopamine levels alone is not sufficient to affect the expression of social play behaviour (Vanderschuren *et al.*, 2008). In summary, dopamine clearly affects different aspects of social play behaviour, although for some of these aspects in opposite directions, making it difficult to postulate one single function of dopamine in social play behaviour. However, these results do emphasize that different aspects of social play behaviour might be regulated via different neurobiological mechanisms.

Enhancement of endogenous noradrenaline levels did not alter the acquisition of CPP, suggesting that this has no influence on the pleasurable aspects of social play behaviour. In contrast, it did reduce responding for social play behaviour in an operant task. The reduction in play behaviour observed after enhancement of endogenous noradrenaline levels, observed in chapter 6 and in previous studies (Beatty *et al.*, 1982; Vanderschuren *et al.*, 2008), might thus be related to a reduced motivation for social play behaviour. Interestingly, the results from chapter 5 demonstrate that the effect of methylphenidate on the expression of social play behaviour is mediated via the habenula and amygdala. This might suggest that proper noradrenaline function in the habenula and amygdala is related to motivational aspects of social play behaviour.

In summary, the results in chapter 6 indicate that dopamine and noradrenaline influence different aspects of social play behaviour. Enhancement of dopamine levels has an opposite effect on the pleasurable and motivational properties, while having no

effect on the expression of social play behaviour. Noradrenaline appears to mainly affect the motivational aspects of social play behaviour and thereby influence the expression of social play behaviour. Methylphenidate influences all three investigated aspects of social play behaviour via different mechanisms: a dopaminergic effect on the acquisition of social play-induced CPP and operant responding and a noradrenergic effect on the expression of social play behaviour. Interestingly, these results indicate that the different aspects of social play behaviour (e.g. pleasurable and motivational) are mediated via multiple neurobiological mechanisms.

Neurobiology of social play behaviour

The aim of this thesis was to further elucidate the neurobiology of social play behaviour. The studies described in this thesis confirmed the involvement of several candidate regions, such as the prefrontal cortex, striatum, thalamus and amygdala, and extend this with several regions such as the habenula, pedunculopontine nucleus, dorsal raphe nucleus, rostromedial tegmental nucleus, VTA, and substantia nigra. Together with previous studies, the results from this thesis can serve as to further delineate a potential 'play' network in the rat brain (Fig.1). The network illustrated here is not meant as a final overview, but rather combines the different systems described in this thesis and speculates about their potential functional connections. This 'play' network plausibly consists of several cortical and subcortical systems that work together to establish the appropriate behavioural components of social play behaviour. Sensory and motor-related information may be processed in the thalamus and relayed to several cortical regions and striatum. The prefrontal cortex might further process this information to establish information about the social context of the interaction, for example regarding the identity and rank of the play partner. The prefrontal cortex also receives additional information from other regions, such as the amygdala, about the emotional value of environmental cues. The information processed in the prefrontal cortex might then be relayed to structures such as the striatum. In turn, the striatum receives input from other modulatory neurotransmitter systems. Monoamines from the dorsal raphe, locus coeruleus and VTA influence signalling in these aforementioned structures (e.g. prefrontal cortex, striatum, and amygdala). The monoamine producing regions are in turn regulated by the habenula, rostromedial tegmental nucleus and pedunculopontine nucleus. The striatum may integrate these afferent information streams, resulting in processing of the rewarding and motivational value of the interaction and select the appropriate motor acts. As a result, this 'play' network establishes the proper behavioural acts in response to social and environmental cues to ensure that the appropriate behaviour is expressed considering the temporal and contextual setting, i.e. proper expression of social play behaviour.

Future directions

The 'play' network described above based on the results from this thesis and previous studies is a very useful start for understanding the neurobiology of social play behaviour. Further studies are required to fill in the caveats in our understanding of social play behaviour.

The present thesis demonstrated that corticostriatal systems are likely involved in

social play behaviour, although their exact role remains to be established. The strong correlations in c-fos activity between prefrontal and striatal target regions provide a good starting point. It will be highly informative to investigate via which pathway the marked reduction in social play behaviour that was observed after prefrontal cortex inactivation occurs. In addition, further studies investigating the origin of the glutamatergic projections that facilitate social play behaviour via the dorsomedial striatum will complete the play network a bit further.

Considering the involvement of the habenula in social play behaviour there are several issues that need to be clarified. For example, determining via which monoamine altered signalling in the habenula influences social play behaviour will be not only informative for understanding how the habenula influences play behaviour, but other behaviours as well. In addition, it is important to understand how monoamines (such as noradrenaline and/or dopamine) influence habenula function, since this will provide knowledge regarding potential methods to interfere with habenula signalling and thereby influence behaviour.

Lastly, in the present thesis we have further elucidated the site and mechanism of action by which methylphenidate modulates different aspects of social play behaviour. The dissociable effects observed via dopaminergic and noradrenergic neurotransmission indicated which aspects of social play behaviour are affected by methylphenidate. It is important for further studies to determine if modulation of behaviour via the habenula and amygdala is related to noradrenergic neurotransmission and to the motivational aspects of social play behaviour. In addition, since the habenula and amygdala were beforehand at first not the most likely candidates for methylphenidate to alter play, these findings stress the importance of both structures in adolescent social behaviour and may stimulate research into their role in the therapeutic and/or side effects of methylphenidate treatment in humans.

In summary, the studies described in this thesis advance our understanding of the neural underpinnings of social play behaviour. Using different methodological approaches, we discovered and elucidated the involvement of several key brain structures and neurotransmitter systems important for social play behaviour. Understanding these neural substrates hopefully benefits investigations into disorders related to adolescent social dysfunction.

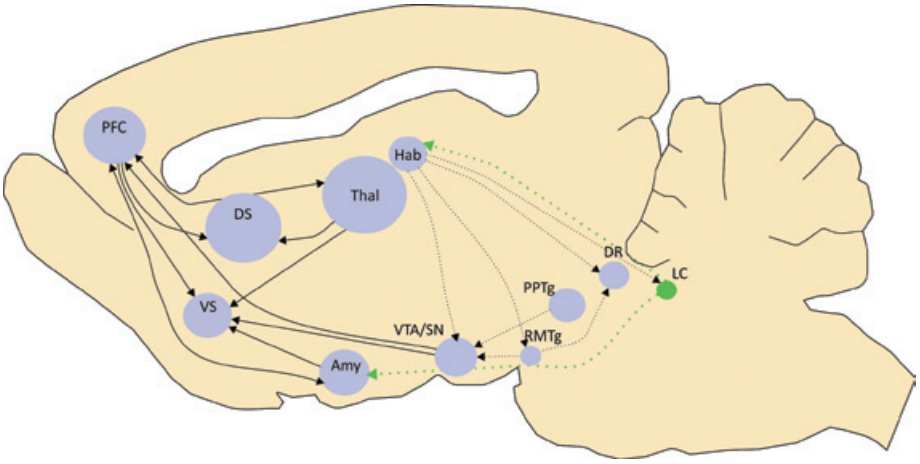


Fig. 1: The results described in this thesis provide important insight into the neural network underlying social play behaviour, i.e. a potential 'play' network. Key brain structures in this network are the prefrontal cortex, striatum, amygdala, habenula and thalamus. Activity in these regions is under control of several modulating neurotransmitters, such as the monoamines. Therefore, the monoamine producing regions (VTA/SN, DR, and LC) are essential brain structures for social play behaviour as well. In turn, these regions are regulated by the habenula, RMTg, and PPTg. Together, these brain structures comprise a network that regulates the expression of all behavioural acts in an appropriate sequential order and in the appropriate environmental context to establish the proper expression of social play behaviour. Black arrows indicate the involvement of projections identified in this thesis by the correlation analysis described in chapter 2. Black dotted arrows indicate projections possibly involved in play behaviour, since these are known anatomical projections connecting key brain regions identified in this thesis (chapter 2 and 4). Green dotted arrows indicate possible noradrenergic projections involved in play behaviour as suggested by the study into methylphenidate's site of action (chapter 5). Abbreviations: PFC = prefrontal cortex, DS = dorsal striatum, VS = ventral striatum, Hab = habenula, Thal = thalamus, Amy = amygdala, VTA/SN = ventral tegmental area and substantia nigra, RMTg = rostromedial tegmental nucleus, PPTg = pedunculopontine nucleus, DR = dorsal raphe nucleus, LC = locus coeruleus.

References

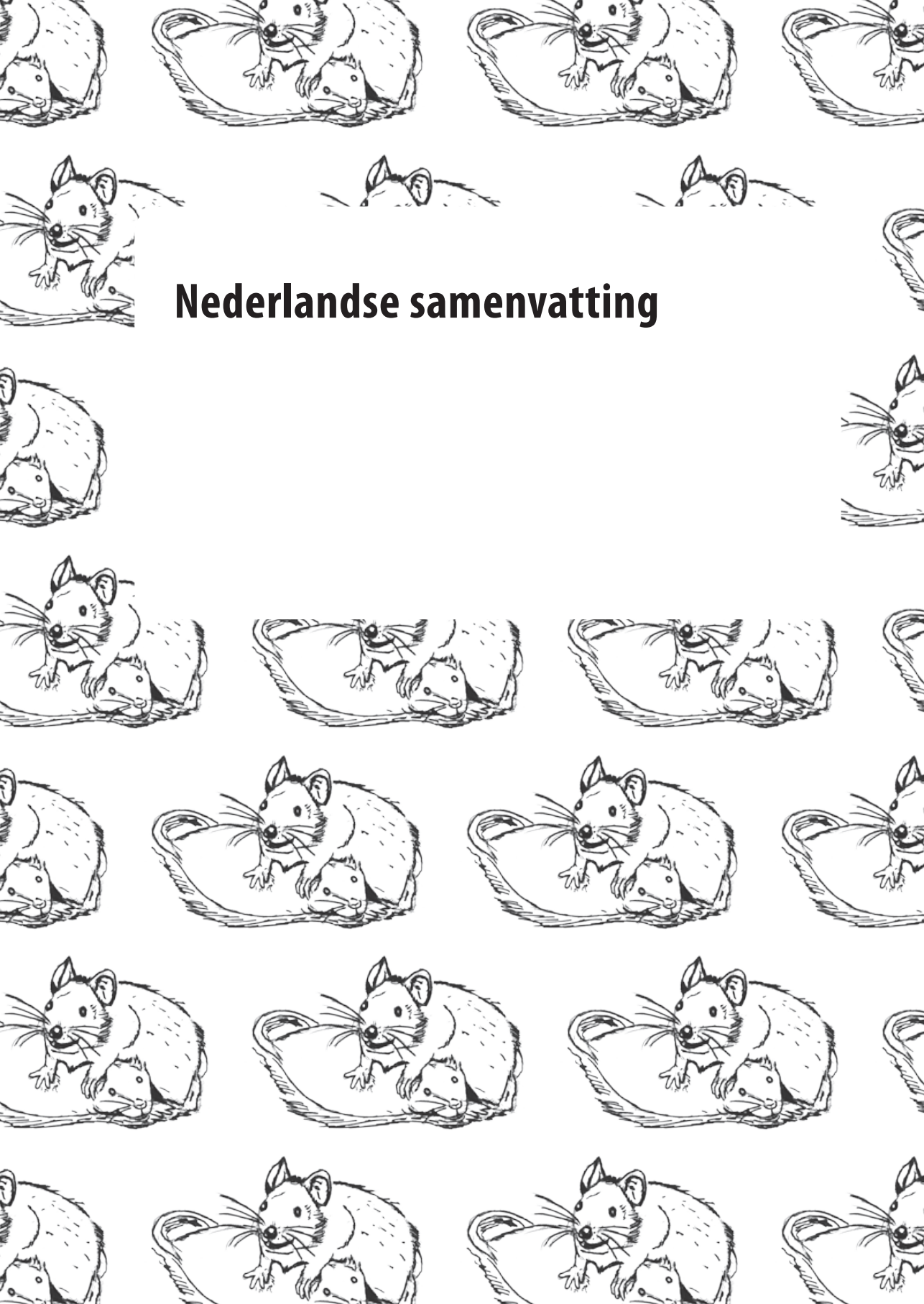
- Alessandri SM (1992) Attention, play, and social behavior in ADHD preschoolers. J Abnorm Child Psychol 20:289-302.
- Aron AR, Dowson JH, Sahakian BJ, Robbins TW (2003) Methylphenidate improves response inhibition in adults with attention-deficit/hyperactivity disorder. Biol Psychiatry 54:1465-1468.
- Azzi JC, Sirigu A, Duhamel JR (2012) Modulation of value representation by social context in the primate orbitofrontal cortex. Proc Natl Acad Sci U S A 109:2126-2131.
- Baarendse PJJ, Counotte DS, O'Donnel P, Vanderschuren LJMJ (2012) Social experience during adolescence is critical for the development of cognitive control and dopamine modulation of prefrontal cortex function. Submitted.
- Baldo BA, Kelley AE (2007) Discrete neurochemical coding of distinguishable motivational processes: insights from nucleus accumbens control of feeding. Psychopharmacology (Berl) 191:439-459.
- Barbano MF, Cador M (2007) Opioids for hedonic experience and dopamine to get ready for it. Psychopharmacology (Berl) 191:497-506.
- Bari A, Mar AC, Theobald DE, Elands SA, Oganya KC, Eagle DM, Robbins TW (2011) Prefrontal and monoaminergic contributions to stop-signal task performance in rats. J Neurosci 31:9254-9263.
- Bault N, Joffily M, Rustichini A, Coricelli G (2011) Medial prefrontal cortex and striatum mediate the influence of social comparison on the decision process. Proc Natl Acad Sci U S A 108:16044-16049.
- Beatty WW, Costello KB, Berry SL (1984) Suppression of play fighting by amphetamine: effects of catecholamine antagonists, agonists and synthesis inhibitors. Pharmacol Biochem Behav 20:747-755.
- Beatty WW, Dodge AM, Dodge LJ, White K, Panksepp J (1982) Psychomotor stimulants, social deprivation and play in juvenile rats. Pharmacol Biochem Behav 16:417-422.
- Bell HC, McCaffrey DR, Forgie ML, Kolb B, Pellis SM (2009) The role of the medial prefrontal cortex in the play fighting of rats. Behav Neurosci 123:1158-1168.
- Bell HC, Pellis SM, Kolb B (2010) Juvenile peer play experience and the development of the orbitofrontal and medial prefrontal cortices. Behav Brain Res 207:7-13.
- Berridge KC (2007) The debate over dopamine's role in reward: the case for incentive salience. Psychopharmacology (Berl) 191:391-431.
- Berridge KC, Kringelbach ML (2008) Affective neuroscience of pleasure: reward in humans and animals. Psychopharmacology (Berl) 199:457-480.
- Cardinal RN, Parkinson JA, Hall J, Everitt BJ (2002) Emotion and motivation: the role of the amygdala, ventral striatum, and prefrontal cortex. Neurosci Biobehav Rev 26:321-352.
- Cenci MA, Kalen P, Mandel RJ, Bjorklund A (1992) Regional differences in the regulation of dopamine and noradrenaline release in medial frontal cortex, nucleus accumbens and caudate-putamen: a microdialysis study in the rat. Brain Res 581:217-228.
- Cheng SY, Taravosh-Lahn K, Delville Y (2008) Neural circuitry of play fighting in golden hamsters. Neuroscience 156:247-256.
- Christakou A, Robbins TW, Everitt BJ (2001) Functional disconnection of a prefrontal cortical-dorsal striatal system disrupts choice reaction time performance: implications for attentional function. Behav Neurosci 115:812-825.
- Claussen CM, Chong SL, Dafny N (2012) Selective bilateral lesion to caudate nucleus modulates the acute and chronic methylphenidate effects. Pharmacol Biochem Behav 101:208-216.
- Corbit LH, Janak PH (2007) Inactivation of the lateral but not medial dorsal striatum eliminates the excitatory impact of Pavlovian stimuli on instrumental responding. J Neurosci 27:13977-13981.

- Dalley JW, Mar AC, Economidou D, Robbins TW (2008) Neurobehavioral mechanisms of impulsivity: fronto-striatal systems and functional neurochemistry. Pharmacol Biochem Behav 90:250-260.
- de Bruin JP, van Oyen HG, Van de Poll N (1983) Behavioural changes following lesions of the orbital prefrontal cortex in male rats. Behav Brain Res 10:209-232.
- Devan BD, McDonald RJ, White NM (1999) Effects of medial and lateral caudate-putamen lesions on place- and cue-guided behaviors in the water maze: relation to thigmotaxis. Behav Brain Res 100:5-14.
- Eagle DM, Baunez C (2010) Is there an inhibitory-response-control system in the rat? Evidence from anatomical and pharmacological studies of behavioral inhibition. Neurosci Biobehav Rev 34:50-72.
- Eagle DM, Robbins TW (2003) Inhibitory control in rats performing a stop-signal reaction-time task: effects of lesions of the medial striatum and d-amphetamine. Behav Neurosci 117:1302-1317.
- Eagle DM, Tufft MR, Goodchild HL, Robbins TW (2007) Differential effects of modafinil and methylphenidate on stop-signal reaction time task performance in the rat, and interactions with the dopamine receptor antagonist cis-flupenthixol. Psychopharmacology (Berl) 192:193-206.
- Economidou D, Theobald DE, Robbins TW, Everitt BJ, Dalley JW (2012) Norepinephrine and dopamine modulate impulsivity on the five-choice serial reaction time task through opponent actions in the shell and core sub-regions of the nucleus accumbens. Neuropsychopharmacology 37:2057-2066.
- Gerall HD, Ward IL, Gerall AA (1967) Disruption of the male rat's sexual behaviour induced by social isolation. Anim Behav 15:54-58.
- Gordon NS, Kollack-Walker S, Akil H, Panksepp J (2002) Expression of c-fos gene activation during rough and tumble play in juvenile rats. Brain Res Bull 57:651-659.
- Hikosaka O (2010) The habenula: from stress evasion to value-based decision-making. Nat Rev Neurosci 11:503-513.
- Homberg JR, Schiepers OJG, Schoffelmeer ANM, Cuppen E, Vanderschuren LJMJ (2007) Acute and constitutive increases in central serotonin levels reduce social play behaviour in peri-adolescent rats. Psychopharmacology (Berl) 195:175-182.
- Hong S, Jhou TC, Smith M, Saleem KS, Hikosaka O (2011) Negative reward signals from the lateral habenula to dopamine neurons are mediated by rostromedial tegmental nucleus in primates. J Neurosci 31:11457-11471.
- Jordan R (2003) Social play and autistic spectrum disorders: a perspective on theory, implications and educational approaches. Autism 7:347-360.
- Kalen P, Lindvall O, Bjorklund A (1989a) Electrical stimulation of the lateral habenula increases hippocampal noradrenaline release as monitored by in vivo microdialysis. Exp Brain Res 76:239-245.
- Kalen P, Strecker RE, Rosengren E, Bjorklund A (1989b) Regulation of striatal serotonin release by the lateral habenula-dorsal raphe pathway in the rat as demonstrated by in vivo microdialysis: role of excitatory amino acids and GABA. Brain Res 492:187-202.
- Kutcher S, Aman M, Brooks SJ, Buitelaar J, van Daalen E, Fegert J, Findling RL, Fisman S, Greenhill LL, Huss M, Kusumakar V, Pine D, Taylor E, Tyano S (2004) International consensus statement on attention-deficit/hyperactivity disorder (ADHD) and disruptive behaviour disorders (DBDs): clinical implications and treatment practice suggestions. Eur Neuropsychopharmacol 14:11-28.
- Lecourtier L, Defrancesco A, Moghaddam B (2008) Differential tonic influence of lateral habenula on prefrontal cortex and nucleus accumbens dopamine release. Eur J Neurosci 27:1755-1762.

- Lecourtier L, Kelly PH (2007) A conductor hidden in the orchestra? Role of the habenular complex in monoamine transmission and cognition. Neurosci Biobehav Rev 31:658-672.
- Lee MJ, Swann AC, Dafny N (2008) Methylphenidate sensitization is prevented by prefrontal cortex lesion. Brain Res Bull 76:131-140.
- Manning MM, Wainwright LD (2010) The role of high level play as a predictor social functioning in autism. J Autism Dev Disord 40:523-533.
- Panksepp J, Normansell L, Cox JF, Siviy SM (1994) Effects of neonatal decortication on the social play of juvenile rats. Physiol Behav 56:429-443.
- Panksepp J, Siviy SM, Normansell L (1984) The psychobiology of play: theoretical and methodological perspectives. Neurosci Biobehav Rev 8:465-492.
- Pellis SM, Castaneda E, McKenna MM, Tran-Nguyen LT, Whishaw IQ (1993) The role of the striatum in organizing sequences of play fighting in neonatally dopamine-depleted rats. Neurosci Lett 158:13-15.
- Pellis SM, Hastings E, Shimizu T, Kamitakahara H, Komorowska J, Forgie ML, Kolb B (2006) The effects of orbital frontal cortex damage on the modulation of defensive responses by rats in playful and nonplayful social contexts. Behav Neurosci 120:72-84.
- Pellis SM, Pellis VC (2009) The Playful Brain. OneWorld Publications.
- Pellis SM, Pellis VC, Whishaw IQ (1992) The role of the cortex in play fighting by rats: developmental and evolutionary implications. Brain Behav Evol 39:270-284.
- Pereira M, Morrell JI (2011) Functional mapping of the neural circuitry of rat maternal motivation: effects of site-specific transient neural inactivation. J Neuroendocrinol 23:1020-1035.
- Rogers RD, Baunez C, Everitt BJ, Robbins TW (2001) Lesions of the medial and lateral striatum in the rat produce differential deficits in attentional performance. Behav Neurosci 115:799-811.
- Rubia K, Halari R, Cubillo A, Mohammad AM, Brammer M, Taylor E (2009) Methylphenidate normalises activation and functional connectivity deficits in attention and motivation networks in medication-naive children with ADHD during a rewarded continuous performance task. Neuropharmacology 57:640-652.
- Rubia K, Halari R, Mohammad AM, Taylor E, Brammer M (2011) Methylphenidate normalizes frontocingulate underactivation during error processing in attention-deficit/hyperactivity disorder. Biol Psychiatry 70:255-262.
- Rudebeck PH, Bannerman DM, Rushworth MF (2008) The contribution of distinct subregions of the ventromedial frontal cortex to emotion, social behavior, and decision making. Cogn Affect Behav Neurosci 8:485-497.
- Rudebeck PH, Walton ME, Millette BH, Shirley E, Rushworth MF, Bannerman DM (2007) Distinct contributions of frontal areas to emotion and social behaviour in the rat. Eur J Neurosci 26:2315-2326.
- Salamone JD, Correa M (2012) The mysterious motivational functions of mesolimbic dopamine. Neuron 76:470-485.
- Schneider M, Koch M (2005) Deficient social and play behavior in juvenile and adult rats after neonatal cortical lesion: effects of chronic pubertal cannabinoid treatment. Neuropsychopharmacology 30:944-957.
- Schoenbaum G, Roesch MR, Stalnaker TK, Takahashi YK (2011) Orbitofrontal Cortex and Outcome Expectancies: Optimizing Behavior and Sensory Perception. In: Neurobiology of Sensation and Reward Boca Raton (FL): CRC Press.

- Siviy SM, Deron LM, Kasten CR (2011) Serotonin, motivation, and playfulness in the juvenile rat. Dev Cogn Neurosci 1:606-616.
- Siviy SM, Fleischhauer AE, Kerrigan LA, Kuhlman SJ (1996) D2 dopamine receptor involvement in the rough-and-tumble play behavior of juvenile rats. Behav Neurosci 110:1168-1176.
- Siviy SM, Panksepp J (1985) Dorsomedial diencephalic involvement in the juvenile play of rats. Behav Neurosci 99:1103-1113.
- Siviy SM, Panksepp J (1987) Juvenile play in the rat: thalamic and brain stem involvement. Physiol Behav 41:103-114.
- Siviy SM, Panksepp J (2011) In search of the neurobiological substrates for social playfulness in mammalian brains. Neurosci Biobehav Rev 35:1821-1830.
- Spencer RC, Klein RM, Berridge CW (2012) Psychostimulants act within the prefrontal cortex to improve cognitive function. Biol Psychiatry 72:221-227.
- Spinka M, Newberry RC, Bekoff M (2001) Mammalian play: training for the unexpected. Q Rev Biol 76:141-168.
- Stern WC, Johnson A, Bronzino JD, Morgane PJ (1979) Effects of electrical stimulation of the lateral habenula on single-unit activity of raphe neurons. Exp Neurol 65:326-342.
- Tannock R, Schachar RJ, Carr RP, Chajczyk D, Logan GD (1989) Effects of methylphenidate on inhibitory control in hyperactive children. J Abnorm Child Psychol 17:473-491.
- Tomasi D, Volkow ND, Wang GJ, Wang R, Telang F, Caparelli EC, Wong C, Jayne M, Fowler JS (2011) Methylphenidate enhances brain activation and deactivation responses to visual attention and working memory tasks in healthy controls. Neuroimage 54:3101-3110.
- Trezza V, Baarendse PJJ, Vanderschuren LJMJ (2010) The pleasures of play: pharmacological insights into social reward mechanisms. Trends Pharmacol Sci 31:463-469.
- Trezza V, Damsteegt R, Achterberg EJM, Vanderschuren LJMJ (2011) Nucleus accumbens mu-opioid receptors mediate social reward. J Neurosci 31:6362-6370.
- Trezza V, Damsteegt R, Manduca A, Petrosino S, Van Kerkhof LWM, Pasterkamp RJ, Zhou Y, Campolongo P, Cuomo V, Di Marzo V, Vanderschuren LJMJ (2012) Endocannabinoids in amygdala and nucleus accumbens mediate social play reward in adolescent rats. J Neurosci 32:14899-14908.
- Van den Berg CL, Hol T, Van Ree JM, Spruijt BM, Everts H, Koolhaas JM (1999) Play is indispensable for an adequate development of coping with social challenges in the rat. Dev Psychobiol 34:129-138.
- Van Kerkhof LWM, Trezza V, Mulder T, Gao P, Voorn P, Vanderschuren LJMJ (2012) Cellular activation in limbic brain systems during social play behaviour in adolescent rats. Submitted.
- Vanderschuren LJMJ, Niesink RJM, Van Ree JM (1997) The neurobiology of social play behavior in rats. Neurosci Biobehav Rev 21:309-326.
- Vanderschuren LJMJ, Trezza V, Griffioen-Roose S, Schiepers OJG, Van Leeuwen N, De Vries TJ, Schoffelmeer ANM (2008) Methylphenidate disrupts social play behavior in adolescent rats. Neuropsychopharmacology 33:2946-2956.
- Von Frijtag JC, Schot M, Van den BR, Spruijt BM (2002) Individual housing during the play period results in changed responses to and consequences of a psychosocial stress situation in rats. Dev Psychobiol 41:58-69.
- Wang F, Zhu J, Zhu H, Zhang Q, Lin Z, Hu H (2011) Bidirectional control of social hierarchy by synaptic efficacy in medial prefrontal cortex. Science 334:693-697.
- Wongwitdecha N, Marsden CA (1996) Social isolation increases aggressive behaviour and alters the effects of diazepam in the rat social interaction test. Behav Brain Res 75:27-32.

• Yang LM, Hu B, Xia YH, Zhang BL, Zhao H (2008) Lateral habenula lesions improve the behavioral response in depressed rats via increasing the serotonin level in dorsal raphe nucleus. Behav Brain Res 188:84-90.
• Zahm DS (1999) Functional-anatomical implications of the nucleus accumbens core and shell subterritories. Ann N Y Acad Sci 877:113-128.



Nederlandse samenvatting

Het ervaren van positieve sociale interacties is van groot belang voor kinderen en adolescenten. Tijdens deze levensfases zijn er veel veranderingen te zien in sociale gedragspatronen. De sociale interesse die eerst voornamelijk op de ouders is gericht verschuift naar leeftijdsgenoten. Kenmerkend voor de kindertijd en vroege adolescentie is de aanwezigheid van veel sociaal speelgedrag, wat zowel te zien is bij kinderen als bij jonge dieren. Sociaal speelgedrag is heel belonend, en het ervaren van sociaal speelgedrag is erg belangrijk voor een goede sociale, emotionele en cognitieve ontwikkeling. Afwijkingen in sociaal speelgedrag worden dan ook gezien bij psychiatrische stoornissen zoals autisme en ADHD (attention deficit/hyperactivity disorder). Bovendien vergroten sociale problemen tijdens de kindertijd en adolescentie de kans op het ontwikkelen van psychische stoornissen op volwassen leeftijd. Dit geeft aan dat het ervaren van goede sociale interacties erg belangrijk is. Echter, het is op dit moment voor een groot deel nog onduidelijk hoe verschillende hersengebieden en signaalstoffen betrokken zijn bij sociaal speelgedrag. Hoe doel van dit proefschrift is om de kennis over de neurobiologie van sociaal speelgedrag te vergroten.

Om een goede indruk te krijgen van de hersengebieden die betrokken zijn bij sociaal speelgedrag hebben we in hoofdstuk 2 de hersenactiviteit in kaart gebracht na sociaal speelgedrag door een 'immediate early gene' te gebruiken. Immediate early genes zijn genen waarvan de expressie verhoogd wordt in neuronen die actief zijn geworden door een stimulus (in dit geval sociaal speelgedrag). Met deze techniek laten we in hoofdstuk 2 zien dat de activiteit veranderd is tijdens spel in verschillende hersengebieden waaronder de prefrontale cortex, striatum, amygdala, thalamus, habenula en pedunculopontine nucleus. Tevens hebben we gekeken of gebieden, waarvan bekend is dat ze met elkaar in verbinding staan, tijdens spel ook met elkaar communiceren door te onderzoeken of de activiteiten in deze gebieden een correlatie vertonen. Significante correlaties werden gevonden tussen mediale prefrontale gebieden en striatum, striatum en amygdala, striatum en ventraal tegmentum gebied (VTA), prefrontale gebieden en amygdala, en tussen prefrontale gebieden en VTA. Deze resultaten leverden nieuwe informatie op met betrekking tot mogelijke neuronale netwerken die betrokken zijn bij sociaal speelgedrag. Van deze netwerken is nl. bekend dat ze een belangrijke functie hebben bij o.a. motivationele, emotionele en cognitieve aspecten van gedrag. Tevens vormden deze resultaten aanleiding om verder te gaan kijken naar corticostriatale systemen, waarvan de projecties van de prefrontale cortex naar het striatum deel uitmaken. Deze corticostriatale systemen zijn belangrijk voor verschillende cognitieve en emotionele processen.

Met behulp van een farmacologische inactivatie techniek, waarbij locaal in het brein GABA receptor-agonisten werden toegediend, hebben we de betrokkenheid van de prefrontale cortex en het striatum bij spel verder onderzocht in hoofdstuk 3. Tijdelijke inactivatie van de prefrontale gebieden resulteerde in een sterke afname van speelgedrag. Eerdere studies waarbij deze gebieden uitgeschakeld werden door middel van lesies lieten zien dat speelgedrag in grote mate nog aanwezig is in afwezigheid van de prefrontale gebieden. Het aanbrengen van lesies op jonge leeftijd beïnvloedt echter de normale ontwikkeling van de hersenen. Wellicht kunnen andere hersengebieden de functies van de prefrontale cortex dan gedeeltelijk overnemen waardoor er alleen kleine veranderingen in speelgedrag gedetecteerd worden. Onze studie laat daarentegen zien

dat de prefrontale gebieden wel degelijk een belangrijke rol spelen tijdens speelgedrag. Inactivatie van het dorsomediale striatum leidde niet tot grote veranderingen in speelgedrag; wel was er een lichte toename te zien in het aantal spelinitiaties (pouncing). Het tijdelijk blokkeren van de glutamaterge projecties naar dit gebied (waaronder die vanuit de prefrontale cortex), door middel van locale toediening van een AMPA-glutamaat receptor antagonist, leidde tot een verhoging van speelgedrag. Dit geeft aan dat glutamaterge inputs een remmende werking hebben op speelgedrag in het dorsomediale striatum. Deze rol van het dorsomediale striatum was nog niet eerder aangetoond bij sociaal speelgedrag. De studies beschreven in hoofdstuk 2 en 3 laten dus zien dat er verschillende neuronale netwerken betrokken zijn bij speelgedrag. Deze netwerken zijn hoogstwaarschijnlijk nauw betrokken bij het reguleren van cognitieve, emotionele en motivationele aspecten van gedrag.

In hoofdstuk 4 hebben we onderzocht wat de rol van de habenula is bij sociale isolatie en sociaal speelgedrag. De habenula nog niet eerder is bestudeerd in relatie tot sociaal gedrag tijdens de adolescentie. Bovendien is er de laatste jaren veel aandacht voor de habenula en de rol die dit hersengebied speelt bij psychiatrische aandoeningen waaronder depressie. Het is dus erg relevant om te begrijpen hoe de habenula functioneert tijdens sociale gedragingen. Eerdere studies hebben uitgewezen dat de habenula een belangrijk gebied is voor de regulatie van de activiteit van monoamines in de hersenen. Monoamines (te weten, dopamine, noradrenaline en serotonine) vormen een klasse van signaalstoffen die een grote rol spelen bij verschillende emoties en gedragingen, waaronder sociaal speelgedrag. Onze studie laat zien dat de habenula actief wordt tijdens sociale isolatie (24 uur) en dat deze activiteit afneemt als dieren de kans krijgen om te spelen. Deze resultaten, in combinatie met eerdere studies naar functies van de habenula suggereren dat de habenula actief word tijdens negatieve situaties (zoals sociale isolatie) en dat het ondergaan van een positieve ervaring (zoals speelgedrag) die activiteit weer wat kan remmen. In deze studie hebben we ook de habenula geïnactiveerd (wederom door het toedienen van GABA receptor agonisten) en dit resulteerde in een vermindering van het speelgedrag. Deze resultaten tonen aan dat de habenula inderdaad een belangrijke rol heeft tijdens sociale isolatie en speelgedrag. Verdere studies zijn nodig om te bepalen welke aspecten van sociaal spel worden beïnvloed en via welke neurale mechanismen deze effecten plaatsvinden, waarbij de monoamines een voor de hand liggende kandidaat zijn.

In hoofdstuk 5 hebben we gekeken via welke neurale mechanismen methylfenidaat spel gedrag beïnvloedt. Methylfenidaat (Ritalin®, Concerta®) is een medicijn dat veel gebruikt wordt bij de behandeling van ADHD. Het is een remmer van de heropname van dopamine en noradrenaline, nadat deze signaalstoffen zijn afgegeven door hersencellen. Als deze heropname geremd wordt door methylfenidaat neemt de sterkte van het signaal dat deze signaalstoffen doorgeven toe. Eerdere studies hebben al laten zien dat ratten die met methylfenidaat behandeld zijn minder speelgedrag vertonen. Aangezien methylfenidaat andere gedragingen (zoals locomotoriek en sociale interesse) niet beïnvloedt, lijkt dit effect specifiek te zijn voor sociaal speelgedrag. Door middel van directe toediening in verschillende hersengebieden hebben we laten zien methylfenidaat speelgedrag beïnvloedt via de amygdala en habenula, terwijl verschillende prefrontale gebieden en de nucleus accumbens shell niet betrokken zijn. Het was verrassend dat de prefrontale

en striatale gebieden niet betrokken zijn, aangezien vele eerdere studies naar de werking van methylfenidaat een belangrijke rol van deze gebieden bij de effecten van deze stof hebben laten zien. Identificatie van de amygdala en habenula als belangrijke gebieden voor het effect van methylfenidaat biedt een nieuwe richting voor toekomstige studies naar het verklaren van de effecten van methylfenidaat.

Speelgedrag bestaat uit meerdere componenten, waaronder motivationele, belonende en cognitieve aspecten van het gedrag. In hoofdstuk 6 hebben we onderzocht of dopamine en noradrenaline twee van deze aspecten van speelgedrag beïnvloeden. Hiervoor hebben we eerst een nieuwe taak opgezet om de motivationele aspecten van sociaal speelgedrag te kunnen onderzoeken. In deze taak leren ratten dat ze op een pedaaltje moeten drukken om toegang te krijgen tot een spelpartner. Vervolgens wordt het aantal pedaaldrukken dat vereist is om die toegang te krijgen verhoogd. Zo kunnen we onderzoeken hoe vaak een rat bereid is te drukken voor een sociale (spel)interactie, wat een indicatie is van motivatie voor sociaal speelgedrag. Om de plezierige effecten van sociaal speelgedrag te onderzoeken hebben we gebruik gemaakt van de plaats preferentie test. In deze test worden dieren herhaaldelijk in een kant van de test kooi geplaatst met een spelpartner. Deze kant is dan afgesloten van de rest van de testkooi. Tevens worden ze herhaaldelijk aan de andere kant geplaatst (die er duidelijk anders uitziet dan de ene kant), maar hier worden ze altijd individueel geplaatst. Op de testdag mogen dieren dan kiezen in welke van de twee kooien ze hun tijd doorbrengen. Eerder onderzoek heeft al aangetoond dat dieren een preferentie ontwikkelen voor de stimuluskant als de stimulus als positief wordt ervaren. Dit is aangetoond voor allerlei positieve stimuli waaronder voedsel, verslavende stoffen en sociale interactie.

In hoofdstuk 6 laten we zien dat dopamine en noradrenaline op een verschillende manier betrokken zijn bij de motivationele en plezierige aspecten van speelgedrag. Zo heeft het verhogen van noradrenaline niveaus, d.m.v. blokkade van de heropname van noradrenaline met de selectieve noradrenaline heropnameremmer atomoxetine (die overigens ook gebruikt wordt bij de behandeling van ADHD) een verlagend effect op de motivatie voor speelgedrag, terwijl het de plezierige effecten niet beïnvloedt. Eerder onderzoek heeft al laten zien dat het verhogen van noradrenaline niveaus leidt tot verminderd speelgedrag; dit lijkt dus voornamelijk te komen door een verminderde motivatie voor spel. Het verhogen van dopamine niveaus, d.m.v. blokkade van de heropname van dopamine met de selectieve dopamine heropnameremmer GBR12909, leidt tot een toename in de hoeveelheid pedaaldrukken die dieren willen maken voor sociale interactie en het voorkomt dat zich een plaats preferentie ontwikkelt voor sociale interactie. Net zoals in eerdere studies had deze behandeling geen effect op de totale hoeveelheid spel die deze dieren laten zien. Dopamine lijkt dus belangrijk te zijn voor meerdere aspecten van sociaal speelgedrag waarbij het deze aspecten tegengesteld lijkt te beïnvloeden: het verhoogt de motivatie en verlaagt de beloning. Tevens laten we in hoofdstuk 6 zien dat na behandeling met methylfenidaat de effecten van dit stofje op de dopamine niveaus zichtbaar zijn, te weten een toename in de hoeveelheid pedaaldrukken en het voorkomen van plaats preferentie voor spel gedrag. Dit is opvallend aangezien eerdere studies hebben laten zien dat de effecten van methylfenidaat op de expressie van speelgedrag ontstaan door de verhoging van noradrenaline niveaus. Dit onderzoek geeft duidelijk aan dat methylfenidaat via verschillende neurale systemen verscheidene

aspecten van sociaal speelgedrag kan beïnvloeden.

De studies beschreven in dit proefschrift geven nieuwe inzichten en kennis met betrekking tot de neurale systemen die ten grondslag liggen aan sociaal speelgedrag. De gebieden en neurotransmitters die onderzocht zijn in dit boekje spelen o.a. een rol bij emotie, motivatie en cognitie. Deze aspecten zijn ook belangrijk voor sociaal speelgedrag, De resultaten van het huidige onderzoeksproject geven ons dus inzicht in hoe deze neurale systemen de verschillende aspecten van sociaal speelgedrag zouden kunnen reguleren. De rol van de neurale gebieden en signaalstoffen beschreven in dit boekje geven bovendien nieuwe aanknopingspunten voor studies naar de pathofysiologie van ziektes bij kinderen en adolescenten waarbij sociaal gedrag is verstoord.

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Curriculum Vitae

Linda Wilhelmina Maria van Kerkhof werd op 28 november 1984 geboren te Nijmegen. In 2003 behaalde zij haar VWO diploma aan het Pax Christi College te Druten. Datzelfde jaar startte zij met de studie Biomedische Wetenschappen aan de Universiteit van Utrecht en vervolgens in 2006 met de Master Neuroscience and Cognition aan dezelfde universiteit. Tijdens deze master werden twee wetenschappelijke stages uitgevoerd. De eerste onder begeleiding van dr. Heidi Lesscher bij het Rudolf Magnus Instituut voor Neurowetenschappen te Utrecht. Dit betrof een studie naar een model voor alcoholverslaving bij muizen. Haar tweede stageproject werd uitgevoerd onder begeleiding van dr. Elena DiDaniel bij GlaxoSmithKline in Harlow, United Kingdom. Hierbij bestudeerde ze de rol van het MAP2 eiwit bij op de stabiliteit van microtubuli als een potentiele target in de behandeling van psychiatrische stoornissen. In 2008 behaalde zij haar master diploma en begon ze aan haar promotieonderzoek onder begeleiding van prof. dr. Louk Vanderschuren. De resultaten van haar onderzoek naar de neurale substraten van sociaal speelgedrag staan beschreven in dit proefschrift.