On the pleasurable, motivational and cognitive aspects of social play behavior: pharmacological studies in rats

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On the pleasurable, motivational and cognitive aspects of social play behavior: pharmacological studies in rats

De plezierige, motivationele en cognitieve aspecten van sociaal spelgedrag: farmacologische studies in ratten

(met een samenvatting in het Nederlands)

Proefschrift

ter verkrijging van de graad van doctor aan de Universiteit Utrecht op gezag van de rector magnificus, prof. dr. G.J. van der Zwaan, ingevolge het besluit van het college voor promoties in het openbaar te verdedigen op donderdag 3 april 2014 des ochtends te 10.30 uur

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“Happiness is never better exhibited than by young animals, such as puppies, kittens, lambs, etc., when playing together, like our own children.”

Charles Darwin, *The Descent of Man* **(1871)**

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Chapter 1

General introduction

Social behavior: relevant for both fundamental and clinical research

Children playing hide-and-seek, a dog chasing away another dog from its territory, having sex, a cat nursing her kittens and apes engaged in mutual grooming are all examples of social behaviors. Social behaviors make up a fundamental part of the behavioral repertoire of mammals, including humans, and are crucial for survival of the individual, group and species. For example, studies show that having poor social interactions in humans is considered a risk factor in a wide range of diseases (Holt-Lunstad et al., 2010) and that depriving animals from interacting with conspecifics has severe consequences for the individual (Arling and Harlow, 1967; Harlow and Suomi, 1971; Moberg and Wood, 1982; Fone and Porkess, 2008). Furthermore, being able to interact with conspecifics is considered one of the aspects that are crucial for maintaining animal welfare, which is also implemented in European legislation (The Community Action Plan on the Protection and Welfare of Animals 2006–2010).

The social repertoire of animals is not rigid and changes throughout life. The first social interaction occurs between mother and infant when nursing; during adolescence, in both humans and social animals, interaction with peers becomes increasingly important (Larson and Richards, 1991; Meaney and Stewart, 1981; Spear, 2000). In adulthood, social behavior mainly consists of affiliative, sexual, parental and aggressive/territorial behavior. Social interactions are often perceived as rewarding; maternal care, sexual behavior and social play behavior are among the best described examples of positive social interactions (Trezza et al., 2011a). The focus of this thesis will be on social play behavior in adolescent rats.

From a fundamental point of view, understanding how social play behavior is generated and which brain areas and neurotransmitter systems modulate this behavior increases the knowledge about normal social behavior. For example, as social play is considered a reward, the involvement of the brain reward-system in social play behavior can not only be compared to other natural rewards such as food and sex but also to artificial rewards such as drugs of abuse.

From a clinical point of view, several neuropsychiatric disorders such as disruptive behavior disorder (DBD), autism-spectrum disorder (ASD), early onset schizophrenia and attention-deficit/hyperactivity disorder (ADHD) are characterized by impairments in social (play) behaviors (Alessandri, 1992; Moller and Husby, 2000; Jordan, 2003; Manning and Wainwright, 2010). Thus, a greater understanding of the underlying neurobehavioral mechanisms of social play is not only of importance to understand social behavior in itself but also in finding (pharmaco)therapies for disorders with impairments in the social domain. In addition, drugs of abuse, such as nicotine, alcohol and cocaine are often used in a social setting and can influence social (play) behavior to a great extent (Boys et al., 2001; Vanderschuren et al., 1997, 2008; Young et al., 2011; Bardo et al., 2013). Therefore, understanding how drugs of abuse affect social (play) behavior is an important issue in addiction research. In addition, social disorders such as DBD and ADHD are an important risk factor for alcohol and drug addiction (Young et al., 1995; Biederman et al., 1998; Disney et al., 1999; Merikangas and Avenevoli 2000; Costello et al., 2003; Kim-Cohen et al., 2003; Rutter et al., 2006; Fergusson et al., 2007)

Having fun in a social context: social play behavior

Social play behavior, also referred to as play-fighting or rough-and-tumble play, is the earliest and most characteristic form of non mother-directed social behavior observed in juvenile mammals. It can be described as highly vigorous, voluntary and containing

exaggerated (or incomplete) forms of adult affiliative, aggressive and sexual behavior. There is, however, a difference in context, form and intensity of the behavior (Bekoff, 1974; Poole and Fish, 1975; Fagen, 1981; Pellis and Pellis, 1991; Panksepp et al., 1984; Vanderschuren et al., 1997; Graham and Burghardt, 2010). Social play can be found from children, young chimpanzees, puppies and kittens to adolescent rats (Bekoff and Byers, 1998). Although adult animals occasionally engage in playful interactions, both with other adults and with young conspecifics (Whaley, 1990, Ciani et al., 2012), both children and juvenile animals spent a significant amount of time (20% of the daily time budget and 10% of the daily energy budget) on social play (Siviy and Atrens, 1992; Pellegrini et al. 1998).

The function of social play behavior

The exact function of social play behavior is still topic of debate and over 30 hypotheses have been put forward to explain why young animals perform play behavior (Panksepp et al., 1984; Vanderschuren et al., 1997; Bekoff and Byers 1998; Pellis and Pellis, 2009). The functions of social play are both proximal (immediate) and distal (delayed). It is performed to develop and maintain social relationships (e.g. maintaining group cohesion) and to reduce stress. Furthermore, as a social species it is of great importance to be able to function in a social environment. By engaging in social play, mammals acquire and improve proficiency of both social and non-social skills necessary in adulthood. They learn how to interact with other members of the group and how to behave and adjust behavior in the appropriate way under changeable circumstances in the environment, i.e. they train behavioral and cognitive flexibility (Špinka et al., 2001; Pellis and Pellis, 2009). During childhood and adolescence, the brain undergoes several changes, both functionally and structurally (Blakemore, 2008; Nelson et al., 2005). Thus, social play is essential for social, physical and cognitive development (Hol et al., 1999; Van den Berg et al., 1999). In support of this idea are studies showing that social isolation during the two weeks in adolescence when social play is most abundant results in impaired patterns of social, agonistic and mating behavior in adult life (Potegal and Einon, 1989; Hol et al., 1999; Van den Berg et al., 1999a,b; Spinka et al., 2001). Furthermore, disrupted impulse control and impaired decision making were found after play deprivation, as well as a loss of sensitivity to dopamine in the mPFC pyramidal neurons (Baarendse et al., 2013a) and an increased sensitivity to cocaine self-administration (Baarendse et al., 2013b).

The structure of social play behavior in the laboratory rat

By far, the most extensive experimental work studying social play in mammals has been performed in the laboratory rat (*Rattus norvegicus*) (Panksepp et al., 1984; Vanderschuren et aI., 1997; Pellis and Pellis, 2009; Trezza et al., 2010). In rats social play follows an inverted U-shaped pattern in ontogeny, it emerges around weaning (approximately post natal day 21), peaks in the juvenile period (day 25-40) and decreases after sexual maturity around 60 days post-natally (Bolles and Woods, 1964; Baenninger, 1967; Meaney and Stewart, 1981; Panksepp, 1981). The structure of social play behavior in rats has previously been described in great detail (Baenninger, 1967; Bolles and Woods, 1964; Panksepp and Beatty, 1980; Pellis and Pellis, 1987; Pellis et al., 1989; Poole and Fish, 1975; for reviews see Panksepp et al., 1984; Pellis and Pellis, 1998; Trezza et al., 2010; Vanderschuren et al., 1997). A typical play bout starts when a rat 'invites' another rat to play by attempting to nose or rub the nape of the neck of a conspecific, i.e. pouncing' (figure 1a) and this behavior is often used as a measure of play initiation. The animal that is pounced upon can respond in different ways. If the animal that is pounced upon responds by evading, the soliciting rat may start to chase

it, making another attempt to launch a play bout. The solicited animal may also rear towards the soliciting animal and the two animals may rapidly push, paw, and grab each other ('boxing'). The animal that is pounced upon can also react by rotating to its back. By rotating to the back the pouncing animals has access to the ventral surface of the body of pounced animal, which can be nuzzled or groomed, i.e. 'pinning' (figure 1b). Pinning is the most characteristic posture displayed by adolescent rats engaged in social play. Pinning is not regarded as an endpoint because the animal on its back can launch a counterattack easily (Poole and Fish, 1976; Pellis et al., 1989). Furthermore, both animals actively engage in play, either by pinning or by allowing to be pinned, and mutual pouncing and rapid role reversals are often reported (Vanderschuren et al., 1997). With increasing age the structure of play changes and the response to being pounced with pinning will occur less, while evasions and partial rotations (see Table 1) will occur more often (Pellis and Pellis, 2009). For a full description of the different behaviors displayed during social play see Table 1 (adapted from Trezza et al., 2010).

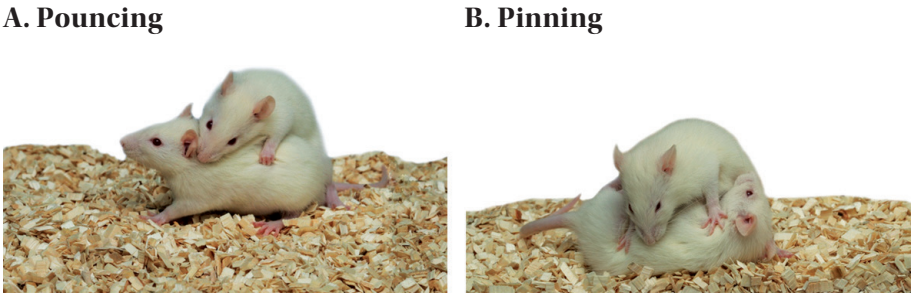

Figure 1: The two most characteristic play behaviors displayed by young rats: A. pouncing B. pinning. Adapted from Trezza et al., (2010) with permission.

Table 1: Ethogram of behaviors displayed during social play. Adapted from Trezza et al., 2010 with permission.

Behavior	Description
Pouncing	Nuzzling the nape of the neck with the tip of the snout, followed by a rubbing movement (figure 1a).
Evasion	Upon solicitation, the recipient animal avoids contact with the nape by leaping, running, or turning away from the partner.
Partial rotation	Upon contact of the nape, the recipient animal begins to rotate along its longitudinal axis, but then stops and keeps one or both hind feet firmly planted on the ground.
Pinning	Upon contact of the nape, the recipient animal fully rotates around the longitudinal axis of its body, ending in a supine position with the other animals standing over it (figure 1b).
Boxing/wrestling	Rearing in an upright position towards the other subject, combined with both rats rapidly pushing, pawing and grabbing at each other, or one rat wrapping around the other subject.
Following/chasing	Moving or running forward in the direction of or pursuing the other subject, who moves away.
Social exploration	Sniffing, licking or grooming any part of the body of the test partner, including the anogenital area. This behavior is and expression of general social interest and is not necessarily a part of social play behavior.

Social play: a natural reward and reinforcer

Social play behavior has a strong emotional component, its most characteristic element being its high reward value (Panksepp et al., 1984; Vanderschuren et al., 1997; Pellis and Pellis, 2009; Trezza et al., 2010). According to Berridge and Kringelbach (2008) a reward consists of several components: 1. Hedonic impact or liking, i.e. the subjective feeling of pleasure; 2. Motivation or wanting (incentive salience), this is what induces approach behavior towards or the willingness to work for it a certain stimulus; 3. Associative learning and memory (cognition), animals are able ascribe salience to social cues and predict that certain social stimuli are positive on the basis of what they experienced before.

The earliest studies reporting that social interaction can be rewarding were done in chimpanzees and showed that these animals learned a discrimination task which was rewarded by the opportunity to groom the experimenters arm (Falk, 1958). Mason et al. (1963) showed that young chimpanzees preferred social play with an experimenter over being groomed by the experimenter, being petted and the opportunity to groom the arm of the experimenter. In rats, the rewarding value of social play has been demonstrated in T-maze learning, place conditioning and operant conditioning set-ups.

T-maze learning

In a T-maze set-up, animals are placed in a 'startbox' at the bottom of the T-shaped maze and after a short delay are allowed to choose which arm of the T they prefer. This paradigm is used to determine preference for certain stimuli as well as to asses memory. Compared to group-raised animals, social isolation-reared adolescent rats chose the opportunity for social interaction more often compared to a palatable food reward (Ikemoto and Panksepp, 1992). In addition, young rats preferred a playing partner compared to a social but non-playing partner (Humphreys and Einon, 1981; Normansell and Panksepp, 1990).

Place conditioning

The rewarding properties of social play behavior have been demonstrated using conditioned place preference (CPP) (Calcagnetti and Schechter, 1992; Crowder and Hutto 1992; Douglas et al., 2004; Thiel et al., 2008, 2009; Trezza et al., 2009; Peartree et al., 2012), a widely used behavioral paradigm to measure both drug and non-drug rewards (Bardo and Bevins, 2000; Tzschentke, 2007). A typical place conditioning set-up consists of three linked chambers, a middle or 'start' compartment and two chambers with different visual and/or tactile cues (see figure 2). It is based on the principle that through coupling of the primary rewarding properties of social play to distinct environmental cues of a particular compartment, a young rat will spent more time in that environment, when allowed to choose, because these distinct environmental cues acquired secondary rewarding properties and elicit approach behavior towards these cues. Animals play in one compartment and are alone in the other compartment. Usually, 24 hours after the last training session animals are placed in the middle compartment and the animals is allowed to choose for a certain amount of time. The time spent in each of the chambers compared to one another is an indication of the preference for that chamber.

Calcagnetti and Schechter (1992) were the first to demonstrate that conditioned place preference (CPP) could be acquired by using social play. Young rats were conditioned twice per day during four days in the place conditioning apparatus. The rats were conditioned with a scopolamine-treated and therefore non-playful partner in one compartment, while rats were coupled with a playful partner in

the other compartment. During testing, it was shown that young rats significantly preferred the compartment previously paired with a playful social partner, showing that social play is rewarding. Douglas et al. (2004) showed that isolated adolescent and adult rats of both sexes demonstrated social CPP, with adolescent males showing strongest preference. No social CPP was found in group-housed adults whereas group-housed adolescents showed preference for the compartment previously paired with similarly housed partners. However, when socially housed adolescents were conditioned with isolated partners, no social CPP developed. These results show that social play is most rewarding for isolated adolescent male rats and that for a social interaction to be rewarding, partners should have a comparable level of sociability. Inconsistent with Douglas et al. (2004), a study by Trezza and colleagues (2009) showed that social play-induced CPP was only found in animals that were socially isolated during conditioning. They also found that animals isolated for 3.5 hours before conditioning showed a trend towards significant place preference. This isolation period induces a half-maximal increase in the amount of social play behavior (Niesink and Van Ree 1989; Vanderschuren et al., 1995c, 2008). No CPP developed in animals that were group housed or housed with an adult rat. Also, the authors showed that eight but not four conditioning sessions of 30 but not 15 minutes were needed to induce CPP for social play. Importantly, it was demonstrated that it is indeed social play rather than social interaction that induces place preference. Rats coupled with a methylphenidate-treated partner, a drug known to selectively reduce play-related behaviors without affecting general social interest (Vanderschuren et al., 2008), did not develop place preference. However, a recent paper (Peartree et al., 2012) showed that social interaction without play is enough to develop place preference. However, social interaction alone is less effective in establishing social CPP compared to social play: indeed, 2 pairings with a playful partner were sufficient to develop CPP, while 8 pairings were necessary to establish CPP when the social partner was confined behind a barrier (so that the testing rats could smell but not play with the social partner) or to have access to a ball (object play). In this study, animals were socially isolated for a period of 4 to 5 days prior to conditioning compared to a maximum of 24 hours of social isolation in the other described experiments. These differences in isolation procedures used in the different studies may explain the discrepancy in results.

Studies by Thiel and colleagues (2008, 2009) have demonstrated that social play can also be used to enhance the rewarding properties of drugs of abuse such as cocaine and nicotine and vice versa. By using a subeffective conditioning paradigm (2 pairings with a play partner and 2 pairings with cocaine/nicotine), in which each condition alone was not sufficient to produce CPP, the two rewards together interacted synergistically to produce CPP, although both nicotine and cocaine reduced play itself. These studies are important for understanding the effects of social context on drug reward during adolescence. All in all, social play can induce place conditioning, providing an opportunity to study the rewarding aspects of social play behavior.

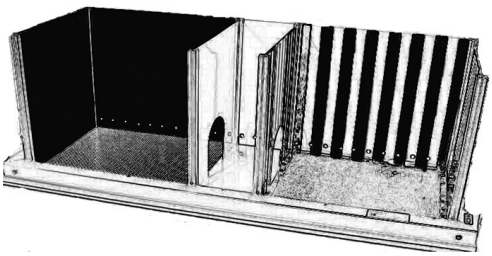

Figure 2: Apparatus used for place conditioning tasks. It consists of two distinct contexts and a neutral 'start' compartment. Adapted from Veeneman-Rijkens (2011) with permission.

Operant conditioning

Social play, like tasty food and drugs of abuse can be used for operant conditioning. In operant conditioning, the animal has to work for rewards by pressing a lever or poking the nose in a hole. Operant conditioning takes place in so-called 'skinner boxes' (named after B.F. Skinner, the instigator of operant conditioning research) which consists of a computer controlled chamber with cue lights and lever protruding from the wall (Figure 3). When an animal makes a response, i.e. a nose poke or a lever-press, the cue light goes on and the animal receives a reward, i.e. food, a drug of abuse or access to a receptive female. In this way the animal learns the contingency between its response (e.g. lever-pressing) and the delivery of the reward. This increases the likelihood that the animal will press the lever to obtain the reward when placed in the box again, a phenomenon known as reinforcement. Depending on preprogrammed schedules lever-presses are reinforced or not. These schedules manipulate the contingency between responses and outcomes. The schedules of reinforcement that are commonly used consist of 2 basic types: (1) the contingency depends on the number of responses given by the animal, so called ratio schedules where ratio of the schedule refers to the number of responses required for each reinforcement and (2) the contingency depends on the timing of the animal, so called interval schedules, in which responses are reinforced only if a pre-determined time interval has elapsed. The ratios and intervals may be either fixed or variable, which results in four main schedules: (a) fixed-ratio (FR): a fixed number of responses must be made before the reinforcement occurs; (b) fixed interval (FI): the reinforcement becomes available upon the first response made after a given time interval; (c) variable-ratio (VR): the number of responses required varies between reinforcements; and (d) variable interval (VI): the interval requirements vary between reinforcements around a specified average value.

The progressive-ratio (PR) schedule was developed to specifically study animals' motivation for rewards (Hodos, 1961; Richardson and Roberts, 1996). In this particular schedule the number of responses to obtain the next reward is increased after every obtained reward, until the animal stops responding. The maximal number of responses performed to obtain one single reward, i.e. the breakpoint, is used as a measure for motivation.

Operant conditioning for social play has, until now, only been studied in primates. Mason et al. (1962) tested in 2 young chimpanzees whether they preferred social play with an experimenter, being groomed by the experimenter, being petted and the opportunity to groom the arm of the experimenter by pressing a lever. All 4 options were presented with either an accessible but passive experimenter or an inaccessible experimenter. They found that these chimpanzees chose social play with an experimenter in 82.6% of the cases whereas the other options were chosen 60% or less of the time. In a follow up study (Mason et al., 1963) chimpanzees could press for food or social interaction. The incentive value of the food was manipulated by testing the animals when hungry of satiated or by changing the palatability of the presented food. Social interaction consisted of being petted by the experimenter or social play with the experimenter. Food was preferred when animals were hungry or highly palatable food was present and the animals preferred play over petting. The most intriguing part of the study was that even when the animals were hungry, they still preferred to play half of the time. Also when satiated or when highly palatable food was present, the animals opted for the opportunity to play on half of the occasions. Together, these results show that social play is a rewarding activity and, more importantly, that operant conditioning can be used to asses motivation for social play behavior, at least in primates.

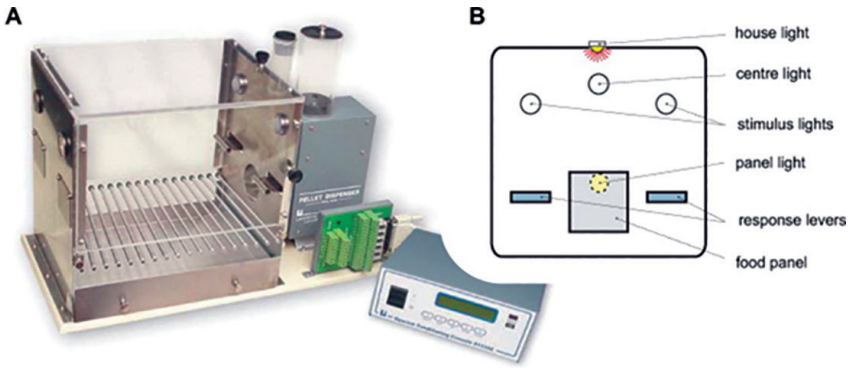

Figure 3: A typical rat Skinner box used for operant conditioning (A), Schematic diagram of the front wall of the box (B). Adapted from Trueman et al., (2012)

Ultrasonic vocalizations

Indirect evidence to show that social play can be considered rewarding comes from the field of ultrasonic vocalizations (USV's). In appetitive situations rats produce high-frequency 50-kHz USV's and especially the frequency-modulated subtype of this call is thought to reflect a positive affective state of the animal (Knutson et al., 1998). In young rats, these sounds are elicited most robustly when engaging in appetitive social interactions and in particular during social play and even when being tickled playfully by an experimenter (Panksepp and Burgdorf, 2000; Burgdorf et al., 2008; Wöhr et al., 2009, 2010) whereas in adults the highest rates of these sounds are emitted during mating (Burgdorf et al., 2008). In addition, these calls are emitted in anticipation of social play (Knutson et al., 1998), result in approach behavior during playback (Panksepp and Burgdorf 2000; Wöhr and Schwarting, 2007) and animals are willing to nosepoke for playback of these calls (Burgdorf et al., 2008). Furthermore, these calls produce activation of brain areas implicated in reward processing, such as the nucleus accumbens (Sadananda et al., 2008). These data seem to indicate that social play is rewarding. However, emission of 50-kHz USV's does not always seem to correlate with social reward (Willey et al, 2009; Willey and Spear, 2012, 2013; Manduca et al., submitted).

Social play and animal welfare

Social play behavior can also be considered a welfare indicator in young animals, including rats. It has been considered an indicator of the current welfare state of an animal (Fagen 1981, Lawrence 1987). According to Held and Špinka (2011), play is a welfare indicator because (1) it is expressed in the absence threats to the survival of the animal, such as under hunger and thirst, in the presence of predators (or odor) and while suffering from an injury. Furthermore, play is reduced when conditions become challenging and stressful for animals, e.g. under bright light conditions and a lack of shelter (Lawrence 1987; Fraser and Duncan 1998; Špinka et al., 2001; Burghardt, 2005; Panksepp et al., 1984; Vanderschuren et al., 1995a; Panksepp and Burgdorf 2010). (2) play increases to act as a buffer against the negative effects of deteriorating conditions at present or in the future, such as in socially instable situations, where social play can function to reduce stress and increase group cohesion or (3) play can be initially reduced but through compensation or 'rebound effect' can increase above baseline when conditions become less challenging, such as when animals have been socially isolated. Social isolation of young animals increases the levels of social play. Interestingly, this

appears to be specific for play as it was found that social separation by a wire mesh, were the rats were still able to smell and interact but not play, also caused increases in play (Holloway and Suter, 2004). However, one should keep in mind that play is only pleasurable when both animals have the same motivation to play (Douglas et al., 2004), therefore the rebound effect could increase welfare for one of the play partners but not necessarily for the other.

Social reward-related memory formation: a cognitive component of social play

Place conditioning experiments have also been used to study cognitive aspects of rewards, such as long-term memory. Memories for events, individuals, places, food and emotions are of critical importance for the survival, well-being and adaptation of all complex organisms (Tronson et al., 2007). When new memories are formed, they are initially labile and susceptible to both facilitation and impairment, but they become progressively stabilized as the transition is made from short term memory (STM) to long term memory (LTM). This transition is termed memory consolidation and is dependent upon transcription and the synthesis of new proteins (McGaugh, 2000). Traditional theories of memory consolidation posit that once memory is fully consolidated, it is insensitive to disruption (McGaugh, 2000). However, several studies have shown that when a well consolidated memory is recalled or retrieved by re-exposure to specific stimuli associated with the memory (e.g. environmental cues), it returns to a labile state during which it becomes vulnerable to interference and after which it has to be reconsolidated (reviewed in Sara, 2000; Dudai, 2004; Tronson et al., 2007; Nader et al., 2009; Inda et al., 2011; Besnard et al., 2012). Memory reconsolidation consists of two phases: a retrieval-dependent destabilization phase followed by a protein synthesis-dependent re-stabilization phase (Nader, 2003). Although reconsolidation seems to have functional similarities (memory storage) with consolidation, it differs from consolidation in temporal profile (post-retrieval instead of post acquisition) and underlying processes (involvement of different intracellular signal transduction pathways) (Tronson et al., 2007; Taubenfeld et al., 2001; Lee et al., 2004; Barnes et al., 2010; Figure 4).

Although it is still topic of debate, the function of memory reconsolidation is generally considered to be the strengthening and the incorporation of new information into an activated memory trace, in addition to the storage of a destabilized memory (e.g. Lee, 2009). Memories are retrieved often and provide additional information to situations that are encountered before. In this way, the capacity for changes in memory strength or content following memory retrieval seems potentially adaptive because the reconsolidation process maintains the relevance of a specific memory in guiding future behavior (Lee, 2009).

Reconsolidation has been studied using a variety of tasks often based on negatively-valenced salient stimuli such as classical, auditory and contextual fear conditioning (e.g. Eisenberg et al., 2003; Nader et al., 2000 and Lee et al., 2004), inhibitory avoidance (e.g. Taubenfeld et al., 2001) and conditioned taste aversion (e.g. Gruest et al., 2004). On the other hand, mnemonic processing of food and drug reward has been intensively investigated as well (Lee et al., 2005; Lee and Everitt 2008; Milton et al., 2008, 2012). In recent years, CPP experiments have been used to study how reward-related memories are consolidated, reconsolidated and extinguished. Most of these studies involved drug-induced CPP (Robinson et al. 2007; Bernardi et al. 2006; Fricks-Gleason et al. 2008).

Although using another paradigm, the social recognition test, one study investigated social reward-related memory (Perrin et al., 2007). Because reconsolidation is protein

synthesis dependent, interfering with protein synthesis by using a protein synthesis inhibitor disrupts the reconsolidation process and therefore animals are unable to recall a specific memory (e.g. Tronson and Taylor, 2007). Perrin and colleagues (2007) used sheep to investigate social recognition memory. They found that ewes treated with the protein synthesis inhibitor cyclohexamide rejected unfamiliar lambs significantly more compared to vehicle-treated animals when long-term memory was assessed but not when short-term memory was assessed. These result suggest that indeed reconsolidation of a social memory was affected by treatment with the protein-synthesis inhibitor.

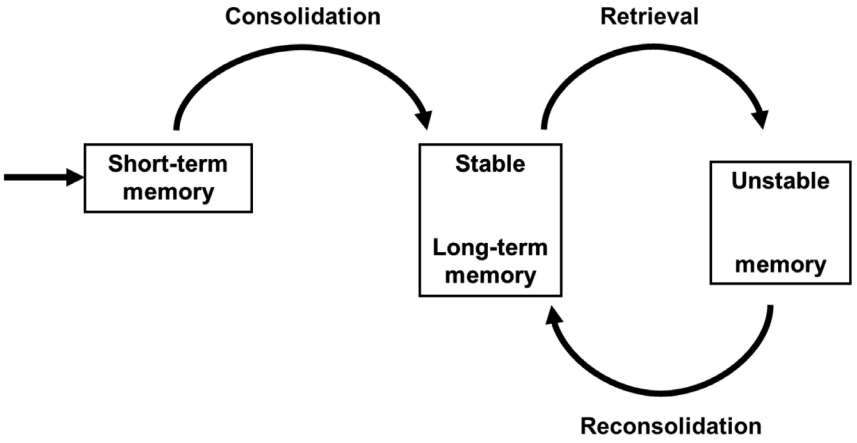

Figure 4: The memory formation process. Modified from Nader et al., (2009)

The neurobiology of social play: a summary

Although substantial progress has been made in recent years, our understanding of the neurobiology of social play behavior is still quite limited. One of the aims of this thesis is to increase this knowledge. Below, a summary is provided about the neurotransmitter systems and brain structures implicated in social play behavior in rats.

Pharmacological studies

Dopamine

Dopamine has been implicated in processes related to reward and motivation and social play behavior is considered a natural reward (Vanderschuren et al., 1997; Trezza et al., 2010, 2011). Dopamine plays an important role in the motivational, but not the pleasurable properties, of rewards (Cardinal et al., 2002; Salamone et al., 2005; Berridge, 2007; Berridge and Kringelbach 2008). Furthermore, social play behavior is associated with increased (forebrain) dopamine release (Panksepp, 1993; Robinson et al., 2011), and according to Trezza and colleagues (2010), an optimal dopamine-level is necessary for the expression of play behavior. It is therefore likely that changes in dopamine signaling modulate the expression of social play behavior. However, the role of dopamine in social play behavior is not straightforward. Whereas dopamine antagonists reduce play behavior, both decreases and increases in play have been reported for dopamine agonists (Niesink and van Ree, 1989; Vanderschuren et al, 2008;

Beatty et al, 1982a, 1984; Siviy et al, 1996; Trezza and Vanderschuren, 2009). In addition, increasing endogenous dopamine levels by dopamine reuptake inhibition did not affect play behavior (Vanderschuren et al, 2008). Intriguingly, increases in social play behavior due to indirect cannabinoid agonists, low doses of nicotine or alcohol could be blocked by a subeffective dose of the non-selective dopamine antagonist alpha-flupenthixol (Trezza et al., 2009a). However, dopamine antagonists were ineffective in restoring the play suppressing effect of the psychostimulants methylphenidate, amphetamine and cocaine, that act on multiple monoaminergic systems, including dopamine.

Noradrenaline

Noradrenergic neurotransmission is important for proper performance of social play behavior. Treatment with the α2-noradrenaline receptor antagonist RX821002 enhances social play behavior, whereas the α2-noradrenaline agonist clonidine was found to reduce social play behavior (Normansell and Panksepp, 1985a; Siviy et al., 1994; Siviy and Baliko, 2000). Siviy and colleagues (1994) also found a possible role of the α1 noradrenaline receptor in the modulation of social play behavior, albeit to a lesser extent than the α2-noradrenaline receptor. In addition, treatment with the β-noradrenaline receptor antagonist has been found to decrease social play behavior (Beatty et al., 1984). Importantly, it was demonstrated that the psychostimulants amphetamine and methylphenidate disrupt play behavior (Beatty et al., 1982a, 1984; Thor and Holloway, 1983; Vanderschuren et al., 2008). Both drugs elevate levels of endogenous dopamine and noradrenaline by acting as neurotransmitter reuptake inhibitors, whereas amphetamine also induces release of these neurotransmitters. The play-reducing effect of methylphenidate was found to be mediated by noradrenergic transmission since play was also suppressed by the selective noradrenaline reuptake inhibitor atomoxetine, but not by the selective dopamine reuptake inhibitor GBR12909 (Vanderschuren et al., 2008). In addition, the play suppressing effect of methylphenidate could be blocked by pretreatment with a subeffective dose of the α2-noradrenaline receptor antagonist RX821002 (Vanderschuren et al, 2008). Furthermore, the effect was not blocked by pretreatment with the α1-noradrenaline receptor antagonist prazosin, the α-noradrenaline receptor antagonist propranolol or dopamine receptor antagonists. Together, these data show that methylphenidate suppresses social play through stimulation of α2 noradrenaline receptors.

Serotonin (5-HT)

It has been difficult to elucidate the exact role of 5-HT in social play behavior, probably because 5-HT projections in the brain are widespread and there are 14 different 5-HT receptor subtypes. In both humans and mammals, 5-HT has been found to be involved in several aspects of social behavior including: establishing an maintaining dominance hierarchies (Huber et al., 2001; Raleigh et al., 1991), defensive behavior (Blanchard et al., 1998; Graeff 2002), aggression (Holmes et al., 2002) but also affective behaviors (Dayan and Huys, 2009; Hariri and Holmes, 2006; Knutson et al., 1997). 5-HT is also involved in the modulation of social play behavior. For example, 5-HT transporter knockout rats were found to display reduced social play (Homberg et al., 2007). Enhancement of 5-HT levels by the selective reuptake inhibitor fluoxetine and MDMA ('ecstasy') the releasing agent fenfluramine or the agonist quipazine inhibit play behavior (Normansell and Panksepp, 1985b; Panksepp et al., 1987; Homberg et al., 2007). Lowering central 5-HT levels by a low tryptophan diet (the 5-HT precursor) or para-chloro-phenylalanine treatment had no effect on play (Panksepp et al., 1987; Knutson et al., 1996). Although the 5-HT1B/2C agonist fluprazine increased social play

behavior (Panksepp, 1993), increasing social play by decreasing 5-HT levels has been difficult (Siviy et al., 2011). Interestingly, changes in 5-HT levels affects play differently depending on the dominance status of rats in an asymmetric play couple (one animal initiating play more that the other). Specifically, pinning in the dominant but not the subordinate rat is affected by altered levels of 5-HT; enhancing 5-HT levels reduced the asymmetry, while depleting 5-HT increased pinning asymmetry (Knutson et al., 1996; Knutson and Panksepp, 1997; Siviy et al., 2011).

Cannabinoids

The endogenous cannabinoids, or endocannabinoids, have been implicated in positive emotions and motivation (Mahler et al., 2007; Solinas et al., 2008). In keeping with this notion, endocannabinoids have been found to modulate social play behavior. Treating rats with indirect cannabinoid agonists (i.e., drugs that prolong endocannabinoid signaling by blocking the enzymatic degradation or the reuptake of the endocannabinoid anandamide) enhanced social play performance, whereas activation of CB1 receptors with direct receptor agonists reduced social play (Trezza and Vanderschuren 2009; Trezza and Vanderschuren 2008a,b). This seems paradoxal, but is probably the result of the peculiar mechanism of action of endocannabinoids. Thus, endocannabinoids are only released on demand after neural depolarization, so inhibiting their deactivation during social play by preventing their degradation, prolongs endocannabinoid signaling in active synapses only (and hence, within brain areas involved in play). This preserves the spatio-temporal specificity of endocannabinoid activity, thereby stimulating play behavior. On the other hand, since cannabinoid receptors are abundantly present throughout the brain, treatment with a direct cannabinoid receptor agonist induces an artificial endocannabinoid signal in brain regions not directly involved in play behavior or where increased endocannabinoid levels evoke processes that are incompatible with play, such as cognitive impairments (Schneider and Koch, 2002; Egerton et al., 2006), thereby creating a mental state incompatible with social play behavior. Recently, the effect of endocannabinoids was shown to be dependent on opioid and dopamine signaling and to be mediated by the nucleus accumbens and basolateral amygdala (Trezza et al., 2012; Trezza and Vanderschuren, 2008a). Interestingly, the endocannabinoid system interacts with the endogenous opioid system in the modulation of social play behavior: thusthe play enhancing effects of the anandamide hydrolysis inhibitor URB597 was completely blocked by pretreatment with the opioid receptor antagonist naloxone (Trezza and Vanderschuren, 2008a). Similarly, the increase in social play induced by systemic administration of morphine was counteracted when animals were pretreated with the cannabinoid receptor antagonist SR142716A (Trezza and Vanderschuren, 2008a).

Opioids

Opioids play an important role in the performance of social play. It has been suggested that they are specifically involved in the rewarding, rather than the motivational properties of play (Trezza et al., 2010; Panksepp et al., 1980). For example, low doses of drugs that mimic the effects of endogenous opioids such as the μ-opioid receptor agonists morphine, methadone and fentanyl, or the endogenous opioid **β**-endorphin enhance social play (Trezza et al., 2010; Trezza and Vanderschuren 2008a,b; Vanderschuren et al., 1997; Vanderschuren et al., 1995c,d; Normansell and Panksepp 1990; Niesink and Van Ree 1989; Panksepp et al., 1985). In contrast, opioid receptor antagonists such as naloxone, naltrexone and beta-funaltrexamine reduce social play (Normansell and Panksepp 1990; Niesink and Van Ree 1989; Jalowiec et al., 1989; Siegel and Jensen 1986; Panksepp et al., 1985; Siegel et al., 1985; Beatty and Costello 1982). In

addition, stimulation of **κ**-opioid receptors disrupted social play, whereas stimulation of δ-opioid receptors did not affect play (Vanderschuren et al., 1995d). The play-enhancing effects of the opioid receptor agonist morphine depended on stimulation of opioid and cannabinoid, but not on dopamine receptors. Animals pretreated with an opioid- or a cannabinoid-receptor antagonist, but not with a dopamine-receptor antagonist, before morphine-treatment did not show changes in play behavior compared to control animals (Trezza and Vanderschuren 2008a). Furthermore, it has been shown that opioid modulation of the rewarding aspects of social play is mediated via the nucleus accumbens (Trezza et al., 2011b).

Brain areas

A complex behavior such as social play involves a wide array of subcortical and cortical neural circuits. Subcortical regions are thought to mediate the execution of the appropriate motor acts and the integration of sensory stimuli as well as encoding the emotional and motivational properties of social play, whereas cortical regions are suggested to facilitate play by guiding its expression in the appropriate temporal and contextual setting (Siviy and Panksepp, 2011; Pellis and Pellis, 2007; Vanderschuren et al., 1997). In the next section, several widely investigated brain areas involved in social play will be discussed.

Subcortical regions

Striatum

The striatum is important for sensorimotor integration, generation of voluntary movement (dorsal striatum), as well as for regulating emotional and motivational aspects of behavior (ventral striatum, including the nucleus accumbens), as well as for certain forms of associative learning (both dorsal and ventral striatum) (Haber and Knutson, 2010; Berridge and Kringelbach, 2008; Salamone et al., 2005; Cardinal et al., 2002). In support of the importance of the striatum in social play, it was shown that the size of the striatum is associated with the amount of time spent on social play behavior in non-human primates, while no association was found for non-social play behavior (Graham, 2011). It has been previously suggested that the striatum is involved in switching between and the serial ordering of behaviors, since it was found that neonatal striatal dopamine depletion resulted in a decrease in play initiation, as well as switching to grooming or sexual behaviors in the middle of play sequences (Pellis et al., 1993). This suggested that the striatum is necessary for prioritizing when certain play behaviors have to be executed. In the nucleus accumbens, a region known to be involved in the pleasurable and motivational aspects of rewards (Berridge and Kringelbach, 2008; Cardinal et al., 2002), increased opioid activity during social play was found (Vanderschuren et al., 1995b).

In studies where the immediate-early gene c-fos was used as a marker for neuronal activity during social play behavior, an increase in cellular activity during social play behavior in the striatum was shown(Van Kerkhof et al., 2013a; Cheng et al., 2008; Gordon et al, 2002). The cellular activation in the dorsal striatum was higher compared to the ventral striatum. Van Kerkhof et al., (2013a) hypothesized that dorsal prefrontal-dorsomedial striatum projections are involved in the sequential en temporal organization of play behavior, whereas the ventral-prefrontal-ventral striatum projections mediate the rewarding aspects of social play. Interestingly, pharmacological inactivation of the nucleus accumbens core or shell did not affect social play (Van Kerkhof et al., 2013b). This was interpreted as indicating that the expression of social play behavior can take place in the absence of a functional nucleus accumbens core

or shell region, so that if output from the core is inhibited, other striatal regions such as the nucleus accumbens shell mediate social play. Indeed, when levels of specific neurotransmitters such as opioids and endocannabinoids in these regions are altered, social play is affected (Trezza et al., 2012; Trezza et al., 2011b).

Inactivating the dorsomedial striatum tended to increase play initiation, whereas blocking AMPA/kainate glutamate receptors in this region increased play (Van Kerkhof et al., 2013b). The dorsomedial striatum is thought to play a role in response selection and response inhibition (Eagle and Robbins, 2003; Devan et al., 1999; Corbit and Janak, 2007), with animals showing disinhibited behavior when functional activity in the dorsomedial striatum is reduced. This led to the hypothesis that this structure, through inhibitory mechanisms, controls the vigor of social play (Van Kerkhof et al., 2013b).

Amygdala

The amygdala is known to be involved in the processing of negative as well as positive emotions (Morrison and Salzman 2010; Phelps and Ledoux, 2005; Cardinal et al., 2002). In humans the amygdala is implicated in emotional processing as well as the recognition of facial expressions (Whalen et al., 2013; Phelps and Ledoux, 2005) and in nonhuman primates, a larger amygdala size was found to be correlated with a higher percentage of time spent on social play behavior (Lewis and Barton, 2006).

Because social play has a high positive emotional value (Trezza et al., 2011a; Trezza et al., 2010; Vanderschuren, 2010; Trezza et al., 2009a,b; Pellis and Pellis, 2009; Vanderschuren, 1997, Panksepp et al., 1984), it is likely that the amygdala is involved in modulating play behavior. Lesioning the amygdala in neonatal and three-week-old rats reduced social play (Deanen et al., 2002; Wolterink et al., 2001) and abolished sex differences in the patterns and levels of social play (Meaney et al., 1981). In addition, recently, endocannabinoids were shown to modulate social play via CB1 cannabinoid receptors in the basolateral amygdala (Trezza et al., 2012).

In a c-fos study by Van Kerkhof et al., (2013a) social play led to an increased expression of c-fos in the lateral amygdala. In addition, social play-induced c-fos expression in the amygdala was found to correlate with activity in several subregions of the prefrontal cortex and the striatum. It was therefore suggested that amygdalo-prefrontal-striatal circuits are involved in social play behavior, perhaps to mediate its rewarding properties.

Siviy and Panksepp (2011) suggested that the amygdala acts a modulator of play by receiving input of social, temporal and contextual cues in a social interaction and to mediate the emotional value to this interaction.

Habenula

The habenula is known to modulate monoaminergic neurotransmission through its inputs into the monoaminergic nuclei such as the ventral tegmental area (VTA, dopamine), dorsal raphe nucleus (serotonin) and the locus coeruleus (noradrenaline) (Hikosaka, 2010; Lecoutier and Kelley, 2007). The habenula is known to be involved in functions that are monoamine-dependent such as reward, punishment, attention, stress, decision-making and learning (Hikosaka, 2010; Lecoutier and Kelley, 2007). Since an optimal balance in levels of monoamines has been suggested to be necessary for proper execution of play behavior (Siviy and Panksepp, 2011; Trezza et al., 2010; Vanderschuren et al., 1997), it is well conceivable that this structure is involved in social play. Recently, increased cellular activity in response to social isolation was found in the habenula and this increase in activity could be reduced by social play, suggesting opposing reactions to aversive (social isolation) and rewarding (social play) stimuli by the habenula (Van Kerkhof et al., 2013c; Hikosaka, 2010). In addition, Van Kerkhof et

al., (2013c) showed that temporal inactivation of the habenula reduced the expression of social play, with pinning being more sensitive to the inactivation than pouncing. These data implicate the habenula in the modulation of social play behavior.

Cortex

Social play behavior is complex and unpredictable, requiring an animal to make an assessment of the emotional state of a conspecific and plan actions on the basis of its partners behavior. Therefore, it is likely cortical areas are be involved in social play behavior (Vanderschuren et al., 1997).

Interestingly, several studies have shown that the cortex is not necessary for the performance of social play behavior. Decorticated rat were still able to play, although the structure of their play behavior showed some abnormalities. For example, pinning was reduced in decorticated rats, but the number of play initiations was not altered. The reduction in pinning in decorticated rats was attributed to their altered pattern of defense, i.e. partial rotation instead of complete rotation (Schneider and Koch, 2005; Panksepp et al., 1994; Pellis et al., 1992). Furthermore, decortication alters the target of play initiations (Pellis et al., 1992). Intact animals mainly directed their attacks at the nape of the neck and only a small portion was directed at the more caudal regions such as the back and rump. In contrast, only a third of the play attacks was directed at the nape by decorticated rats. However, although decortication altered the pattern of response and the target of play initiation, it did not affect the number of play initiations (Schneider and Koch, 2005; Panksepp et al., 1994; Pellis et al., 1992).

The frontal cortex can be divided in the prefrontal cortex (PFC) and the orbitofrontal cortex (OFC). These regions have been implicated in higher cognitive functions that influence motivational and rewarding processes, such as attention, decision making, and coding the expected values of planned behavior (Robbins and Arnsten 2009; Miller 2000; Schoenbaum et al., 2009). The effect of specific lesions in these particular regions on social play have been studied as well. Depending on de social status (dominant vs. subordinate) and the sex of their play partner, rats modulate their play behavior accordingly. Full rotations are more common when encountering a dominant male rat, whereas an encounter with a subordinate or female rat more often results in partial rotation (Pellis et al., 2010, 2006). Neonatal lesions of the OFC results in a failure of rats to modify their social behavior, both playful and non-playful, in response to the identity of their partner (Pellis et al., 2006). These animals did not respond in an appropriate way (they rotated less to a supine position) to play initiation. Animals with neonatal lesions of the medial PFC showed less playful responses and more evasions to play initiation and rotated less to supine, often shortening the play bout (Bell et al., 2009; Schneider and Koch, 2005). Bell and al., (2009) suggested that the mPFC is necessary for the organization of movements in play behavior. A study looking at the effect of lesions in the motor cortex on play behavior found that it eliminated the normal, age-related modulation in defensive tactics (Kamitakahara et al., 2007). To summarize, these studies indicate that the prefrontal cortex is not of critical importance for the expression of social play behavior, it rather fine-tunes its expression in relation to social, contextual and temporal cues.

In c-fos studies heterogeneous activity patterns were found in the mPFC and the OFC, suggesting that distinct frontal subregions are active during the performance of social play and probably modulate play behavior differently (Van Kerkhof et al., 2013a,b; Cheng et al., 2008; Gordon et al., 2002). In addition, c-fos activity in mPFC correlated with c-fos activity in their striatal target regions, which suggests that a medial PFC-striatum projection is active during social play (Van Kerkhof et al., 2013a,b). Furthermore, temporary inactivation of specific subregions of the

PFC (prelimbic and infralimbic cortex) and OFC (medial/ventral orbitofrontal cortex) decreased play behavior (Van Kerkhof et al., 2013c). These effects were more pronounced than the previously described lesion studies as both play responsiveness as well as play initiation were reduced by inactivation. The inactivation data suggests that indeed specific subregions in both the PFC and OFC contribute to the expression of social play behavior.

Together, these studies suggest that the medial prefrontal and orbitofrontal cortex modulate play behavior as these regions are important for the ability of animals to respond appropriately and flexibly to changeable social conditions (Van Kerkhof et al., 2013a,b; Bell et al., 2010, 2009; Pellis et al., 2006).

Aims and outline

Given the importance of social play for behavioral, emotional and cognitive development and its relevance for child and adolescent psychiatry, it is important to understand the brain areas and neurotransmitter systems that mediate and modulate this behavior. The overall aim of this thesis was to elucidate the neurotransmitter systems and neural substrates involved in the pleasurable, motivational and cognitive aspects of social play behavior with a specific focus on monoamines, opioids and cannabinoids. To this aim different pharmacological and behavioral techniques were used.

In **Chapter 2** we investigate the pharmacological underpinnings of the play suppressive effects of psychostimulants that are known to elevate levels of monoamines, i.e. amphetamine and cocaine. We pretreated animals with several (combinations of) monoaminergic receptor antagonists to counteract the play-inhibitory effect of amphetamine and cocaine. We also administered subeffective doses of selective (combinations of) monoaminergic reuptake inhibitors in combination with a subeffective dose of cocaine to mimic its effect on play. Next, in **chapter 3**, we determine via which neural substrates methylphenidate (MPH) reduces play behavior. MPH is the first choice medication for treatment of ADHD and it enhances monoamine levels. Previously, it was shown that MPH inhibits play via **α**2-noradrenaline receptors (Vanderschuren et al., 2008). However, the brain areas mediating this effect are still unknown. Therefore, MPH was locally administered into the nucleus accumbens shell, amygdala and habenula and several subregions of the PFC and OFC. To verify whether the reduction of social play is dependent on noradrenergic neurotransmission, atomoxetine is infused in brain regions in which MPH reduces social play behavior. Subsequently, in **chapter 4**, we investigated whether motivational and/or pleasurable aspects of social play are influenced by dopaminergic and noradrenergic neurotransmission. A newly established operant conditioning task, in which rats were trained to press a lever for access to a play partner, and a CPP task for social play (Trezza et al., 2009b) were used to asses whether motivational and/or pleasurable aspects of social play behavior were affected after treatment with several drugs that modulate dopaminergic or noradrenergic neurotransmission. The effects of opioids and cannabinoids on motivational and/or pleasurable aspects of social play are summarized in **Chapter 5**. **Chapter 6** describes the more cognitive aspect of social play: here we investigated the effect of a β-adrenoceptor antagonist, administered at several time-points critical for memory processing, on social play-related memory using social play-induced CPP. These studies were followed up and in **chapter 7**, it was assessed whether pharmacological compounds known to disrupt a specific aspect of memory processing, i.e. reconsolidation, in both non-social appetitive and aversive memories would disrupt social rewarding memory reconsolidation. This was done using social play-induced CPP. Finally, in **chapter 8**, the results acquired in this thesis and their implications are summarized and discussed.

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Chapter 2

Amphetamine and cocaine suppress social play behavior in rats through distinct mechanisms

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Abstract

Rationale

Social play behavior is a characteristic form of social behavior displayed by juvenile and adolescent mammals. This social play behavior is highly rewarding and of major importance for social and cognitive development. Social play is known to be modulated by neurotransmitter systems involved in reward and motivation. Interestingly, psychostimulant drugs, such as amphetamine and cocaine, profoundly suppress social play, but the neural mechanisms underlying these effects remain to be elucidated.

Objective

In this study, we investigated the pharmacological underpinnings of amphetamine- and cocaine-induced suppression of social play behavior in rats.

Results

The play-suppressant effects of amphetamine were antagonized by the alpha-2 adrenoreceptor antagonist RX821002 but not by the dopamine receptor antagonist alpha-flupenthixol. Remarkably, the effects of cocaine on social play were not antagonized by alpha-2 noradrenergic, dopaminergic, or serotonergic receptor antagonists, administered either alone or in combination. The effects of a subeffective dose of cocaine were enhanced by a combination of subeffective doses of the serotonin reuptake inhibitor fluoxetine, the dopamine reuptake inhibitor GBR12909, and the noradrenaline reuptake inhibitor atomoxetine.

Conclusions

Amphetamine, like methylphenidate, exerts its play-suppressant effect through alpha-2 noradrenergic receptors. On the other hand, cocaine reduces social play by simultaneous increases in dopamine, noradrenaline, and serotonin neurotransmission. In conclusion, psychostimulant drugs with different pharmacological profiles suppress social play behavior through distinct mechanisms. These data contribute to our understanding of the neural mechanisms of social behavior during an important developmental period, and of the deleterious effects of psychostimulant exposure thereon.

Introduction

The young of many mammalian species, including humans, display a characteristic form of social interaction known as social play behavior or rough-and-tumble play (Panksepp et al. 1984; Vanderschuren et al. 1997; Pellis and Pellis 2009). Social play behavior is of major importance for social and cognitive development (Potegal and Einon 1989; Van den Berg et al. 1999; Baarendse et al. 2013). Furthermore, social play is highly rewarding. It is an incentive for maze learning, operant conditioning, and place conditioning in rats and primates (for reviews, see Vanderschuren 2010; Trezza et al. 2011), and it is modulated through neurotransmitter systems implicated in the positive subjective and motivational effects of food, sex, and drugs of abuse (Trezza et al. 2010; Siviy and Panksepp 2011). However, the underlying neurobiological mechanisms of social play behavior are still incompletely understood.

The abundance of social play behavior is an expression of the marked changes in social behavior that take place during post-weaning development (Spear 2000; Nelson et al. 2005). Interestingly, the increased importance of interactions with peers during this phase of life (i.e., the juvenile and adolescent stages in rodents, roughly equivalent to childhood and adolescence in humans) coincides with other changes in behavior, such as increased risk-taking and experimenting with drugs of abuse (Casey and Jones 2010; Blakemore and Robbins 2012). Especially in the early stages of use, drugs are often experienced in a social setting (Boys et al. 2001; Newcomb and Bentler 1989) because of their presumed ability to facilitate interaction with peers, peer acceptance, and group cohesion. However, drug use can have negative consequences for social behavior (for review, see Young et al. 2011). Therefore, investigating the effects of drugs of abuse on social play behavior serves two purposes. First, it increases our knowledge of the neural substrates of social play behavior. Second, it provides important information about how drugs of abuse affect the quality of social interactions during an important period of social development.

In rodent and primate studies, the psychostimulant drugs amphetamine, methylphenidate, and cocaine have been shown to interfere with various social behaviors (Schiørring 1979; Mizcek and Yoshimura 1982; Beatty et al. 1982, 1984; Thor and Holloway 1983; Sutton and Raskin 1986; Ferguson et al. 2000; Vanderschuren et al. 2008; Liu et al. 2010). In particular, these psychostimulants profoundly decrease social play behavior in adolescent rats, without affecting general social interest (Beatty et al. 1982, 1984; Thor and Holloway 1983; Sutton and Raskin 1986; Ferguson et al. 2000; Vanderschuren et al. 2008). We have previously found that the play-suppressant effects of methylphenidate are mediated by stimulation of alpha-2 adrenoceptors, but that they are independent of dopaminergic neurotransmission (Vanderschuren et al. 2008). However, the mechanisms by which amphetamine and cocaine inhibit social play behavior are unknown (Beatty et al. 1984).

It is well established that amphetamine and cocaine increase the synaptic concentrations of dopamine, noradrenaline, and serotonin (5-HT), by stimulating their release and inhibiting their reuptake, respectively (Heikkila et al. 1975; Ritz and Kuhar 1989; Rothman et al. 2001). In addition, there is recent evidence to suggest that amphetamine and cocaine also facilitate exocytotic dopamine release (Venton et al. 2006; Aragona et al. 2008; Daberkow et al. 2013). The relative effectiveness of amphetamine and cocaine on monoamine neurotransmission differs, however. Whereas amphetamine preferentially enhances noradrenaline and dopamine neurotransmission, cocaine most profoundly inhibits the reuptake of 5-HT and dopamine (Ritz and Kuhar 1989; Rothman et al. 2001). Therefore, we investigated the pharmacological mechanisms through which amphetamine and cocaine reduce social

play behavior in rats. On the basis of our previous findings (Vanderschuren et al. 2008), and the pharmacological profiles of amphetamine and cocaine, we hypothesized that amphetamine suppresses social play through stimulation of alpha-2 adrenoceptors, but that the effect of cocaine on social play relies on dopamine and/or 5-HT mechanisms.

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 (temperature 20–21 °C, 55±15 % relative humidity, and 12/12-h light cycle with lights on at 0700 hours). Food and water were available ad libitum. All animals were experimentally naive and were used only once (i.e., different groups of rats were used for each experiment). All experiments were approved by the Animal Ethics Committee of Utrecht University and were conducted in agreement with Dutch laws (Wet op de Dierproeven 1996) and European regulations (Guideline 86/609/EEC).

Drugs

(+)-Amphetamine sulfate (0.05–0.5 mg/kg, s.c.) was obtained from O.P.G. (Utrecht, The Netherlands). Cocaine hydrochloride (0.5–7.5 mg/kg, s.c.), the dopamine receptor antagonist alpha-flupenthixol dihydrochloride (0.125 mg/kg, i.p.), the 5-HT1a receptor antagonist WAY100635 maleate (0.1 mg/kg, s.c.), and the 5-HT2a receptor antagonist M100907 (0.2 mg/kg, s.c.) were obtained from Sigma-Aldrich (Schnelldorf, Germany). The alpha-2 adrenoreceptor antagonist RX821002 hydrochloride (0.2mg/kg i.p.), the 5-HT2 receptor antagonist amperozide hydrochloride (0.5 mg/kg, i.p.), the 5-HT1/2 receptor antagonist methysergide maleate (2.0 mg/kg, s.c.), the 5-HT3 receptor antagonist ondansetron hydrochloride (2.0 mg/kg, i.p.), the 5-HT reuptake inhibitor fluoxetine hydrochloride (1–3 mg/kg, s.c.), the dopamine reuptake inhibitor GBR12909 dihydrochloride (3 mg/kg, s.c.), and the noradrenaline reuptake inhibitor atomoxetine hydrochloride (0.1–0.3 mg/kg, i.p.) were obtained from Tocris Bioscience (Avonmouth, UK). All drugs were dissolved in saline, except for GBR12909 which was dissolved in sterile water andM100907 which was dissolved in saline containing 10 % Tween 80 (Sigma-Aldrich, Schnelldorf, Germany). Amphetamine and cocaine were injected 30 min before the test. The antagonists were administered 30 min before amphetamine or cocaine except for RX821002, which was administered 15 min before amphetamine and cocaine. The reuptake inhibitors were injected 30 min before the test. We used doses of dopamine-, 5-HT-, and noradrenaline receptor antagonists and reuptake inhibitors that had no effect ton social play by themselves (Homberg et al. 2007; Trezza and Vanderschuren 2008b; Vanderschuren et al. 2008). Drug doses and pretreatment intervals were based on our previous work, literature, and pilot experiments. Solutions were freshly prepared on the day of the experiment and administered in a volume of 2 ml/kg. When an experiment involved a combination of antagonists or reuptake inhibitors, the different compounds were dissolved and injected separately to prevent interaction of two or more drugs in the same solution. Because of the importance of the neck area in the expression of social play behavior (Pellis and Pellis 1987; Siviy and Panksepp 1987), subcutaneous injections were administered in the flank.

Procedures

All behavioral procedures were conducted as previously described (Vanderschuren et al. 2008; Trezza et al. 2008a). Briefly, the experiments were performed in a sound-

attenuated chamber under dim light conditions. The testing arena consisted of a Plexiglas cage measuring 40×40×60 cm (l ×w ×h), with approximately 2 cm of wood shavings covering the floor. At 26–28 days of age, rats were individually habituated to the test cage for 10 min on each of the 2 days before testing. On the test day, the animals were socially isolated for 3.5 h before testing, to enhance their social motivation and thus facilitate the expression of social play behavior during testing. This isolation period has been shown to induce a half-maximal increase in the amount of social play behavior (Niesink and Van Ree 1989; Vanderschuren et al. 1995a, 2008). At the appropriate time before testing, pairs of animals were treated with drugs or vehicle. The test consisted of placing two animals into the test cage for 15 min. The animals in a pair did not differ more than 10 g in body weight. Since dominance status has a profound influence on the intensity and structure of social play (Pellis et al. 1997), and drug effects can be different in dominant versus subordinate animals (e.g., Panksepp et al. 1985; Knutson et al. 1996), animals in a test pair had no previous common social experience (i.e., they were not cage mates), to minimize the influence of dominance/subordination relationships on social play and the effects of drugs thereon. The behavior of the animals was videotaped, and analysis from the video tape recordings was performed afterwards by an observer blind to treatment. Behavior was assessed per pair of animals using Observer 3.0 software (Noldus Information Technology BV, Wageningen, The Netherlands).

In rats, a bout of social play behavior starts with one rat soliciting ("pouncing") another animal, by attempting to nose or rub the nape of its neck. The animal that is pounced upon can respond in different ways: if the animal fully rotates to its dorsal surface, "pinning" is the result, i.e., one animal lying with its dorsal surface on the floor with the other animal standing over it. From this position, the supine animal can initiate another play bout, by trying to gain access to the other animal's neck. Thus, during social play, pouncing is considered an index of play solicitation, while pinning functions as a releaser of a prolonged play bout (Panksepp and Beatty 1980; Pellis and Pellis 1987; Poole and Fish 1975). Pinning and pouncing frequencies can be easily quantified and are considered the most characteristic parameters of social play behavior in rats (Panksepp and Beatty 1980; Trezza et al. 2010). During the social encounter, animals may also display social behaviors not directly associated with play, such as sniffing or grooming the partner's body (Panksepp and Beatty 1980; Vanderschuren et al. 1995a, b). Since social play behavior in rats strongly depends on the playfulness of its partner (Pellis and McKenna 1992; Trezza and Vanderschuren 2008a), both animals in a play pair received the same drug treatment, and a pair of animals was considered as one experimental unit. The following parameters were therefore scored per pair of animals:

• Social behaviors related to play:

– Frequency of pinning
– Frequency of pouncing

• Social behaviors unrelated to play:

– Time spent in social exploration: the total amount of time spent in non-playful forms of social interaction (i.e., one animal sniffing or grooming any part of the partner's body).

Statistical analysis

Data are expressed as mean ± SEM. To assess the effects of single or combined treatments on social play behavior, data were analyzed using one- or two-way ANOVA. ANOVAs were followed by Tukey's post-hoc test, where appropriate.

Results

The play-suppressant effects of amphetamine are mediated by activation of alpha-2 noradrenergic but not dopamine receptors

Amphetamine (amph; 0.2 and 0.5 mg/kg) significantly reduced pinning and pouncing, with no effect on social exploration [pinning: $F(amph)3,28=16.58$, $p<0.001$; pouncing: $F(amph)3,28=23.12$, $p<0.001$; social exploration: $F(amph)3,28=0.53$, NS, Fig. 1a–c]. We previously found that the reduction in social play behavior induced by treatment with methylphenidate was prevented by pretreatment with the alpha-2 adrenoceptor antagonist RX821002, but not the dopamine receptor antagonist alpha-flupenthixol (Vanderschuren et al. 2008). Therefore, we investigated whether RX821002 and

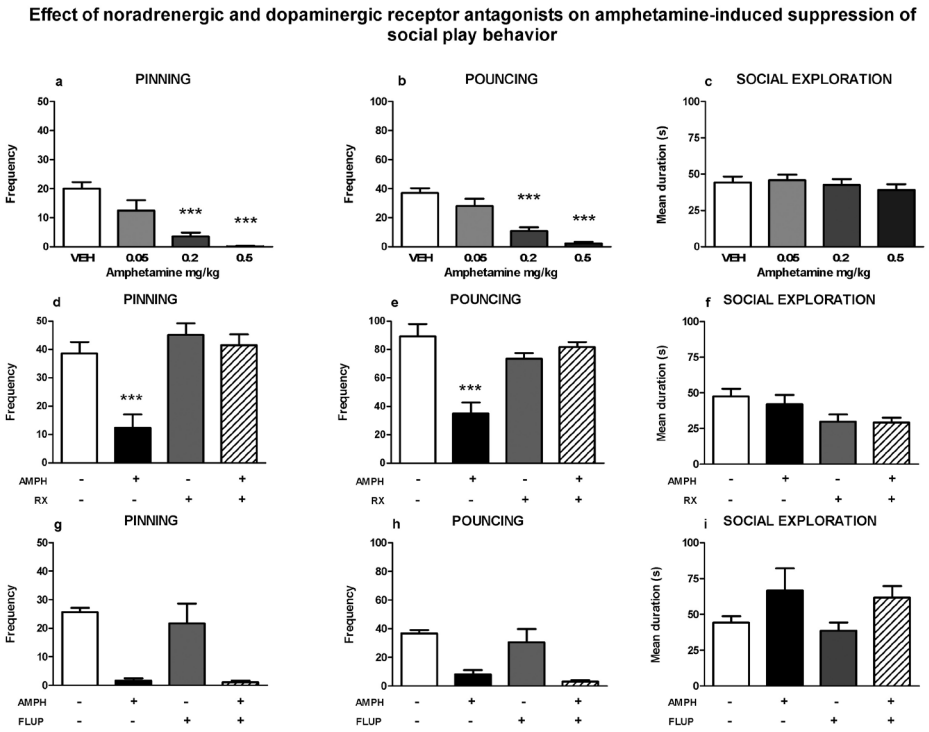

Fig. 1 Effect of noradrenaline and dopamine receptor antagonists on amphetamine-induced suppression of social play behavior. Amphetamine (amph, s.c.) dose-dependently reduced pinning (a) and pouncing (b) without affecting social exploration (c). The effect of amphetamine (0.2 mg/kg) was blocked by the alpha-2 adrenoreceptor antagonist RX821002 (rx, 0.2 mg/kg, i.p.), pinning (d), pouncing (e), and social exploration (f). The effect of amphetamine on pinning (g), pouncing (h), and social exploration (i) was not blocked by the dopamine receptor antagonist alpha-flupenthixol (flup, 0.125 mg/kg, i.p.). Bars show the frequency (mean + SEM) of pinning and pouncing and themean (+ SEM) duration of social exploration (seconds) of the different treatment groups. Plus sign indicates couples of animals treated with the test compound; minus sign indicates couples treated with the corresponding vehicle. N=6–8 couples per treatment group. One- or two-way ANOVA with Tukey post-hoc test, ***$p<0.001$, different from vehicle.

alpha-flupenthixol altered the effect of the lowest effective dose of amphetamine (0.2 mg/kg) on social play. Pretreatment with RX821002 (0.2 mg/kg) blocked the effects of amphetamine on social play behavior (Fig. 1d, e). After saline pretreatment, amphetamine significantly reduced pinning and pouncing frequencies, whereas no significant differences between amphetamine- and vehicle-treated rats were found after pretreatment with RX821002 [pinning: $F(RX)1,28=18.09$, $p < 0.001$; $F(amph)1,28=12.72$, $p =0.001$; $F(RX\times amph)1,28= 7.29$, $p =0.01$; pouncing: $F (RX)1,28=5.94$, $p =0.02$; $(amph)1,28=12.86$, $p =0.001$; $F(RX\times amph)1,28=23.75$, $p < 0.001$]. RX821002 reduced social exploration, but amphetamine did not influence this effect [social exploration: $F(RX)1,28=8.40$, $p =0.01$; $F(amph)1,28=0.36$, NS; $F(RX\times amph)1,28=0.21$, NS; Fig. 1f]. Pretreatment with alpha-flupenthixol (flup; 0.125 mg/kg) did not affect the reduction in pinning and pouncing induced by amphetamine [pinning: $F(flup)1,20=0.42$, NS; $F(amph)1,20=38.57$, $p <0.001$; $F(flup\times amph)1,20=0.22$, NS; pouncing: $F(flup)1,20=0.28$, NS; $F(amph)1,20=30.31$, $p <0.001$; $(flup\times amph)1,20=0.01$, NS; Fig. 1g–h]. In this experiment, amphetamine-treated rats spent more time in social exploration than vehicle-treated animals [social exploration: $F(flup)1,20=0.30$, NS; $F(amph)1,20=5.71$, $p =0.03$; $F(flup\times amph)1,20=0.001$, NS; Fig. 1i].

The play-suppressant effects of cocaine are not blocked by administration of dopamine, noradrenaline, or 5-HT receptor antagonists

Cocaine (5.0–7.5 mg/kg) reduced pinning [$F(coc)4,35=8.91$, $p <0.001$] and pouncing [$F (coc)4,35=10.12$, $p <0.001$; Fig. 2a, b], whereas 2.5 mg/kg cocaine increased social exploration [$F(coc)4,35=5.86$, $p =0.001$; Fig. 2c]. Since pretreatment with the RX821002, but not alpha-flupenthixol, blocked the effects of methylphenidate (Vanderschuren et al. 2008) and amphetamine (above) on social play, we next investigated whether these drugs also altered the effect of cocaine on social play. The reduction in social play induced by the lowest effective dose of cocaine (5.0 mg/kg) was not altered by pretreatment with RX821002 [0.2 mg/kg, pinning: $F(RX)1,31=0.90$, NS; $F(coc)1,31=71.00$, $p <0.001$; $F(RX\times coc)1,31=0.15$, NS; pouncing: $F(RX)1,31=0.90$, NS; $F(coc)1,31=76.78$, $p <0.001$; $F(RX\times coc)1,31=0.16$, NS; social exploration: $F(RX)1,31= 0.99$, NS; $F(coc)1,31=1.45$, NS; $F(RX\times coc)1,31=0.04$, NS; Fig. 2d–f] or alpha-flupenthixol [0.125 mg/kg, pinning: $F(flup)1,20=0.26$, NS; $F(coc)1,20=42.11$, $p <0.001$; $F(flup\times coc)1,20=0.37$, NS; pouncing: $F (flup)1,20=0.45$, NS; $F(coc)1,20=37.66$, $p <0.001$; $F(flup\times coc)1,20=0.32$, NS; social exploration: $F(flup)1,20=0.82$, NS; $F(coc)1,20=3.42$, NS, $F(flup\times coc)1,20=0.85$, NS; Fig. 2g–i].

Next, we assessed the involvement of 5-HT receptor stimulation in the play-suppressant effect of cocaine. Neither the 5-HT1/2 receptor antagonist methysergide [mts; 2 mg/kg, pinning: $F(mts)1,28=0.30$, NS; $F(coc)1,28=44.00$, $p <0.001$; $F(mts\times coc)1,28=0.19$, NS; pouncing: $F(mts)1,28=0.20$, NS; $F(coc)1,28=48.64$, $p <0.001$; $F(mts\times coc)1,28=0.29$, NS; Fig. 3a, b] nor the 5-HT2 receptor antagonist amperozide [apz; 0.5 mg/kg, pinning: $F(apz)1,20=1.50$, NS; $F(coc)1,20= 49.55$, $p <0.001$; $F(apz\times coc)1,20=0.57$, NS; pouncing: $F(apz)1,20=0.40$, NS; $F(coc)1,20=58.62$, $p <0.001$; $F(apz\times coc)1,20=0.03$, NS; Fig. 3c, d], the 5-HT3 receptor antagonist ondansetron [ond; 1.0 mg/kg, pinning: $F(ond)1,28=2.04$, NS; $F(coc)1,28=55.59$, $p <0.001$; $F(ond\times coc)1,28=1.22$, NS; pouncing: $F(ond)1,28=1.27$, NS; $F(coc)1,28=62.68$, $p < 0.001$; $F(ond\times coc)1,28=0.42$, NS; Fig. 3e, f], the 5-HT1a receptor antagonist WAY100365 [way; 1 mg/kg, pinning: $F(way)1,28=3.99$, NS; $F(coc)1,28=68.00$, $p <0.001$; $F(way\times coc)1,28=3.50$, NS; pouncing: $F (way)1,28=2.66$, NS; $F(coc)1,28=96.05$, $p <0.001$; $F(way\times coc)1,28=3.08$, NS; Fig. 3g, h], or the 5-HT2a receptor antagonist M100907 (m100; 0.2 mg/kg, Fig. 3i, j) altered the effect of cocaine on social play, with no effect on social exploration (Table 1). M100907 itself reduced

pinning [F(m100)1,28=4.77, p = 0.04; F(coc)1,28=17.26, p <0.001; (m100×coc)1,28=4.77, p =0.04; Fig. 3i], but not pouncing [F(m100)1,28=1.98, NS; F(coc)1,31=37.71, p <0.001; F(m100×coc)1,28=0.81, NS; Fig. 3j] or social exploration (Table 1), whereas ondansetron altered social exploration (Table 1).

We then hypothesized that the effect of cocaine is mediated by redundant monoaminergic mechanisms. To test this possibility, we investigated the effect of pretreatment with combinations of two or three monoamine receptor antagonists on the play-suppressant effect of cocaine. Pretreatment with a combination of RX821002 (0.2 mg/kg) and methysergide (2 mg/ kg, Fig. 4a, b), a combination of alpha-flupenthixol (0.125 mg/kg) and methysergide (2 mg/kg, Fig. 4c, d), or a combination of RX821002 (0.2 mg/kg), alpha-flupenthixol (0.125 mg/kg), and methysergide (2 mg/kg, Fig. 4e, f) did not affect the reduction in pinning F(mts+rx)1,25=0.57, NS; F(coc)1,25=16.59, p <0.001; F(mts+rx×coc)1,25=0.12, NS; F(flup+mts)1,28=1.39, NS; F(coc)1,28=50.56, p

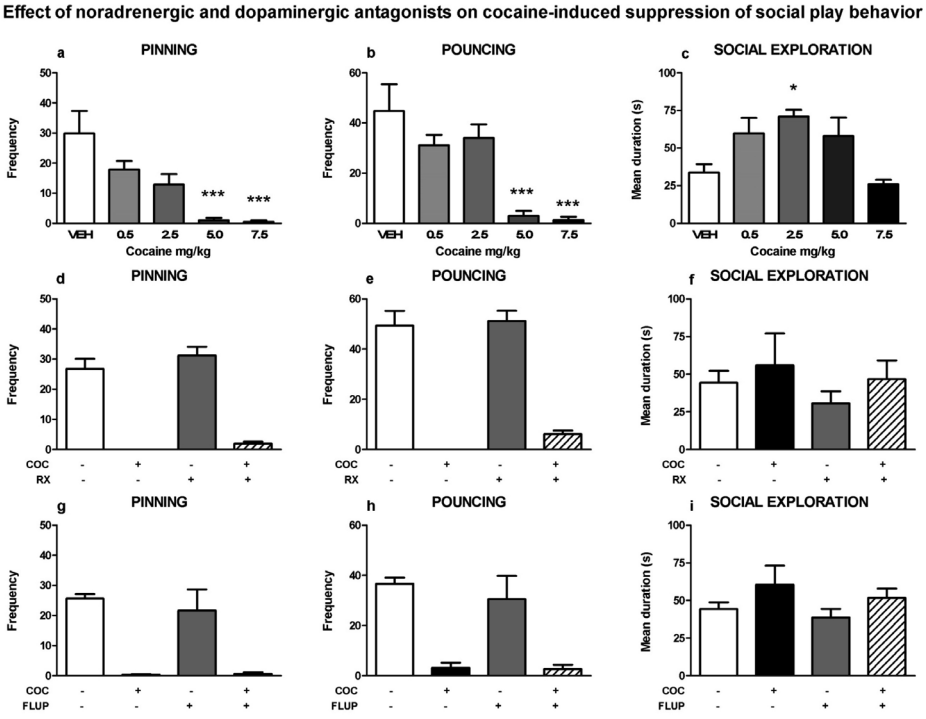

Fig. 2 Effects of noradrenaline and dopamine receptor antagonists on cocaine-induced suppression of social play behavior. Cocaine (coc, s.c.) dose-dependently suppressed pinning (a) and pouncing (b) and increased social exploration (c). The alpha-2 adrenoreceptor antagonist RX821002 (rx, 0.2 mg/kg, i.p.) and the dopamine receptor antagonist alpha-flupenthixol (flup, 0.125 mg/kg, i.p.) did not counteract the effects of cocaine (COC, 5 mg/kg) on pinning (d, g) and pouncing (e, h). Social exploration was unaffected by the treatments (f,i). Bars show the frequency of pinning and pouncing and the duration of social exploration (seconds) of the different treatment groups (mean + SEM). Plus sign indicates couples of animals treated with the test compound; minus sign indicates couples treated with the corresponding vehicle. N=4–8 couples per treatment group. One- or two-way ANOVA with Tukey post-hoc test, *p <0.05, ***p <0.001, different from vehicle.

<0.001; F(flup+mts×coc)1,28=1.39, NS; F(rx+flup+mts)1,28=0.15, NS; F(coc)1,28=27.47, p <0.001; F(rx+flup+mts×coc)1,28= 0.35, NS] and pouncing [F (mts+rx)1,25=0.82, NS; F(coc)1,25=17.99, p <0.001; F(mts+rx×coc)1,25=0.14, NS; F(flup+mts)1,28=1.37, NS; F(coc)1,20=51.51, p <0.001; F(flup+mts×coc)1,28=1.37, NS; F(rx+flup+mts)1,28=1.47, NS; F(coc)1,28=30.57, p <0.001; F(rx+flup+mts×coc)1,28=0.06, NS], induced by cocaine (5.0 mg/kg). These drug combinations did not affect social exploration (Table 1).

The play-suppressant effects of cocaine are mediated by simultaneous blockade of dopamine, noradrenaline, and 5-HT neurotransmission

The data presented in Figs. 2, 3, and 4 did not identify the dopamine, noradrenaline, or 5-HT receptor mechanism through which cocaine exerts its effect on social play. To test whether monoamine reuptake is at all involved in the effect of cocaine, we investigated the effects of combined subeffective doses of cocaine and monoamine reuptake inhibitors on social play. The effect of a subeffective dose of cocaine (0.5 mg/kg)on pinning and pouncing was not changed by treatment with either a subeffective dose of the 5-HT reuptake inhibitor fluoxetine [f3; 3 mg/kg, pinning: F(f3)1,27=2.44, NS; F(coc)1,27=0.04, NS; F(f3×coc)1,28=0.17, NS; pouncing: F(f3)1,27=2.41, NS; F(coc)1,28=0.05, NS; F(f3×coc)1,27= 0.11, NS; Fig. 5a, b] or by a combination of subeffective doses of the 5-HT reuptake inhibitor fluoxetine (3 mg/kg) and the dopamine reuptake inhibitor GBR12909 [g3; 3 mg/kg, pinning: F(f3+g3)1,28=0.30, NS; F(coc)1,28=1.23, NS; F(f3+g3×coc)1,28=0.09, NS; pouncing: F(f3+g3)1,28=0.10, NS; F (coc)1,28=0.68, NS; F (f3+g3×coc)1,28=0.68, NS; Fig. 5c, d]. Combined administration of fluoxetine, GBR12909, and the noradrenaline reuptake inhibitor atomoxetine (a0.1; 0.1 mg/kg) reduced pinning [F(f3+g3+a0.1)1,26=20.08, p <0.001; F(coc)1,26=3.23, NS] and pouncing [F(f3+g3+a0.1)1,26=23.72, p <0.001; F(coc)1,26=2.67, NS; F(f3+g3+a0.1×coc)1,26=3.51, NS], and increased social exploration (Table 1). Importantly, a significant interaction between the combination of reuptake inhibitors and a subeffective dose of cocaine was found for pinning [F(f3+g3+a0.1×coc)1,26=4.46, p =0.05; Fig. 5e, f]. Post-hoc analyses revealed that pinning was reduced in animals treated with the reuptake inhibitors plus a subeffective dose of cocaine compared to the other groups (Fig. 5e). These results suggest that combined blockade of the reuptake of dopamine, noradrenaline, and 5-HT underlies the effect of cocaine on social play behavior in rats.

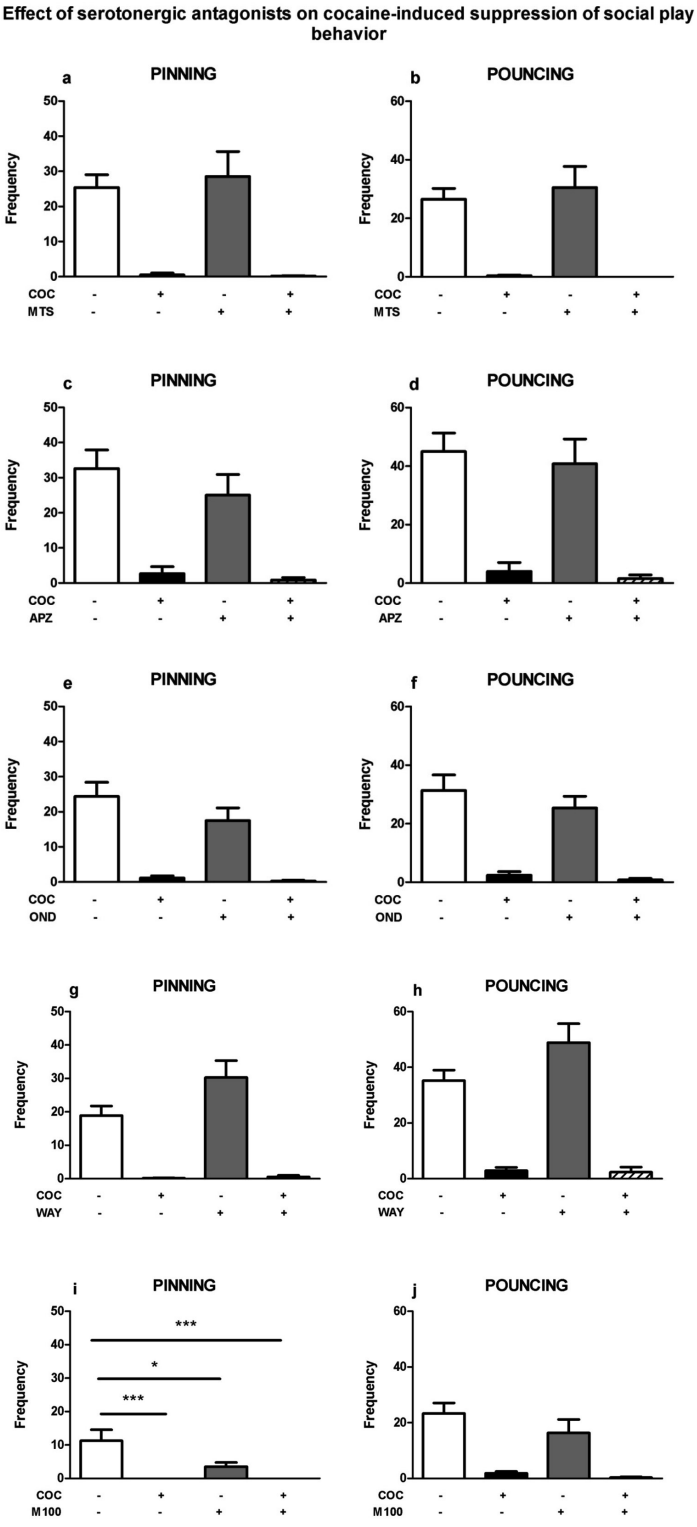

Fig. 3:
Effects of 5-HT receptor antagonists on cocaine-induced sup-pression of social play behavior. 5-HT antagonists (methysergide: mts, 5HT1/2 receptor antagonist, 2 mg/kg, s.c.; amperozide: apz, 5HT2 receptor antagonist, 0.5 mg/kg, i.p.; ondansetron: ond, 5HT3 receptor antagonist, 1.0 mg/kg, i.p.; WAY100365: way, 5HT1a receptor antagonist, 0.1 mg/kg, s.c.; M100907: m100, 5HT2a receptor antagonist, 0.2 mg/kg, s.c.) did not counteract the suppression of social play behavior induced by cocaine (coc, 5 mg/kg, s.c.): pinning (a, c, e, g, i) and pouncing (b, d, f, h, j). Bars show the frequency (mean + SEM) of pinning and pouncing of the different treatment groups. Plus sign indicates couples of animals treated with the test compound; minus sign indicates couples treated with the corresponding vehicle. N =5–8 couples per treatment group. Two-way ANOVA with Tukey post-hoc test, *p <0.05, ***p <0.001.

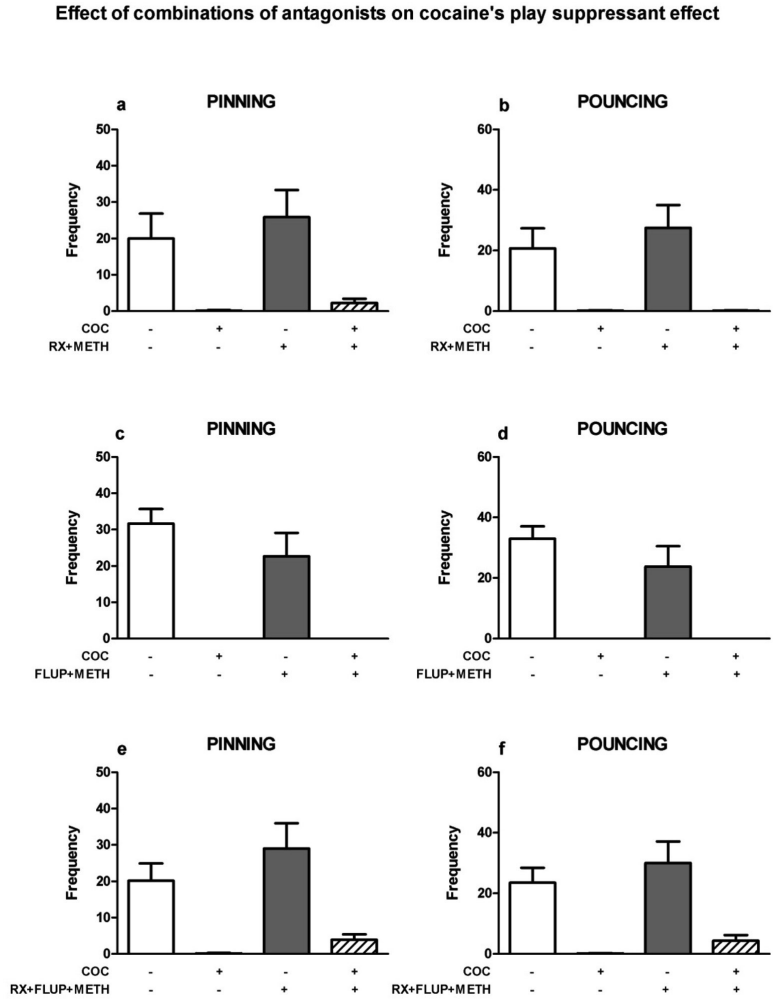

Fig. 4: Effects of combinations of monoamine receptor antagonists on the play suppressant effects of cocaine (coc, 5 mg/kg, s.c.). A combination of RX821002 (rx, α2-adrenoreceptor antagonist, 0.2 mg/kg, i.p.) + methysergide (mts, 5-HT1/2 receptor antagonist, 2 mg/kg, s.c.), a combination of α-flupenthixol (flup, dopamine receptor antagonist, 0.125 mg/kg, i.p.) + methysergide (mts, 5-HT1/2 receptor antagonist, 2 mg/kg, s.c.), and a combination of RX821002 (rx, α2-adrenoreceptor antagonist, 0.2 mg/kg, i.p.)+α-flupenthixol (flup, dopamine receptor antagonist, 0.125 mg/kg, i.p.) + methysergide (mts, 5-HT1/2 receptor antagonist, 2 mg/kg, s.c.) did not antagonize the reduction in pinning (a, c, e) and pouncing (b, d, f) induced by cocaine. Bars show the frequency (mean + SEM) of pinning and pouncing of the different treatment groups. Plus sign indicates couples of animals treated with the test compounds; minus sign indicates couples treated with the corresponding vehicles, N=7–8 couples per treatment group.

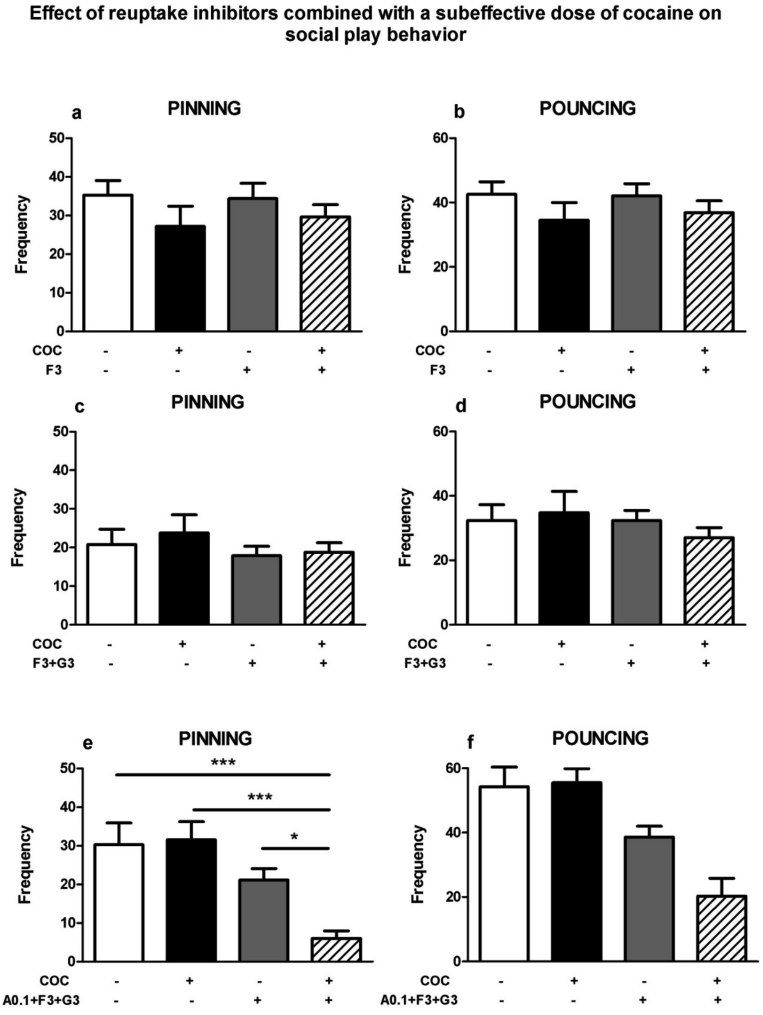

Fig. 5: Effect of (combinations of) subeffective doses of monoamine reuptake inhibitors and a subeffective dose of cocaine on social play. Combined administration of a subeffective dose of fluoxetine (f3, serotonin reuptake inhibitor, 3 mg/kg, s.c.) and a subeffective dose of cocaine (coc, 0.5mg/kg, s.c.) or combined administration of a subeffective dose of fluoxetine (f3, serotonin reuptake inhibitor, 3 mg/kg, s.c.) and GBR12909 (g3, dopamine reuptake inhibitor, 3 mg/kg, s.c.) together with a subeffective dose of cocaine (coc, 0.5 mg/kg, s.c.) had no effects on pinning (a, c) and pouncing (b, d). Combined administration of a subeffective dose of fluoxetine (f3, serotonin reuptake inhibitor, 3 mg/kg, s.c.) + GBR12909 (g3, dopamine reuptake inhibitor, 3 mg/kg) + atomoxetine (a0.1, noradrenaline reuptake inhibitor, 0.1 mg/kg, i.p.) together with a subeffective dose of cocaine (COC, 0.5 mg/kg, s.c.) significantly reduced pinning (e) but not pouncing (f). Bars show the frequency (mean + SEM) of pinning and pouncing of the different treatment groups. Plus sign indicates couples of animals treated with the test compounds; minus sign indicates couples treated with the corresponding vehicles. N=6–8 couples per treatment group. Two-way ANOVA with Tukey post-hoc test, *p <0.05, ***p < 0.001.

Table 1: Social exploration data and statistics

Drug class	Drug	Mean ± SEM	Statistics
5-HT antagonists	**Methysergide** (mts, 5HT1/2 antagonist, 2 mg/kg, s.c.)	veh-veh: 294,49 ± 35,13 veh-coc: 341,31 ± 22,99 mts-veh: 283,20 ± 28,67 mts-coc: 305,89 ± 39,85	$F(mts)_{1,28}$=0.52, NS $F(coc)_{1,28}$=1.16, NS $F(mts \times coc)_{1,28}$=0.14, NS
	Amperozide (apz, 5HT2 antagonist, 0.5 mg/kg, i.p.)	veh-veh: 44,98 ± 11,88 veh-coc: 44,49 ± 7,75 apz-veh: 38,66 ± 6,23 apz-coc: 40,86 ± 7,32	$F(apz)_{1,20}$=0.30, NS $F(coc)_{1,20}$=0.01, NS $F(apz \times coc)_{1,20}$=0.02, NS
	Ondansetron (ond, 5HT3 antagonist, 1.0 mg/kg, i.p.)	veh-veh: 212,67 ± 12,22 veh-coc: 274,41 ± 17,12 ond-veh: 239,98 ± 16,20 ond-coc: 219,10 ± 23,49	$F(ond)_{1,28}$=5.43, p= 0.03 $F(coc)_{1,28}$=1.33, NS $F(ond \times coc)_{1,28}$=0.62, NS
	WAY100365 (way, 5HT1a antagonist, 0.1 mg/kg, s.c.)	veh-veh: 92,18 ± 10,94 veh-coc: 74,95 ± 8,15 way-veh: 70,17 ± 10,08 way-coc: 56,25 ± 13,29	$F(way)_{1,28}$=3.12, NS $F(coc)_{1,28}$=0.19, NS $F(way \times coc)_{1,28}$=0.02, NS
	M100907 (m100, 5HT2a antagonist, 0.2 mg/kg, s.c.)	veh-veh: 147,70 ± 13,91 veh-coc: 137,75 ± 23,40 m100-veh: 186,13 ± 25,88 m100-coc: 105,48 ± 17,24	$F(m100)_{1,28}$=0.02, NS $F(coc)_{1,28}$=4.81, NS $F(m100 \times coc)_{1,28}$=2.93, NS
Combinations of antagonists	**RX821002** (rx, α2-adrenoreceptor antagonist, 0.2 mg/kg, i.p.) **methysergide** (meth, 5HT1/2 antagonist, 2 mg/kg, s.c.)	veh-veh: 304,00 ± 25,72 veh-coc: 216,22 ±19,02 rx + meth-veh: 281,77 ± 29,44 rx + meth-coc: 281,28 ± 34,05	$F(rx + meth)_{1,25}$=0.59, NS $F(coc)_{1,25}$=2.51, NS $F(rx + meth \times coc)_{1,28}$=2.46, NS
	α-flupenthixol (flup, dopamine antagonist, 0.125 mg/kg, i.p.) **methysergide** (meth, 5HT1/2 antagonist, 2 mg/kg, s.c.)	veh-veh: 282,61 ± 27,66 veh-coc: 267,1825 ± 21,71 flup + meth-veh: 314,69 ± 34,75 flup + meth-coc: 298,90 ± 27,10	$F(flup + meth)_{1,28}$=1.28, NS $F(coc)_{1,28}$=0.31, NS $F(flup + meth \times coc)_{1,28}$=0.00, NS
	RX821002 (rx, α2-adrenoreceptor antagonist, 0.2 mg/kg, i.p.) **α-flupenthixol** (flup, dopamine antagonist, 0.125 mg/kg, i.p.) **methysergide** (meth, 5HT1/2 antagonist, 2 mg/kg, s.c.)	veh-veh: 291,49 ± 22,24 veh-coc: 264,89 ± 22,06 rx + flup + meth-veh: 326,00 ± 41,38 rx + flup + meth-coc: 368,29 ± 43,57	$F(rx + flup + meth)_{1,28}$=4.14, NS $F(coc)_{1,28}$=0.05, NS $F(rx + flup + meth \times coc)_{1,28}$=1.03, NS
Combinations of reuptake inhibitors	**fluoxetine** (f3, SSRI*, 3 mg/kg, s.c.)	veh-veh: 243,06 ± 27,31 veh-coc: 339,91 ± 20,70 f3-veh: 319,86 ± 39,745 f3-coc: 322,99 ± 31,36	$F(f3)_{1,27}$=3.40, NS $F(coc)_{1,27}$=1.79, NS $F(f3 \times coc)_{1,27}$=0.65, NS
	fluoxetine (f3, SSRI, 3 mg/kg, s.c.) **GBR12909** (g3, DARI# 3 mg/kg, s.c.)	veh-veh: 241,31 ± 19,18 veh-coc: 256,80 ± 20,32 f3 + g3-veh: 270,42 ± 35,49 f3 + g3-coc: 282,83 ± 26,07	$F(f3 + g3)_{1,28}$=1.12, NS $F(coc)_{1,28}$=0.29, NS $F(f3 + g3 \times coc)_{1,28}$=0.00, NS
	fluoxetine (f3, SSRI, 3 mg/kg, s.c.) **GBR12909** (g3, DARI 3 mg/kg) **atomoxetine** (a0.1, NARI$, 0.1 mg/kg, i.p.)	veh-veh: 291,49 ± 20,80 veh-coc: 264,89 ± 20,64 f3 + g3 + a0.1-veh: 326,00 ± 38,70 f3 + g3 + a0.1-coc: 368,29 ± 40,76	$F(f3 + g3 + a0.1)_{1,26}$=9.35, p= 0.01 $F(coc)_{1,26}$= 0.03, p= NS $F(f3 + g3 + a0.1 \times coc)_{1,26}$=1.85, p= NS

*SSRI: selective serotonin reuptake inhibitor, #DARI: dopamine reuptake inhibitor, $NARI: noradrenaline reuptake inhibitor.

Discussion

The present study investigated the pharmacological mechanisms underlying the effects of amphetamine and cocaine on social play behavior. We found that low doses of amphetamine and cocaine suppressed social play behavior in adolescent rats. These effects were behaviorally specific, since both psychostimulants did not consistently alter social exploratory behavior. The effects of amphetamine on social play depended on stimulation of alpha-2 noradrenaline but not dopamine receptors. In contrast, the effects of cocaine on social play depended on simultaneous increases in dopamine, noradrenaline, and 5-HT neurotransmission.

We have previously shown that the reduction in social play induced by the dopamine and noradrenaline reuptake inhibitor methylphenidate was reversed by pretreatment with the alpha-2 adrenoceptor antagonist RX821002, but not the alpha-1 adrenoceptor antagonist prazosine, the beta-adrenoceptor antagonist propranolol or the dopamine receptor antagonist alpha-flupenthixol. Furthermore, the play-suppressant effect of methylphenidate was mimicked by the selective noradrenaline reuptake inhibitor atomoxetine but not by the selective dopamine reuptake inhibitor GBR12909 or the dopamine receptor agonist apomorphine (Vanderschuren et al. 2008). In line with these findings, the play-suppressant effects of amphetamine were blocked by RX821002, but not alpha-flupenthixol. These findings are consistent with previous observations that the dopamine receptor antagonist haloperidol, the alpha-1 adrenoreceptor antagonist phenoxybenzamine, the beta-adrenoreceptor antagonist propranolol, and the combined alpha-1 and dopamine D2-receptor antagonist chlorpromazine were ineffective in counteracting the effects of amphetamine on social play behavior (Beatty et al. 1984), and that haloperidol and chlorpromazine did not counteract the disruptive effects of amphetamine and cocaine on social behavior in primates (Miczek and Yoshimura 1982). Together, these data show that the play suppressant effects of amphetamine, like methylphenidate, are mediated by activation of alpha-2 adrenoreceptors, and are independent of dopaminergic neurotransmission.

Cocaine inhibits the reuptake of dopamine, 5-HT and, to a lesser extent, noradrenaline (Heikkila et al. 1975; Ritz and Kuhar 1989; Rothman et al. 2001). We found that administration of the dopamine receptor antagonist alpha-flupenthixol, the alpha-2 adrenoreceptor antagonist RX821002, or the 5-HT receptor antagonists amperozide (5-HT2), methysergide (5-HT1/2), ondansetron (5-HT3), WAY100365 (5-HT1a), and M100907 (5-HT2a) did not antagonize the reduction in social play behavior induced by cocaine, indicating that it is not likely that one single monoamine receptor mechanism underlies this effect of cocaine. In keeping with these findings, it has previously been found that the reduction in social interaction induced by cocaine in rats was not antagonized by pretreatment with amperozide (Rademacher et al. 2002), and that cocaine-induced social deficits in primates were not altered by pretreatment with chlorpromazine or haloperidol (Miczek and Yoshimura 1982). Interestingly, combinations of methysergide and RX821002, methysergide and alpha-flupenthixol, or a combination of methysergide, RX821002, and alpha-flupenthixol did not counteract the effect of cocaine on social play, which suggests that the play-suppressant effect of cocaine is not exerted through redundant monoamine receptor mechanisms. Since at least 14 subtypes of 5-HT receptors exist (Boess and Martin 1994), the possibility remains that a combination of drugs antagonizing different 5-HT receptors is effective in counteracting the play-suppressant effects of cocaine. To identify which monoamines were involved in the effects of cocaine on social play, we tested the effects of subeffective doses of monoamine reuptake inhibitors administered in combination with a subeffective dose of cocaine. We found that a combination of subeffective

doses of the dopamine, noradrenaline, and serotonin reuptake inhibitors GBR12909, atomoxetine, and fluoxetine modestly reduced social play, which was potentiated by a subeffective dose of cocaine. However, fluoxetine alone or a combination of fluoxetine and GBR12909 were ineffective, and co-administration of a subeffective dose of cocaine with either fluoxetine or fluoxetine plus GBR12909 did not reduce social play either. Since cocaine has much lower affinity for the noradrenaline transporter than for the dopamine or 5-HT transporter (Ritz and Kuhar 1989; Rothman et al. 2001), we did not test the effects of atomoxetine combined with fluoxetine,GBR12909, and/or cocaine. We have previously shown that atomoxetine and fluoxetine, at doses higher than those used here, reduced play behavior, whileGBR12909 did not alter social play (Homberg et al. 2007; Vanderschuren et al. 2008). Together, these findings suggest that simultaneous increases in synaptic concentrations of all three monoamines underlie the inhibitory effect of cocaine on social play behavior, although the specific receptors involved remain to be elucidated. Intracranial infusion studies may be helpful in clarifying the mechanism of action by which cocaine inhibits social play behavior.

Social play behavior is a highly vigorous form of social behavior with a strong locomotor component (Panksepp et al. 1984; Vanderschuren et al. 1997; Pellis and Pellis 2009), and amphetamine and cocaine are known to evoke locomotor hyperactivity (Wise and Bozarth 1987). It may therefore be that the psychostimulant-induced suppression of play is the result of behavioral competition, i.e., that the exaggerated hyperactivity induced by amphetamine and cocaine prevents the animals from engaging in a meaningful social interaction. However, we think that this possibility is unlikely, for two reasons. First, the reduction in social play behavior was induced by lower doses of amphetamine and cocaine than those typically used to induced psychomotor hyperactivity (e.g., Sahakian et al. 1975; White et al. 1998), even when taking into account that the sensitivity to psychostimulant drugs may be different for periadolescent vs adult rats (for review, see Schramm-Sapyta et al. 2009). Second, the psychomotor hyperactivity induced by amphetamine and cocaine strongly depends on dopaminergic neurotransmission (e.g., Kelly et al. 1975; White et al. 1998), whereas their effects on social play behavior are dopamine-independent (Beatty et al. 1984; Vanderschuren et al. 2008; present study). Third, we have previously shown that the effects of methylphenidate on social play and its psychomotor stimulant effects can be dissociated (Vanderschuren et al. 2008).

One may argue that the play-suppressant effects of amphetamine and cocaine reflect an occlusion of social reward Thus, the positive subjective effects of the psychostimulants could substitute for rewarding effects of social play, so that the animals would no longer need to seek out a social source of positive emotions. Along similar lines, it has been suggested that amphetamine may substitute for the rewarding effects of pair bond formation in prairie voles, and vice versa (Liu et al. 2010, 2011).We do not think that this is the explanation for the present findings, however, for two reasons. First, whereas the rewarding effects of psychostimulant drugs rely on dopaminergic mechanisms (e.g., Veeneman et al. 2011, 2012; for reviews, see Wise 2004; Pierce and Kumaresan 2006), their effects on social play do not. Second, non-psychostimulant drugs of abuse, such as opiates, nicotine, and ethanol, as well as drugs that enhance endocannabinoid signaling, actually enhance social play (for reviews, see Trezza et al. 2010; Siviy and Panksepp 2011). It would then be difficult to conceive why some euphorigenic drugs increase whereas others reduce social play if their positive subjective effects would substitute for those of social play behavior.

An alternative interpretation of the play-suppressant effect of amphetamine and cocaine is that these psychostimulants are anxiogenic (File and Seth 2003). However, amphetamine and cocaine did not affect social exploratory behavior, which is the

standard parameter used in the social interaction test of anxiety (File and Seth 2003). Moreover, pharmacological analysis of social play behavior has consistently shown that anxiolytic or anxiogenic drugs do not invariably increase or reduce social play, respectively (Vanderschuren et al. 1997). On the contrary, our recent experiments have shown that doses of nicotine and ethanol, that increase social play in both familiar and unfamiliar environments, did not affect anxiety as tested on the elevated plus maze. Conversely, the standard anxiolytic drug diazepam, which did have an anxiolytic effect on the elevated plus maze, reduced social play, but increased social exploration (Trezza et al. 2009). Thus, it is highly unlikely that the reduction in social play induced by cocaine and amphetamine is secondary to an anxiogenic effect of these drugs.

Several hypotheses can be put forward to explain the effects of psychostimulants on social play behavior. First, on the basis of their effectiveness in attention-deficit/hyperactivity disorder, and the comparable pharmacological profile of the effects of amphetamine and methylphenidate on social play behavior (Vanderschuren et al. 2008; present study) and the stop signal task (Eagle and Baunez 2010), it can be hypothesized that the play suppressant effects of psychostimulants are the result of enhanced or exaggerated behavioral inhibition. That is, through increasing inhibitory control over behavior, psychostimulant drugs may enhance attention for non-social stimuli in the environment, causing the animals to engage less in vigorous playful interactions, that are accompanied by reduced attention for, potentially important, environmental stimuli. Second, and in contrast, psychostimulant-induced increases in tonic noradrenergic neurotransmission may promote disengagement from ongoing (playful) behaviors and facilitate the switching of behaviors (Aston-Jones and Cohen 2005). This may impact social play behavior that requires appropriate behavioral responses from both partners of a dyad. Third, the play-suppressant effect of psychostimulants can be explained by the notion that they increase the intensity of behavior. As not all behaviors can be intensified to the same degree, this causes a narrowing down of the behavioral repertoire with simple behaviors being favored over complex behaviors, such as social play (Lyon and Robbins 1975). One needs to bear in mind though that the present findings add a layer of complexity over the possible behavioral mechanisms of psychostimulant-induced suppression of social play. That is, since amphetamine and methylphenidate reduce social play through a distinct pharmacological mechanism of action than cocaine does, it is well conceivable that the behavioral underpinnings of these effects also differ between different psychostimulant drugs. In this regard, it is worth mentioning that amphetamine and cocaine suppressed pouncing (i.e., play solicitation) and pinning (the most prominent response to pouncing in rats this age, i.e., response to play solicitation) with comparable potency. This is somewhat in contrast with a previous study that showed that amphetamine affects pouncing at lower doses than responding to pouncing by rotating to supine (i.e., pinning; Field and Pellis 1994). It may therefore well be that subcomponents of social play (i.e., pouncing, and the different possible defense strategies) are also differentially affected by amphetamine and cocaine.

In summary, we show here that amphetamine, like methylphenidate, exerts its play-suppressant effect through stimulation of alpha-2 adrenoreceptors. Cocaine, on the other hand, exerts its effect through simultaneous increases in dopamine, noradrenaline, and 5-HT neurotransmission. Positive social interactions in young individuals are essential for emotional well-being and for social and cognitive development. Moreover, the inability to assign a positive subjective value to social stimuli may be a key process in the pathophysiology of childhood and adolescent psychiatric disorders characterized by aberrant social interactions. Our present study advances our understanding of how psychostimulant drugs negatively impact upon social interactions in young individuals.

This work has relevance for our understanding of the neural mechanisms of normal social development, as well as childhood and adolescent psychiatric disorders with a prominent social dimension, such as autism, disruptive behavior disorders, and schizophrenia. Moreover, given the emergence of drug use during early adolescence, increasing our understanding of how psychostimulant drugs affect social behavior has obvious repercussions for the etiology of substance abuse disorders.

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Chapter 3

Methylphenidate and atomoxetine inhibit social play behavior in rats through prefrontal, amygdala and habenula mechanisms

In preparation

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Abstract

Positive social interactions during the juvenile and adolescent phases of life, in the form of social play behavior, are essential for proper social and cognitive development. We have previously shown that treatment with methylphenidate suppressed social play behavior in rats, through a noradrenergic mechanism. The aim of the present study was to identify the neural substrates of the play-suppressant effect of methylphenidate. Methylphenidate is thought to exert its effects on cognition and behavior via limbic corticostriatal systems. Therefore, methylphenidate was infused into prefrontal and orbitofrontal cortical regions and the nucleus accumbens shell, amygdala and habenula, regions also implicated in social play behavior. Infusion of methylphenidate (5.0 μg) into the anterior cingulate cortex, infralimbic cortex, amygdala and habenula inhibited social play, while social exploratory behavior and locomotor activity were unaffected. The noradrenergic nature of the reduction in social play by methylphenidate was confirmed by the observation that infusion of the noradrenaline reuptake inhibitor atomoxetine (10.0 μg) into these same regions also reduced social play. Methylphenidate administration into the prelimbic, medial/ventral orbitofrontal and ventrolateral orbitofrontal cortex or nucleus accumbens shell was ineffective. Our data show that the inhibitory effects of methylphenidate and atomoxetine on social play are mediated through a network of prefrontal and limbic subcortical regions implicated in cognitive control and emotional processes. These findings increase our understanding of the neural underpinnings of this developmentally important social behavior, as well as the mechanism of action of methylphenidate and atomoxetine, widely used treatments for childhood and adolescent attention-deficit/hyperactivity disorder.

Introduction

Social play behavior is a highly vigorous form of social interaction, abundantly expressed in juvenile and adolescent animals, including humans (Panksepp et al., 1984; Vanderschuren et al., 1997; Pellis and Pellis, 2009). It is thought that social play behavior plays a critical role in social, cognitive and emotional development (Van den Berg et al., 1999; Potegal and Einon, 1989; Baarendse et al., 2013; Vanderschuren and Trezza, 2013). Therefore, identifying the neural underpinnings of social play behavior increases our understanding of normal development as well as of the etiology of childhood and adolescent psychiatric disorders characterized by social impairments, such as autism and attention deficit/hyperactivity disorder (ADHD).

Investigation of the neural substrates of social play behavior has revealed that psychomotor stimulants, such as amphetamine and methylphenidate, profoundly inhibit social play behavior (Beatty et al., 1982; -1984; Thor and Holloway, 1983; Sutton and Raskin, 1986; Vanderschuren et al., 2008; Achterberg et al., 2013). We have previously shown that methylphenidate inhibits social play behavior through a noradrenergic mechanism, since the effect of methylphenidate was mimicked by the noradrenaline reuptake inhibitor atomoxetine and blocked by the α-2 adrenoceptor antagonist RX821002 (Vanderschuren et al., 2008). Interestingly, both methylphenidate and atomoxetine are widely used for the treatment of ADHD (Kutcher et al., 2004; Kratochvil et al., 2006). However, despite the therapeutic efficacy in ADHD, their mechanisms of action are incompletely understood.

We previously hypothesised that the effects of methylphenidate and atomoxetine on social play behavior were related to enhanced behavioral inhibition (Vanderschuren et al., 2008), since these drugs enhance several aspects of cognitive control in rats and humans (Chamberlain and Sahakian, 2007; Eagle et al., 2008; Pattij and Vanderschuren, 2008). The effects of methylphenidate and atomoxetine on behavioral inhibition are thought to be mediated through limbic corticostriatal circuits (Arnsten, 2011; del Campo et al., 2011). For example, administration of atomoxetine into the dorsal prelimbic cortex and orbitofrontal cortex improved stop-signal task performance (Bari et al., 2011), and atomoxetine administration into the nucleus accumbens shell reduced premature responding in the 5-choice serial reaction time task in rats (Economidou et al., 2012).

The prefrontal cortex, orbitofrontal cortex and nucleus accumbens shell have also been implicated in social play behavior (Bell et al., 2009; Panksepp et al., 1994; Pellis et al., 2006; Schneider and Koch, 2005; Van Kerkhof et al., 2013a; -2013b; Trezza et al., 2011a). Therefore, we investigated whether infusion of methylphenidate into prefrontal and orbitofrontal regions, the nucleus accumbens shell, amygdala, and the habenula inhibited social play. We included the amygdala and habenula because of their involvement in cognitive and emotional processes (Baxter and Murray, 2002; Phelps and LeDoux, 2005; Lecourtier and Kelly, 2007; Hikosaka, 2010), including social play behavior (Trezza et al., 2012; Van Kerkhof et al., 2013c), and their well-characterized noradrenergic innervation (Moore and Bloom, 1979; Gottesfeld, 1983; Unnerstall et al., 1984; Lecourtier and Kelly, 2007). To test whether the effect of methylphenidate was of noradrenergic origin, we also infused atomoxetine into brain regions in which methylphenidate inhibited social play behavior.

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 12/12h 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 legislation (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., 2011a; Trezza et al., 2012; Van Kerkhof et al., 2013b; -2013c). 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 anterior cingulate cortex (coordinates: anterior-posterior (AP) +2.6 mm from Bregma; medial-lateral (ML) ± 0.8 mm from the midline; dorsal-ventral (DV) -2.4 mm from skull surface), prelimbic cortex (coordinates: AP +2.6 mm from Bregma; ML ± 0.8 mm from the midline; DV -3.2 mm from skull surface), infralimbic cortex (coordinates: AP +2.6 mm from Bregma; ML ± 0.8 mm from the midline; DV -4.1 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., 2011a; -2012; Van Kerkhof et al., 2013b, 2013c) 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. Atomoxetine-HCL (Tocris Bioscience, Avonmouth, UK) was dissolved in 50% DMSO and 50% saline. In all regions 10.0 µg/0.3 µl was administered. Infusion procedures were as previously described (Trezza et al., 2011; -2012; Van Kerkhof et al., 2013b; -2013c). 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 drug or vehicle solution 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.

Behavioral testing

Experiments were performed, as previously described (Trezza and Vanderschuren, 2008; Vanderschuren et al., 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. On the third day, which was the first test day, rats were isolated for 2.5 h. Both rats in a pair were then simultaneously infused with either drug (methylphenidate oratomoxetine) or vehicle before testing. On the second test day, the animals were also isolated for 2.5 h, and treatments were reversed, so that animals that received drug (methylphenidate or atomoxetine) treatment on the first test day now received vehicle and vice versa. The first and second test day were separated by a wash-out day during which the animals received no treatment and were not tested. This within-subjects design was used in all experiments, except for experiment in which methylphenidate was infused into the amygdala, in which two independent groups of animals were used, that received either vehicle or methylphenidate.

Testing consisted of placing a pair of animals into the testing arena for 15 min. Behavior of the animals was recorded using a camera with zoom lens, video tape recorder and television monitor. The behavior of the rats was assessed using the Observer 5.1 software (Noldus Information Technology B.V., The Netherlands). The structure of social play behavior in rats has been previously described in detail (Baenninger, 1967; Bolles and Woods, 1964; Panksepp and Beatty, 1980; Pellis and Pellis, 1987; Pellis et al, 1989; Poole and Fish, 1975; for reviews see Panksepp et al, 1984; Pellis and Pellis, 1998; Trezza et al, 2010; Vanderschuren et al, 1997). In rats, a bout of social play behavior starts with one rat soliciting ('pouncing') another animal, by attempting to nose or rub the nape of its neck. The animal that is pounced upon can respond in different ways. If the animal that is pounced upon responds by evading, the soliciting rat may start to chase it, thus making another attempt to launch a play bout. The solicited animal may also rear towards the soliciting animal and the two animals may rapidly push, paw, and grab each other ('boxing'). If the animal that is pounced upon fully rotates to its dorsal surface, 'pinning' is the result, i.e. one animal lying with its dorsal surface on the floor with the other animal standing over it. From this position, the supine animal can initiate another play bout, by trying to gain access to the other animal's neck. Thus, during social play, pouncing is considered an index of play solicitation, while pinning functions as a releaser of a prolonged play bout (Panksepp and Beatty, 1980; Pellis and Pellis, 1987; Pellis et al, 1989; Poole and Fish, 1975). Pinning and pouncing frequencies can be easily quantified and are considered the most characteristic parameters of social play behavior in rats (Panksepp and Beatty, 1980). During the social encounter, animals may also display social behaviors not directly associated with play, such as sniffing or grooming the partner's body (Panksepp and Beatty, 1980; Vanderschuren et al, 1995). Since social play behavior in rats strongly depends on the playfulness of its partner (Pellis and McKenna, 1992; Trezza and Vanderschuren, 2008), in the present study, both animals in a play pair were similarly treated and a pair of animals was considered as one experimental unit. The following parameters were therefore scored per pair of animals:

Social behaviors related to play:
- Frequency of pinning
- Frequency of pouncing

Social behaviors unrelated to play:
- Time spent in social exploration: the total amount of time spent in non playful forms of social interaction (i.e., one animal sniffing or grooming any part of the partner's body).

Pinning, pouncing and other playful behaviors usually occur very rapidly and they are of short duration. Therefore, scoring their individual frequency is more informative than scoring their duration. Moreover, we have also found that pinning and pouncing are very reliable play parameters, that occur consistently and with considerable frequency during playful encounters (see also Panksepp and Beatty, 1980; Vanderschuren et al, 1995), whereas the occurrence of chasing and boxing can be quite variable.

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). 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 (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).

Statistical analysis

Pinning and pouncing frequencies and time spent on social exploration (s) were expressed as mean ± SEM. To assess the effect of methylphenidate and atomoxetine administration on social play behavior, 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 independent Student's t-test. Horizontal locomotor activity was expressed as mean ± SEM travelled distance (cm) in 5 min bins. The effects of methylphenidate and atomoxetine administration on locomotor activity were analysed using a two-way repeated measures ANOVA.

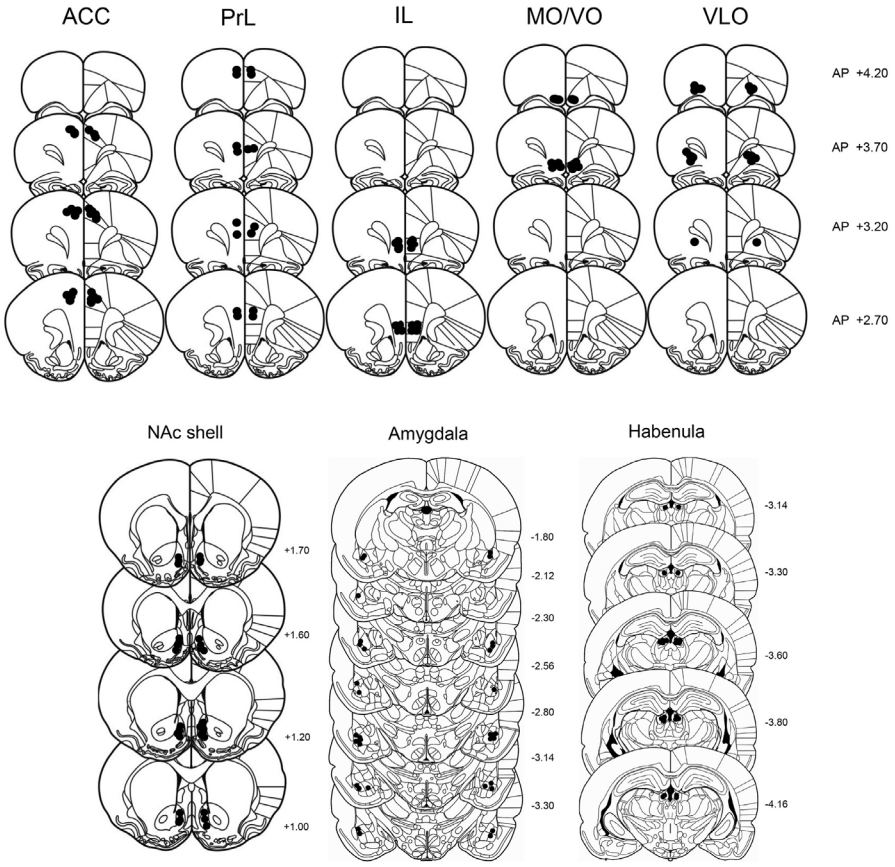

Fig. 1: Schematic representation of brain sections with microinjection placements for the methylphenidate experiments in the (A): anterior cingulate cortex (ACC), prelimbic cortex (PrL), infralimbic cortex (IL), medial/ventral orbitofrontal cortex (MO/VO), ventrolateral orbitofrontal cortex (VLO) and (B): nucleus accumbens shell (NAc shell), amygdala and habenula. AP= anterior-posterior level in mm from Bregma. Adapted from Paxinos and Watson (2007).

Results

Methylphenidate infusion into medial prefrontal but not orbitofrontal cortical regions inhibits social play

Infusion of methylphenidate into the anterior cingulate cortex reduced pinning (t=3.10, df=6, p=0.02, n=7) and pouncing (t=2.49, df=6, p=0.05) (Fig 2A-B). A trend towards an increase in social exploration (t=-2.24, df=6, p=0.07) but no effect on locomotor activity was found ($F_{treatment}(1,14)=0.43$, p=0.78; $F_{time}(5,70)=19.11$, p<0.001; $F_{time\,x\,treatment}(5,70)=0.49$, p=0.78, n=8 per treatment) (Fig. 2C-D). After infusion of methylphenidate into the infralimbic cortex, a reduction in pinning (t=2.46, df=11, p= 0.03, n=12) and pouncing (t=2.47, df=11, p=0.03) (Fig. 2E-F) but not social exploration (t=-1,16, df=11, p=0.13) or locomotor activity ($F_{treatment}(1,14)=0.43$, p=0.78; Ftime(5,70)=16.05, p<0.001; $F_{time\ x\ treatment}(5,70)=0.44$, p=0.84, vehicle n=7, methylphenidate n=9) (Fig. 2G-H) was found.

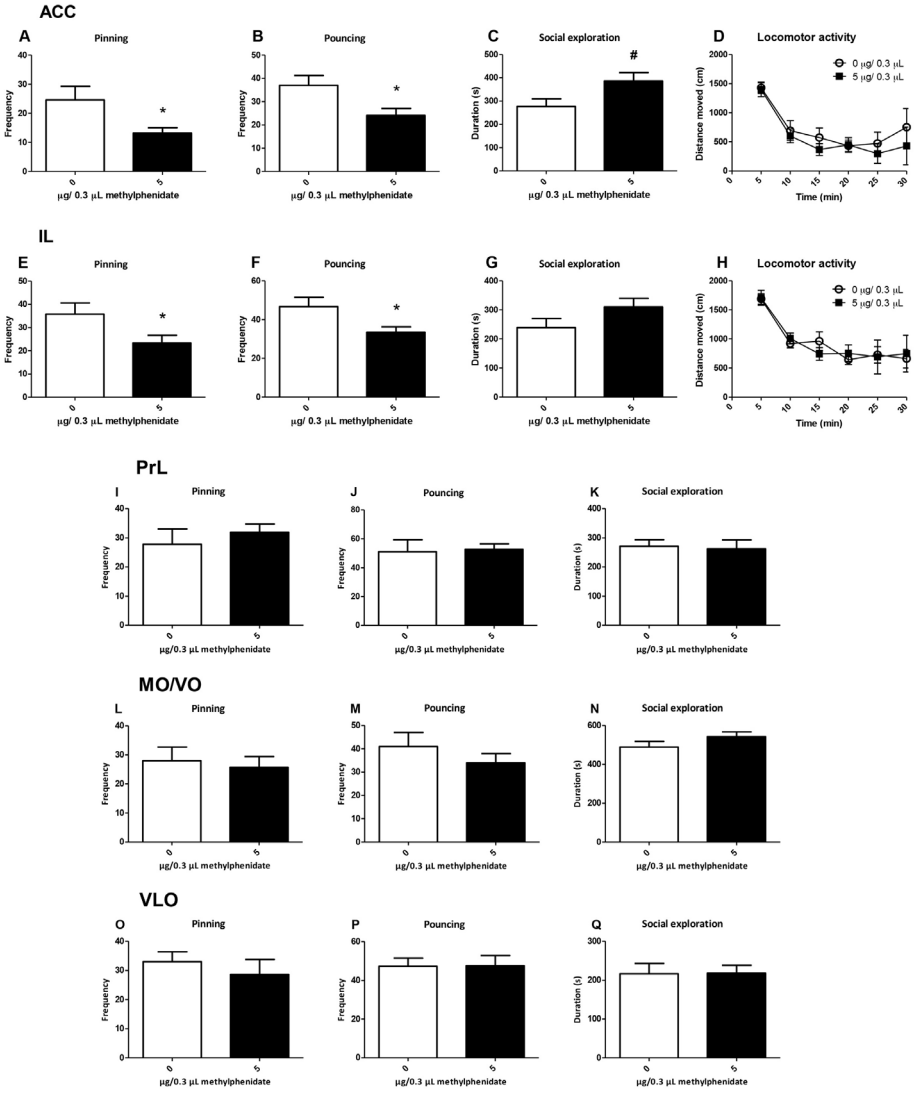

Fig 2: The effect of methylphenidate administration (5.0 μg/0.3 μL) into prefrontal and orbitofrontal regions on social play behavior. Infusions were given in the anterior cingulate cortex (ACC: A-D), infralimbic cortex (IL: E-H), prelimbic cortex (PrL: I-K), medial/ventral orbitofrontal cortex (MO/VO: L-N) and the ventrolateral orbitofrontal cortex (VLO: O-Q). Data are presented as mean + SEM. Methylphenidate infusion reduced pinning (A,E), pouncing (B,F) and tended to increase social exploration after infusion into the ACC (C) but not the IL (G). In both brain regions methylphenidate did not affect locomotor activity (D,H). In the PrL, MO/VO and VLO methylphenidate had no effect on play behavior or social exploration (I-Q). * $p<0.05$, # $p<0.08$.

Treatment with methylphenidate in the prelimbic cortex did not affect pinning (t=-0.80, df=8, p=0.45), pouncing (t=-0.19, df=8, p=0.79; n=9) or social exploratory behavior (t=0.28, df=8, p=0.79) (Fig. 2I-K). Social play behavior and social exploration was unaffected after methylphenidate infusion into the medial/ventral orbitofrontal cortex (pinning: t=0.45, df=6, p=0.67; pouncing: t=0.78, df=6, p=0.47; social exploratory behavior: t=-1.53, df=6, p=0.18; n=7) (Fig. 2L-N) or the ventrolateral orbitofrontal cortex (pinning: t=0.74, df=6, p=0.49; pouncing: t=0.06, df=6, p=0.96; social exploratory behavior: t=0.49, df=6, p=0.65, n=7) (Fig. 2O-Q).

Methylphenidate infusion into the amygdala and habenula, but not nucleus accumbens shell reduces social play

Infusion of methylphenidate into the amygdala (n=6 per group) reduced the frequency of pinning (t=2.73, df=10, p=0.02) and pouncing (t=2.82, df=10, p=0.02), without changing social exploration (t=0.86, df=10, p=0.41) or locomotor activity ($F_{treatment}(1,17)=0.22$, p=0.65; $F_{time}(5,85)=26.48$, p<0.001; $F_{time\ x\ treatment}(5,85)=0.75$, p=0.59) (Fig. 3A-D).

A reduction in the frequency of both play parameters was also observed after administration of methylphenidate into the habenula (pinning: t=4.87, df=8, p=0.001; pouncing: t=5.58, df=8, p=0.001; n=9) (Fig. 3E-F). No changes were observed in the time spent on social exploration (t=-0.19, df=8, p=0.85) or in locomotor activity ($F_{treatment}(1,18)=0.15$, p=0.71; $F_{time}(5,90)=49.34$, p<0.001; $F_{time\ x\ treatment}(5,90)=0.218$, p=0.95) (Fig. 4E-H).

Administration of methylphenidate into the nucleus accumbens shell did not affect social play behavior, since the frequency of pinning and pouncing was not altered (pinning: t=0.51, df=9, p=0.62; pouncing: t=0.15, df=9, p=0.89; n=10) (Fig. 3J-K). In addition, no effects were observed on the time spent on social exploration (t=-0.24, df=9, p=0.25) (Fig. 3I-K).

Atomoxetine infusion into the anterior cingulate cortex, infralimbic cortex, amygdala and habenula decreases social play

Infusion of atomoxetine into the anterior cingulate cortex reduced pinning (t=7.68, df=7, p<0.001, n=8) and pouncing (t=7.74, df=7, p<0.001) and increased the time spent on social exploration (t=-3.84, df=7, p=0.01) (Fig 5A-C). However, no effect of atomoxetine was found on locomotor activity ($F_{treatment}(1,17)=0.16$, p=0.70; $F_{time}(5,85)=31.69$, p<0.001; $F_{time\ x\ treatment}(5,85)=0.34$, p=0.89; vehicle: n=8, atomoxetine n=11) (Fig. 6D).

Treatment with atomoxetine in the infralimbic cortex reduced pinning (t=2.91, df=9, p=0.02, n=10) and pouncing (t=3.55, df=9, p=0.01) and increased social exploration (t=-2.27, df=9, p=0.05) (Fig 5E-G). Intra-infralimbic cortex atomoxetine did not alter locomotor activity ($F_{treatment}(1,19)=0.14$, p=0.71; $F_{time}(5,95)=26.78$, p<0.001; $F_{time\ x\ treatment}(5,95)=0.44$, p=0.82; vehicle n=12, atomoxetine: n=9) (Fig. 5H).

After infusion of atomoxetine into the amygdala, a reduction in pinning (t=3.34, df=5, p<0.001, n=6) and pouncing (t=3.38, df=5, p=0.02) (Fig 5I-J) was found. Intra-amygdala atomoxetine did not affect social exploration (t=-3.84, df=7, p=0.01) or locomotor activity ($F_{treatment}(1,14)=0.89$, p=0.36; $F_{time}(5,70)=19.64$, p<0.001; $F_{time\ x\ treatment}(5,70)=1.95$, p=0.10; vehicle: n=9, atomoxetine n=7) (Fig. 5K-L).

When atomoxetine was infused into the habenula, pinning (t=2.39, df=6, p=0.05, n=7) and pouncing (t=3.53, df=6, p=0.02) (Fig 5M-N) were reduced, whereas social exploration (t=-1.42, df=6, p=0.20) and locomotor activity were not affected ($F_{treatment}(1,16)=2.72$, p=0.12; $F_{time}(5,80)=50.75$, p<0.001; $F_{time\ x\ treatment}(5,80)=1.03$, p=0.40, n=8 per treatment) (Fig. 5O-P).

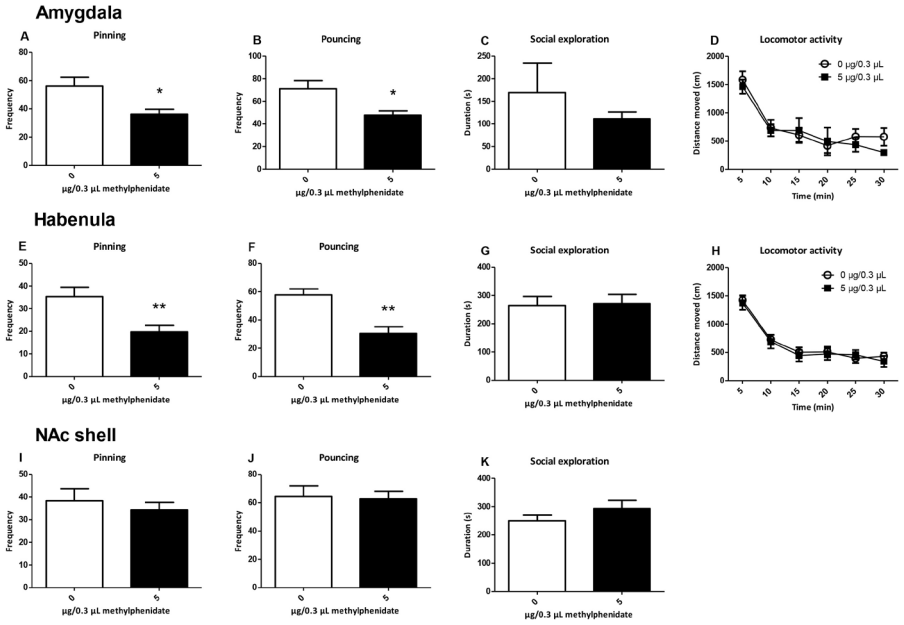

Fig 3: The effect of methylphenidate administration (5.0 µg/0.3 µL) into the amygdala (A-D), habenula (E-H) and the nucleus accumbens shell (NAc shell: I-K). on social play behavior. Data are presented as mean + SEM. Methylphenidate infusion into the amygdala and habenula reduced pinning (A,E) and pouncing (B,F), while it did not affect social exploration (C,G) or locomotor activity (D,H). Methylphenidate did not affect play behavior or social exploration after infusion into the NAc shell (I-K). * $p<0.05$, ** $p<0.01$.

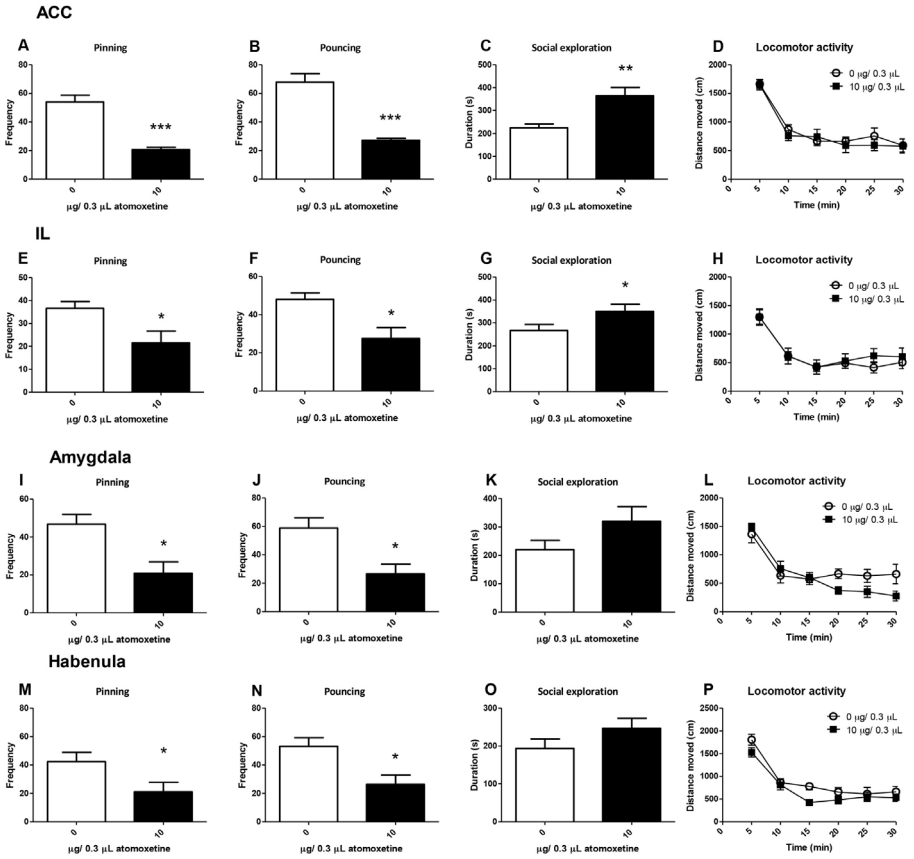

Fig 4: The effect of atomoxetine administration (10.0 µg/0.3 µL) into the anterior cingulate cortex (ACC), infralimbic cortex (IL), amygdala and habenula on social play behavior. Data are presented as mean + SEM. Atomoxetine infusion into all the brain regions decreased pinning (A,E,I) and pouncing (B,F,J), while it increased social exploration (C,G) or had no effect (K,O). No effect on locomotor activity was found (D,H,L,P). * $p<0.05$, ** $p<0.01$, *** $p<0.001$.

Discussion

In the present investigation, we found that infusion of methylphenidate and atomoxetine into the anterior cingulate and infralimbic cortex, amygdala and habenula inhibited social play behavior in rats. These effects were behaviorally specific, since methylphenidate and atomoxetine did not inhibit social exploratory behavior and locomotor activity. Moreover, these effects were anatomically specific, since infusion of methylphenidate into the prelimbic, medial/ventral orbitofrontal and ventrolateral orbitofrontal cortex and nucleus accumbens shell did not alter social play behavior.

Prefrontal mechanisms underlying the inhibition of social play behavior by methylphenidate

In the present study, methylphenidate and atomoxetine reduced social play behavior after administration into the anterior cingulate and infralimbic cortex. The main function of the prefrontal cortex is thought to be the mediation of executive functions, such as attention, planning, cognitive flexibility, and decision making (Miller and Cohen, 2001; Robbins and Arnsten, 2009). Since social interactions are inherently complex and unpredictable, it is likely that frontal cortical regions subserve executive functions in social situations (Adolphs, 2003; Blakemore, 2008; Rilling et al., 2008), including social play behavior (Siviy and Panksepp, 2011; Vanderschuren and Trezza, 2013).

We have previously hypothesized that methylphenidate reduces social play by improving behavioral inhibition, that is, by suppressing a vigorous form of social behavior that is associated with diminished attention for the environment (Vanderschuren et al., 2008). Indeed, systemic methylphenidate and atomoxetine are known to improve behavioral inhibition in rats and humans, in paradigms such as the stop signal task and the 5-choice serial reaction time task (Chamberlain and Sahakian, 2007; Eagle et al., 2008; Pattij and Vanderschuren, 2008). However, it has recently been reported that atomoxetine increases performance in the stop signal task via the dorsal prelimbic and (ventrolateral) orbitofrontal, but not anterior cingulate or infralimbic cortex (Bari et al. 2011). Moreover, infusion of atomoxetine into the infralimbic cortex did not affect premature responding in the 5-choice serial reaction time task (Economidou et al., 2012). This suggests that if methylphenidate and atomoxetine reduce social play through enhanced inhibition of behavior, this aspect of inhibition is distinct from the constructs analyzed in the stop signal and 5-choice serial reaction time tasks. Alternatively, the prefrontally mediated inhibition of social play by methylphenidate and atomoxetine may be related to impaired behavioral flexibility. Thus, depletion of noradrenaline from the ventromedial prefrontal, including the infralimbic cortex (McGaughy et al., 2008) or medial prefrontal, including both the anterior cingulate and prelimbic cortex (Tait et al., 2007) disrupted extradimensional shifting in an attentional set-shifting task. Remarkably, while atomoxetine reversed the set-shifting deficit produced by noradrenergic depletion, it disrupted performance in sham-lesioned rats (Newman et al., 2008). In the context of social play, this suggests that noradrenergic mechanisms in the prefrontal cortex subserve the cognitive flexibility necessary to be able to respond to the changeable, often unpredictable behavior of a conspecific.

We have previously reported that functional inactivation of medial prefrontal subregions, i.e. the prelimbic, infralimbic, and medial/ventral orbitofrontal cortex, using a mixture of the GABA-A receptor agonist muscimol and the GABA-B receptor agonist baclofen, inhibits social play (Van Kerkhof et al., 2013b). Interestingly, of these regions, in the present study, the infralimbic, but not the prelimbic and medial/ventral orbitofrontal cortex was shown to be involved in the play-reducing effects of

methylphenidate and atomoxetine. Together, these findings provide a glimpse into the heterogeneity of the prefrontal functions involved in social play (see also Schneider and Koch, 2005; Pellis et al., 2006; Bell et al., 2009). Thus, in keeping with the reported functional heterogeneity of the prefrontal cortex (Chudasama et al., 2003; Gourley et al., 2010; Killcross and Coutureau, 2003; Peters et al., 2009), noradrenergic mechanisms may underlie the functional involvement of the infralimbic and anterior cingulate, but not prelimbic and orbitofrontal cortex, suggesting that these prefrontal subregions are involved in distinct executive aspects of social play behavior.

Limbic subcortical mechanisms underlying the inhibition of social play behavior by methylphenidate

Methylphenidate and atomoxetine infused into the amygdala and the habenula suppressed social play behavior, without affecting social exploration or in locomotor activity. However, intra-nucleus accumbens shell methylphenidate treatment did not alter social play.

Noradrenaline has been shown to reduce neuronal activity in the lateral and basolateral amygdala via α2-adrenoceptors (Buffalari and Grace, 2007; Ferry et al., 1997; Johnson et al., 2011). Since stimulation of α2-adrenoceptors underlies the play-suppressant effect of methylphenidate (Vanderschuren et al., 2008), the inhibition of social play by intra-amygdala methylphenidate and atomoxetine may be the result of reduced amygdaloid activity. The hypothesis that reduced amygdala function decreases social play is consistent with previous findings that amygdala lesions reduce social play in male rats (Daenen et al., 2002; Meaney et al., 1981). In addition, systemic treatment with low doses of methylphenidate has been reported to decrease glucose metabolism in the habenula (Porrino and Lucignani, 1987), suggesting that enhancement of noradrenergic neurotransmission by methylphenidate and atomoxetine results in decreased habenula activity. Consistent, we have recently shown that inactivation of the habenula decreased social play behavior (Van Kerkhof et al., 2013c).

Social play is a highly rewarding activity (Trezza et al., 2011b; Vanderschuren, 2010), and both the amygdala and habenula are involved in reward processes (Baxter and Murray, 2002; Bromberg-Martin and Hikosaka, 2011; Cardinal et al., 2002; Hikosaka, 2010; Lecourtier and Kelly, 2007; Morrison and Salzman, 2010). Indeed, we have recently found that endocannabinoid-mediated facilitation of social play, which may be related to an increased reward value of social play, depends on the basolateral amygdala (Trezza et al., 2012). Therefore, functional inhibition of the the amygdala and habenula by methylphenidate and atomoxetine may have reduced the positive emotional properties of social play. Conversely, both the habenula and amygdala are involved in stress- and anxiety-related behaviors (Phelps and LeDoux, 2005; Hikosaka, 2010; Roozendaal et al., 2009; Shin and Liberzon, 2010). Indeed, increased noradrenaline levels in the amygdala have been associated with stress and anxiety (Tanaka et al., 2000). However, intra-amygdala and intra-habenula methylphenidate and atomoxetine did not affect social exploratory behavior, which is the standard parameter used in the social interaction test of anxiety (File and Seth 2003). Moreover, pharmacological analysis of social play behavior has consistently shown that anxiolytic or anxiogenic drugs do not invariably increase or reduce social play, respectively (Vanderschuren et al. 1997; Trezza et al., 2009). Therefore, it is unlikely that changes in stress and/or anxiety explain the effects of methylphenidate and atomoxetine on social play behavior. Methylphenidate and atomoxetine in the amygdala and habenula may also have influenced cognitive aspects of social play. For example, habenula lesions have been shown to disrupt attention (Lecourtier and Kelly, 2005), and the amygdala has been implicated

in behavioral flexibility (Churchwell et al., 2009; Schoenbaum et al., 2003).

Methylphenidate did not alter social play after infusion into the nucleus accumbens shell. Systemic methylphenidate suppressed social play behavior in a noradrenaline-dependent way (Vanderschuren et al., 2008). Importantly, the nucleus accumbens shell is the only part of the striatum that receives a noradrenergic innervation (Berridge et al., 1997; Delfs et al., 1998). Therefore, it is unlikely that the play-suppressant effect of methylphenidate is mediated within the striatum.

Neurocircuitry of social play behavior

On the basis of the present findings, it is a challenging thought that the infralimbic cortex, anterior cingulate cortex, amygdala and habenula are part of a functional network involved in the modulation of social play behavior. Indeed, all four regions have reciprocal connections with the locus coeruleus (Jodo et al., 1998; Moore and Bloom, 1979; Gottesfeld, 1983; Unnerstall et al., 1984; Lecourtier and Kelly, 2007; Radley et al., 2008; Pitkänen, 2000; Vertes, 2004; Hoover and Vertes, 2007). Furthermore, the intralimbic cortex has reciprocal connections with the anterior cingulate cortex, and both have reciprocal connections with the amygdala (Pitkänen, 2000; Vertes, 2004; Hoover and Vertes, 2007). The infralimbic cortex also sends a moderate innervation to the habenula. Last, both the amygdala and habenula send distributed outputs to the thalamus, which in turn innervates the prefrontal cortex (Groenewegen, 1988; De Olmos et al., 2004; Lecourtier and Kelly, 2007). Thus, the four structures implicated in the effects of methylphenidate and atomoxetine on social play are intricately linked.

Conclusion

This study provides new insights into the neural underpinnings of a developmentally important social activity, as well as the behavioral mechanism of action of two drugs widely used for the treatment of ADHD. Our findings suggest that an interplay between limbic cortical and subcortical structures underlies the integration of cognitive and emotional information during the proper execution of a playful social encounter.

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Chapter 4

Contrasting roles of dopamine and noradrenaline in social play reward in rats

Submitted

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Abstract

Social play behavior is a vigorous form of social interaction, abundant in the young of many mammalian species, including humans. Social play is highly rewarding, and as such, the expression of social play depends on its pleasurable and motivational properties. Because dopamine and noradrenaline have been implicated in both social play and in reward processes, we here investigated the role of dopamine and noradrenaline in the pleasurable and motivational properties of social play behavior in rats. To assess social play motivation, we developed a setup in which rats responded for social play under a progressive ratio schedule of reinforcement. The pleasurable properties of social play were assessed using place conditioning. The dopamine/noradrenaline reuptake inhibitor methylphenidate increased responding for social play, suppressed its expression, but did not alter its pleasurable properties. The noradrenaline reuptake inhibitor atomoxetine decreased both social play motivation and expression, but spared social play-induced place conditioning. The dopamine reuptake inhibitor GBR12909 increased motivation for social play, did not affect its expression, but reduced its pleasurable properties. The effect of methylphenidate on social play motivation was blocked by the dopamine receptor antagonist α-flupenthixol, but not the α-2 adrenoceptor antagonist RX821002, whereas the reverse was the case of the effect of methylphenidate on social play expression. These data demonstrate dissociable roles for dopamine and noradrenaline in social play behavior: dopamine is involved in the motivational and pleasurable properties of social play, whereas noradrenaline modulates the motivation for play and its expression.

Introduction

The experience of social interactions during post-weaning development (i.e. childhood and adolescence in humans, roughly equivalent to the juvenile and adolescent stages in rodents) is critical for social and cognitive development (Baarendse et al., 2013a; Potegal and Einon, 1989; Van den Berg et al., 1999). Throughout this developmental period, a particular, highly vigorous form of social interaction, i.e. social play behavior, is abundantly expressed (Panksepp et al., 1984; Pellis and Pellis, 2009; Vanderschuren et al., 1997). Social play behavior is highly rewarding (Trezza et al., 2011a; Vanderschuren, 2010) and it is modulated through neural systems implicated in other rewards such as food, sex, and drugs of abuse (Berridge, 2003; Siviy and Panksepp, 2011; Trezza et al., 2010). Reward processes comprise pleasurable ('hedonic'), incentive motivational, and learning effects, which are mediated via different neural systems (Berridge et al., 2009). For example, opioids and endocannabinoids have been implicated in the pleasurable properties of a reward, whereas dopamine is thought to mediate its motivational aspects (Barbano and Cador, 2007; Berridge et al., 2009; Kelley, 2004; Salamone and Correa, 2012).

Previous studies have indicated that social play behavior is modulated by dopaminergic and noradrenergic neurotransmission. For example, treatment with dopamine receptor agonists and antagonists alters the expression of social play behavior (Niesink and Van Ree, 1989; Siviy et al, 1996; Trezza and Vanderschuren, 2009; Vanderschuren et al, 2008). In addition, the stimulation of social play by endocannabinoids, ethanol and nicotine depends upon dopamine receptor stimulation (Trezza et al., 2009a; Trezza and Vanderschuren, 2008; Trezza and Vanderschuren, 2009). Administration of the α-2 adrenoceptor agonist clonidine and the α-2 adrenoceptor antagonist RX821002 reduced and enhanced social play, respectively (Normansell and Panksepp, 1985; Siviy et al., 1994; Siviy and Baliko, 2000). In addition, the dopamine/noradrenaline reuptake inhibitor methylphenidate, and the noradrenaline reuptake inhibitor atomoxetine reduced social play, through stimulation of α-2 adrenoceptors (Beatty et al., 1982; Vanderschuren et al., 2008). However, whether dopamine and noradrenaline are involved in the pleasurable and motivational properties of social play behavior is unknown.

In the present study, we therefore investigated whether dopamine and noradrenaline are involved in the pleasurable and/or motivational aspects of social play behavior. To address this aim, we tested the effects of the dopamine/noradrenaline reuptake inhibitor methylphenidate, the noradrenaline reuptake inhibitor atomoxetine and dopamine reuptake inhibitor GBR12909 on the expression of social play behavior, and on its motivational and pleasurable properties. To measure the motivational properties of social play behavior, we developed an operant conditioning task, in which rats responded for access to a playful partner under a progressive ratio schedule of reinforcement. Observation of behavior during reinforced periods also allowed for the assessment of expression of social play after drug treatment. To assess the pleasurable properties of social play, we investigated the acquisition of social play-induced conditioned place preference (CPP). In this task, rats develop a preference for an environment previously associated with social play if the play encounter is perceived as pleasurable (Calcagnetti and Schechter, 1992; Thiel et al., 2008; -2009; Trezza et al., 2009b). We hypothesized that dopaminergic and noradrenergic neurotransmission play dissociable roles in these different aspects of social play behavior.

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 ($l \times w \times h$) Macrolon cages under controlled conditions (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 Dierproeven, 1996) and European regulations (Guideline 86/609/EEC).

Drugs

Methylphenidate hydrochloride (BUFA, Castricum, The Netherlands), atomoxetine hydrochloride, RX821002 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, α-flupenthixol and RX821002 were administered intraperitoneally (i.p.). Drug doses and pretreatment intervals were based on previous studies (Baarendse et al., 2013a; -b; Baarendse and Vanderschuren, 2012; Trezza and Vanderschuren, 2009; Vanderschuren et al., 2008). Drug doses were calculated as salt. Drugs were administered 30 min before testing, except when methylphenidate treatment was combined with α-flupenthixol or RX821002 treatment, in which case α-flupenthixol and RX821002 were administered 30 and 15 min prior to methylphenidate administration, respectively. In view of the importance of the neck area in the expression of social play behavior (Pellis and Pellis, 1987; Siviy and Panksepp, 1987), s.c. injections were administered in the flank.

Operant conditioning

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 by more than 10 grams in body weight at the start of the experiment. A test pair consisted of one experimental animal and its stimulus partner. At 24 days of age, test pairs were habituated to the test cage for 10 min (see Supplementary Materials and Methods for a description of the apparatus). During the habituation session, the animals could freely explore the entire apparatus. After the habituation session, animals were isolated for 24 h/day for 5 consecutive days/week, except in the first validation experiment, in which we also included a group of animals isolated for 2 h/day for 5 days/week. Next, the animals received two shaping sessions on two consecutive days. During these shaping sessions, the cue light was presented, the lever retracted and the door opened when the experimental animal approached the active lever. Rats were allowed to interact for two minutes after which the door closed and each rat was placed back into its starting compartment by the experimenter. 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 an additional shaping session in the afternoon or on the next day.

On the fourth day, the lever pressing sessions (20 min) 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 inter-trial interval. During this interval, the experimenter placed each rat back into its starting compartment. 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; Hodos, 1961; Richardson and Roberts, 1996). When rats met the response requirement, 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 received one session per day, for 5 consecutive days/week. During the other 2 days/week animals were socially housed with their original cage-mates. After responding had stabilized, defined as obtaining at least six rewards on three consecutive days with a variation of no more than two rewards, drug treatment started according to a Latin Square design. Inactive lever presses were recorded, but had no programmed consequences.

During earned social interactions, behavior 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, behavior of the animals was recorded using a camera with zoom lens, video tape recorder and television monitor. Three behavioral elements were scored (Panksepp et al., 1984; Trezza et al., 2010; Vanderschuren et al., 1997). 1. Frequency of pinning: one animal lying with its dorsal surface on the floor with the other animal is standing over it. 2. Frequency of pouncing: one animal attempts to nose/rub the nape of the neck of the partner, which is an index of play solicitation (Fig. S1-3). Pinning and pouncing frequencies are considered the most characteristic parameters of social play behavior in rats (Panksepp and Beatty, 1980). 3. Time spent in social exploration: one animal sniffing or grooming any part of the partner's body. This is a measure of general social interest.

Place conditioning

Place conditioning was performed as previously described (Achterberg et al., 2012; Trezza et al., 2009b; -2011b, see Supplementary Materials and Methods).

Statistical analysis

Data were analysed using SPSS software 15.0 for Windows and expressed as mean ± SEM. To correct for differences in earned social interaction time, the frequency of pinning and pouncing and the duration of social exploration during operant conditioning were calculated per minute or as a percentage of the interaction time, respectively. Pinning, pouncing, social exploration, rewards obtained and inactive lever presses were analysed using a paired Student's t-test or repeated measures ANOVA with drug dose as within-subjects factor followed by a paired Student's t-test when appropriate. Breakpoints under the PR schedule of reinforcement are derived from an escalating curve, which violates the homogeneity of variance. Therefore, they were analysed using the non-parametric Friedman test, followed by a post-hoc Wilcoxon signed ranks test when appropriate. Place conditioning data were expressed as mean time spent in the social paired and non-social paired compartment, and analysed using two-way ANOVA, with compartment and treatment as factors, followed by paired Student's t-test when appropriate.

Results

Validation of the operant conditioning task

In order to verify that our operant conditioning task was sensitive to differences in social motivation, we compared rats that were socially isolated for 2h or 24h, which is known to induce moderate and maximal increases in social play behavior, respectively (Niesink and van Ree, 1989; Vanderschuren et al., 1995; 2008). All rats acquired the task, i.e. pressed the active lever for the opportunity for a social interaction under the FR-1 schedule of reinforcement. However, only after 24 h of isolation did all tested animals (6/6) reach performance criterion under the FR-1 schedule of reinforcement within 8 days of training, whereas only one third (2/6) of the animals isolated for 2 h reached criterion (Fig. S1A). Under the PR schedule of reinforcement, rats isolated for 24 h obtained more rewards ($t=12.87$, $df=5$, $p<0.001$) (Fig. 1A), and reached a higher breakpoint ($Z=-2.20$, $p=0.03$) (Fig. 1B) and made more inactive lever presses compared to animals isolated for 2h (Supplementary Table 1). Frequencies of pinning ($t=2.15$, $df=5$, $p=0.09$; Fig. 1C) and social exploration ($t=-1.06$, $df=5$, $p=0.34$; Fig. 1D) did not differ between the groups. These data indicate that social isolation increases responding for social play behavior.

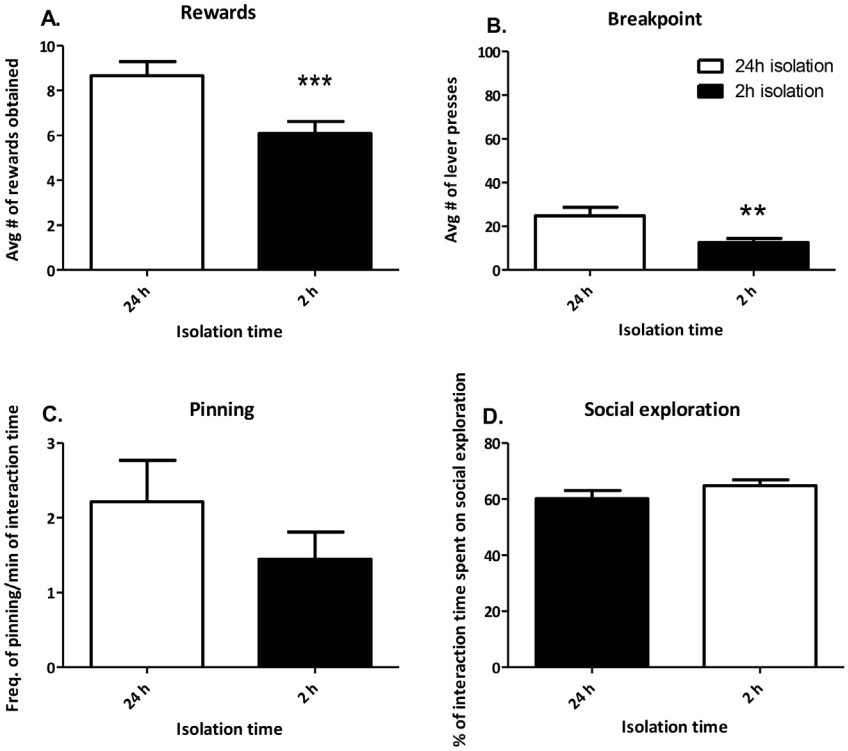

Fig. 1: Duration of social iso-lation influenced responding for social play behavior. Short isolation (2h) reduced ope-rant responding (n=6) as reflected in the number of rewards ob-tained (A), and the breakpoint (B). Frequency of pinning and the time spent on social ex-ploration did not differ due to isolation time (C-D). Data are presented as mean + SEM. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

The dopamine/noradrenaline reuptake inhibitor methylphenidate enhanced responding for social play behavior

The dopamine/noradrenaline reuptake inhibitor methylphenidate (1-3 mg/kg) enhanced the number of rewards obtained ($F_{treatment}(2,10)=19.94$, $p<0.001$) the breakpoint ($X^2=8.27$, df= 2, $p=0.02$) (Fig. 2A-B), but not inactive lever presses (Supplementary Table 1). However, methylphenidate treatment decreased the frequency of pinning ($F_{treatment}(2,10)=65.97$, $p<0.001$) (Fig. 2C) and increased the duration of social exploration ($F_{treatment}(2,10)=8.73$, $p=0.01$) (Fig. 2D). These results show that, despite decreasing the expression of social play methylphenidate enhanced responding for play.

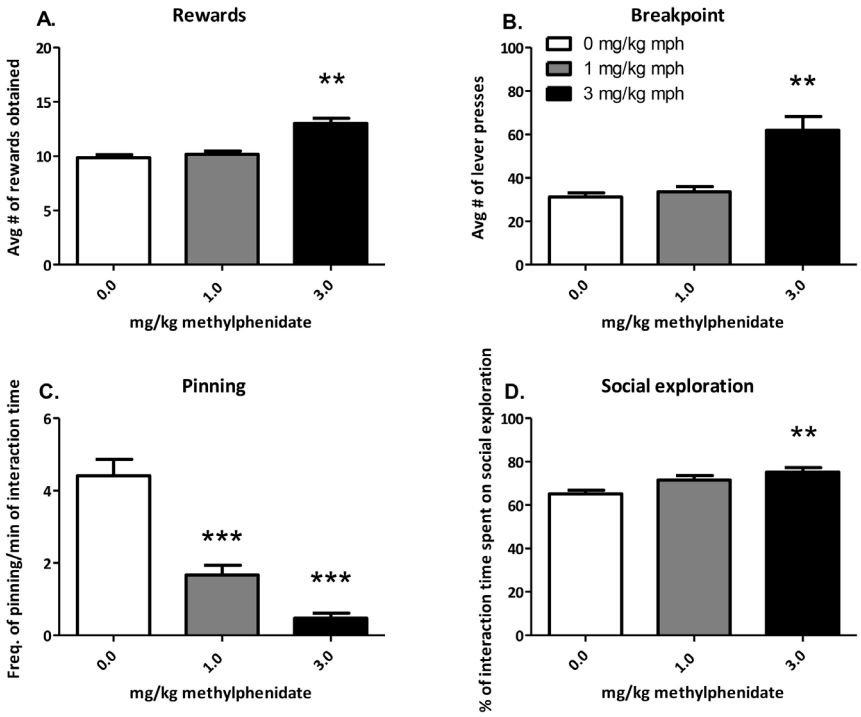

Fig. 2: Methylphenidate enhanced operant responding for social play behavior, but inhibited the expression of social play. Treatment with methylphenidate (1-3 mg/kg, s.c.) enhanced the number of rewards obtained (A), the breakpoint (B) and the time spent on social exploratory behavior (D), while it reduced the frequency of pinning (C) (n=6). Data are presented as mean + SEM. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, relative to saline treatment.

Selective dopamine or noradrenaline reuptake inhibition differentially affected responding for social play

To investigate the role of dopamine and noradrenaline neurotransmission in the motivation for social play, we treated rats with the selective dopamine reuptake inhibitor GBR-12909 or the noradrenaline reuptake inhibitor atomoxetine. GBR-12909 (10 mg/kg) increased the number of rewards obtained (t=-2.93, df=6, p=0.03) and the breakpoint (Z = -2.21, p = 0.03) (Fig. 3A-B), but not inactive lever presses (Supplementary Table 1). GBR-12909 treatment did not affect pinning (t=0.89, df=6, p=0.43) (Fig. 3C) or social exploration (t=1.14, df=6, p=0.30) (Fig. 3D).

Administration of atomoxetine (1-3 mg/kg) reduced the number of rewards obtained ($F_{treatment}(2,14)=48.31$, $p<0.001$), the breakpoint ($X^2=15.00$, df=2, $p<0.001$) (Fig. 3E-F) and inactive lever presses (Supplementary Table 1). Atomoxetine reduced pinning ($F_{treatment}(2,14) = 9.65$, $p = 0.002$) (Fig. 3G) but not social exploration ($F_{treatment}(2,14)=2.01$, p=0.17) (Fig. 3H).

Doubly dissociable roles for dopamine and noradrenaline receptors in the effects of methylphenidate of social play motivation and expression

The data presented above, combined with our previous work (Vanderschuren et al, 2008) suggest that the effects of methylphenidate on the motivation and the expression of social play are the result of increases in dopamine and noradrenaline neurotransmission, respectively. To test this possibility, we investigated the effect of methylphenidate on social play motivation and expression after pretreatment with the dopamine receptor antagonist α-flupenthixol and the alpha2-adrenoceptor antagonist RX821002, respectively. At the doses used, α-flupenthixol and RX821002 had no effect on the parameters measured (Figs. S4 and S5).

The treatments affected the number of rewards obtained ($F_{treatment}(3,21)=10.51$, $p<0.001$), breakpoint ($X^2=13.50$, df=2, p=0.004), pinning ($F_{treatment}(3,21)=10.09$, p=0.002) and social exploration ($F_{treatment}(3,21)=5.07$, p=0.002) but not inactive lever presses (Supplementary Table 1). Consistent with the previous experiment, 3 mg/kg methylphenidate increased responding (i.e., rewards and breakpoint), decreased pinning and increased social exploratory behavior. Pretreatment with RX821002 (0.2 mg/kg, i.p.) did not antagonize the increase in rewards obtained and breakpoint induced by methylphenidate, but it counteracted the effects of methylphenidate on pinning and social exploration. In contrast, pretreatment with α-flupenthixol (0.125 mg/kg, i.p.) antagonized the effects of methylphenidate on responding for social play, but not the effects of methylphenidate on pinning and social exploration (Fig 4A-D). Together, these results demonstrate a double dissociation in the effects of methylphenidate on social play behavior. The effects of methylphenidate on social play motivation are mediated through stimulation of dopamine receptors, its effects on expression of social play behavior rely alpha-2 adrenoceptor stimulation.

All manipulations tested altered pinning and pouncing in the same direction (for pouncing data see Fig. S1-3).

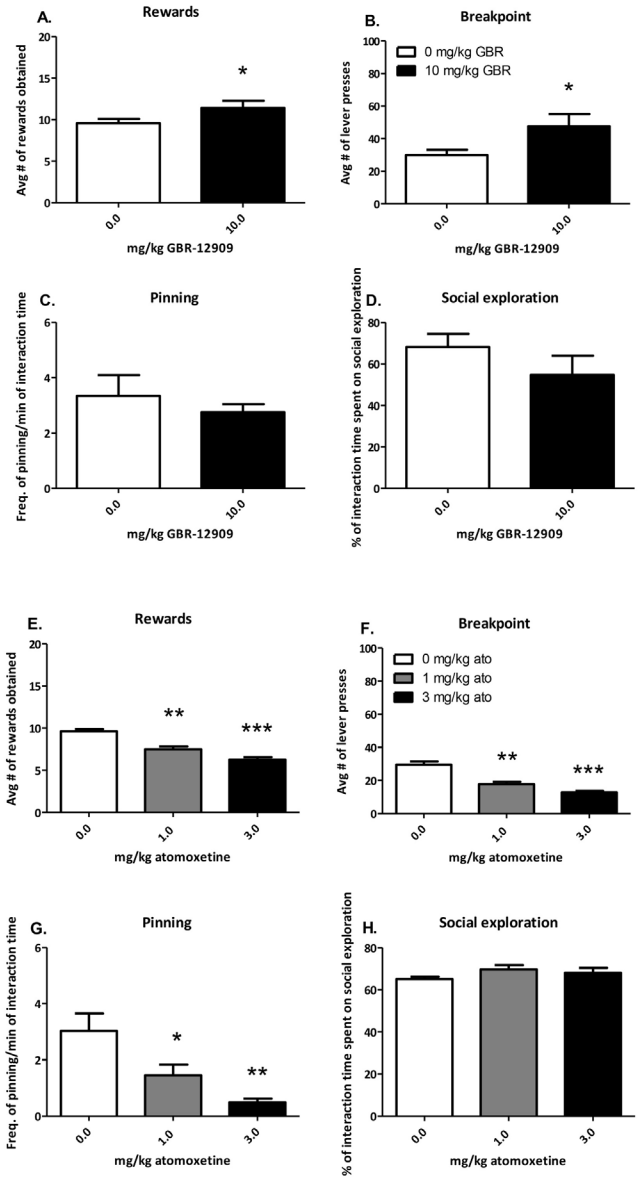

Fig. 3: The effect of GBR-12909 (n=7) and atomoxetine (n=8) on operant responding for social play behavior. Treatment with GBR-12909 (10 mg/kg, s.c.) enhanced responding for social play. GBR-12909 increased the number of rewards obtained (A) and the breakpoint (B). Administration of GBR-12909 did not affect the frequency of pinning (C), or the time spent on social exploration (D). Treatment with atomoxetine (1-3 mg/kg, i.p.) reduced operant responding and social play behavior. The number of rewards obtained was reduced (E) and the breakpoint was lower (F). In addition, the frequency of pinning (G) was reduced. The time spent on social exploration was unaffected (H). Data are presented as mean + SEM. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, relative to saline treatment.

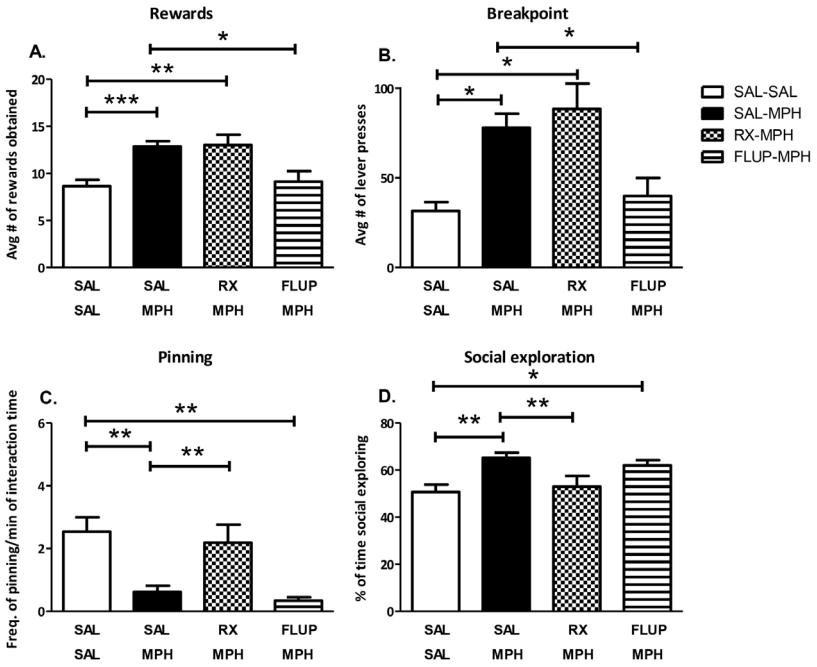

Fig. 4: A double dissociation in the effect of methylphenidate on operant responding for social play behavior (n = 8). Methylphenidate (MPH: 3 mg/kg, s.c.) increased the number of obtained rewards (A) and the breakpoint reached (B); this effect could be prevented by pre-treatment with α-flupenthixol (FLUP: 0.125 mg/kg, i.p.) but not RX821002 (RX: 0.2 mg/kg, i.p.). Methylphenidate reduced the frequency of pinning (C) and the time spent on social exploration (D); this effect could be prevented by pre-treatment with RX821002 but not α-flupenthixol. Data are presented as mean + SEM. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

Role of dopamine and noradrenaline neurotransmission in the acquisition of social play-induced CPP

Methylphenidate (1-3 mg/kg) altered the acquisition of social play-induced CPP ($F_{compartment}(1,114)=163.79$, $p<0.001$; $F_{treatment}(2,114)=0.01$, $p=0.99$; $F_{compartment\ x\ treatment}(2,114)=8.73$, $p<0.001$). At the lowest dose, it did not affect social play-induced CPP, but treatment with 3 mg/kg methylphenidate increased CPP (Fig. 5A). In contrast, atomoxetine did not affect the acquisition of social play-induced CPP ($F_{compartment}(1,70)=28.05$, $p<0.001$; $F_{treatment}(2,70)=0.20$, $p=0.82$; $F_{compartment\ x\ treatment}(2,70)=0.73$, $p=0.49$; Fig. 5B). The acquisition of social play-induced CPP was blocked by GBR-12909 ($F_{compartment}(1,40)=12.65$, $p=0.001$; $F_{treatment}(1,40)<0.001$, $p=0.99$; $F_{compartment\ x\ treatment}(1,40)=4.630$, $p=0.04$; Fig. 5C) but not by α-flupenthixol ($F_{compartment}(1,104)=42.89$, $p<0.001$; $F_{treatment}(3,104)=0.24$, $p=0.85$; $F_{compartment\ x\ treatment}(3,104)=0.97$, $p=0.41$; Fig. 5D). Together, these data indicate that dopaminergic rather than noradrenergic neurotransmission underlies pleasurable aspects of social play.

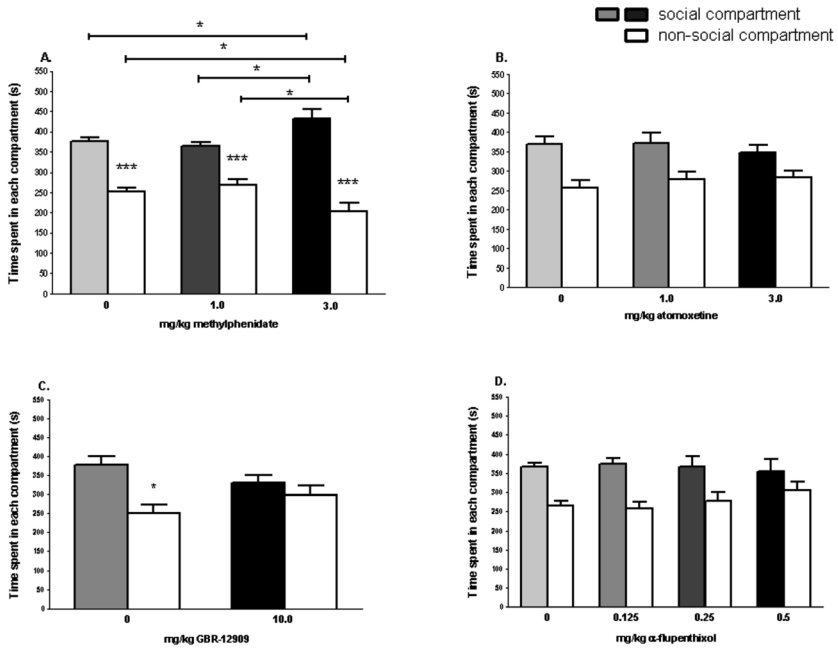

Fig. 5: Effect of methylphenidate, atomoxetine, GBR-12909 and α-flupenthixol on the acquisition of social play-induced CPP. At the dose of 1 mg/kg, methylphenidate did not affect social play-induced CPP; at the dose of 3 mg/kg, methylphenidate increased the time spent in the social compartment and reduced the time spent in the non-social compartment. (A; n=33/19/8). Treatment with GBR-12909 (10 mg/kg, s.c.) disrupted the acquisition of social play-induced CPP (C; n=12/10), while treatment with atomoxetine (1-3 mg/kg, i.p.) (B; n=18/10/10) and α-flupenthixol (0.125-0.5 mg/kg, i.p.) (D; n=24/12/10/10) had no effect on the acquisition of social play-induced CPP. Data are presented as mean ± SEM time (sec) spent in each compartment. Lines with stars indicate differences between social compartments or non-social compartments between different treatments; stars alone indicate differences between social and non-social compartments within treatment groups. * $p < 0.05$, *** $p < 0.001$.

Discussion

An operant conditioning task for social play

The aim of this study was to investigate the role of dopamine and noradrenaline in the pleasurable and motivational aspects of social play behavior in rats, using behavioral tasks to dissociate these different components of social play reward. Responding under a progressive ratio schedule of reinforcement is a widely used method to measure the motivational properties of rewards (Hodos, 1961; Richardson and Roberts, 1996). We therefore developed a novel setup in which rats were trained to lever-press for social play behavior. T-maze tasks have previously been used to assess motivational aspects of social play behavior in rats (Humphreys and Einon, 1981; Normansell and Panksepp, 1990), but to the best of our knowledge, ours is the first study to show that rats perform an operant conditioning task reinforced with social play. There is a close relationship between the length of social isolation and the amount of social play behavior expressed during testing (Niesink and Van Ree, 1989; Vanderschuren et al., 1995; -2008). Therefore, we assumed that longer social isolation would enhance social play motivation. Indeed, animals isolated for 24 h acquired the operant task faster and reached a higher breakpoint than animals isolated for 2 h. In addition, after 24 h of isolation, the majority of rats acquired the task within 5-8 sessions, reaching a near maximal number of rewards. These results show that it is possible to measure differences in social motivation using an operant conditioning task. In contrast to previous findings (Niesink and Van Ree, 1989; Vanderschuren et al., 1995; -2008), the levels of social play were not significantly higher in animals isolated for 24 h. This likely resulted from the small group size in this experiment, which precludes a firm statistical analysis of the effect of social isolation on social play expression. However, this was not the main goal of this experiment, which aimed to validate the operant conditioning task.

Dissociable roles of dopamine and noradrenaline in social play motivation and expression

Blocking dopamine and noradrenaline reuptake with methylphenidate enhanced responding for social play behavior. Conversely, the expression of social play behavior itself was reduced, consistent with previous reports (Beatty et al., 1982; Vanderschuren et al., 2008). One may argue that the effects of methylphenidate on responding for play were the result of an extinction overshoot (i.e., the animals responded more because they received less social play reward after acute methylphenidate treatment). However, our preliminary data (not shown) indicate that the effects of methylphenidate on responding for social play (as well as on social play itself, see also Vanderschuren et al., 2008) do not decline with repeated treatment, which rules out that the effect of methylphenidate on responding for social play resulted from an extinction overshoot. Administration of the dopamine reuptake inhibitor GBR-12909 or the dopamine receptor antagonist α-flupenthixol did not affect the expression of social play behavior, as previously reported (Vanderschuren et al., 2008; Trezza and Vanderschuren, 2009). However, similar to methylphenidate treatment, GBR-12909 enhanced the motivation for social play behavior, whereas α-flupenthixol reduced responding. These results suggest that the enhancement of motivation for social play induced by methylphenidate is mediated via a dopaminergic mechanism. Indeed, the effects of methylphenidate on responding were antagonized by pretreatment with α-flupenthixol, whereas the reduction in social play behavior remained unaffected.

The role of dopamine in the motivation for social play is consistent with its involvement in incentive motivation for rewards. 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; Salamone and Correa, 2012). For example, administration of amphetamine into the nucleus accumbens enhances operant responding for food (e.g. Zhang et al., 2003), but not food consumption (e.g. Hanlon et al., 2004). Our observations are therefore consistent with the view that dopaminergic neurotransmission plays a critical role in incentive 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.

Treatment with the noradrenaline reuptake inhibitor atomoxetine reduced the expression of social play behavior as well as operant responding for social play. We have previously shown that the reduction in the expression of social play behavior induced by methylphenidate depended upon stimulation of α2-adrenoceptors (Vanderschuren et al., 2008). These results indicate that enhanced noradrenaline signalling reduces the motivation for and expression of social play behavior. However, pre-treating animals with the α2-adrenoceptor antagonist RX821002 prevented the reduction in social play evoked by methylphenidate, leaving the methylphenidate-induced increase in operant responding unchanged. This suggests that elevated dopamine levels may overshadow the suppressant effects of increased noradrenaline signalling on motivation for social play or that the dopaminergic effect on social play motivation occurs downstream of the noradrenergic effect. Alternatively, the effect of increased noradrenaline on motivational parameters may be secondary to the reduction in play, i.e., the performance of social play was reduced by atomoxetine and therefore the motivation to work for it decreased as well.

Role of dopaminergic and noradrenergic neurotransmission in the acquisition of social play-induced CPP

Next, we determined the role of dopamine and noradrenaline neurotransmission in the pleasurable aspects of social play, using social play-induced CPP. Our data indicate that dopamine, but not noradrenaline is involved in the acquisition of social play-induced CPP. Administration of GBR-12909 prior to social conditioning sessions disrupted the establishment of CPP, whereas treatment with methylphenidate, atomoxetine or α-flupenthixol did not affect the acquisition of CPP. These results indicate that enhanced dopamine levels are incompatible with the acquisition of social play-induced CPP.

It is unlikely that treatment with GBR-12909 interfered with learning processes during conditioning. For example, treatment with methylphenidate itself induces CPP, which shows that adolescent rats under conditions of elevated dopamine signalling are capable of acquiring a place-reward association (Trezza et al., 2009b). Moreover, there is a vast literature showing that treatment with psychostimulant drugs, that increase dopaminergic neurotransmission, induces CPP in adult rats (for review see Tzschentke, 2007). A more plausible explanation for our data is that enhancement of endogenous dopamine levels reduced the pleasurable effects of social play, 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 may reduce the hedonic value of a reward. Possibly, increases in dopamine resulted in an enhanced motivation to play but a reduction of its pleasurable properties, thus leaving the expression of social play behavior unaltered. If dopamine plays a distinct role in the motivational and pleasurable aspects of social play, this may explain why treatment with drugs that increase dopamine neurotransmission has such variable effects on social play (Trezza et al., 2010). Furthermore, it supports the notion that

optimal levels of dopamine are required for social play behavior to occur (Trezza et al., 2010).

In contrast with our previous findings (Trezza et al., 2009), methylphenidate did not impair the acquisition of social play-induced CPP. In fact, animals treated with 3 mg/kg methylphenidate actually showed a stronger preference for the social compartment compared to control animals. These apparently discrepant data may be explained by a trade-off between the reduction of social play induced by methylphenidate (Beatty et al, 1982; Vanderschuren et al, 2008) and the rewarding properties of the drug itself (Trezza et al., 2009). The enhancement of CPP could therefore be due to the rewarding properties of methylphenidate having the upper hand, since the animals were treated with methylphenidate only before the social session. In addition, it has previously been shown that the positive effects of low doses of drugs of abuse, that itself reduce social play, may add up to the rewarding properties non-playful social interaction to induce CPP (Thiel et al., 2008; -2009). Enhancing noradrenergic neurotransmission, by blocking its reuptake reduces social play (Vanderschuren et al., 2008; present study). However, 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 suggests that at the higher dose of atomoxetine the magnitude of CPP was slightly reduced, which may be related to the large reduction in social play behavior with this dose of atomoxetine (Vanderschuren et al., 2008). These data suggest that increased endogenous noradrenaline inhibits social play, but not as a result of a change in its pleasurable effects.

Concluding remarks

The present study adds a new dimension to the analysis of social play behavior, by introducing a method with which the incentive motivational properties of social play can be explicitly assessed. Furthermore, our data show that dopaminergic and noradrenergic signalling affect different aspects of social play behavior. Enhancement of endogenous dopamine levels increases the motivation for social play, but reduces its pleasurable effects. Increases in noradrenergic neurotransmission reduce the expression of social play, and possibly also the motivation for social play, leaving its pleasurable properties unaffected. These data therefore provide new insights into the intricate mechanisms by which catecholamines modulate social play behavior in rats. Elucidating the neural underpinnings of social behavior in the young may increase our understanding of normal, adaptive social development, and may shed light on the pathophysiology of childhood and adolescent psychiatric disorders characterized by aberrant social behavior.

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Supplementary Materials and Methods

Operant conditioning paradigm

Apparatus

Behavioral testing was conducted in an operant conditioning chamber (Med Associates, Georgia, VT, 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 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, Georgia, VT, USA).

Place conditioning paradigm

Apparatus

The place conditioning boxes (TSE Systems, Bad Homburg, Germany), consisted 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

At 26 days of age (day 1), each rat was placed in the middle compartment of the apparatus and was allowed to move freely in the three compartments for 15 min. On the basis of their pre-test preference scores, rats were assigned to a compartment in which they would be allowed social interaction during conditioning. Place conditioning was performed according to a counterbalanced design (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-test performance, some rats were conditioned in their preferred compartment, but others were conditioned in their non-preferred compartment. This procedure rules out the possibility that preference shifts as a result of conditioning are the result of decreased avoidance of the non-preferred compartment. Subsequently, the 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; Trezza et al., 2009b; -2011b). 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.

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Supplementary Results

Supplementary figure 1

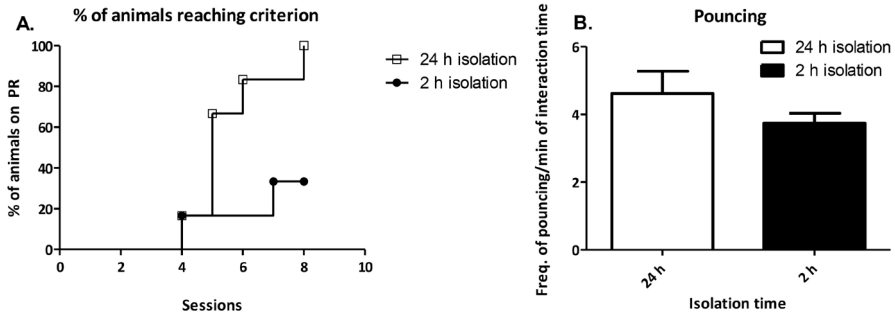

Fig S1: Acquisition curve and pouncing frequency after 2h and 24h of social isolation in an operant conditioning task for social play.
A. All (6/6) rats isolated for 24 h reach criterion under the FR-1 schedule of reinforcement to progress to the PR schedule of reinforcement within 8 days of training, whereas only one third (2/6) of the animals isolated for 2 h reached the FR-1 criterion within 8 days. B. No differences in the amount of pounces/min were found between the two isolation periods ($t=1.28$, $df=5$, $p=0.26$, $n=6$). Data are presented as percentage of animals reaching criterion or as mean + SEM.

Supplementary figure 2

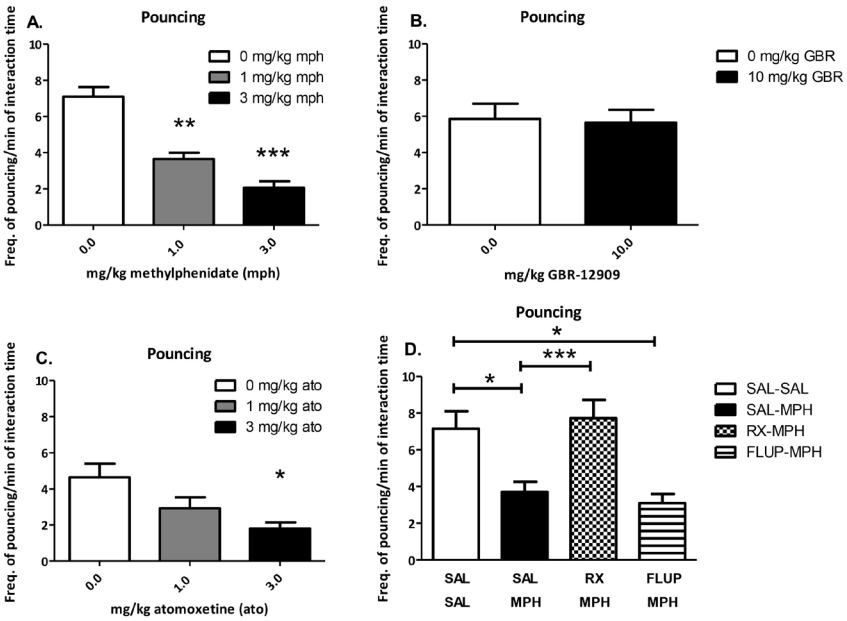

Fig S2: Effect of methylphenidate, GBR-12909 and atomoxetine or methylphenidate in combination with α-flupenthixol and RX821002 on pouncing frequency.

Treatment with methylphenidate (mph: 1-3 mg/kg) reduced pouncing: $F_{treatment}(2,10)=49.04$, $p<0.001$, n=6) (A). GBR-12909 treatment did not affect pouncing ($t=0.20$, $df=6$, $p=0.85$, n=7) (B). Atomoxetine-treated animals showed decreased pouncing ($F_{treatment}(2,14)=6.63$, $p=0.01$, n=8) (C). Pretreatment with α-flupenthixol or RX821002 before methylphenidate differentially affected pouncing ($F_{treatment}(3,21)=10.62$, $p<0.001$, n=8) (D). Data are presented as mean + SEM. * $p<0.05$, ** $p<0.01$, *** $p<0.001$.

Supplementary figure 3

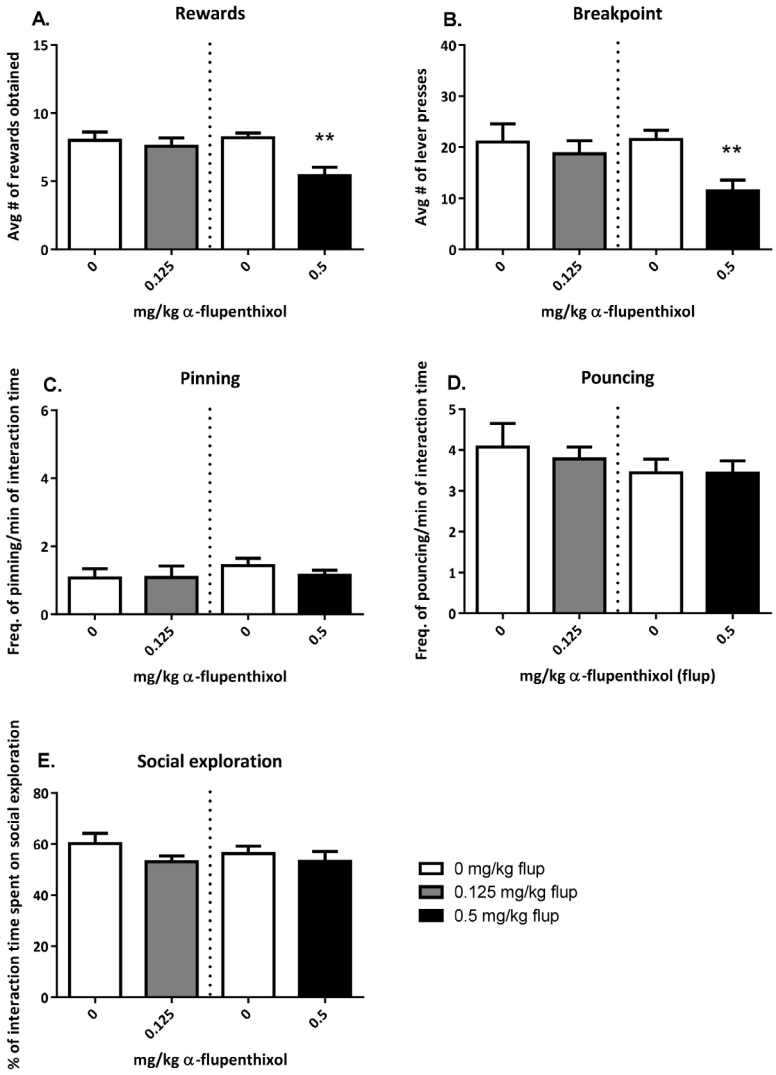

Fig. S3: Effect of the dopamine receptor antagonist α-flupenthixol on operant responding for social play behavior

Animals treated with 0.125 mg/kg α-flupenthixol (flup) did not differ in the amount of rewards obtained (t=0.89, df=6, p=0.41), the breakpoint (Z=4.00, p=0.34) (A-B) and inactive lever presses (see Supplementary Table 1). Furthermore, this dose did not affect pinning (t=-0.08, df=6, p=0.94), pouncing (t=0.49, df=6, p=0.64) or social exploration (t= 1,30, df=6, p=0.24, n=7) (C-E). The 0.5 mg/kg dose of α-flupenthixol reduced the number of rewards obtained (t=3.93, df=14, p=0.001) and breakpoint (Z=7.50, p=0.01) (A-B) but did not affect inactive lever presses (see Supplementary Table 1), pinning (t=1.25, df=14, p=0.23), pouncing (t=0.01, df= 14, p=0.99) or social exploration (t=0.64, df=14, p=0.53, n=15) (C-E). These data show that blocking dopamine receptors with 0.5 mg/kg but not 0.125 mg/kg α-flupenthixol reduces the motivation for social play without affecting play behavior itself. Data are presented as mean + SEM. ** p<0.01.

Supplementary figure 4

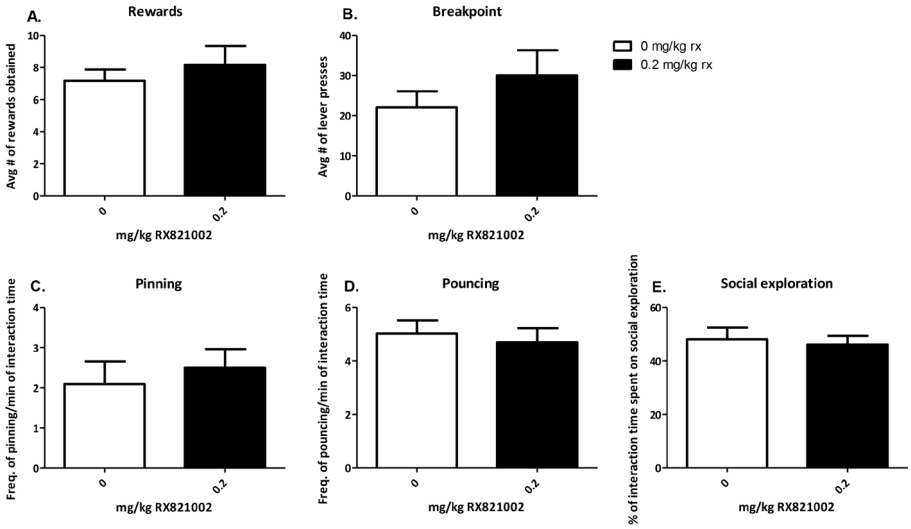

Fig. S4: Effect of the α-2 noradrenaline receptor antagonist RX821002 on operant responding for social play behavior

Administration of 0.2 mg/kg of the α-2 noradrenaline receptor antagonist RX821002 (rx) did not affect the number of rewards obtained (t=-0.91, df=5, p=0.40), breakpoint (Z=12.00, p=0.23) (A-B) or inactive lever presses (see Supplementary Table1). Social play behavior (pinning: t=-0.74, df=5, p=0.49; pouncing: t=0.39, df=5, p=0.72) and social exploration (t=0.45, df=5, p=0.67) were also unaffected by treatment with RX821002 (C-E, n=6). These data show that at this dose, RX821002 does not affect the motivation for social play or its expression. Data are presented as mean + SEM.

Supplementary Table 1: Number of inactive lever presses under the PR schedule of reinforcement

Experiment	Mean ± SEM	Statistics
24h vs 2h social isolation on acquisition and performance	24h:13,92 ± 3,37 2h: 3,92 ± 0,34	t=2.81, df= 5, p= 0.04
Methylphenidate (dopamine/noradrenaline reuptake inhibitor)	0 mg/kg: 15,25 ± 1,55 1 mg/kg: 13,67 ± 1,23 3 mg/kg: 20,00 ± 3,59	Ftreatment(2,10)= 4.21, p= 0.05 0 vs 1 mg/kg: t=0.60, df= 5, p=NS 0 vs 3 mg/kg: t=-1.89, df= 5, p=NS 1 vs 3 mg/kg: t=-2.20, df= 5, p=NS
GBR-12909 (dopamine reuptake inhibitor)	0 mg/kg: 21,43 ± 2,93 10 mg/kg: 14,86 ± 2,86	t=1.67, df=7, p=NS
Atomoxetine (noradrenaline reuptake inhibitor)	0 mg/kg: 15,25 ± 3,57 1 mg/kg: 7,25 ± 2,36 3 mg/kg: 4,875 ± 1,93	Ftreatment(2,14)=6.00, p=0.01 0 vs 1 mg/kg: t=2.08, df= 7, p=NS 0 vs 3 mg/kg: t=3.41, df= 7, p=0.01 1 vs 3 mg/kg: t=1.01, df= 7, p=NS
α-flupenthixol (dopamine receptor antagonist)	0 mg/kg: 7,71 ± 2.10 0,125 mg/kg: 15,28 ± 4.26	t=-1.63, df= 6, p=NS
	0 mg/kg: 7,47 ± 1,54 0.5 mg/kg: 7,00 ± 2,02	t=0.23, df=14, p=NS
RX821002 (α2 noradrenaline receptor antagonist)	0 mg/kg: 7,00 ± 2,12 0,2 mg/kg: 6,33 ± 1,59	t=0.27, df=5, p=NS
Pretreatment with α-flupenthixol (flup) or RX821002 (rx) followed by methylphenidate (mph)	sal-sal: 11,50 ± 1,50 sal-mph: 11,88 ± 2,79 rx-mph: 24,25 ± 7,00 flup-mph: 8,38 ± 2,38	Ftreatment(3,21)=3.83, p=NS

photo: D.J. Houwing

Chapter 5

Role of opioids and endocannabinoids in the expression and the pleasurable and motivational properties of social play behavior in rats

In preperation

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Abstract

Social play behavior is a vigorous form of social interaction abundant during the juvenile and adolescent phases of life in many mammalian species, including humans. Social play is thought to be important for social and cognitive development. Being a rewarding activity, the expression of social play depends on its pleasurable and motivational properties. Since opioids and endocannabinoids have been widely implicated in reward processes, in the present study, we investigated the role of opioids and endocannabinoids in the pleasurable and motivational properties of social play behavior in rats. To assess social play motivation, an operant conditioning setup was used in which rats responded for social play under a progressive ratio schedule of reinforcement. The pleasurable properties of social play were assessed using the place conditioning paradigm. Blocking opioid receptors with naloxone reduced responding for social play, the expression of social play and blocked the development of social play-induced conditioned place preference (CPP). The cannabinoid-1 (CB1) receptor antagonist rimonabant non-specifically reduced operant responding, due to its pruritic effect, without affecting social play expression or CPP. Treatment with the opioid receptor agonist morphine disrupted operant responding, whereas enhancing endocannabinoid levels with URB597, that inhibits the hydrolysis of the endocannabinoid anandamide, reduced operant responding at specific doses. Both morphine and URB597 did not affect the expression of social play in the operant task, which may be related to methodological constraints of this paradigm. These data demonstrate that blocking opioid receptors affects the pleasurable and motivational aspects of social play, whereas enhancing opioid neurotransmission does not have a clear effect on the motivation for play. Endocannabinoids do not seem to be involved in the motivational and pleasurable aspects of social play behavior as measured in these experimental conditions.

Introduction

Social play behavior is abundantly expressed throughout the juvenile and adolescence periods in life (Panksepp et al., 1984; Pellis and Pellis, 1998; Spear, 2000). It is a highly vigorous form of social interaction, in which components of other social behaviors are present, although expressed in an adapted and/or out-of-context manner (Pellis and Pellis, 2009; Vanderschuren et al., 1997). Engaging in social play behavior is important for social and cognitive development (Baarendse et al., 2013a; Potegal and Einon, 1989; Van den Berg et al., 1999) as it equips animals and humans with a rich behavioral repertoire to flexibly adapt to challenges in the (social) environment (Špinka et al., 2001).

Social play behavior 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).

revious studies have indicated that the expression of social play behavior is modulated by opioid and cannabinoid neurotransmission. It has been suggested that opioids are specifically involved in the pleasurable aspects, rather than the motivation for social play (Trezza et al., 2010; Panksepp et al., 1980). For example, low doses of drugs that mimic the effects of endogenous opioids (e.g. morphine) enhance social play (Trezza et al., 2010; Trezza and Vanderschuren 2008a,b; Vanderschuren et al., 1997; Vanderschuren et al., 1995a,b; Normansell and Panksepp 1990; Niesink and Van Ree 1989; Panksepp et al., 1985). In contrast, opioid receptor antagonists (e.g. naloxone) reduce social play (Normansell and Panksepp 1990; Niesink and Van Ree 1989; Jalowiec et al., 1989; Siegel and Jensen 1986; Panksepp et al., 1985; Siegel et al., 1985; Beatty and Costello 1982; Trezza and Vanderschuren, 2009a). Furthermore, it has been shown that antagonizing μ-opioid receptors in the nucleus accumbens prevented the development of social play-induced conditioned place preference (CPP) (Trezza et al., 2011b). Endocannabinoids have been implicated in positive emotions and motivation (Mahler et al., 2007; Barbano et al., 2009). Treating rats with indirect cannabinioid agonists, i.e. drugs that prolong endocannabinoid signaling, such as URB597 (which inhibits FAAH, the enzyme that degrades the endocannabinoid anandamide) or VDM11 (which blocks anandamide reuptake) enhanced social play (Trezza and Vanderschuren 2009; Trezza and Vanderschuren 2008a,b). Interestingly, the effects of endocannabinoids on social play were found to depend on opioid receptor stimulation, and vice versa (Trezza and Vanderschuren, 2008a).

In the present study, we investigated whether opioids and endocannabinoids are involved in the pleasurable and motivational properties of social play behavior. To measure the motivational aspects of social play behavior we used an operant conditioning task that we recently developed, in which rats pressed a lever for access to a playful partner under a progressive ratio schedule of reinforcement (Chapter 4). In addition, we investigated whether changes in opioid and cannabinoid neurotransmission affected the acquisition of social play-induced 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 environment 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 opioid and cannabinoid neurotransmission in distinct aspects of social play behavior.

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 ($l \times w \times h$) 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

URB597 (Tocris Cookson, Avonmouth, UK) and rimonabant (National Institute of Mental Health's Chemical Synthesis and Drug Supply Program, Bethesda, MD, USA) were dissolved in 5% Tween-80/5% polyethylene glycol/saline. Morphine (O.P.G. Utrecht, The Netherlands), naloxone (Tocris Cookson, Avonmouth, UK), were dissolved in saline. Morphine and naloxone were administered subcutaneously (s.c.), 1h and 30 min before testing, respectively. URB597 and rimonabant were administered intra-peritoneally (i.p.), 2h and 30 min before testing, respectively. Drug doses and pre-treatment intervals were based on previous studies (Trezza and Vanderschuren 2008a,b). Drug doses were calculated as salt. In view of the importance of the neck area in the expression of social play behavior (Pellis and Pellis, 1987; Siviy and Panksepp, 1987), s.c. injections were administered in the flank.

Operant conditioning paradigm

Apparatus

Behavioral testing was conducted in an operant conditioning chamber (Med Associates, Georgia, VT, 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, Georgia, VT, 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 by more than 10 grams in body weight at the start of the experiment. A test pair consisted of one experimental animal and its stimulus partner. At 24 days of age, test pairs were habituated to the test cage for 10 min. During the habituation session, the

animals could freely explore the entire apparatus. After the habituation session, animals were isolated for 24 h/day for 5 consecutive days/week, except in the first validation experiment, in which we also included a group of animals isolated for 2 h/day for 5 days/week. Next, the animals received two shaping sessions on two consecutive days. During these shaping sessions, the cue light was presented, the lever retracted and the door opened when the experimental animal approached the active lever. Rats were allowed to interact for two minutes after which the door closed and each rat was placed back into its starting compartment by the experimenter. 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 an additional shaping session in the afternoon.

On the fourth day, the lever pressing sessions (20 min) 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 inter-trial interval. During this interval, the experimenter placed each rat back into its starting compartment. 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; Hodos, 1961; Richardson and Roberts, 1996). When rats met the response requirement, 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 received one session per day, for 5 consecutive days/week. During the other 2 days/week animals were socially housed with their original cage-mates. After responding had stabilized, defined as obtaining at least six rewards on three consecutive days with a variation of no more than two rewards, drug treatment started according to a Latin Square design. Inactive lever presses were recorded, but had no programmed consequences.

Analysis of social play behavior

During earned social interactions, behavior 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, behavior of the animals was recorded using a camera with zoom lens, video tape recorder and television monitor. Three behavioral elements were scored (Panksepp et al., 1984; Trezza et al., 2010; Vanderschuren et al., 1997). 1. Frequency of pinning: one animal lying with its dorsal surface on the floor with the other animal is standing over it. 2. Frequency of pouncing: one animal attempts to nose/rub the nape of the neck of the partner, which is an index of play solicitation. Pinning and pouncing frequencies are considered the most characteristic parameters of social play behavior in rats (Panksepp and Beatty, 1980). 3. Time spent in social exploration: one animal sniffing or grooming any part of the partner's body. This is a measure of general social interest. Because of the pruritic action of the cannabinoid-1 (CB1) receptor antagonist rimonabant (Cook et al. 1998; Rubino et al. 2000; Tallett et al. 2007; Vickers et al. 2003), time spent scratching was also scored in the experiment where rimonabant was tested. Scratching was scored separately during lever pressing (scratching alone) and during social interaction (scratching together).

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 (in 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 (experimental 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, some of the rats were conditioned in their preferred compartment, while some 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 and the duration of rimonabant-induced scratching was calculated as a percentage of time. These data were analysed using a repeated measures ANOVA with drug dose as within-subjects factor 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 when 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

Effects of the opioid receptor antagonist naloxone and the CB-1 cannabinoid receptor antagonist rimonabant on operant responding for social play

Animals treated with naloxone (0.1-1.0-3.0 mg/kg) showed reduced responding for social play under a PR schedule of reinforcement ($F_{treatment}(3,21)=10.07$, $p<0.001$, $n=8$). Animals discriminated between the active and inactive lever ($F_{lever}(1,7)=40.33$, $p<0.001$). After treatment with naloxone, there was a significant, dose-dependent reduction in the number of active responses with no change in responses on the inactive lever ($F_{lever\ x\ treatment}(3,21)=8.94$, $p=0.001$) (Fig. 1A). Furthermore, the number of rewards obtained as well as the breakpoint was dose-dependently reduced (rewards: $F_{treatment}(3,21)=5.94$, $p=0.004$; breakpoint: $X^2=10.09$, $df=3$, $p=0.02$) (Fig. 1B-C). In addition to the reduction in operant responding, treatment with naloxone decreased the frequency of pinning ($F_{treatment}(3,21)=10.48$, $p<0.001$) and pouncing ($F_{treatment}(3,21)=15.58$, $p<0.001$) but did not affect the time spent on social exploration ($F_{treatment}(3,21)=1.14$, $p=0.36$) (Fig. 1D-F).

After treatment with the CB1 receptor antagonist rimonabant (0.1-0.3-1.0 mg/kg), responding for social play was reduced ($F_{treatment}(3,21)=3.59$, $p=0.03$, $n=8$). Animals discriminated between the active and inactive lever ($F_{lever}(1,7)=136.81$, $p<0.001$). Treatment with the highest dose of rimonabant (1 mg/kg), tended to affect the number of active and inactive responses differently ($F_{lever\ x\ treatment}(3,21)=2.72$, $p=0.07$) (Fig. 2A). In addition, the number of rewards obtained was significantly reduced at the highest dose ($F_{treatment}(3,21)=4.16$, $p=0.02$) (Fig. 2B), and no effect on the breakpoint ($X^2=6.46$, $df=3$, $p=0.09$) (Fig. 2C). Treatment with rimonabant did not affect the frequency of pinning ($F_{treatment}(3,21)=2.60$, $p=0.08$) and pouncing ($F_{treatment}(3,21)=0.58$, $p=0.63$) or the time spent on social exploration ($F_{treatment}(3,21)=0.58$, $p=0.64$) (Fig. 2D-F). The time spent scratching during lever pressing was significantly increased by 0.3 and 1.0 mg/kg rimonabant ($F_{treatment}(3,21)=6.67$, $p=0.03$) (Fig. 2G), and scratching during social interaction was significantly enhanced after treatment with 1.0 mg/kg rimonabant ($F_{treatmen}t(3,21)=6.45$, $p=0.02$) (Fig. 2H).

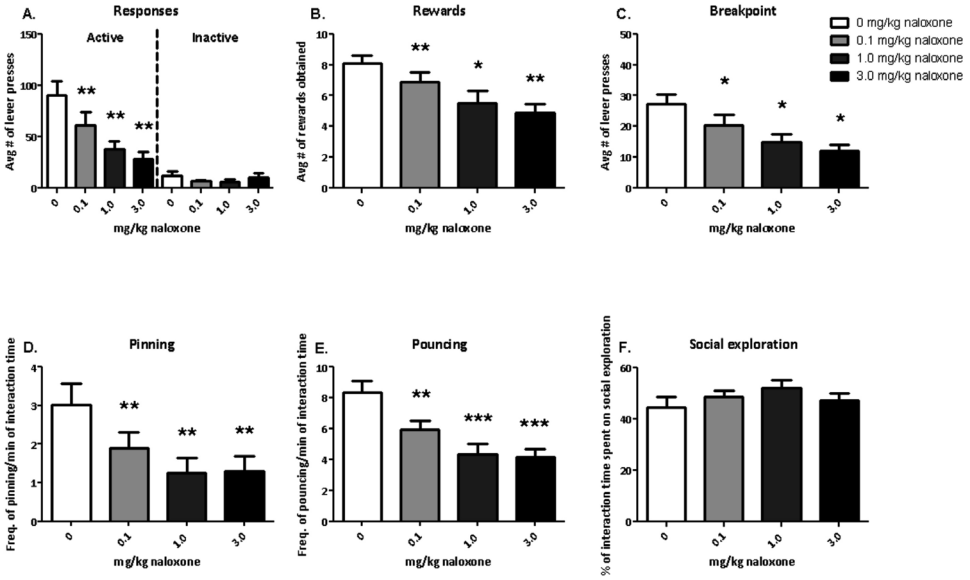

Fig. 1: The effect of the opioid receptor antagonist naloxone (0.1-1.0-3.0 mg/kg, n=8) on operant responding for social play behavior. Treatment with naloxone reduced the number of active responses without affecting inactive responses (A). Breakpoint (B) and rewards obtained (C) were also reduced. Naloxone-treatment reduced the expression of social play behavior, i.e pinning (D) and pouncing (E) without affecting social exploration (F). Data are presented as mean + SEM. * p< 0.05, ** p< 0.01, *** p< 0.001, relative to vehicle treatment.

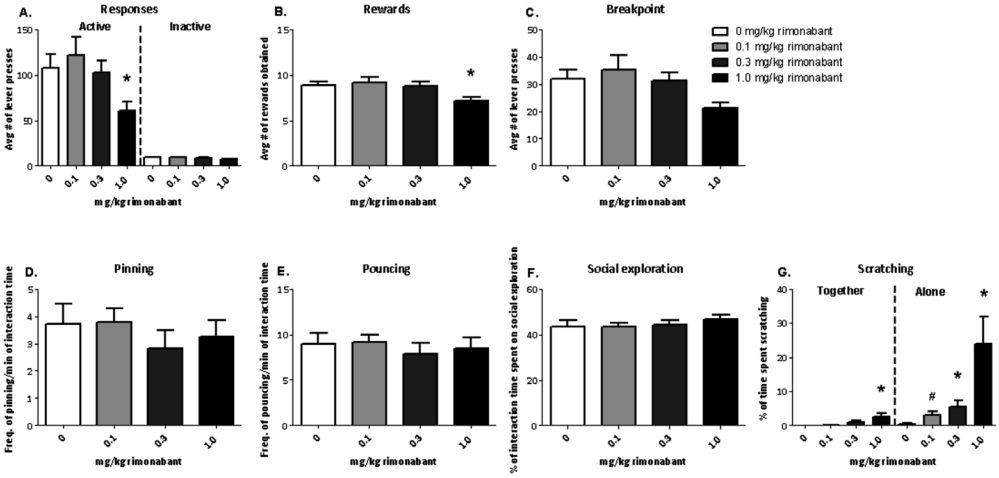

Fig. 2: The effect of the CB1 cannabinoidreceptor antagonist rimonabant (0.1-0.3-1.0 mg/kg, n=8) on operant responding for social play behavior. Treatment with the highest dose of rimonabant reduced the number of active but not inactive responses (A) and the number of rewards obtained (B). There was a tendency for a reduced breakpoint after rimonabant (C). Rimonabant did not alter the expression of social play, i.e. pinning (D) and pouncing (E) or social exploratory behavior (F). Rimonabant induced a marked increase in scratching, which was most pronounced during responding for social play (G). Data are presented as mean + SEM. * p< 0.05, # p= 0.05-0.08, relative to vehicle treatment.

Effects of the opioid receptor agonist morphine and the FAAH inhibitor URB597 on responding for social play

At the highest dose tested, (3.0 mg/kg), morphine reduced responding for social play ($F_{treatment}(3,21)=23.53$, $p<0.001$). Although the animals discriminated between the levers ($F_{lever}(1,7)=84.33$, $p<0.001$), the highest dose of morphine reduced the number of both active and inactive responses ($F_{lever\ x\ treatment}(3,21)=14.56$, $p<0.001$) (Fig. 3A). Furthermore, the number of rewards obtained as well as the breakpoint was reduced at the highest dose of morphine (rewards: $F_{treatment}(3,21)=36.12$, $p<0.001$; breakpoint: $X^2=15.64$, df=3, p=0.001) (Fig. 3B-C). Morphine did not affect the frequency of pinning ($F_{treatment}(3,21)=1.09$, p=0.38), pouncing ($F_{treatment}(3,21)=0.66$, p=0.59) or the time spent on social exploration ($F_{treatment}(3,21)=0.81$, p=0.50) (Fig. 3D-F).

The FAAH inhibitor URB597 (URB: 0.05-0.1-0.2 mg/kg) altered operant responding for social play ($F_{treatment}(3,21)=3.47$, p=0.03). Administration of URB597 tended to reduce responding on the active lever at the lowest dose and significantly decreased it at the highest dose, but did not affect inactive lever presses ($F_{lever}(1,7)=86.95$, $p<0.001$) ($F_{lever\ x\ treatment}(3,21)=4.42$, p=0.02) (Fig. 4A). In addition, URB597 tended to reduce the number of rewards obtained at the lowest dose and significantly decreased it at the highest dose ($F_{treatment}(3,21)=3.62$, p=0.03) (Fig. 4B). There was a tendency for URB597 treatment to reduce the breakpoint ($X^2=7.34$, df=3, p=0.06) (Fig. 4C). Treatment with URB597 did not alter pinning ($F_{treatment}(3,21)=0.14$, p=0.93), pouncing ($F_{treatment}(3,21)=0.66$, p=0.59) and social exploration ($F_{treatment}(3,21)=1.58$, p=0.23) (Figure 4D-F).

Effects of the opioid receptor antagonist naloxone and the CB1 cannabinoid receptor antagonist rimonabant on acquisition of social play-induced CPP

For animals treated with naloxone (0.1-1.0-3.0 mg/kg), the two-way ANOVA revealed a significant effect of compartment ($F_{compartment}(1,120)=33.00$, $p<0.001$), no effect of dose ($F_{dose}(3,120)=0.01$, p=0.99) and a significant compartment by dose interaction ($F_{compartment\ x\ dose}(3,120)=25.27$, $p<0.001$). Post hoc analysis showed that treatment with 1.0 and 3.0 mg/kg naloxone blocked the acquisition of social play-induced CPP (Fig. 5A). Rimonabant treatment (0.1-0.3 mg/kg) did not affect acquisition of social play-induced CPP. The two-way ANOVA showed a significant effect of compartment ($F_{compartment}(1,58)=43.72$, $p<0.001$) but no effect of dose ($F_{dose}(2,58)=0.15$, p=0.86) or a compartment by dose interaction ($F_{compartment\ x\ dose}(2,58)=2.48$, p=0.09) (Fig.5B).

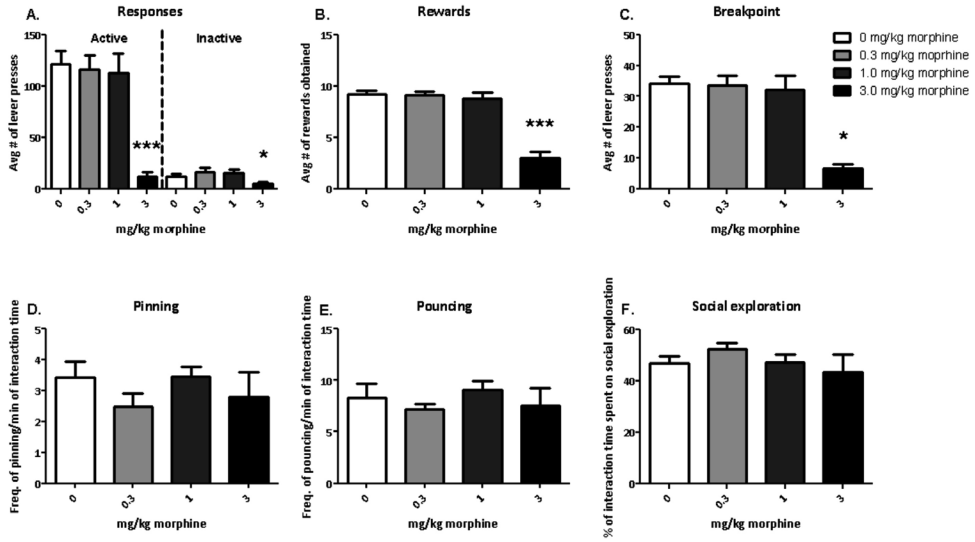

Fig. 3: The effect of the opioid receptor agonist morphine (0.3-1.0-3.0 mg/kg, n=8) on responding for social play. The highest dose of morphine reduced both active and inactive responses (A), rewards (B) and breakpoint (C), without affecting pinning (D), pouncing (E) social exploration (F). Data are presented as mean + SEM. * p< 0.05, *** p< 0.001, relative to vehicle treatment.

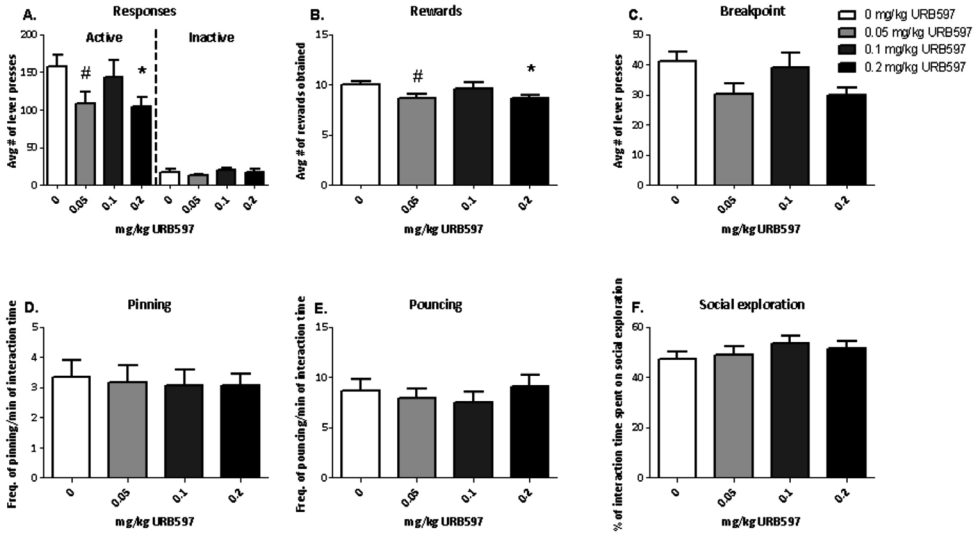

Fig. 4: The effect of the FAAH inhibitor URB597 (0.05-0.1-0.2 mg/kg, n=8) on responding for social play. URB597 reduced active but not inactive responses (A), the number of rewards obtained (B) and tended to decrease breakpoint (C). Pinning (D), pouncing (E) as well as social exploration (F) were unaffected by URB597-treatment. Data are presented as mean + SEM. * p< 0.05, # p= 0.05-0.08, relative to vehicle treatment.

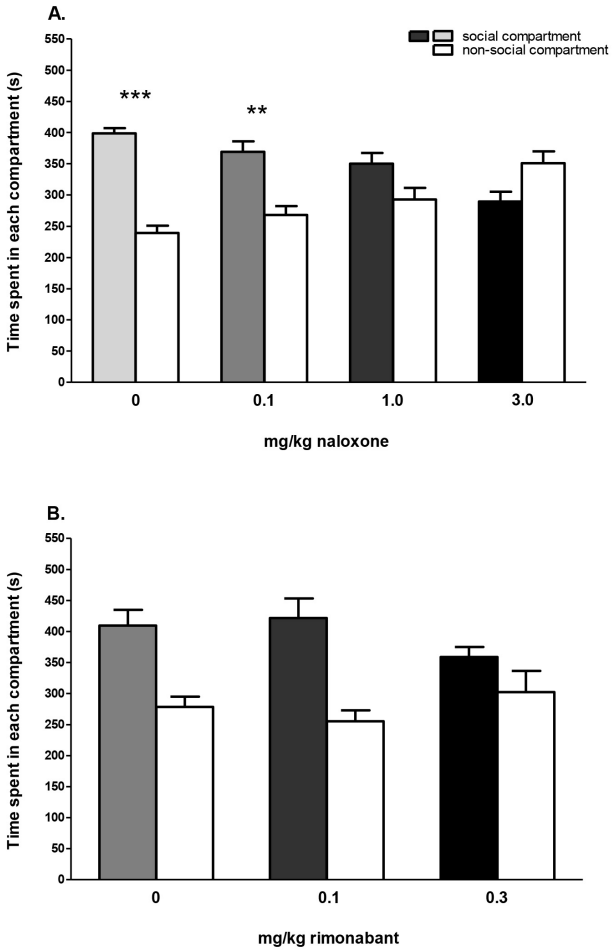

Fig. 5: Effect of the opioid receptor antagonist naloxone (0.1-1.0-3.0 mg/kg) and the CB1 cannabinoid receptor antagonist rimonabant (0.1-0.3 mg/kg) on acquisition of social play-induced CPP. Treatment with naloxone disrupted acquisition of social play-induced CPP (A; n = 28/10/10/16). Treatment with rimonabant did not affect the acquisition of social play-induced CPP (B; n = 12/10). Data are presented as mean + SEM time (sec) spent in each compartment. Grey bars indicate the time spent in the social compartment, white bars indicate the time spent in the non-social compartment. ** $p < 0.01$, *** $p < 0.001$, social vs. non-social compartment.

Discussion

The aim of the present study was to investigate the role of opioids and endocannabinoids in the motivational and pleasurable aspects of social play behavior. The present study shows that: (1) blocking opioid receptors with naloxone disrupts expression of social play, as well as its pleasurable and motivational properties; (2) blocking CB1 cannabinoid receptors with rimonabant non-specifically reduced responding for play, probably due to its pruritic properties, and did not affect acquisition of social play-induced CPP; (3) at the highest does tested, the opioid receptor agonist morphine reduced responding for social play, probably as a result of its disruptive effects on operant behavior, whereas expression of social play was unaffected; (4) enhancing endocannabinoid levels with the indirect agonist URB597 reduced the motivation to play but did not affect its expression.

Naloxone reduced responding for social play, expression of social play and the development of social play-induced CPP

Blocking opioid receptors with naloxone reduced responding for social play behavior as well as its expression. The naloxone-induced reduction in responding for social play is in line with studies on the effects of naloxone (Cleary et al., 1996; Solinas and Goldberg, 2005; Barbano and Cador, 2007; Barbano et al., 2009; Schneider et al., 2010), and genetic deletion of μ-opioid receptors (Papaleo et al., 2007) on operant responding for food. Together, this implicates opioid receptor stimulation in the motivational properties of natural rewards, such as food and social play behavior. Interestingly, using a play-rewarded T-maze task, Normansell and Panksepp (1990) found that treatment with naloxone or morphine did not affect the motivational parameter in the task (i.e. latency to enter the goal-box), but did affect the expression of social play. This discrepancy in findings can be due to differences in setup (runway vs. operant responding), especially the difference in time and effort necessary to obtain the reward. That is, running down a T-maze requires less time and effort compared to lever pressing under a progressive ratio schedule of reinforcement. Therefore, a runway task may be less sensitive to motivational factors than operant responding. Indeed, we have previously found that time to traverse a runway for social play is not sensitive to duration of social isolation (Trezza and Vanderschuren, unpublished data), whereas operant responding for social play is (chapter 4).

In line with previous reports, a reduction in play was found after naloxone treatment in the operant setup (Trezza and Vanderschuren, 2009; Normansell and Panksepp, 1990; Siegel and Jensen, 1986; Panksepp et al., 1985; Beatty and Castello, 1980). Moreover, in keeping with the notion that naloxone affects the pleasurable aspects of rewards (Delameter et al., 2000; Imazumi et al., 2001; Kelley, 2004; Jarosz et al., 2006; Barbano and Cador, 2007; Berridge et al., 2009; Schneider et al., 2010; Salamone and Correa, 2012), naloxone treatment disrupted the acquisition of social play-induced CPP. Consistent, we have previously found that infusion of the μ-opioid antagonist CTAP into the nucleus accumbens prevented the development of social play-induced CPP (Trezza et al., 2011b). This suggests that blocking opioid receptors affected the pleasurable properties of social play in such a way that it no longer supports learning.

Rimonabant did not affect responding for social play, expression of social play or social play-induced CPP

Blocking CB1 cannabinoid receptors with rimonabant reduced operant responding for social play but did not affect the expression of social play behavior. However, the reduction in operant responding induced by rimonabant may be secondary

to its pruritic effect (Cook et al. 1998; Rubino et al. 2000; Tallett et al. 2007; Vickers et al. 2003). Indeed, during lever pressing, animals treated with the highest dose of rimonabant (1.0 mg/kg) spent about 25% of their time scratching, which may have interfered with operant responding. Thus, the reduction in operant responding is likely the result of behavioral competition, which has been proposed to underlie certain other behavioral effects of rimonabant as well (e.g. Tallett et al., 2007). Interestingly, rimonabant-induced scratching did not interfere with the expression of social play behavior, as animals treated with 1.0 mg/kg rimonabant spent only 4% of their time on scratching during reinforced periods in the operant task. This is probably due to the high motivation of the animals to play. The animals' only opportunity to play is during earned social interaction in the operant task, suggesting that competing behaviors may also reduce the influence of the pruritic effects of rimonabant on behavior.

Blocking CB1 receptors did not affect acquisition of social play-induced CPP, although numerically, the social play-induced CPP after treatment with 0.3 mg/kg rimonabant was somewhat reduced. Thus, rimonabant may have a modest effect on the pleasurable properties of social play, consistent with the findings that food-induced CPP was inhibited after CB1 receptor blockade (Chaperon et al., 1998; Mendez-Diaz et al., 2012).

In previous studies, rimonabant was found to reduce operant responding under both PR and second order schedules of reinforcement for food (Solinas et al., 2005; Evenden and Ko, 2007; Ward et al., 2008; Meye et al., 2013) and chocolate-drinks (Maccioni et al., 2008). Furthermore, it also reduced the amount of food intake and chocolate-drink consumed, suggesting that the reduction in motivation is related to the pleasurable aspects of rewards being affected by rimonabant. For social play behavior, however, these data suggest that the effects of CB1 cannabinoid receptor blockade on the motivational and pleasurable properties of social play are modest at best, at least in the experimental conditions used in the present study

The effect of morphine and URB597 on responding for social play and expression of social play

Treatment with morphine reduced operant responding at the highest dose (3.0 mg/kg) but did not affect social play expression. Although morphine has been found to increase responding for food a PR schedule (Solinas and Goldberg, 2005), suppressant effects of morphine on operant behavior are well-documented (Thompson et al., 1970; Leander et al., 1975; Adams and Holtzman, 1990). Indeed, this dose of morphine reduced inactive lever presses as well, which suggests that non-specific, rate-decreasing effects of morphine underlie this effect.

Enhancing endocannabinoid levels using the FAAH inhibitor URB597 modestly reduced responding for social play at the highest dose tested. In contrast, URB597 treatment has previously been found not to affect responding for food (Oleson et al., 2012) or nicotine (Forget et al., 2009). Moreover, inhibiting the reuptake of anandamide had no effect on responding for food (Gamaleddin et al., 2013). Together, these data do not support a general role for endocannabinoid signaling in the motivational properties of rewards.

Remarkably, in contrast to previous studies (Trezza and Vanderschuren 2008a; -2008b; -2009), social play behavior in the operant conditioning task was not altered by morphine, URB597 or rimonabant. Previous studies have shown that morphine enhances social play according to an inverted U-shaped dose-effect curve, whereby 1 mg/kg induced robust increases in both pinning and pouncing (Trezza and Vanderschuren 2008a; Vanderschuren et al., 1995b-1996). In addition, treatment with URB597 increased social play after both systemic (Trezza and Vanderschuren 2008a,b)

and central (nucleus accumbens and basolateral amygdala) administration (Trezza et al., 2012), whereas rimonabant reduced social play (Trezza and Vanderschuren, 2009). Several factors could explain the discrepancies in findings. First, in the present study, only the experimental animal was treated, and not its stimulus partner. Trezza and Vanderschuren (2008b) previously showed that treating one animal of a couple with morphine results in an increase in pouncing (play initiations) but not pinning, whereas treating one animal with URB597 does not enhance social play, when behavior of a test couple was analyzed. Thus, treating only one animal in a test couple may not be sufficient to observe a robust increase (or decrease, for that matter) in social play. Second, here we socially isolated animals for 24 hours, whereas most previous studies used 3.5 hours of social isolation. Social isolation for 24 hours causes a maximal increase in the amount of social play (Niesink and Van Ree 1989; Vanderschuren et al., 1995b, 2008), which may obscure the play-enhancing properties of morphine and URB597 because of a ceiling effect (but see Vanderschuren et al., 1995b). Obviously, the lack of effect of rimonabant can not be explained on the basis of a ceiling effect. Third, the behavior of the stimulus animal should be considered as well. Possibly, drug-treatment of the experimental animal in combination with the 24 hours of social isolation may cause a difference in the willingness to play between both animals, so that the play interaction is less rewarding for the stimulus animals, thereby blunting the effects of morphine, URB597 and rimonabant. Indeed, social play has been found to be most pleasurable when both animals have a similar motivation to play (Douglas et al., 2004). Fourth, in the operant setup, animals have only one minute to play per reinforcement, whereas our previous studies on the expression of social play analysed this behavior for 15 minutes continuously. It could therefore be that stimulating effects on social play are blunted because the playful interaction is interrupted after 1 min. The present data, together with our previous findings (chapter 4) therefore indicate that social play expression in our operant setup may be more sensitive to manipulations that decrease social play than to those that increase this behavior. Possibly, adjustments to this setup may facilitate the detection of increases in social play expression, such as using a shorter isolation time, or longer interaction time per reinforcement.

In summary, a high dose of morphine disrupts operant responding for social play because of its rate-decreasing effects, whereas URB597 modestly reduced operant responding. Both treatments did not affect the expression of social play, which may be the result of the methodological constraints of the operant setup.

Conclusion

In the present study, we found that blocking opioid receptors affects the pleasurable and motivational aspects, as well as the expression of social play, as measured in operant and place conditioning tasks. Altering endocannabinoid signaling has no marked consequences for the motivational and pleasurable aspects of social play behavior, as measured in these behavioral tasks. Using the operant and place conditioning setup gives us the opportunity to gain more insight into the neural mechanisms involved in the motivational and the pleasurable aspects of social play behavior.

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Chapter 6

β-Adrenoreceptor stimulation mediates reconsolidation of social reward-related memories

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Abstract

Background

In recent years, the notion that consolidated memories become transiently unstable after retrieval and require reconsolidation to persist for later use has received strong experimental support. To date, the majority of studies on reconsolidation have focused on memories of negative emotions, while the dynamics of positive memories have been less well studied. Social play, the most characteristic social behavior displayed by young mammals, is important for social and cognitive development. It has strong rewarding properties, illustrated by the fact that it can induce conditioned place preference (CPP). In order to understand the dynamics of positive social memories, we evaluated the effect of propranolol, a β-adrenoreceptor antagonist known to influence a variety of memory processes, on acquisition, consolidation, retrieval and reconsolidation of social play-induced CPP in adolescent rats.

Methodology/Principal Findings

Systemic treatment with propranolol, immediately before or after a CPP test (i.e. retrieval session), attenuated CPP 24h later. Following extinction, CPP could be reinstated in saline- but not in propranolol-treated rats, indicating that propranolol treatment had persistently disrupted the CPP memory trace. Propranolol did not affect social play-induced CPP in the absence of memory retrieval or when administered 1h or 6h after retrieval. Furthermore, propranolol did not affect acquisition, consolidation or retrieval of social play-induced CPP.

Conclusions/Significance

We conclude that β-adrenergic neurotransmission selectively mediates the reconsolidation, but not other processes involved in the storage and stability of social reward-related memories in adolescent rats. These data support the notion that consolidation and reconsolidation of social reward-related memories in adolescent rats rely on distinct neural mechanisms.

Introduction

A newly acquired memory is initially unstable and prone to both facilitation and impairment. Memory consolidation progressively stabilizes the memory, making it resistant to interference (McGaugh, 2000). However, retrieval of a consolidated memory has been found to cause the memory to become unstable, in the sense that it is again vulnerable to interference. Reconsolidation is the process by which a retrieved memory is stabilized again (Misanin et al., 1968; Przybyslawski et al., 1999; Nader et al., 2000; for reviews see: Tronson and Taylor, 2007 Nader and Hardt, 2009; Inda et al., 2011). The function of memory reconsolidation is a topic of debate. Recent studies propose that reconsolidation is a process for maintaining and strengthening memory to prevent forgetting (Nader and Hardt, 2009) or to incorporate new information into the reactivated memory-trace (Lee et al., 2009). Reconsolidation is usually studied using aversive memories. There is also a substantial literature about the reconsolidation of food and drug memories, but reconsolidation of memories of physiologically relevant natural rewards such as social stimuli, has received little attention (Perrin et al., 2007). Social play is the most characteristic social behavior in adolescent mammals, which serves to facilitate social, physical and cognitive development (Panksepp et al., 1984; Vanderschuren et al., 1997; Špinka et al., 2001; Pellis and Pellis, 2009) Social play is highly rewarding for adolescent rats (Vanderschuren et al., 1997; Trezza et al., 2010; Trezza et al., 2011a) as exemplified by its capacity to induce conditioned place preference (CPP) (Calcagnetti and Schechter, 1992; Crowder and Hutto, 1992; Thiel et al., 2008; Trezza et al., 2009). Because place conditioning relies on an associative mechanism, it can be used to study the dynamics of emotionally charged memories (Bernardi et al., 2006; Fricks-Gleason and Marshall, 2008).

The β-adrenergic receptor has been implicated in memory reconsolidation for aversive as well as for pleasurable stimuli and events. For example, systemic administration of β-adrenergic antagonists such as propranolol (PROP) induces a memory impairment in rats in tasks such as fear conditioning (e.g. Debiec and Ledoux, 2004), conditioned stimulus-induced cocaine or sucrose seeking (Diergaarde et al., 2006; Milton et al., 2008; Robinson and Franklin, 2007), and drug-induced CPP (Bernardi et al., 2006; Fricks-Gleason and Marshall, 2008; Robinson and Franklin, 2007). PROP has also been shown to disrupt reconsolidation of fear memory in humans (Kindt et al., 2009).

In the present study, we investigated whether retrieved social reward-related memories in a social play-induced CPP paradigm could be disrupted by administration of PROP in adolescent rats. We hypothesized that if social reward-related memories reconsolidate following memory retrieval, PROP would attenuate preference for a social play-paired environment by disrupting the memory trace. This would prevent reinstatement of CPP following extinction and retraining. We also investigated the period of instability of the social play memory after retrieval (reconsolidation-window). Furthermore, since β-adrenergic signaling has also been implicated in other aspects of learning and memory (McGaugh, 2000, Cahill et al., 2000), we also tested whether PROP affected the acquisition, consolidation and retrieval of social play-induced CPP.

Materials and Methods

Ethics statement

All experiments were approved by the Animal Ethics Committee of the Utrecht University (license no. 2010.I.04.057) and were in agreement with Dutch laws (Wet op Dierproeven 1996) and European regulations (Guideline 86/609/EEC).

Animals
Male Wistar rats (Charles River, Sulzfeld, Germany) arrived in our animal facility at 21 days of age and were housed in groups of three or four in 40 x 26 x 20 cm (*l x w x h*) Macrolon cages under controlled conditions (i.e. temperature 20-24°C, 60-65% relative humidity and 12/12 h light cycle with lights on at 7.00 AM). Upon arrival, the animals were allowed at least 5 days of acclimatization to the facility and were handled for 3 days before the start of the experiment. Food and water were available *ad libitum*. All animals were experimentally naïve and were used only once.

Apparatus
Place conditioning was performed as previously described (Trezza et al., 2009; Veeneman et al., 2011). The place conditioning setup (TSE System, Bad Homburg, Germany) comprised 8 boxes, each consisting of three compartments with removable Plexiglas lids; two equally sized large conditioning compartments (30 x 25 x 30 cm *l x w x h*) separated by a smaller, neutral compartment (10 x 25 x 30 cm; *l x w x h*). The two conditioning compartments had different visual and tactile cues, which also differed from the cues in the middle compartment. The position of the animal in the apparatus was monitored by an array of photobeam sensors located 2.5 cm above the floor. A computer recorded the time (in msec) the animals spent in each compartment. All experiments with this setup were performed in a sound attenuated and dimly lit room.

Drugs
(±)-Propranolol HCl (PROP, Sigma-Aldrich, Germany) was dissolved in saline and administered *i.p.* (10 mg/kg, injection volume 2 ml/kg). At doses up to 10 mg/kg, PROP has been shown not to influence social play behavior (Vanderschuren et al., 2008), spontaneous locomotor activity or exploratory behavior (Sara et al., 1995).

Statistical analysis
Data were analyzed using SPSS software 15.0 for Windows. For each experiment, the time spent in the social paired and non-social paired compartments were expressed as mean ± SEM. Data were analyzed using ANOVA (mixed-model or two-way, depending on the experiment), using compartment (social or non-social) and treatment (PROP or saline) as between-subjects factor and test-day as repeated-measures factor. ANOVA was followed by Student's paired t-tests when appropriate, to investigate differences between the time spent in the social and non-social compartment.

Experimental procedures

1. Effects of acute post-retrieval PROP on social play-induced CPP.
The aim of this experiment was to investigate the effect of an acute post-retrieval PROP injection on the reconsolidation and reinstatement of social play-induced CPP. At 26 days of age (day 1), each rat was placed in the middle compartment of the CPP apparatus and pre-conditioning side preference was determined by allowing the rats to move freely around the three compartments of the apparatus for 15 min (Pretest). On the basis of their Pretest scores, rats were assigned to a compartment in which they would be allowed social interaction during conditioning. We used a counterbalanced place conditioning design (Tzschentke, 2007), 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 Pretest performance, some rats were conditioned in their preferred compartment, but others were conditioned in their non-

preferred compartment. This procedure rules out the possibility that preference shifts are the result of decreased avoidance of the non-preferred compartment. After the Pretest, rats were individually housed to increase their motivation for social interaction and to facilitate the development of social play-induced CPP (Trezza et al., 2009).

Place conditioning began on day 2. Rats underwent eight consecutive days of conditioning, with two conditioning sessions per day. On days 2, 4, 6 and 8 of the experiment, 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 days 3, 5, 7 and 9, the order of sessions was reversed, i.e. rats were placed alone in one side of the CPP apparatus during the morning session, and were placed in the other compartment with the social partner in the afternoon session. Social and non-social conditioning-sessions were separated by at least one hour. On day 10, rats were placed in the middle compartment and were allowed to explore the entire apparatus for 15 min (retrieval, RETR), and time spent in each compartment was recorded. Immediately after the retrieval session, the animals were randomly assigned to either the saline- or PROP-treatment group and injected. The next day, the animals were placed in the middle compartment again and were again allowed to move freely in the apparatus for 15 min to investigate the effect of the injection (TEST); this test is also considered the first extinction session. This procedure was repeated once a day for the following days to extinguish place preference, i.e., until the mean difference between the time spent in the social-paired and the non-social-paired compartments was no longer statistically significant for four consecutive days in all the experimental groups. This took between 8 and 22 extinction sessions. Twenty-four hours after the last extinction session, the rats received a reconditioning session. Each rat was placed in the social compartment with a social partner for 30 min (social session) and at least 1 hour later, it was placed in the non-social compartment alone for 30 min (non social session). The next day, the animals were exposed to the whole apparatus for 15 min and preference was determined again (reinstatement, REIN).

2. Effects of delayed post-retrieval PROP on reconsolidation of social play-induced CPP.

This experiment was designed to determine the period of instability of the social play-related memory trace after memory retrieval. Animals were conditioned as described in experiment 1. On day 10, one group of animals received PROP or saline 1h after retrieval while another group of animals received PROP or saline 6h after memory retrieval. The next day, i.e. 18h and 23h after injection, rats were tested (TEST) as described in experiment 1.

3. Effects of PROP on social play-induced CPP in the absence of memory retrieval.

This experiment investigated whether memory retrieval is essential for PROP to affect reconsolidation of social play-induced CPP. Animals were conditioned as described in experiment 1. On day 10, instead of a memory retrieval session, animals were treated with PROP or saline in their homecage. The next day, both groups were tested (TEST) as described in experiment 1.

4. Effects of PROP on retrieval of social play-induced CPP.

This experiment was designed to investigate the effect of PROP on retrieval of memory for social play-induced CPP. Animals were conditioned as described in experiment 1. PROP or saline was injected 30 min before the memory retrieval session. Animals were

tested for reconsolidation (TEST) and reinstatement (REIN) as described in experiment 1.

5. Effects of PROP on acquisition and consolidation of social play-induced CPP.

These experiments investigated the effects of PROP on acquisition and consolidation of social play-induced CPP. Animals were conditioned as described in experiment 1. Thirty minutes before or immediately after each conditioning session, animals were treated with PROP or saline, to investigate the effect of PROP on acquisition and consolidation of social play-induced CPP, respectively. On day 10, the animals were tested as described in experiment 1 (TEST).

Results

1. Effects of acute post-retrieval PROP on social play-induced CPP.

The mixed-model ANOVA revealed an effect of compartment ($F_{(1,50)}$= 45.78, p< 0.01), test-day ($F_{(2,100)}$= 5.88, p< 0.01) and a compartment per treatment interaction ($F_{(1,50)}$= 6.65, p< 0.05). No other main or interaction effects were found. Post-hoc tests revealed that the 'to be' saline-treated animals, and the 'to be' PROP-treated animals showed a significant preference for the social-play paired compartment on day 10 (RETR: PROP-treated rats: n= 8, t= 2.36, p= 0.05; saline-treated rats: n= 18, t= 7.35, p< 0.001; Figure 1). Twenty-four hours later (TEST, Figure 1), saline-treated animals still showed a preference for the social play-paired compartment (t= 5.18, p< 0.001), whereas PROP-treated animals did not (t= 1.72, p= 0.13). Following the reconditioning session, saline-treated animals showed reinstatement of social-play induced CPP (REIN: t= 3.69, p< 0.01), while PROP-treated rats did not (REIN: t= 0.40, p= 0.70; Figure 1). These findings indicate that PROP treatment interferes with memory reconsolidation immediately following retrieval of the social reward memory.

2. Effects of delayed post-retrieval PROP on reconsolidation of social play-induced CPP.

The mixed-model ANOVA revealed an effect of compartment ($F_{(1,74)}$= 150.71, p< 0.05). No other main or interaction effects were found. Post-hoc tests revealed that all three groups showed a significant preference for the social-paired compartment (RETR: saline-treated rats: n= 17, t= 7.09, p< 0.001; 1h delayed PROP-treated rats: n= 13, t= 9.89, p< 0.001; 6h delayed PROP-treated rats: n= 10, t= 2.82, p< 0.05; Figure 2). The next day, all groups continued to show a significant preference for the social-paired compartment (TEST: saline-treated rats: t= 3.30, p< 0.01; 1h delayed PROP-treated rats: t= 2.29, p< 0.05; 6h delayed PROP-treated rats: t= 2.49, p< 0.05). These data suggest that β-adrenoceptor-dependent reconsolidation of social reward-related memories takes place within 1h after memory retrieval.

3. Effects of PROP on social play-induced CPP in the absence of memory retrieval.

A two-way ANOVA revealed an effect of compartment (F(1,60)= 44.74, p< 0.05). No other main or interaction effects were found. Post-hoc tests showed that twenty-four hours after PROP or saline administration in the home-cage, animals showed a significant preference for the social-paired compartment (TEST: PROP-treated animals: n= 16, t= 3.36, p< 0.01; saline-treated animals: n= 16, t= 4.03, p< 0.01; Figure 3). These results indicate that memory retrieval is required for PROP to affect reconsolidation of social reward-related memories.

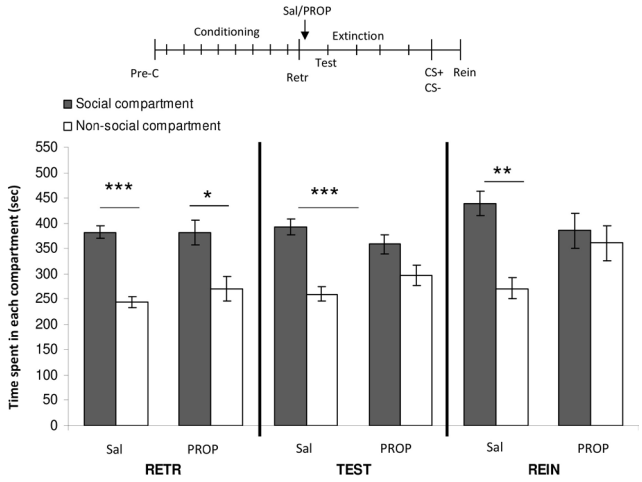

Figure 1 Effects of acute post-retrieval PROP on social play-induced CPP. The experimental protocol is depicted above the graph (Pre-C: pre-conditioning test, CS+: conditioning session with a play-partner, CS-: conditioning session alone). Data represent the mean time (sec ± SEM) spent in the social compartment (grey bars) and the non-social compartment (white bars) during 15min retrieval- (RETR), test- (TEST) and reinstatement- (REIN) sessions. Saline-treated animals (2ml/kg, i.p., n= 18), PROP-treated animals (10 mg/kg, i.p., n= 8). Post-hoc Student's paired t-tests for difference in time spent in the social- and non-social compartment *p< 0.05, **p< 0.01, ***p< 0.001.

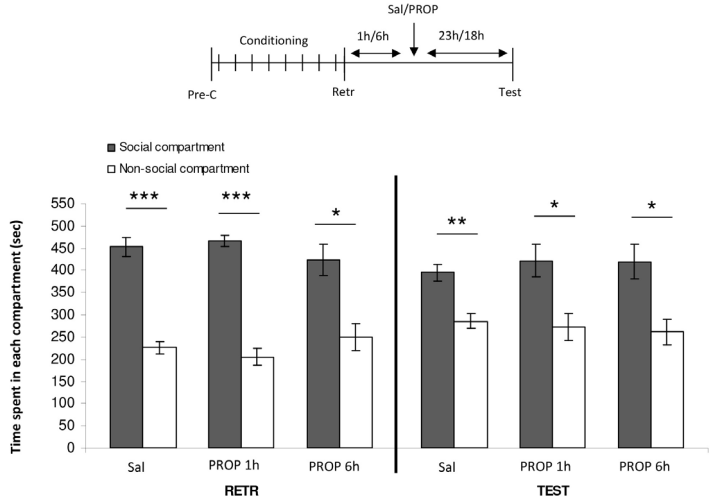

Figure 2 Effects of 1h and 6h delayed post-retrieval PROP on reconsolidation of social play-induced CPP. The experimental protocol is depicted above the graph (Pre-C: pre-conditioning test, CS+: conditioning session with a play-partner, CS-: conditioning session alone). Data represent the mean time (sec ± SEM) spent in the social compartment (grey bars) and the non-social compartment (white bars) during 15min retrieval- (RETR) and test- (TEST) sessions. Saline-treated animals (2ml/kg, i.p., n= 17), 1h delayed PROP-treated animals (10 mg/kg, i.p., n= 13), 6h delayed PROP-treated animals (10 mg/kg, i.p., n= 10). Post-hoc Student's paired t-tests for difference in time spent in the social- and non-social compartment *p< 0.05, **p< 0.01, ***p< 0.001.

4. Effects of PROP on retrieval of social play-induced CPP.

The mixed model ANOVA revealed an effect of compartment ($F_{(1,70)}$= 34.09, p< 0.05), test-day ($F_{(1,140)}$= 6.01, p< 0.05) and a compartment per treatment interaction ($F_{(1,70)}$= 13.24, p< 0.05). No other main or interaction effects were found. Post-hoc tests revealed that both the saline- and PROP-treated animals showed a significant preference for the social-paired compartment at retrieval (RETR: saline-treated animals: n= 15, t= 7.09, p< 0.001; PROP-treated animals: n= 22, t= 2.70, p= 0.01; Figure 4). These results suggest that PROP does not affect retrieval of social reward-related memories. Twenty-four hours later, saline-treated animals continued to show a significant preference for the social-paired compartment (TEST: t= 3.61, p< 0.01), while PROP-treated animals no longer showed CPP (TEST: t= 0.86, p= 0.40). After extinction and reconditioning, animals were tested for reinstatement. Saline-treated animals showed significant reinstatement of CPP whereas PROP-treated animals did not reinstate their preference (REIN: saline-treated animals: t= 2.46, p< 0.05; PROP-treated animals: t= 0.11, p= 0.92). These results suggest that instead of retrieval, reconsolidation is affected by PROP, consistent with the results of experiment 1.

5. Effects of PROP on acquisition and consolidation of social play-induced CPP.

Two-way ANOVAs revealed an effect of compartment (acquisition: $F_{(1,60)}$= 114.93, p< 0.05; consolidation: $F_{(1,44)}$= 85.40, p< 0.05). No other main or interaction effects were found. Post-hoc tests revealed that both the PROP- and the saline-treated animals showed a robust preference for the social-paired compartment after 8 days of conditioning (Figure 5A: acquisition: RETR: PROP-treated animals: n= 16, t= 5.24, p< 0.01; saline-treated animals: n= 16, t= 7.40, p< 0.01; Figure 5B: consolidation: RETR: PROP-treated animals: n= 12, t= 5.40, p< 0.01; saline-treated animals: n= 12, t= 4.98, p< 0.01). These results show that PROP does not affect acquisition and consolidation of social play-induced CPP.

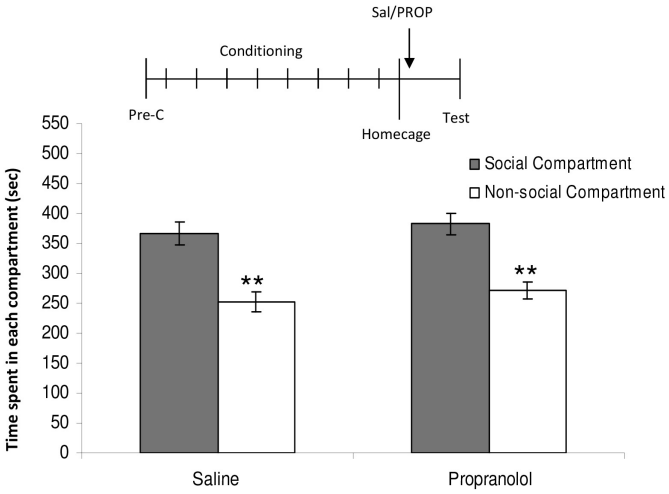

Figure 3 Effects of PROP on social play-induced CPP in the absence of memory-retrieval. The experimental protocol is depicted above the graph (Pre-C: pre-conditioning test). Data represent the mean time (sec ± SEM) spent in the social compartment (grey bars) and the non-social compartment (white bars) during a 15 min test session. Saline-treated animals (2ml/kg, *i.p.*, n= 16), PROP-treated animals (10 mg/kg, *i.p.*, n= 16). Post-hoc Student's paired t-tests for difference in time spent in the social- and non-social compartment **p< 0.01.

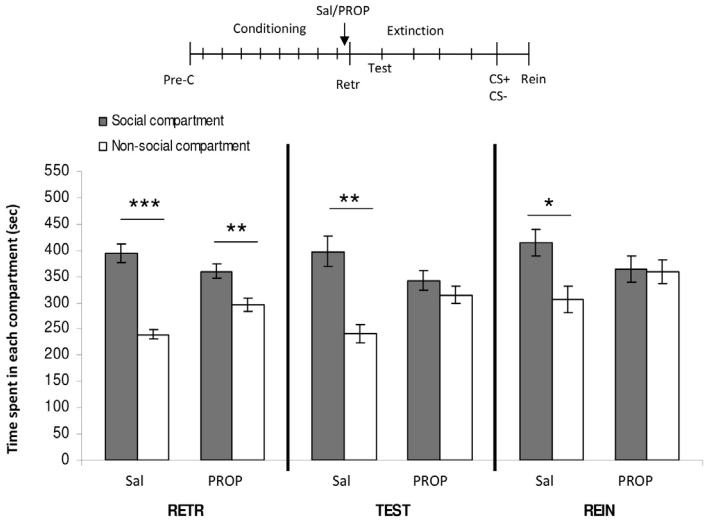

Figure 4 Effects of PROP on memory-retrieval of social play-induced CPP. The experimental protocol is depicted above the graph (Pre-C: pre-conditioning test, CS+: conditioning session with a play-partner, CS-: conditioning session alone). Data represent the mean time (sec ± SEM) spent in the social compartment (grey bars) and the non-social compartment (white bars) during 15 min retrieval- (RETR), test-(TEST) and reinstatement- (REIN) sessions. Saline-treated animals (2ml/kg, *i.p.*, n= 22), PROP-treated animals (10 mg/kg, *i.p.*, n= 15). Post-hoc Student's paired t-tests for difference in time spent in the social- and non-social compartment *p< 0.05, **p< 0.01, ***p< 0.001.

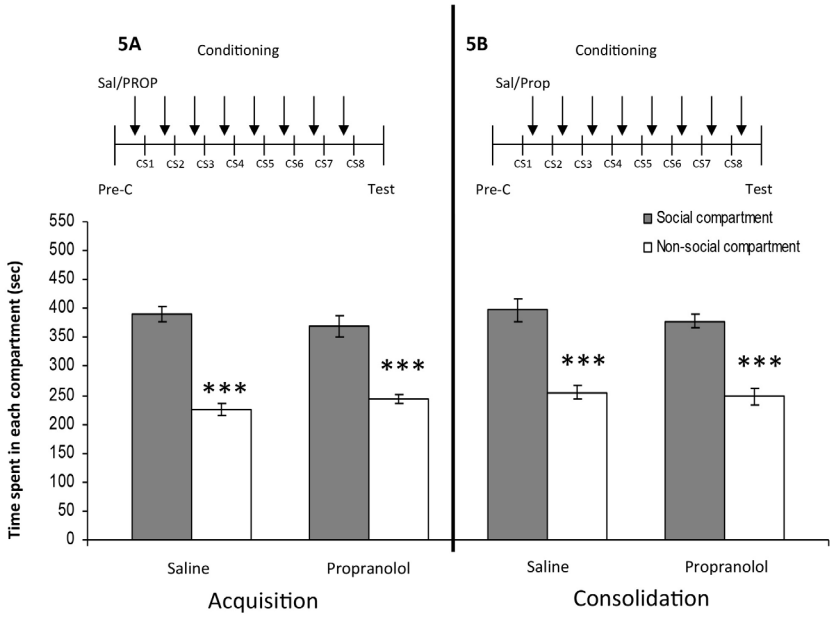

Figure 5 Effects of PROP on acquisition (panel A) and consolidation (panel B) of social play-induced CPP. The experimental protocol is depicted above the graph (Pre-C: pre-conditioning test, CS: daily conditioning sessions, consisting of one session with and one session without a play-partner present). PROP was administered either 30 min before (acquisition) or immediately after (consolidation) each conditioning session. Data represent the mean time (sec ± SEM) spent in the social compartment (grey bars) and the non-social compartment (white bars) during a 15 min retrieval-session. Saline-treated animals (2ml/kg, *i.p.*, acquisition: n= 16, consolidation: n= 12), PROP-treated animals (10 mg/kg, *i.p.*, acquisition: n= 16, consolidation: n= 12). Post-hoc Student's paired t-tests for difference in time spent in the social- and non-social compartment $^{**}p< 0.01$, $^{***}p< 0.001$.

Discussion

In this study, we investigated the involvement of noradrenergic neurotransmission in reconsolidation of social reward-related memories in adolescent rats. Our hypothesis was that, following memory-retrieval, the β-adrenergic receptor antagonist PROP would disrupt the reconsolidation of social play-induced CPP. We show that: (1) the reconsolidation process, which has previously been observed in rat pups (Languille et al., 2009) and adults (Nader et al., 2000), also occurs in adolescent rats; (2) systemic pre- or post-retrieval treatment with PROP impaired the reconsolidation of social play-induced CPP; (3) CPP could be reinstated after extinction in vehicle- but not PROP-treated rats; (4) the reconsolidation-window for social reward-related memories is less than 1h; (5) memory retrieval is necessary for PROP to affect the stability of social reward-related memories; (6) PROP does not affect acquisition, consolidation or retrieval of social reward-related memories. Together, our data show that, concerning the dynamics of social reward-related memories, β-adrenergic neurotransmission specifically mediates the reconsolidation of social play-induced CPP.

In the first experiment, saline-treated animals showed a preference for the social-

paired compartment 24h after post-retrieval treatment, whereas PROP-treated animals did not. This effect of PROP was not the result of a non-specific memory impairment, since PROP treatment in the absence of retrieval did not alter social play-induced CPP (Tronson and Taylor, 2007; Nader and Hardt, 2009). Furthermore, following extinction of CPP, saline-treated animals reinstated their preference 24h after a reconditioning session, whereas PROP-treated animals did not. Post-retrieval PROP administration has been found to impair memory when animals are re-tested 24h after retrieval in a variety of paradigms (Nader et al., 2000; Bernardi et al., 2006; Fricks-Gleason and Marshall, 2008; Diergaarde et al., 2006; Milton et al., 2008; Robinson and Franklin, 2007). The inability to reinstate the social play-induced CPP response in the PROP-treated group suggests that acute post-retrieval PROP persistently disrupted the social play-CPP memory trace, rather than inducing a retrieval deficit. PROP may have facilitated extinction learning instead of disrupting reconsolidation. However, since extinguished memories can be reinstated after retraining (Bouton, 1993), and PROP seems to impair rather than facilitate extinction (Cain et al., 2004, Cohen and Gotthard, 2011), this explanation is rather unlikely. Somewhat consistent with our results, post-retrieval PROP treatment has previously been shown to disrupt the reconsolidation and reinstatement of cocaine-induced CPP, albeit that a single PROP treatment interfered with reconsolidation, but that repeated post-retrieval PROP treatments were necessary for blockade of reinstatement (Fricks-Gleason and Marshall, 2007). In the case of morphine-induced CPP, PROP disrupted reconsolidation but not reinstatement (Robinson and Franklin, 2007). An important difference between our experiments and these previous studies is the way in which reinstatement was evoked, i.e. a single reconditioning session in the present study vs a drug prime in the previous studies. Another possible explanation for the differences between the abovementioned findings and our results could be that drug reward-context associations might be stronger than natural reward-context associations, so that repeated interference with reconsolidation is necessary to persistently disrupt a drug-induced CPP memory trace (Sadler et al., 2007). Together, these findings show that β-noradrenergic neurotransmission, involved in reconsolidation of memory for drug (Bernardi et al., 2006; Fricks-Gleason and Marshall, 2007; Robinson and Franklin, 2007; Milton et al., 2008) and food rewards (Diergaarde et al., 2006; Milton et al., 2008) is also involved in reconsolidation of social reward memories in adolescent rats. Furthermore, PROP persistently disrupted the social-play CPP memory trace as social play-induced CPP could be reinstated in saline- but not PROP-treated animals.

Our results show that the period of instability for social reward-related memories lasted less than 1h. Using different paradigms, amnesic agents and species, a window of about 6h after which amnesic treatment no longer affects reconsolidation has often been reported (Nader et al., 2000; Przybyslawski et al., 1999; Suzuki et al., 2004). Consistent, we found that post-retrieval PROP treatment after a 6h delay did not impair social play-induced CPP. Interestingly, and in keeping with our findings, two recent studies have shown that amnesic treatments 1 hr post-retrieval do not affect reconsolidation of amphetamine-induced CPP or fear memory (Suzuki et al., 2004; Kim et al., 2010). Our data therefore suggest that memory reconsolidation for social play-induced CPP occurs quite quickly. This is not surprising from a mechanistic point of view. Reconsolidation is thought to depend on restabilization of existing synaptic networks (Nader et al., 2000), and to serve as an updating mechanism for existing memory traces (Lee et al., 2009). In this light, a brief reconsolidation-window for social memories may be beneficial for social animals, including humans. Because social animals live in a complex, rapidly changing social environment and social interaction can be very brief, the updating of social information must be rapid in order for social animals to function properly.

Administration of PROP 30 min before the CPP test did not alter the expression of CPP, showing that PROP did not affect retrieval of social reward-related memories. The PROP-treated animals, however, did show an absence of preference 24h after the test for retrieval, suggesting that, consistent with our first experiment, PROP affected reconsolidation instead of retrieval. Furthermore, in contrast to saline-treated animals, PROP-treated rats did not reinstate their preference for the social-paired compartment. In PROP-treated animals across the different tests in this experiment, the presence and absence of CPP was comparable to that of rats receiving a post-retrieval PROP injection. These findings show that β-noradrenergic neurotransmission is not involved in the retrieval of social reward-related memories, but that blockade of β-adrenoceptors during the retrieval session, and perhaps briefly after, interfered with the reconsolidation of social play-induced CPP. In contrast to our results, PROP has been shown to impair memory retrieval in different paradigms in adult rats and mice (Murchison et al., 2004; De Quervain et al, 2007; Otis and Mueller, 2011), but not in humans (Tollenaar et al., 2008). Thus, the involvement of β-noradrenergic signaling in memory retrieval likely depends on the type of memory, species and age of the subjects. Since noradrenergic neurotransmission is known to be involved in acquisition and consolidation of certain types of memories, we tested whether β-adrenoreceptors are involved in the acquisition and consolidation of social play-induced CPP as well. However, daily pre-training or post-training administration of PROP did not affect social play-induced CPP. These results indicate that PROP interferes with synapse-remodeling when the social reward-related memory is reactivated but not when it is formed. Administration of PROP has previously been shown to impair the acquisition of aversive memories in rats and humans (Beatty and Rush, 1983; Cahill and Setlow, 2000; Sara et al., 1999). Apparently, involvement of β-adrenoceptors in memory acquisition does not extend to positive emotional memories, although more research is needed to support this suggestion. Unlike memory acquisition, the literature about the effect of PROP on memory consolidation is inconclusive. Post-training administration of PROP has been found to disrupt memory consolidation in some studies (Beatty and Rush, 1983; Sara et al., 1999), but not in others (Debiec and Ledoux, 2004; Murchison et al., 2004; Kroon and Carobrez, 2009). Again, most of these studies used aversive paradigms to investigate the effect of PROP on memory consolidation, whereas we used an appetitive paradigm. Also, none of these studies used adolescent animals, like the present study. Thus, β-noradrenergic neurotransmission appears to be involved in memory consolidation, but this depends on the type of memory studied and age of the subjects used.

The present study demonstrates that, comparable to adult animals, PROP impairs memory reconsolidation processes in adolescent rats as well. However, unlike the present data, as summarized above, PROP has been shown to disrupt memory acquisition, consolidation (Beatty and Rush, 1983; Sara et al., 1999) or retrieval (Murchison et al., 2004; De Quervain et al., 2007) in adult rats, at least in certain studies. The discrepancies between the role of β-adrenoceptors in these memory processes in adolescent and adult animals may be associated with the age-related changes in noradrenergic innervation of brain structures implicated in learning and memory, such as the hippocampus, amygdala and frontal cortex (Everitt et al., 1999; Maren, 2011). Thus, β-adrenoceptor binding has been shown to decline between adolescence and adulthood in cortex (Pittman et al., 1980). Furthermore, the density of the noradrenaline transporter, likely reflecting noradrenergic innervation, decreases between adolescence and adulthood in frontal cortex and amygdala, but only very modestly so in hippocampus (Moll et al., 2000; Sanders et al., 2005). Although the relationship between noradrenaline transporter and β-adrenoreceptor density during

development and their involvement in memory processes is not straightforward, it is not unlikely that some of the discrepancies noted here are the result of developmental changes in noradrenergic innervation. On a more general note, the fact that memory reconsolidation has previously been observed in rat pups (Languille et al., 2009) and adults (Nader et al., 2000), may lead to the intuitive assumption that this also occurs in adolescent rats. The present data are, to the best of our knowledge, the first demonstration that this is indeed the case, indicating that memory reconsolidation is a relevant part of memory dynamics throughout the entire lifespan of animals.

Our results demonstrate that in adolescent rats, β-adrenergic neurotransmission mediates the reconsolidation but not the acquisition, consolidation or retrieval of social reward-related memories. This supports the notion that consolidation and reconsolidation of social reward-related memories rely on distinct neural mechanisms. Indeed, several differences in the molecular pathways underlying consolidation and reconsolidation of fear memories have been found (Taubenfeld et al., 2001; Barnes et al., 2010; Lee et al., 2004). In keeping with these findings, our results suggest that a distinction between the neural mechanisms of consolidation and reconsolidation also holds for positive emotional memories.

In conclusion, the present study extends our knowledge about memory reconsolidation, showing that social reward-related memories in adolescent rats are subject to reconsolidation after retrieval. In particular, we have demonstrated that treatment with PROP impairs the reconsolidation, but not the acquisition, consolidation and retrieval of social play-induced CPP in adolescent rats. Together, these data show that β-adrenoceptor stimulation is specifically involved in the reconsolidation of social reward memories in adolescent rats. Future studies should determine the neural site of action of β-adrenoceptor-dependent reconsolidation of social play-induced CPP.

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author contributions

Conceived and designed the experiments: E.J.M.A., V.T., L.J.M.J.V. Performed the experiments: E.J.M.A. Analyzed the data: E.J.M.A., V.T. Wrote the paper: E.J.M.A., V.T., L.J.M.J.V.

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Chapter 7

Glucocorticoid receptor antagonism disrupts reconsolidation of social reward-related memories in rats

In revision

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Abstract

Reconsolidation is the process whereby consolidated memories are destabilized upon retrieval and restabilized to persist for later use. Although the neurobiology of reconsolidation of both appetitive and aversive memories has been intensively investigated, reconsolidation of memories of physiologically relevant social rewards has received little attention. Social play, the most characteristic social behaviour displayed by young mammals, is highly rewarding, illustrated by the fact that it can induce conditioned place preference (CPP). Here, we investigated the role of signaling mechanisms implicated in memory processes including reconsolidation, i.e. glucocorticoid, mineralocorticoid, NMDA glutamatergic and CB1 cannabinoid receptors, in the reconsolidation of social play-induced CPP in rats. Systemic treatment with the glucocorticoid receptor antagonist mifepristone before, but not immediately after retrieval, disrupted the reconsolidation of social play-induced CPP. Mifepristone did not affect social play-induced CPP in the absence of memory retrieval. Treatment with the NMDA receptor antagonist MK-801 modestly affected reconsolidation of social play-induced CPP. However, reconsolidation of social play-induced CPP was not affected by treatment with the mineralocorticoid and CB1 cannabinoid receptor antagonists spironolactone and rimonabant, respectively. We conclude that glucocorticoid neurotransmission mediates the reconsolidation of social reward-related memories in rats. These data indicate that the neural mechanisms of the reconsolidation of social reward-related memories only partially overlap with those underlying reconsolidation of other reward-related memories.

Introduction

Reconsolidation is the process whereby a retrieved memory enters a destabilized state and is subsequently restabilized (Nader et al., 2000). It has been suggested that this process provides an opportunity for updating or strengthening of existing memory traces (Lee, 2009; Inda et al., 2011). During the last decade, an extensive body of literature has emerged on the neural mechanisms underlying the reconsolidation of aversive memory traces, as well as appetitive food and drug memories. However, reconsolidation of memories of physiologically relevant natural rewards, such as social behaviour, has received little attention (Perrin et al., 2007).

To address this issue, we have recently demonstrated a long-term impairing effect of the beta-adrenoceptor antagonist propranolol on reconsolidation of social reward-related memory using social play behaviour-induced conditioned place preference (CPP) (Achterberg et al., 2012). Social play, the most characteristic social behaviour in juvenile and adolescent mammals, serves to facilitate social, physical and cognitive development (Panksepp et al., 1984; Vanderschuren et al., 1997; Špinka et al., 2001; Pellis and Pellis, 2009; Baarendse et al., 2013). Social play is highly rewarding (Vanderschuren et al., 1997; Trezza et al., 2010, -2011a), as is apparent from the observations that it can induce CPP (Calcagnetti and Schechter, 1992; Crowder and Hutto, 1992; Thiel et al., 2008; Trezza et al, 2009, -2011b). Because place conditioning relies on an associative mechanism, it can be used to study the dynamics of emotionally charged memories (Bernardi et al., 2006; Fricks-Gleason and Marshall, 2008).

Studies into the neural underpinnings of the reconsolidation process have identified a number of signaling mechanisms involved, including the beta noradrenergic, N-methyl-D-aspartate (NMDA), cannabinoid 1 (CB1) and glucocorticoid receptors in several paradigms and species (for reviews see Tronson and Taylor, 2007; Besnard et al., 2012). There is a large amount of literature showing that glucocorticoid hormones, such as corticosterone, strengthen memory of emotionally arousing experiences (De Quervain et al., 1998, -2009; Roozendaal et al., 2008). These hormones bind to glucocorticoid and mineralocorticoid receptors in brain areas involved in learning and memory, such as the hippocampus, amygdala and prefrontal cortex (De Kloet et al., 2005). Blocking glucocorticoid receptors has been found to impair reconsolidation of aversive events (Jin et al., 2007; Wang et al., 2008; Taubenfeld et al., 2009; Pitman et al., 2011; Nikzad et al., 2011), whereas blocking the mineralocorticoid receptor was found to interfere with the retrieval of fear memory in mice (Zhou et al., 2011). Interestingly, there is substantial evidence that the release of glucocorticoids is initiated not only in response to aversive stimuli but also in response to rewarding stimuli such as food, drugs of abuse, sex and social play (Piazza and Le Moal, 1997; Gordon et al., 2002; Koolhaas et al., 2011; Buwalda et al., 2012). Indeed, increased glucocorticoid levels have been shown to improve the acquisition and consolidation of appetitive memories (Micheau et al., 1981, 1985; Zorawski and Killcross, 2002; Wichmann et al. 2012).

Glutamatergic NMDA receptors have been widely implicated in the acquisition, (re)consolidation and extinction of both aversive and appetitive memory traces (Przybyslawski and Sara, 1997; Suzuki et al., 2004; Lee et al., 2006a; Lee and Everitt, 2008). In particular, blockade of NMDA receptors was found to interfere with reconsolidation of drug-induced CPP (Kelley et al., 2007; Sadler et al., 2007, Zhai et al., 2008; Wu et al., 2012). Cannabinoid CB1 receptors are expressed in brain regions involved in memory processing, including the hippocampus, amygdala and prefrontal cortex (Katona et al., 2001; Wilson and Nicoll, 2002; Li et al., 2008), and treatment with the CB1 receptor antagonist rimonabant has been shown to impair the reconsolidation process for both aversive and appetitive memories (Bucherelli et al. 2006; Yu et al.

2009, Fang et al. 2011). To the best of our knowledge, however, the effect of blocking glucocorticoid, mineralocorticoid, NMDA or CB1 receptors has not been investigated with respect to the reconsolidation of social reward-related memories.

In the present study, we therefore investigated whether retrieved social reward-related memories in a social play-induced CPP paradigm could be disrupted by administration of the glucocorticoid receptor antagonist mifepristone, the mineralocorticoid receptor antagonist spironolactone, the NMDA receptor antagonist MK-801 or the CB1 receptor antagonist rimonabant, in rats. We hypothesized that when social reward-related memories reconsolidate following memory retrieval, mifepristone, spironolactone, MK-801 and rimonabant would attenuate CPP on a subsequent test by persistently disrupting the memory trace. We predicted that this would also prevent reinstatement of CPP following extinction and retraining.

Methods

Ethics statement

All experiments were approved by the Animal Ethics Committee of Utrecht University and were in agreement with Dutch laws (Wet op Dierproeven 1996) and European regulations (Guideline 86/609/EEC).

Animals

Male Wistar rats (Charles River, Sulzfeld, Germany) arrived in our animal facility at 21 days of age and were housed in groups of three or four in 40 x 26 x 20 cm (*l x w x h*) Macrolon cages under controlled conditions (i.e. temperature 20-24°C, 60-65% relative humidity and 12/12 h light cycle with lights on at 7.00 AM). Upon arrival, the animals were allowed at least 5 days of acclimatization to the facility and were handled for 3 days before the start of the experiment. Food and water were available *ad libitum*. All animals were experimentally naïve and were used only once.

Apparatus

Place conditioning was performed as previously described (Trezza et al., 2009; -2011b; Achterberg et al., 2012). The place conditioning setup (TSE System, Bad Homburg, Germany) comprised 8 boxes, each consisting of three compartments with removable Plexiglas lids: two equally sized large conditioning compartments (30 x 25 x 30 cm; *l x w x h*) separated by a smaller, neutral compartment (10 x 25 x 30 cm; *l x w x h*). The two conditioning compartments had different visual and tactile cues, which also differed from the cues in the middle compartment. The position of the animal in the apparatus was monitored by an array of photobeam sensors located 2.5 cm above the floor. A computer recorded the time (in msec) the animals spent in each compartment. All place conditioning experiments were performed in a sound attenuated and dimly lit room.

Drugs

The glucocorticoid receptor antagonist mifepristone (RU38486, Tocris Bioscience, UK) and the mineralocorticoid receptor antagonist spironolactone (Tocris Bioscience, UK) were dissolved in propylene glycol (Sigma-Aldrich, Germany) and administered s.c. (mifepristone, 30 mg/kg; spironolactone, 50 mg/kg). The noncompetitive NMDA receptor antagonist (+)-5-methyl-10,11-dihydro-SH-dibenzo[a,d]cyclohepten-5,10-imine maleate (MK-801, Tocris Bioscience, UK) was dissolved in saline and administered *i.p.* (0.1 or 0.2 mg/kg). The CB1 cannabinoid receptor antagonist rimonabant (SR141716A, National Institute of Mental Health's Chemical Synthesis and

Drug Supply Program, National Institutes of Health, Bethesda, MD, USA) was dissolved in 5% Tween 80, 5% polyethylene glycol/saline and administered *i.p.* (1.0 mg/kg). In all the experiments, the injection volume was 2 ml/kg. Drug doses are based on literature about memory processing in rats (Pitman et al., 2011; Vafaei et al., 2011; Yu et al., 2009; Brown et al., 2008; Lee et al., 2006b). See Experimental procedures for timing of drug administration.

Experimental procedures

1. Effects of pre- or post-retrieval mifepristone on social play-induced CPP.
The aim of this experiment was to investigate the effect of pre- or post-retrieval mifepristone treatment on reconsolidation and reinstatement of social play-induced CPP. At 26 days of age (day 1), each rat was placed in the middle compartment of the CPP apparatus and pre-conditioning side preference was determined by allowing the rats to move freely around the three compartments of the apparatus for 15 min (Pretest). On the basis of their Pretest scores, rats were assigned to a treatment group and to the compartment in which they would be allowed social interaction during conditioning. We used a counterbalanced place conditioning design (Tzschentke, 2007; Veeneman et al., 2011), meaning that the pre-conditioning preference in each experimental group for the to be social-paired or non-social paired compartment approximated 50%. As a result, based on their Pretest performance, half of the rats in each experimental group was conditioned in their preferred compartment and half was conditioned in their non-preferred compartment. This procedure rules out the possibility that preference shifts are the result of decreased avoidance of the non-preferred compartment. After the Pretest, rats were individually housed throughout the conditioning period to increase their motivation for social interaction and to facilitate the development of social play-induced CPP (Trezza et al., 2009).

Place conditioning began on day 2. Rats underwent eight consecutive days of conditioning, with two conditioning sessions per day. On days 2, 4, 6 and 8 of the experiment, 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. The composition of the pairs of rats during the social sessions was changed daily. As a result, the animals interacted with the same partner on every third conditioning session, in order to prevent the development of a dominance/subordination relationship within a test pair. All animals were used for analysis of CPP, i.e., no neutral 'stimulus animals' were used. On days 3, 5, 7 and 9, the order of sessions was reversed, i.e. rats were placed alone in one side of the CPP apparatus during the morning session, and were placed in the other compartment with the social partner in the afternoon session. Social and non-social conditioning-sessions were separated by at least one hour. On day 10, rats were placed in the middle compartment, where they were allowed to explore the entire apparatus for 15 min (retrieval; RETR). The time spent in each compartment was recorded. The animals were treated with vehicle or mifepristone (30 mg/kg, s.c.) either 30 min before (pre-retrieval treatment) or immediately after the retrieval session (post-retrieval treatment). The next day, the animals were placed in the middle compartment again and were again allowed to move freely in the apparatus for 15 min to investigate the effect of mifepristone treatment (TEST); this test is also considered the first extinction session. This procedure was repeated once a day for the following days to extinguish place preference, i.e., until the mean difference between the time spent in the social-paired and the non-social-paired compartments was no longer statistically significant for four consecutive days in all the experimental groups. This took between 5 and 10 extinction sessions. Twenty-four hours after the last extinction session, the rats received

a reconditioning session. Each rat was placed in the social compartment with a social partner for 30 min (social session) and at least 1 hour later, it was placed in the non-social compartment alone for 30 min (non-social session). The next day, the animals were exposed to the whole apparatus for 15 min and preference was determined again (reinstatement, REIN). As the pre-retrieval and the post-retrieval vehicle groups did not differ significantly in the time they spent in each compartment, the data of these groups were collapsed.
We also investigated whether memory retrieval is necessary for mifepristone to affect reconsolidation of social play-induced CPP. To that aim, the animals were conditioned as described above. On day 10, instead of a memory retrieval session, animals were treated with mifepristone or vehicle in their home cage. The next day, both groups were tested (TEST) as above.

2. Effects of pre- or post-retrieval spironolactone on social play-induced CPP.
This experiment was designed to investigate the effect of administration of the mineralocorticoid receptor antagonist spironolactone (50 mg/kg, *s.c.*) on retrieval and reconsolidation of memory for social play-induced CPP. The animals were treated with vehicle or spironolactone either 30 min before (pre-retrieval treatment) or immediately after the retrieval session (post-retrieval treatment). Animals were trained and tested for retrieval (RETR), reconsolidation (TEST) and reinstatement (REIN) as in experiment 1.

3. Effects of pre- or post-retrieval MK-801 on social play-induced CPP.
This experiment was designed to investigate the effect of treatment with the NMDA receptor antagonist MK-801 (0.1 or 0.2 mg/kg, *i.p.*) on retrieval and reconsolidation of memory for social play-induced CPP. The animals were treated with vehicle or MK-801 either 30 min before (pre-retrieval treatment) or immediately after the retrieval session (post-retrieval treatment). The 0.2 mg/kg dose was only used post-retrieval because of its disruptive effect on behaviour, which could interfere with memory processing and with the expression of CPP. Animals were trained and tested for retrieval (RETR), reconsolidation (TEST) and reinstatement (REIN) as in experiment 1.

4. Effects of pre- or post-retrieval rimonabant on social play-induced CPP.
This experiment was designed to investigate the effect of treatment with the cannabinoid CB1 receptor antagonist rimonabant (1.0 mg/kg, *i.p.*) on retrieval and reconsolidation of memory for social play-induced CPP. The animals were treated with vehicle or rimonabant either 30 min before (pre-retrieval treatment) or immediately after the retrieval session (post-retrieval treatment). Animals were trained and tested for retrieval (RETR), reconsolidation (TEST) and reinstatement (REIN) as in experiment 1. Because rimonabant is known to have pruritic effects (Cook et al. 1998; Rubino et al. 2000; Tallett et al. 2007; Vickers et al. 2003), which may interfere with the expression of memory retrieval, scratching behaviour was scored for the animals that received rimonabant prior to retrieval.

Statistical analysis
Data were analyzed using SPSS software 15.0 for Windows. For each experiment, the time spent in the social paired and non-social paired compartments was expressed as mean ± SEM. Data were analyzed using ANOVA (mixed-model or two-way, depending on the experiment), using compartment (social or non-social) and treatment (mifepristone/ spironolactone/MK-801/rimonabant or vehicle) as between-subjects factor and test-day as repeated-measures factor. The ANOVA was followed by Student's

paired t-tests when appropriate, to investigate differences between the time spent in the social and non-social compartment. Differences in the time spent scratching were analyzed by a independent-samples t-test.

Results

1. Pre-retrieval treatment with the glucocorticoid receptor antagonist mifepristone disrupted reconsolidation but not retrieval of social reward-related memories

The mixed-model ANOVA revealed an effect of test day ($F_{2,248}$= 5.07, p=0.01) and compartment ($F_{1,124}$= 78.38, p<0.001). Also, a significant compartment x treatment interaction ($F_{2,124}$= 10.39, p<0.001) and a test day x compartment x treatment interaction ($F_{4,248}$= 2.96, p=0.02) was found. No main effect of treatment was found ($F_{2,124}$= 0.88, n.s.) and no other interaction effects were found (test day x compartment: $F_{2,248}$= 1.57, n.s. and test day x treatment: $F_{4,248}$= 0.23, n.s., figure 1a). Post hoc analysis revealed that on day 10 all the groups showed a significant social play-induced CPP (RETR: veh: t(31)= 7.41, p<0.001, n=32; pre: t(8)= 2.40, p=0.04, n=9; post: t(23)= 8.40, p<0.001, n=24), indicating that mifepristone treatment did not affect retrieval of social play-induced CPP. Twenty-four hours later (TEST), the vehicle- and the post-retrieval mifepristone-treated animals still showed a significant preference for the play-paired compartment

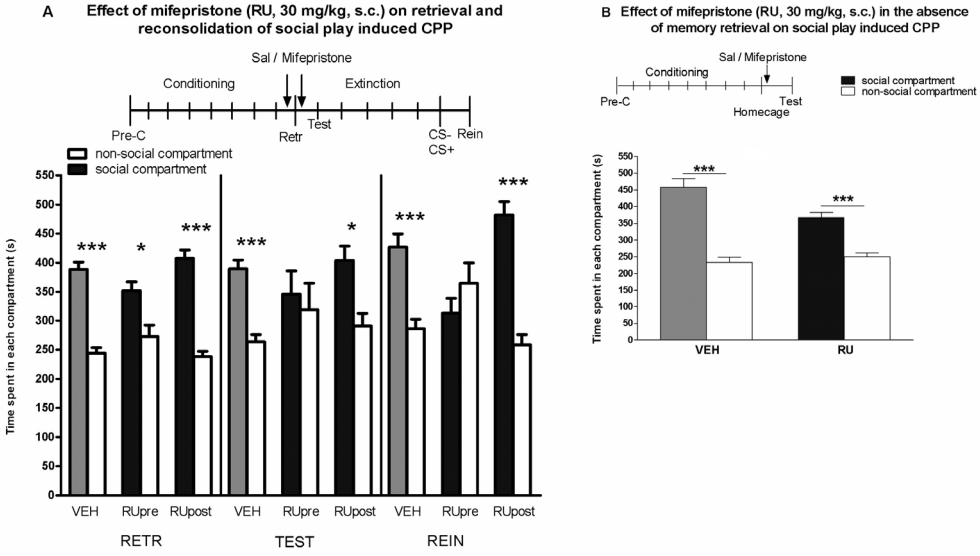

Fig. 1: (A) Effects of pre- and post-retrieval mifepristone (RU486; RU) on social play-induced CPP. The experimental protocol is depicted above the graph (Pre-C: pre-conditioning test, CS+: conditioning session with a play-partner, CS-: conditioning session alone). Data represent the mean time (sec + SEM) spent in the social compartment (grey and black bars) and the non-social compartment (white bars) during 15 min retrieval- (RETR), test- (TEST) and reinstatement- (REIN) sessions. Vehicle-treated animals (VEH: 2ml/kg, *s.c.*, n= 32), mifepristone-treated animals (30 mg/kg, *s.c.*, treatment pre-retrieval: RUpre n= 9, treatment post-retrieval: RUpost: n= 24). (B) Effects of mifepristone on social play-induced CPP in the absence of memory-retrieval. Vehicle-treated animals (VEH; 2ml/kg, *i.p.*, n= 6), mifepristone-treated animals (RU; 30 mg/kg, *i.p.*, n= 10). Post-hoc Student's paired t-tests for difference in time spent in the social- and non-social compartment *p<0.05, **p<0.01, ***p<0.001.

(veh: t(31)= 4.81, p<0.001, post: t(24)= 2.55, p=0.001), whereas the pre-retrieval mifepristone-treated animals no longer showed a preference (pre: t(8)= 0.32, n.s.). Following the reconditioning session, both the vehicle-treated and the post-retrieval mifepristone-treated animals showed a significant social play-induced CPP (REIN: veh: t(31)= 3.88, p=0.001, post: t(24)= 5.65, p<0.001), whereas no significant reinstatement of CPP was found in the animals treated with mifepristone before retrieval (pre: t(8)= 0.88, n.s.). These findings indicate that the glucocorticoid receptor antagonist mifepristone disrupts reconsolidation of social reward-related memory when administered before, but not when administered immediately after a retrieval session.

Treatment with mifepristone did not affect reconsolidation of social reward-related memories in the absence of memory retrieval (figure 1b). Twenty-four hours after administration of mifepristone in the home cage (i.e., without a retrieval session), both the vehicle and the mifepristone-treated rats showed a significant preference for the social compartment. The two-way ANOVA revealed a significant effect of compartment ($F_{1,28}$= 120.25, p<0.001) and treatment ($F_{1,28}$= 8.45, p=0.01) and a compartment x treatment interaction ($F_{1,28}$= 14.02, p=0.001). Post-hoc analysis showed that both the vehicle- and the mifepristone-treated animals showed a significant preference for the social-paired compartment (veh: t(5)= 6.98, p=0.001, n=6; mifepristone: t(9)= 5.06, p=0.001, n=10). These results indicate that mifepristone treatment without a retrieval session does not affect reconsolidation of social play-induced CPP.

2. The mineralocorticoid receptor antagonist spironolactone did not affect retrieval or reconsolidation of social reward-related memories.

The mixed-model ANOVA showed an effect of compartment ($F_{1,52}$= 69.92, p<0.001) and an effect of test day ($F_{2,104}$= 3.70, p=0.03). No other main or interaction effects were found (treatment: $F_{2,52}$= 0.04; compartment x treatment: $F_{2,52}$= 0.43; testday x compartment: $F_{2,104}$= 0.89; test day x treatment: $F_{4,104}$= 0.05; test day x treatment x compartment: $F_{4,104}$= 1.01, all n.s.). All the treatment-groups showed a significant preference for the play-paired compartment at RETR and TEST and reinstatement of social play-induced CPP (figure 2, vehicle: n=12, pre-retrieval spironolactone: n=10, post-retrieval spironolactone: n=7). These results indicate that administering spironolactone either 30 min before or immediately after a retrieval session does not affect retrieval or reconsolidation of social play-induced CPP (figure 2).

3. Effect of the NMDA receptor antagonist MK-801 on retrieval and reconsolidation of social reward-related memories.

In the experiment where the effect of 0.1 mg/kg MK-801 was tested, the mixed-model ANOVA revealed an effect of compartment ($F_{1,172}$= 146.53, p<0.001) and an effect of test day ($F_{2,344}$= 4.42, p=0.02). Also, a compartment x treatment interaction ($F_{2,178}$= 10.33, p<0.001), a test day x compartment interaction ($F_{2,344}$= 6.83, p=0.002) and a test day x compartment x treatment interaction ($F_{4,344}$= 3.36, p=0.02) were found. No main effect of treatment ($F_{2,172}$= 1.16, n.s.) and no test day x treatment interaction was found (test day x treatment: $F_{4,344}$= 0.56, n.s., figure 3a). Post hoc analysis revealed that at RETR and TEST, all groups showed a significant preference for the play-paired compartment (RETR: veh: t(39)= 9.12, p<0.001, n=40; pre: t(28)= 2.48, p=0.02, n=29; post: t(20)= 7.21, p<0.001, n=19; TEST: veh: t(39)= 6.83, p<0.001, pre: t(28)= 2.19, p=0.04, post: t(19)= 2.31, p=0.03). The vehicle and post-retrieval MK-801 treated animals showed significant reinstatement of social play-induced CPP (REIN: veh: t(39)= 2.27, p=0.03, post: t(20)= 3.21, p=0.01), whereas the pre-retrieval MK-801 treated animals did not (REIN: pre: t(28)= 0.79, n.s.).

In the experiment where the effect of 0.2 mg/kg MK-801 was tested, the mixed-model

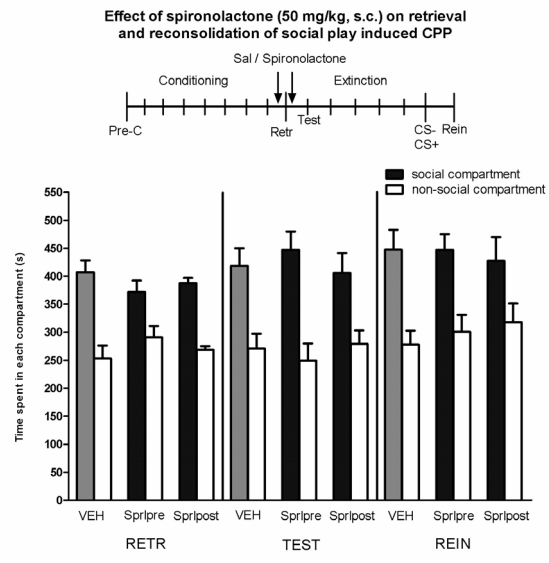

Fig. 2:
Effects of pre- and post-retrieval spironolactone on social play-induced CPP. The experimental protocol is depicted above the graph (Pre-C: pre-conditioning test, CS+: conditioning session with a play-partner, CS-: conditioning session alone). Data represent the mean time (sec + SEM) spent in the social compartment (grey and black bars) and the non-social compartment (white bars) during 15 min retrieval- (RETR), test- (TEST) and reinstatement- (REIN) sessions. Vehicle-treated animals (VEH: 2ml/kg, *s.c.*, n= 12), spironolactone-treated animals (30 mg/kg, *s.c.*, treatment pre-retrieval: Sprlpre n= 10; treatment post-retrieval Sprlpost: n= 7).

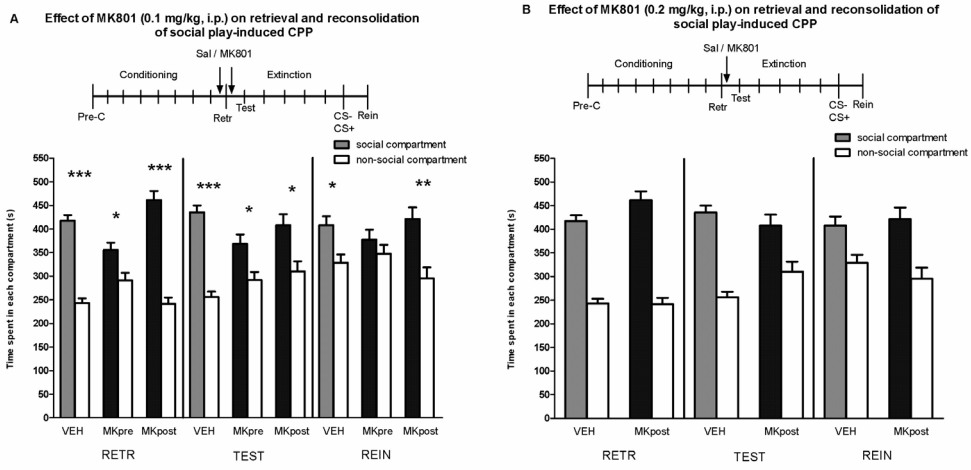

Fig. 3: Effects of MK-801 treatment on social play-induced CPP. The experimental protocol is depicted above the graph (Pre-C: pre-conditioning test, CS+: conditioning session with a play-partner, CS-: conditioning session alone). Data represent the mean time (sec + SEM) spent in the social compartment (grey and black bars) and the non-social compartment (white bars) during 15 min retrieval- (RETR), test- (TEST) and reinstatement- (REIN) sessions. (A) Effects of pre- and post-retrieval MK-801 (0.1 mg/kg). Vehicle-treated animals (VEH: 2ml/kg, *s.c.*, n= 40), MK-801-treated animals (0.1 mg/kg, *i.p.*, treatment pre-retrieval: MKpre: n= 29; treatment post-retrieval: MKpost: n= 19). Post-hoc Student's paired t-tests for difference in time spent in the social- and non-social compartment *p<0.05, **p<0.01, ***p<0.001. (B) Effects of post-retrieval MK-801 (0.2 mg/kg). Vehicle-treated animals (VEH: 2ml/kg, *i.p.*, n= 8), MK-801-treated animals (0.2 mg/kg, *i.p.*, MKpost: n= 8).

ANOVA revealed an effect of compartment ($F_{1,28}$= 53.00, p<0.001). No other main or interaction effects were found (test day: $F_{2,56}$= 1.20; treatment: $F_{1,28}$= 0.08; test day x compartment: $F_{2,56}$= 1.02; test day x treatment: $F_{2,56}$= 0.21; test day x compartment x treatment: $F_{2,56}$= 0.02, all n.s., figure 3b). All groups showed a significant preference for the social-paired compartment at RETR, TEST and REIN (Figure 3b, vehicle: n=8, post-retrieval MK-801: n=8). These results indicate that treatment with 0.2 mg/kg MK-801 immediately after a retrieval session does not affect reconsolidation of social play-induced CPP.

4. The cannabinoid receptor antagonist rimonabant did not affect retrieval or reconsolidation of social reward-related memories.

The mixed-model ANOVA revealed an effect of compartment ($F_{1,68}$= 55.59, p<0.001) but no other main or interaction effects (test day: $F_{2,136}$= 1.16; treatment: $F_{2,68}$= 0.23; treatment x compartment: $F_{2,68}$= 1.19; test day x compartment: $F_{2,136}$= 0.27; test day x treatment: $F_{4,136}$= 0.85; test day x compartment x treatment: $F_{4,136}$= 0.17, n.s.). All groups showed a significant preference for the social-paired compartment at RETR, TEST and REIN (Figure 4a, vehicle: n= 19, pre-retrieval rimonabant: n=10, post-retrieval rimonabant: n=8). These results show that treatment with rimonabant (1.0 mg/kg) either 30 min before or immediately after a retrieval session does not affect retrieval, reconsolidation

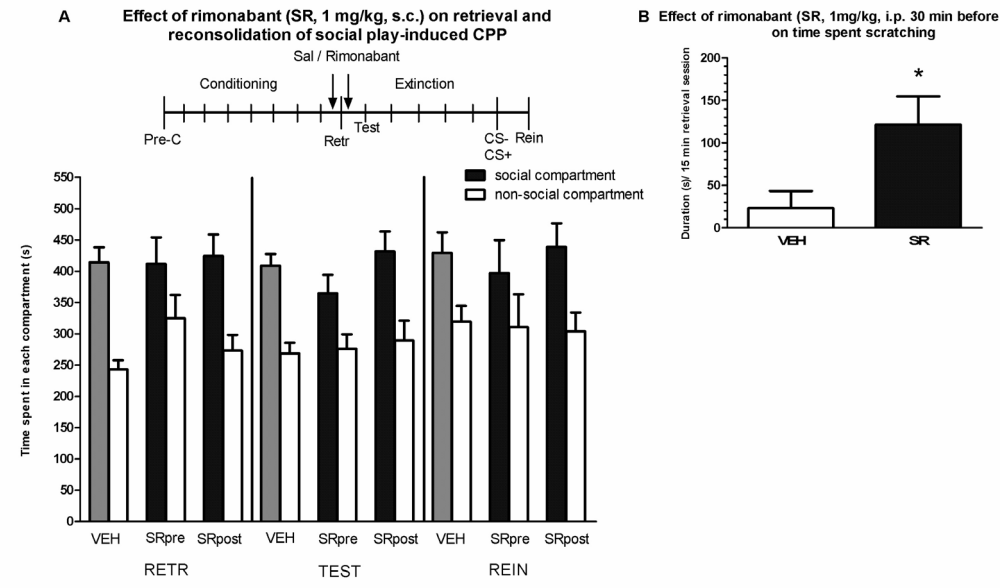

Fig. 4: (A) Effects of pre- and post-retrieval rimonabant (SR141716; SR) on social play-induced CPP. The experimental protocol is depicted above the graph (Pre-C: pre-conditioning test, CS+: conditioning session with a play-partner, CS-: conditioning session alone). Data represent the mean time (sec + SEM) spent in the social compartment (grey and black bars) and the non-social compartment (white bars) during 15 min retrieval- (RETR), test- (TEST) and reinstatement- (REIN) sessions. Vehicle-treated animals (VEH: 2ml/kg, *i.p.*, n= 19), rimonabant-treated animals (1.0 mg/kg, *i.p.*, treatment pre-retrieval: SRpre: n= 10; treatment post-retrieval: SRpost: n= 8). (B) Time spent scratching during the 15 min test in pre-retrieval rimonabant-treated animals. Independent samples t-test, *p<0.05.

or reinstatement of social play-induced CPP. We also found that rimonabant-pretreated animals spent significantly more time scratching during the 15 min test compared to vehicle-treated animals (t(12.87)= -2.52, p=0.03, figure 4b).

Discussion

In this study, we investigated the involvement of glucocorticoid, mineralocorticoid, NMDA and cannabinoid CB1 receptors in retrieval and reconsolidation of social reward-related memories in rats. Our hypothesis was that blocking these receptors would disrupt the reconsolidation of social play-induced CPP. We show that: (1) the glucocorticoid receptor antagonist mifepristone disrupts reconsolidation of social play-induced CPP when administered before a retrieval session; (2) neither the mineralocorticoid receptor antagonist spironolactone, nor the CB1 cannabinoid receptor antagonist rimonabant affected retrieval or reconsolidation of social play-induced CPP, whereas pre-retrieval treatment with the NMDA receptor antagonist MK-801 modestly affected social play-induced CPP. Together, our data show that glucocorticoid neurotransmission mediates the reconsolidation of social play-induced CPP without affecting the retrieval process whereas mineralocorticoid, NMDA and CB1 cannabinoid receptors are not primarily involved in the dynamics of social reward-related memories.

In the first experiment, vehicle- and post-retrieval mifepristone treated animals showed a preference for the social-paired compartment 24h after retrieval, whereas pre-retrieval mifepristone-treated animals did not. This effect of mifepristone was not the result of a non-specific memory impairment, since mifepristone-treatment in the absence of retrieval did not alter social play-induced CPP (Tronel and Alberini, 2007; Jin et al, 2007; Taubenfeld et al., 2009; Nikzad et al., 2011; Pitman et al., 2011). Furthermore, following extinction of CPP, vehicle- and post-retrieval mifepristone-treated animals showed reinstatement of CPP 24h after a reconditioning session, whereas pre-retrieval mifepristone-treated animals did not. The inability to reinstate social play-induced CPP in the pre-retrieval mifepristone-treated group suggests that acute pre-retrieval mifepristone persistently disrupted the social play-CPP memory trace, rather than inducing a retrieval deficit or facilitating extinction learning (for discussion see Achterberg et al., 2012). Our findings are consistent with previous reports showing that mifepristone treatment (either systemic or intra-amygdala/hippocampus) blocks reconsolidation of fear memories, while sparing retrieval (Tronel and Alberini, 2007; Jin et al, 2007; Taubenfeld et al., 2009; Nikzad et al., 2011; Pitman et al., 2011), although it should be noted that most of these previous studies employed post-retrieval mifepristone treatment, which was ineffective in our study. One likely explanation for this apparent discrepancy is that we used a relatively long retrieval session, because in our experience, the expression of CPP is difficult to detect using shorter retrieval sessions. In this scenario, post-retrieval mifepristone is less effective in interfering with reconsolidation since the glucocorticoid receptor-dependent processes involved in the reconsolidation process may take less than 15 min. Interestingly, all the above studies that showed glucocorticoid receptor involvement in reconsolidation were conducted in fear-learning paradigms. Therefore, the present study extends the involvement of glucocorticoid receptors to reconsolidation of appetitive memories. Pleasurable stimuli such as food, drugs of abuse or sex are known to cause a rise in corticosterone levels (Piazza and Le Moal, 1997; Koolhaas et al., 2011; Buwalda et al., 2012). Indeed, an episode of social play also evokes an increase in corticosterone levels in rats (Gordon et al., 2002). Moreover, increasing glucocorticoid levels improves acquisition and/or consolidation of appetitive memory (Micheau et al., 1981, 1985; Zorawski and Killcross,

2002; Wichmann et al. 2012) suggesting a role for glucocorticoid receptors in the initial stages of appetitive memory formation. Our data add to this by demonstrating that reconsolidation of reward-related memory can be disrupted by antagonizing glucocorticoid receptors. Whether other reward-related memories, such as drug-reward memory, are affected by antagonizing glucocorticoid receptors remains to be elucidated. The mineralocorticoid receptor antagonist spironolactone did not interfere with retrieval or reconsolidation of social reward-related memories. Consistent with our findings, Vafaei et al. (2011) found no effect of spironolactone (either systemically and intra-hippocampus) on reconsolidation of inhibitory avoidance memory. On the other hand, in a fear conditioning paradigm, blocking the mineralocorticoid receptors with spironolactone before a brief context retrieval-session, but not a cue-tone retrieval session, disrupted subsequent expression of fear, although post-retrieval treatment with spironolactone was ineffective (Zhou et al., 2011). Thus, mineralocorticoid receptors may be involved in the reconsolidation of certain aversive rather than appetitive memories. However, the contribution of other factors to the discrepancies between the studies (i.e. reliance on cues vs. contextual information, and species and age differences of the animals tested) can at this point not be ruled out, since literature on the role of the mineralocorticoid receptor in reconsolidation is very limited.

Treatment with MK-801 modestly affected reconsolidation of social play-induced CPP. Thus, post-retrieval treatment with MK-801 did not alter the expression of social play-induced CPP during the tests for reconsolidation and reinstatement. After pre-retrieval treatment with 0.1 mg/kg MK-801, there was significant CPP during retrieval and the test for reconsolidation, albeit of a lesser magnitude than seen in the vehicle-treated rats. Interestingly, after reconditioning, there was no reinstatement of CPP in the animals treated with 0.1 mg/kg MK-801 pre-retrieval. This suggests that pre-retrieval NMDA receptor blockade impaired the integrity of the memory trace to some extent. Previously, systemic blockade of NMDA receptors has been found to block reconsolidation of aversive (Suzuki et al., 2004; Lee et al., 2006b) as well as drug- and food reward memory (Kelley et al, 2007; Sadler et al, 2007; Brown et al, 2008; Itzak 2008; Lee and Everitt, 2008; Milton et al, 2008). There are several explanations for our findings that MK-801 treatment did not profoundly disrupt reconsolidation of social play-induced CPP in the present study. Thus, Ben Mamou et al. (2006) and Milton et al. (2013) have shown a role for different subtypes of NMDA receptors in the destabilization and reconsolidation of memory. Blocking NR2B-containing NMDA receptors in the basolateral amygdala prevents the reactivation of a conditioned fear memory, whereas that NR2A-containing NMDA receptors are specifically implicated in reconsolidation of fear memory. It is therefore possible that pre-retrieval MK-801 administration inhibited the reactivation of the social play-CPP memory trace. As a result, reconsolidation could not be completely blocked because the memory trace was not in a fully active state. This retrieval-inhibition explanation is consistent with the reduced magnitude of CPP after pre-retrieval MK-801 treatment. Furthermore, treatment with NMDA receptor antagonists disrupts extinction learning (Suzuki et al, 2004; Lee et al, 2006b; Chan and McNally, 2009). According to Suzuki et al. (2004) there is a brief time window for reconsolidation after retrieval (approximately 3 min), whereas extinction only occurs after prolonged exposure (30 min). As explained above, we used a 15 min reactivation session, which may result in competing reconsolidation and extinction processes, whereby MK-801 administration could affect both, so that the social play CPP memory trace would remain relatively intact.

Neither retrieval nor reconsolidation of social play-induced CPP was disrupted by administration of the CB1 receptor antagonist rimonabant. There is no consensus in the literature on the effect of CB1 antagonists on aversive memory as disruption

(Bucherelli et al. 2006), facilitation (De Oliviera Alvares et al. 2008) and lack of an effect (Suzuki et al. 2008) on reconsolidation has been found. Interestingly, systemic treatment with rimonabant has been shown to disrupt reconsolidation of nicotine-induced and methamphetamine-induced CPP (Fang et al., 2011; Yu et al., 2009). However, these studies used a higher dose of rimonabant (3.0 mg/kg), which leaves the possibility open that this reconsolidation blockade occurred through a non-CB1 receptor-dependent mechanism of action of rimonabant. Moreover, rimonabant is known to be pruritogenic (Cook et al. 1998; Rubino et al. 2000; Tallett et al. 2007; Vickers et al. 2003). Indeed, we found a significant increase in scratching in rimonabant-treated animals. We therefore did not test the 3.0 mg/kg dose of rimonabant, since scratching severely disrupts behaviour, which may interfere with memory processing in the CPP box. Treatment with CB1 receptor antagonists has been shown to disrupt extinction learning in aversive paradigms (Marsicano et al. 2002; Suzuki et al. 2004; Niyuhire et al. 2007) but their role in extinction of appetitive memories is not clear (Hernandez and Cheer, 2011, Manwell et al. 2009). This makes it unlikely that the lack of effect of rimonabant on social play-induced CPP is the result of interference with reconsolidation and extinction at the same time. However, CB1 receptors are thought to be required for memory destabilization (Suzuki et al. 2004; -2008). In conclusion, our data do not support a role for CB1 receptors in the reconsolidation of social reward memories, but the contribution of a destabilization blockade in our findings can as yet not be excluded.

In conclusion, the present study extends our knowledge about reconsolidation of social reward-related memories in rats, showing that this type of reward memory is subject to the impairing effects of glucocorticoid receptor antagonism. However, our data do not support a primary role for mineralocorticoid, NMDA or CB1 receptors in reconsolidation of social reward-related memories in rats.

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Ch.7

Chapter 8

General discussion

Social play behavior is important for the behavioral, emotional and cognitive development of young mammals, including humans. Engaging in social play gives young mammals the opportunity to develop a rich, adaptive and flexible behavioral repertoire to cope with the challenges of adult life (Vanderschuren et al., 1997; Panksepp et al., 1984; Pellis and Pellis et al., 2009; Špinka et al., 2001; Van den Berg et al., 1999a,b; Baarendse et al., 2013a,b; Potegal and Einon, 1989). Lacking or aberrant social play behavior produces animals that are less able to properly behave in a changeable (social) environment (Einon and Morgan, 1997; Einon et al, 1987;Potegal and Einon, 1989; Hol et al., 1999; Van den Berg et al., 1999a,b,c; Spinka et al., 2001; Von Frijtag et al., 2002; Lukkes et al., 2009a,b; Meng et al., 2010; Whitaker et al., 2013; Vanderschuren and Trezza, 2013). For example, play-deprived animals show disrupted impulse control, impaired decision-making (Baarendse et al., 2013a) and an increased sensitivity to cocaine self-administration (Baarendse et al., 2013b). In humans, abnormalities in social play behavior are a feature of childhood and adolescent psychiatric disorders, such as disruptive behavior disorders (DBD), autism-spectrum disorder (ASD), early onset schizophrenia and attention-deficit/hyperactivity disorder (ADHD) (Alessandri, 1992; Moller and Husby, 2000; Jordan, 2003; Manning and Wainwright 2010). Social play behavior is rewarding, since it can be used as an incentive for maze-learning (Humphreys and Einon, 1981; Normansell and Panksepp, 1990) as well as place and operant conditioning (Calcagnetti and Schechter, 1992; Crowder and Hutto 1992; Douglas et al., 2004; Thiel et al., 2008, 2009; Trezza et al., 2009; Peartree et al., 2012). From a more fundamental perspective, by comparing social play to other natural rewards such as food and sex but also to artificial rewards such as drugs of abuse, this could provide us with a better understanding of the involvement of the brain reward-system during social play behavior.

In view of the importance of social play behavior in behavioral development, its relevance for child and adolescent psychiatry, and to get a better understanding of social reward processing in general, the aim of this thesis was to elucidate the neurotransmitter systems and neural substrates involved in the rewarding, motivational and cognitive aspects of social play behavior with a focus on monoamines (dopamine, noradrenaline and serotonin), glucocorticoids, opioids and cannabinoids. In this chapter, the main findings presented in this thesis will be summarized and discussed.

Social play behavior and the effects of psychostimulant drugs

Amphetamine and cocaine

The results provided in **chapter 2** of this thesis demonstrate that amphetamine and cocaine suppress social play behavior in rats, acting through distinct mechanisms. In particular, the play-suppressant effects of amphetamine, like those of methylphenidate (Vanderschuren et al., 2008), were antagonized by the α2-adrenoreceptor antagonist RX821002 but not by the dopamine receptor antagonist α-flupenthixol. In contrast, the effects of cocaine on social play were not antagonized by α2-noradrenergic, dopaminergic or serotonergic receptor antagonists, administered either alone or in combination. However, the effects of a subeffective dose of cocaine were enhanced by a combination of subeffective doses of the serotonin reuptake inhibitor fluoxetine, the dopamine reuptake inhibitor GBR12909 and the noradrenaline reuptake inhibitor atomoxetine. These results indicate that simultaneous increases in the endogenous levels of all three monoamines are involved in the play suppressant effects of cocaine, although the specific receptors mediating these effects remain to be elucidated.

The effects of psychostimulants on social play behavior can be explained by several

hypotheses. First, on the basis of their effectiveness in ADHD, and the comparable effects of amphetamine and methylphenidate on social play behavior (Vanderschuren et al. 2008; chapter 2) and the stop signal task (Eagle and Baunez 2010), it can be hypothesized that the play suppressant effects of psychostimulants are the result of enhanced or exaggerated behavioral inhibition. That is, through increasing inhibitory control over behavior, psychostimulant drugs may enhance attention for non-social stimuli in the environment, causing the animals to engage less in playful interactions, that are accompanied by reduced attention for, potentially important, environmental stimuli. Second, in contrast to the first hypothesis, psychostimulant-induced increases in tonic noradrenergic neurotransmission may promote disengagement from ongoing (playful) behaviors and facilitate the switching of behaviors (Aston-Jones and Cohen 2005). This may impact social play behavior, since appropriate behavioral responses are required from both partners of a play-dyad. Third, increases in tonic noradrenergic neurotransmission due to psychostimulant-treatment may also impair behavioral flexibility. Noradrenergic signalling in the prefrontal cortex has been implicated in attentional set-shifting (McGaughy et al., 2008; Newman et al., 2008; Tait et al., 2007). Since frontal cortical areas are involved in social play (Chapter 3; Siviy and Panksepp, 2011; Van Kerkhof et al., 2013a,b), this suggests that noradrenergic mechanisms in the prefrontal cortex subserve the cognitive flexibility necessary to be able to respond to the changeable, often unpredictable behavior of a conspecific. Last, the play-suppressant effect of psychostimulants can be explained by the notion that they increase the intensity of behavior in general. As not all behaviors can be intensified to the same degree, this causes a narrowing down of the behavioral repertoire with simple behaviors being favored over complex behaviors, such as social play (Lyon and Robbins 1975). However, so far we are unable to distinguish between these hypotheses.

Our results extend previous findings (Vanderschuren et al., 2008) by providing more insight into the disruptive effects of psychostimulants on social play behavior in adolescent rats. Furthermore, these data contribute to our understanding of the neural mechanisms of social behavior during an important developmental period. Our results suggest that acute exposure to psychostimulants has severe consequences for an important form of social behavior in young rats. This may be of relevance for addiction research in humans as well, since little work has been done on the consequences of initial drug use on social behavior in youth. In fact, one has to keep in mind that we investigated the effect of acute exposure to psychostimulants. The effects of repeated or chronic psychostimulant exposure on social behavior may even be more disruptive (for review see Young et al., 2011), but more research is needed to investigate this.

Methylphenidate and social play: mechanism and site of action, dissociable roles of noradrenaline and dopamine

In **chapter 3** we investigated the brain regions involved in the play-suppressant effects of the psychostimulant methylphenidate. In **chapter 4** we used an operant conditioning task to investigate which aspects of social play behavior (motivation to play, pleasurable effects of play, expression of play) were affected by methylphenidate. Methylphenidate is the first-choice pharmacological treatment for ADHD (Kutcher et al., 2004), a psychiatric disorder highly prevalent during childhood and adolescence. Understanding the mechanism and site of action through which methylphenidate influences social play behavior is therefore of considerable clinical relevance.

The prefrontal cortex, orbitofrontal cortex and nucleus accumbens shell have been implicated in social play behavior (Bell et al., 2009; Panksepp et al., 1994; Pellis et al., 2006; Schneider and Koch, 2005; Van Kerkhof et al., 2013a; -2013b; Trezza et al., 2011a). Therefore, in chapter 3, we investigated whether infusion of methylphenidate

into prefrontal and orbitofrontal regions, the nucleus accumbens shell, amygdala, and the habenula inhibited social play. We included the amygdala and habenula because of their involvement in cognitive and emotional processes (Baxter and Murray, 2002; Phelps and LeDoux, 2005; Lecourtier and Kelly, 2007; Hikosaka, 2010), including social play behavior (Trezza et al., 2012; Van Kerkhof et al., 2013c), and their well-characterized noradrenergic innervation (Moore and Bloom, 1979; Gottesfeld, 1983; Unnerstall et al., 1984; Lecourtier and Kelly, 2007). We found that the effects of methylphenidate on social play were mediated through the anterior cingulate cortex, infralimbic cortex, habenula and amygdala, since administration of methylphenidate into these regions reduced the expression of social play behavior, whereas administration into the other regions had no effect (chapter 3). In addition, infusion of the noradrenaline reuptake inhibitor atomoxetine into these regions also reduced social play, indicating that enhanced noradrenaline levels in the anterior cingulate cortex, infralimbic cortex, habenula and amygdala cause a reduction in social play behavior. This is consistent with the finding that methylphenidate reduced social play behavior through a noradrenergic mechanism of action (Vanderschuren et al., 2008; chapter 4).

The main function of the prefrontal cortex is thought to be the mediation of executive functions, such as attention, planning, cognitive flexibility, and decision making (Miller and Cohen, 2001; Robbins and Arnsten, 2009). Since social interactions are inherently complex and unpredictable, it is likely that frontal cortical regions subserve executive functions in social situations (Adolphs, 2003; Blakemore, 2008; Rilling et al., 2008), including social play behavior (Siviy and Panksepp, 2011; Vanderschuren and Trezza, 2013). We have previously reported that functional inactivation of medial prefrontal subregions, i.e. the prelimbic, infralimbic, and medial/ventral orbitofrontal cortex, inhibits social play (Van Kerkhof et al., 2013b). Interestingly, of these regions, the infralimbic, but not the prelimbic and medial/ventral orbitofrontal cortex was shown to be involved in the play-reducing effects of methylphenidate and atomoxetine. Together, these findings therefore underscore the heterogeneity of the prefrontal functions involved in social play (see also Schneider and Koch, 2005; Pellis et al., 2006; Bell et al., 2009). In keeping with the reported functional heterogeneity of the prefrontal cortex (Chudasama et al., 2003; Gourley et al., 2010; Killcross and Coutureau, 2003; Peters et al., 2009), noradrenergic mechanisms may underlie the functional involvement of the infralimbic and anterior cingulate, but not prelimbic and orbitofrontal cortex, suggesting that these prefrontal subregions are involved in distinct executive aspects of social play behavior.

Methylphenidate and atomoxetine, through stimulation of noradrenergic neurotransmission, may have reduced neuronal activity in the amygdala and habenula (Buffalari and Grace, 2007; Ferry et al., 1997; Johnson et al., 2011; Porrino and Lucignani, 1987). Therefore, the decrease in social play after administration of these drugs into the amygdala and habenula may have resulted from a functional inhibition of these regions. This is consistent with previous findings that amygdala lesions reduce social play in male rats (Daenen et al., 2002; Meaney et al., 1981), and that inactivation of the habenula decreased social play behavior (Van Kerkhof et al., 2013c).

Social play is a highly rewarding activity (Trezza et al., 2011b; Vanderschuren, 2010), and both the amygdala and habenula are involved in reward processes (Baxter and Murray, 2002; Bromberg-Martin and Hikosaka, 2011; Cardinal et al., 2002; Hikosaka, 2010; Lecourtier and Kelly, 2007; Morrison and Salzman, 2010). Therefore, functional inhibition of the amygdala and habenula by methylphenidate and atomoxetine may have reduced the positive emotional properties of social play. Conversely, both the habenula and amygdala are involved in negative emotions, such as stress and anxiety (Phelps and LeDoux, 2005; Hikosaka, 2010; Roozendaal et al., 2009; Shin

and Liberzon, 2010). However, intra-amygdala and intra-habenula methylphenidate and atomoxetine did not affect social exploratory behavior, which is the standard parameter used in the social interaction test of anxiety (File and Seth 2003). Moreover, our pharmacological analysis of social play behavior has shown a marked dissociation between anxiolytic/anxiogenic effects of drugs and their influence on the expression of social play behavior (Vanderschuren et al. 1997; Trezza et al., 2009). We therefore consider it unlikely that interference from negative emotions explains the effects of intra-amygdala and intra-habenula methylphenidate and atomoxetine on social play behavior. Last, methylphenidate and atomoxetine in the amygdala and habenula may also have influenced cognitive aspects of social play, such as attention (Lecourtier and Kelly, 2005), and behavioral flexibility (Churchwell et al., 2009; Schoenbaum et al., 2003).

Interestingly, the results described in chapter 4 indicate that methylphenidate administration enhanced operant responding but reduced the expression of social play behavior in rats. This rather counterintuitive effect of methylphenidate on operant responding could be doubly dissociated: the enhancement in operant responding was reduced to control levels by pretreatment with the dopamine receptor antagonist α-flupenthixol, while the expression of social play remained disrupted. Conversely, the disruption in play expression induced by methylphenidate was blocked by pretreatment with the α2 adrenoceptor antagonist RX821002, while the increase in operant responding was not. These experiments provide new information about the mechanism of action of methylphenidate, i.e. the increased motivation for a playful interaction due to elevated endogenous dopamine levels, and the reduced expression of social play through a noradrenergic mechanism of action.

On the basis of the results from chapters 2 and 3, since amphetamine also suppresses social play behavior through stimulation of α2-adrenoceptors, we hypothesize that the same brain areas are involved in the effects of both methylphenidate and amphetamine on social play behavior. In contrast, other neurotransmitters and brain regions might be involved in the play suppressant effects of cocaine.

Dissociating motivation, reward and expression of social play

Dissociable roles of dopamine and noradrenaline

In **chapter 4** we investigated how pleasurable and motivational aspects of social play behavior are modulated by dopamine and noradrenaline. To address this aim, we used the social play-induced conditioned place preference (CPP) task and an operant conditioning task specifically designed to investigate motivational aspects of social interaction.

As mentioned above, methylphenidate enhanced operant responding for social play behavior through dopaminergic neurotransmission, while it reduced the expression of social play behavior through α2-adrenoceptors. The selective noradrenaline reuptake inhibitor atomoxetine lowered both the motivation for and expression of play, while acquisition of CPP was unaltered. On the other hand, treatment with the selective dopamine reuptake inhibitor GBR-12909 disrupted the acquisition of CPP and increased operant responding for social play behavior, while the expression of social play behavior was not altered. This study indicates that dopamine and noradrenaline affect different aspects of social play behavior 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). This is supported by the data in chapter 4 where we found that the increase in operant responding induced by methylphenidate was blocked by pretreatment with the dopamine receptor antagonist α-flupenthixol, while the reduction of play behavior induced by methylphenidate was not. Motivation can be dissociated into directional (i.e. behavior being directed towards or away from certain stimuli) and activational components (i.e. the invigoration of behavior directed at rewards, in terms of speed, persistence, and work output) (see Salamone and Correa, 2012). Dopamine has been particularly implicated in the latter, and although the data in chapter 4 do not allow for a strict distinction between the two, the finding that increasing and decreasing dopamine neurotransmission enhanced and reduced lever pressing for social play, respectively, is consistent with the notion that the activational component of social play motivation is mediated by dopamine. The disruption of CPP acquisition by a selective dopamine reuptake inhibitor suggests that enhancement of endogenous dopamine levels alters the pleasurable effect of social play behavior in such a way that it no longer leads to CPP. Apparently, elevated dopamine levels enhances motivation for social play and reduced its pleasurable properties, and these effects may cancel each other out. This could explain why GBR-12909 did not alter the expression of social play in the operant conditioning setup (chapter 4), or during a dyadic encounter (Vanderschuren et al., 2008). It may also explain why dopamine receptor agonists have such variable effects on play. In summary, the effect of dopaminergic drugs on play expression is not straightforward (see introduction). This study provides the first evidence for a direct role of dopamine in the motivational aspects of social play behavior, although we cannot rule out an effect on its pleasurable properties.

Enhancement of endogenous noradrenaline levels using the noradrenaline reuptake inhibitor atomoxetine did not alter the acquisition of CPP, suggesting that noradrenaline does not play a major role in the pleasurable aspects of social play behavior. In contrast, atomoxetine treatment did reduce responding for social play behavior in an operant task, as well as its expression. The lack of effect of the α2-adrenoreceptor antagonist RX821002 on the methylphenidate-induced enhancement of operant responding suggests that noradrenaline does not play a primary role in the motivation to engage in social play. Therefore, the decrease in responding for social play after atomoxetine treatment may be the result of the reduction in expression of social play. Together, ttese data suggest that increased endogenous noradrenaline inhibits social play, but not as a result of a change in its pleasurable effects.

In summary, the results in chapter 4 indicate that dopamine and noradrenaline influence different aspects of social play behavior. Enhancement of dopamine levels seem to have an opposite effect on the pleasurable and motivational properties, while having no effect on the expression of social play behavior. Noradrenaline appears to mainly affect the expression of social play behavior and thereby influences motivational aspects of social play behavior. Methylphenidate influences both the motivation and expression of social play behavior, but via different mechanisms: a dopaminergic effect on operant responding and a α2 noradrenergic effect on the expression of social play behavior. These results emphasize that different aspects of social play behavior (e.g. pleasurable and motivational) are mediated via multiple neurobiological mechanisms.

Opioids and cannabinoids

In **chapter 5**, we investigated the role of opioid and cannabinoid neurotransmission in the motivational and pleasurable aspects of social play behavior, as well as in play expression. We found that blocking opioid receptors with naloxone reduced operant responding for social play behavior, the expression of social play as well as

social play-induced CPP. The CB1 cannabinoid receptor antagonist rimonabant reduced operant responding for social play likely due to its pruritic effect, without affecting play expression or social play-induced place conditioning. Morphine-treatment non-specifically reduced operant responding at the highest dose (3.0 mg/kg) but did not affect social play expression. Although morphine has been found to increase responding for food a PR schedule (Solinas and Goldberg, 2005), suppressant effects of morphine on operant behavior have been reported (Thompson et al., 1970; Leander et al., 1975; Adams and Holtzman, 1990). Enhancing endocannabinoid levels using the FAAH inhibitor URB597 modestly reduced responding for social play at the highest dose tested. In contrast, URB597 treatment has previously been found not to affect responding for food (Oleson et al., 2012) or nicotine (Forget et al., 2009). Moreover, inhibiting the reuptake of anandamide had no effect on responding for food (Gamaleddin et al., 2013). Together, these data do not support a general role for endocannabinoid signaling in the motivational properties of rewards.

Remarkably, in contrast to previous studies (Trezza and Vanderschuren 2008a; -2008b; -2009), social play behavior in the operant conditioning task was not altered by morphine, URB597 or rimonabant. Previous studies have shown that morphine enhances social play according to an inverted U-shaped dose-effect curve, whereby 1 mg/kg induced robust increases in both pinning and pouncing (Trezza and Vanderschuren 2008a; Vanderschuren et al., 1995b-1996). In addition, treatment with URB597 increased social play after both systemic (Trezza and Vanderschuren 2008a,b) and central (nucleus accumbens and basolateral amygdala) administration (Trezza et al., 2012), whereas rimonabant reduced social play (Trezza and Vanderschuren, 2009). Several factors could explain the discrepancies in findings. First, in the present study, only the experimental animal was treated, and not its stimulus partner. Trezza and Vanderschuren (2008b) previously showed that treating one animal of a couple with morphine results in an increase in pouncing (play initiations) but not pinning, whereas treating one animal with URB597 does not enhance social play, when behavior of a test couple was analyzed. Thus, treating only one animal in a test couple may not be sufficient to observe a robust increase (or decrease) in social play. Second, in chapter 5 we socially isolated animals for 24 hours, whereas most previous studies used 3.5 hours of social isolation. Social isolation for 24 hours causes a maximal increase in the amount of social play (Niesink and Van Ree 1989; Vanderschuren et al., 1995b, 2008), which may obscure the play-enhancing properties of morphine and URB597 because of a ceiling effect (but see Vanderschuren et al., 1995b). Third, the behavior of the stimulus animal should be considered as well. That is, social play has been found to be most pleasurable when both animals have a similar motivation to play (Douglas et al., 2004). Possibly, drug-treatment of the experimental animal in combination with the 24 hours of social isolation may cause a difference in the willingness to play between both animals, so that the play interaction is less rewarding for the stimulus animals, thereby blunting the effects of morphine, URB597 and rimonabant. Fourth, in the operant setup, animals have only one minute to play per reinforcement, whereas our previous studies on the expression of social play analysed this behavior for 15 minutes continuously. It could therefore be that stimulating effects on social play are blunted because the playful interaction is interrupted after 1 min. The present data, together with our previous findings (chapter 4) therefore indicate that social play expression in our operant setup may be more sensitive to manipulations that decrease social play than to those that increase this behavior. Possibly, adjustments to this setup may facilitate the detection of increases in social play expression, such as using a shorter isolation time, or a longer interaction time per reinforcement.

Altogether, the results from chapter 4 and 5 show that the operant conditioning

paradigm provides important information about the neuropharmacology of social play behavior, which may prove to be of importance in elucidating the neural mechanisms underlying the motivational and rewarding aspects of social play behavior. In particular, the present studies shed more light on the effects of methylphenidate on social play. Conversely, setup adjustments may be necessary to better elucidate the role of play-enhancing treatments in this task.

Social reward-related memory processing

The majority of studies on memory processing have focused on memories of negative events, while the dynamic of positive memories is mostly studied in relation to drug exposure (Tronson and Taylor, 2007; Besnard et al., 2012). In fact, processing of memories of physiologically relevant natural rewards such as social stimuli, has received little attention (Perrin et al., 2007).

In **chapter 6** we studied how processing of social reward-related memory, that is, memory for a context previously associated with social play in a place conditioning setup, was influenced by the β-adrenoceptor antagonist propranolol, a compound known to disrupt memory processing of both pleasurable and aversive stimuli and events (Bernardi et al., 2006; Fricks-Gleason and Marshall, 2008; Debiec and Ledoux, 2004; Diergaarde et al., 2006; Milton et al., 2008; Robinson and Franklin, 2007). The results of this study indicate that propranolol specifically and persistently disrupts memory reconsolidation of social reward-related memories in rats, without affecting the other phases of memory processing, such as acquisition, consolidation or retrieval of social play-induced CPP. In addition, we show that the reconsolidation-window for this type of memory is less than one hour. Reconsolidation is thought to depend on restabilization of existing synaptic networks (Nader et al., 2000), and to serve as an updating mechanism for existing memory traces (Lee, 2009). In this light, a brief reconsolidation-window for social memories may be beneficial for social animals, including rats and humans. Because social animals live in a complex, rapidly changing social environment and social interactions can be very brief, the updating of social information must be rapid in order for social animals to function properly.

The results in chapter 6 demonstrate that in adolescent rats, β-adrenergic neurotransmission mediates the reconsolidation but not the acquisition, consolidation or retrieval of social reward-related memories. Propranolol has been shown to disrupt memory acquisition, consolidation (Kroon and Carobrez, 2011; Cahill et al., 1994) or retrieval (Otis and Mueller, 2011; Murchison et al., 2004) in adult rats, at least in certain studies. The discrepancies between the role of β-adrenoceptors in these memory processes in adolescent and adult animals may be associated with the age-related changes in noradrenergic innervation of brain structures implicated in learning and memory, such as the hippocampus, amygdala and frontal cortex (Everitt et al., 1999; Maren, 2011). However, our data do indicate that reconsolidation is an important process throughout life, since we show that, in addition to pups (Languille et al., 2009) and adults (Nader et al., 2000), this process occurs adolescent rats as well. Furthermore, the specificity of propranolol to affect reconsolidation in our paradigm supports the notion that consolidation and reconsolidation of social reward-related memories rely on distinct neural mechanisms. Indeed, several differences in the molecular pathways underlying consolidation and reconsolidation of fear memories have been found (Taubenfeld et al., 2001; Barnes et al., 2010; Lee et al., 2004) and our results suggest that a distinction between the neural mechanisms of consolidation and reconsolidation also holds for positive social-emotional memories.

In **chapter 7** we investigated the involvement of corticosteroid, NMDA glutamatergic and CB1 cannabinoid receptors in memory retrieval and reconsolidation of social-

reward related memories. Glucocorticoids are predominantly involved in aversive memory reconsolidation (Jin et al., 2007; Wang et al., 2008; Taubenfeld et al., 2009; Pitman et al., 2011; Nikzad et al., 2011) Furthermore, glutamatergic NMDA (Kelley et al., 2007; Sadler et al., 2007, Zhai et al., 2008 and Wu et al., 2012) and CB1 cannabinoid receptors (Yu et al. 2009, Fang et al. 2011) have been implicated in reconsolidation of drug-reward memory. The data presented in chapter 7 show that, concerning the dynamics of social reward-related memories, glucocorticoid neurotransmission specifically mediates the reconsolidation of social play-induced CPP, when administered before retrieval and without affecting the retrieval process. Conversely, the mineralocorticoid, NMDA and CB1 receptors are not involved in the retrieval and reconsolidation of social reward-related memories, since both these processes were not affected by treatment with the mineralocorticoid, NMDA and CB1 receptor antagonists.

Pleasurable stimuli such as food, drugs of abuse or sex are known to cause a rise in corticosterone levels (Piazza and Le Moal, 1997; Koolhaas et al., 2011; Buwalda et al., 2012) and increasing glucocorticoid levels improves acquisition and/or consolidation of appetitive memory (Micheau et al., 1981, 1985; Zorawski and Killcross, 2002; Wichmann et al. 2012) suggesting a role for glucocorticoid receptors in the initial stages of appetitive memory formation. Our data add to this scenario by demonstrating that reconsolidation of reward-related memory can be disrupted by antagonizing glucocorticoid receptors. Whether other reward-related memories, such as drug-reward memory, are affected by antagonizing glucocorticoid receptors remains to be elucidated.

Although the place conditioning paradigm allowed us to gain valuable information about the dynamics of memories for physiologically relevant natural rewards, such as social play behavior, this task also has a drawback for appetitive reconsolidation research. According to Suzuki et al. (2004), different processes are started based on the length of the retrieval session. A short retrieval session (±3 min) triggers reconsolidation whereas longer sessions (±30 min) trigger extinction learning. Since we used a 15 min retrieval session, one could argue that we interfere with both processes. However, in CPP experiments a 15 min test session is required to assess if animals acquired place preference for a context previously associated with a specific unconditioned stimulus, and shorter retrieval sessions are usually insufficient to reveal robust expression of CPP (Tzschentke et al., 2007).

In summary, the results obtained in chapter 6 and 7 extend our knowledge of the cognitive aspects of social play and memory function in general. Social reward-related memories are subject to reconsolidation after retrieval and this process is susceptible to impairment by β-noradrenaline and glucocorticoid receptors. In addition, the reconsolidation process occurs throughout the entire lifespan of rats, as we show that interference with the reconsolidation process for an appetitive type of memory is possible in adolescent rats. Furthermore, the results from chapter 6 also highlight the importance of noradrenergic functioning for more cognitive aspects of social play behavior.

Future directions

Since amphetamine and methylphenidate reduce social play through a distinct pharmacological mechanism than cocaine, it is possible that the behavioral underpinnings of these effects also differ between different psychostimulant drugs. Investigating the brain areas underlying the play-suppressant effects of amphetamine and cocaine could not only provide insights into the differences in the mechanism of action of these psychostimulants, but also which brain areas are involved in the generation of normal social play behavior.

As mentioned before, it has been demonstrated that the effect of systemic administration of methylphenidate on social play behavior is mediated via the α2-adrenoceptors (Vanderschuren et al., 2008). Therefore, it is likely that the effects of intracranial administration of methylphenidate are also mediated by α2-adrenoceptors. A conclusive experiment to prove this hypothesis would be to test whether the effects of systemic administration of methylphenidate are antagonized by the α2-adrenoceptor antagonist RX821002 infused into the anterior cingulate cortex, infralimbic cortex, habenula and amygdala.

Considering the involvement of the anterior cingulate cortex, infralimbic cortex, habenula and amygdala, one might speculate about a functional pathway involved in the regulation of social play behavior. In line with Siviy and Panksepp (2011), our data suggest that the thalamus may be an important relay station in the processing of information from the infralimbic and anterior cingulate cortex and the striatum in the expression of social play. The thalamus shares bidirectional projects with the PFC (Groenewegen, 1988) and these projections were found to be involved in play behavior (Siviy et al., 1985, -1987). The thalamus also receives input from the amygdala and habenula (De Olmos et al., 2004; Lecourtier and Kelly, 2007) and projects to the nucleus accumbens (NAc) (Voorn et al., 2004). Therefore, the next step to take would be to infuse methylphenidate and atomoxetine into the thalamus. As a follow up of the brain areas mediating the effect of methylphenidate on play expression, administration of methylphenidate in specific brain regions in rats tested in the operant conditioning paradigm may shed more light on the brain regions mediating of the effects of this drug on motivational aspects of social play behavior in the operant conditioning task. Furthermore, disconnection studies could give more insight in the exact pathways involved both in play expression tests as well as the operant conditioning task. In addition, also the type of neurons involved would be valuable and could answer the question why the anterior cingulate and infralimbic but not the prelimbic cortex is involved in the effect of methylphenidate on social play behavior. Van Kerkhof et al. (2013b) started to address this question and showed that glutamatergic dorsomedial striatal projections inhibit social play behavior.

Glucocorticoids affect memory formation of experiences that are emotionally arousing (Okuda et al., 2004; Roozendaal et al., 2006). Furthermore, arousal-induced noradrenergic activity is found to be required for the effects of glucocorticoids on memory consolidation (Quirarte et al., 1997; Roozendaal et al. 2006; Barsegyan et al., 2010). In chapter 6, we demonstrated that systemic blockade of the β-adrenoceptor with propranolol either before or immediately after retrieval specifically and persistently disrupted reconsolidation of social reward-related memories in a place conditioning set-up, without affecting memory retrieval. On the basis of the results from chapters 6 and 7, I hypothesize that β-noradrenergic and glucocorticoid systems interact in reconsolidation of social reward-related memories. To test this hypothesis, one could investigate whether subeffective doses of β-noradrenergic and glucocorticoid receptor antagonists affect reconsolidation of social reward-related memories. Because of the timing of the effect after administration of the glucocorticoid receptor antagonist and the β-adrenoceptor antagonist, this subeffective dose should be administered before the retrieval session.

Key structures mediating memory processing are the hippocampus, amygdala and prefrontal cortex (Tronson and Taylor, 2007; Besnard et al., 2012). Administration of either the β-adrenoceptor antagonist or the glucocorticoid receptor antagonist (or combined subeffective doses of these drugs) into each of these brain areas would give valuable information with regard to the involvement of these brain regions in reward-related memory processing.

Concluding remarks

The aim of this thesis was to elucidate the neurotransmitter systems and neural substrates involved in the rewarding, motivational and cognitive aspects of social play behavior. In particular, we focused on the role of monoamines, opioids, cannabinoids and glucocorticoids in different aspects of social play behavior.

We found that amphetamine, like methylphenidate, exerts its play suppressant effects via α2-adrenoceptors. In contrast, cocaine reduces social play by simultaneous increases in dopamine, noradrenaline and serotonin neurotransmission. We subsequently found that specific prefrontal (anterior cingulate and infralimbic cortex) and subcortical (amygdala and habenula) regions underlie the effects on methylphenidate on social play, through noradrenergic neurotransmission.

Using place conditioning and operant set-ups, we further elucidate the role of monoamines, opioids and cannabinoids in the several aspects of social play behavior. With regard to monoamines, noradrenaline seems to play a role in mediating and modulating the expression of and the motivation for social play behavior. For dopamine, clear but opposite roles were found on motivation for and pleasurable aspects of social play. We found that the endogenous opioid system is involved in pleasurable and motivational aspects of social play. Conversely, altering endocannabinoid neurotransmission does not seem to affect the motivational and pleasurable aspects of social play behavior in these tasks.

By analyzing the dynamics of social reward-related memories in place conditioning experiments, we found that this type of memories undergoes reconsolidation that depends on β-noradrenergic as well as glucocorticoid receptors, whereas mineralocorticoid, NMDA glutamatergic and CB1 cannabinoid receptors do not seem to be involved.

Collectively, the studies outlined in this thesis advance our understanding of the neural mechanisms involved in several aspects of social play behavior in rats. Since social play behavior is essential for proper functioning in adult life, the presence or absence of social play may be indicative of the welfare state of the animal. However, social play as an indicatior of welfare should only be used in combination with other welfare parameters and measured over a longer period of time. This because of the rebound effect after a stressful event (Held and Špinka, 2011) and differences in motivation (and therefore welfare differences) between individuals in a couple or group of animals (Douglas et al., 2004). Furthermore, despite the limitations of preclinical research in understanding social dysfunctions in psychiatric disorders and the fact that the experiments in this thesis were conducted in healthy animals, these studies provide important information to understand the neurobiology of social behavior and may give directions for the development of pharmacotherapies for social dysfunctions in psychiatric diseases. For example, the differences on the motivational aspects of social play found between methylphenidate and atomoxetine together with our intracranial administration data of these substances may advance our understanding of why certain ADHD-patients respond to one but not the other treatment and how these medications work in the brain to influence social behavior. Furthermore, our studies may help in finding ways to stimulate social behavior in disorders such as autism and schizophrenia or increasing the pleasurable/motivational aspects of social behavior combined with influencing social memory processing in patients suffering from social phobias.

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Addendum

Nederlandse samenvatting

Sociale interacties zijn een fundamenteel onderdeel van het leven van mens en dier. Het ervaren van positieve sociale interacties is zeer belangrijk voor alle jonge mensen en dieren; dit geldt zowel voor kinderen als adolescenten. Sociale gedragspatronen veranderen gedurende het leven. In de kindertijd bijvoorbeeld, verschuift de sociale interesse van kinderen van de ouders naar leeftijdsgenoten. Sociale interacties in de kindertijd en de vroege adolescentie, bij zowel kinderen als jonge dieren, worden gekenmerkt door de aanwezigheid van veel sociaal spelgedrag. Sociaal spelgedrag is zeer belonend en het draagt bij aan een goede sociale, motorische, cognitieve en emotionele ontwikkeling. Dit blijkt enerzijds uit het feit dat sociale problemen tijdens de kindertijd en adolescentie de kans op het ontwikkelen van psychische stoornissen op volwassen leeftijd vergroten. Anderzijds worden afwijkingen in sociaal speelgedrag veelvuldig gezien bij psychische stoornissen bij kinderen en adolescenten zoals autisme en ADHD (attention deficit/hyperactivity disorder). Het is echter op dit moment grotendeels onduidelijk welke hersengebieden en signaalstoffen betrokken zijn bij verschillende aspecten van sociaal spelgedrag. In deze dissertatie hebben we gekeken naar sociaal spelgedrag bij jonge ratten. Specifiek hebben we gekeken naar de hersengebieden en signaalstoffen die betrokken zijn bij de plezierige, motivationele en cognitieve aspecten van sociaal spelgedrag.

Vanuit een fundamenteel oogpunt willen we begrijpen hoe sociaal spelgedrag tot stand komt en welke hersengebieden en signaalstoffen verschillende aspecten van dit gedrag beïnvloeden. Dit vergroot onze kennis over normaal, adaptief sociaal gedrag. Vanuit een klinisch oogpunt kan een grotere kennis van de onderliggende hersenmechanismen van sociaal spelgedrag bijdragen aan de ontwikkeling van (farmacologische) therapieën voor aandoeningen die gekenmerkt worden door afwijkend sociaal gedrag. Daarnaast kan de kennis over sociaal spelgedrag een belangrijke bijdrage leveren aan het begrip van verslaving aan middelen. verslavende middelen zoals tabak, alcohol, cannabis en cocaïne immers vaak in een sociale omgeving gebruikt en ze beïnvloeden sociaal (spel) gedrag in sterke mate. Bovendien worden deze verslavende middelen vaak in de vroege adolescentie voor het eerst gebruikt. Verder zijn sociale stoornissen in de jeugd, zoals bij ADHD en disruptieve gedragsstoornissen, een risicofactor voor het ontwikkelen van een alcohol of drugsverslaving. Daarom is het belangrijk om een beter beeld te krijgen van hoe deze verslavende middelen sociaal spelgedrag beïnvloeden.

In hoofdstuk 2 hebben we onderzocht welke signaalstoffen en receptoren in de hersenen betrokken zijn bij de spel-onderdrukkende effecten van de psychostimulantia amfetamine en cocaïne. Beide stoffen verhogen de hoeveelheid van de signaalstoffen noradrenaline, dopamine en serotonine in het brein. Door het blokkeren van specifieke receptoren voor deze stoffen d.m.v. een voorbehandeling van specifieke receptor antagonisten hebben we geprobeerd het spelverlagende effect van amfetamine en cocaïne teniet te doen. De experimenten lieten zien dat het spel-onderdrukkende effect van amfetamine werd veroorzaakt door stimulatie α2-noradrenaline receptoren. We vonden namelijk dat een voorbehandeling van de dieren met de α2-noradrenaline receptor antagonist RX821002 het spel-onderdrukkende effect van amfetamine teniet deed, waardoor deze dieren weer evenveel speelden als een groep dieren die geen amfetamine hadden gehad. De receptoren die betrokken zijn bij het spel-onderdrukkende effect van cocaïne zijn niet opgehelderd omdat zowel noradrenaline, dopamine en serotonine antagonisten op zichzelf en in combinatie het spel-onderdrukkende effect van cocaïne niet konden voorkomen. Het is wel duidelijk geworden dat zowel noradrenaline, dopamine als serotonine betrokken zijn bij het effect van cocaïne op sociaal spelgedrag omdat we een niet-effectieve

dosering van cocaine effectief, en dus spel-onderdrukkend, konden maken door er een combinatie van noradrenaline, dopamine en serotonine heropname remmers bij te geven. De resultaten in dit hoofdstuk laten zien dat een eenmalige blootstelling aan psychostimulantia gevolgen heeft voor een belangrijke vorm van sociaal gedrag bij jonge ratten. Deze resultaten zijn ook van belang voor verslavingsonderzoek bij mensen, aangezien er nog maar weinig bekend is over de gevolgen van initieel drugsgebruik op sociaal gedrag bij jongeren.

In hoofdstuk 3 hebben we geprobeerd om in kaart te brengen welke hersengebieden betrokken zijn bij het spel-onderdrukkende effect van methylfenidaat (Ritalin®, Concerta®), een dopamine en noradrenaline heropname remmer die veel gebruikt wordt voor de behandeling van ADHD. Hiervoor hebben we canules geplaatst in verschillende delen van de hersenen. Vervolgens hebben we methylfenidaat ingebracht in een bepaald hersengebied en hebben we gekeken naar de hoeveelheid spelgedrag. We vonden dat er twee gebieden in de prefrontale hersenschors, te weten de anterior cingulaire cortex en de infralimbische cortex en twee subcorticale gebieden, te weten de habenula en amygdala, betrokken waren bij het effect van methylfenidaat op sociaal spelgedrag. Toediening van methylfenidaat in de prelimbische cortex, medio-orbitale/ventro-orbitale cortex, ventro-laterale orbitale cortex en de schil van de nucleus accumbens had geen effect op spelgedrag. Wij denken dat het effect van methylfenidaat in de anterior cingulaire cortex, de infralimbische cortex, de amygdala en habenula het gevolg is van verhoogde noradrenaline neurotransmissie, aangezien toediening van de specifieke noradrenaline heropname remmer atomoxetine (Strattera®; dit middel wordt ook gebruikt als medicijn tegen ADHD) ook spelgedrag onderdrukte. Deze resultaten suggereren dat een samenwerking van limbische corticale en subcorticale hersengebieden ten grondslag ligt aan de integratie van cognitieve en emotionele informatie tijdens het uitvoeren van normaal sociaal spelgedrag en dat dit proces verstoord wordt als de concentraties noradrenaline in deze hersengebieden te hoog zijn. Deze resultaten dragen hiermee bij aan de identificatie van een spel-netwerk in de hersenen. Tevens geven de resultaten inzicht in het werkingsmechanisme van methylfenidaat en atomoxetine.

Sociaal spelgedrag bestaat uit meerdere componenten, zoals de plezierige, motivationele en cognitieve aspecten van het gedrag. In hoofdstuk 4 hebben we onderzocht hoe dopamine en noradrenaline deze plezierige en motivationele aspecten beïnvloeden. Hiervoor hebben we eerst een nieuwe taak opgezet om motivatie voor sociaal spelgedrag te kunnen onderzoeken. In deze taak leren jonge ratten dat ze op een pedaaltje moeten drukken om toegang te krijgen tot een soortgenoot waarmee ze kunnen spelen. Vervolgens wordt het aantal pedaaldrukken dat vereist is om toegang te krijgen tot de spelpartner verhoogd. Op deze manier hebben we onderzocht hoe vaak een rat bereid is te drukken voor een sociale (spel)interactie, wat een indicatie is van motivatie voor sociaal spelgedrag. Tevens hebben we de hoeveelheid spel (spel-expressie) gemeten, wanneer de dieren toegang kregen tot een partner. Om de plezierige aspecten effecten van sociaal spelgedrag te onderzoeken, hebben we gebruik gemaakt van de geconditioneerde plaats-preferentie test. In deze test worden dieren herhaaldelijk aan een zijde van de test-kooi geplaatst met een spelpartner. Deze zijde is afgesloten van de rest van de kooi. Tijdens een andere sessie op dezelfde dag, zit het dier zonder een soortgenootje om mee te spelen aan de andere zijde (die er duidelijk anders uitziet en anders aanvoelt). Door herhaaldelijke blootstelling aan spel aan een bepaalde zijde van de test-kooi, zullen de dieren de omgevingskenmerken van die zijde met de (plezierige) eigenschappen van spel gaan associëren. Na herhaaldelijke blootstelling aan beide zijden van het apparaat wordt het dier in het midden van de test-kooi gezet en wordt de tijd opgemeten die het dier doorbrengt aan elk van de twee zijden. Dit geeft

aan voor welke zijde het dier een voorkeur heeft, dit noemen we geconditioneerde plaats-preferentie. We vonden dat dopamine en noradrenaline de motivationele en plezierige aspecten van sociaal spelgedrag verschillend beïnvloedden. Het verhogen van dopamine-concentraties in de hersenen, door toediening van de dopamine heropname remmer GBR1209, verhoogde de motivatie voor sociaal spel, verlaagde het plezierige aspect en had geen invloed op de expressie van spel zelf. Het verhogen van noradrenaline concentraties, door toediening van de noradrenaline heropname remmer atomoxetine, verlaagde de expressie van spel en mogelijk ook de motivatie, maar had geen invloed op het plezierige aspect van sociaal spelgedrag. Deze resultaten geven een extra dimensie aan het onderzoek naar sociaal spelgedrag omdat nu ook specifiek de motivatie voor sociaal spel gemeten kan worden op een vergelijkbare manier bij andere beloningen, zoals verslavende middelen en voedsel. Tevens geven deze resultaten nieuwe inzichten in hoe dopamine en noradrenaline verschillende aspecten van spelgedrag beïnvloeden.

In hoofdstuk 5 hebben we de in hoofdstuk 4 besproken tests gebruikt om te kijken naar de rol van opioïden en endocannabinoïden op de plezierige en motivationele aspecten van sociaal spelgedrag. Het blokkeren van opioïd receptoren verlaagde de plezierige en motivationele aspecten van sociaal spelgedrag en verlaagde eveneens de expressie van spel. Van opioïden is bekend dat ze voornamelijk de plezierige aspecten van beloningen beïnvloeden. Op basis van de gevonden resultaten kunnen we daar sociaal spel als beloning aan toevoegen. Het beïnvloeden van endocannabinoïde transmissie had weinig invloed op de motivationele en plezierige aspecten van sociaal spelgedrag. Deze resultaten geven nieuwe inzichten in hoe opioïden en endocannabinoïde neurotransmissie spelgedrag moduleren.

In hoofdstuk 6 hebben we de cognitieve aspecten van sociaal spelgedrag bestudeerd, namelijk het geheugen voor de plek waar dieren hebben gespeeld. Dit soort geheugenprocessen zijn vooral onderzocht voor onderzoek naar angst(stoornissen) en beloningen, zoals voedsel en verslavende middelen, maar er is weinig bekend over hoe geheugen voor sociale stimuli wordt verwerkt en opgeslagen in het brein. Om het geheugen te onderzoeken hebben we gebruik gemaakt van de hierboven beschreven geconditioneerde plaats-preferentie test. We hebben onderzocht wat de invloed is van het blokkeren van beta-noradrenaline receptoren op verschillende fases in de geheugenvorming, namelijk de acquisitie (vorming korte-termijn geheugen), de consolidatie (vorming en opslag van lange-termijn geheugen), het terughalen (het actief maken van het lange-termijn geheugen, waardoor het gedrag kan beïnvloeden en aangepast kan worden aan de nieuwe situatie) en de reconsolidatie (hernieuwde opslag van het teruggehaalde lange-termijn geheugen). We hebben de beta-noradrenerge receptor antagonist propranolol gebruikt, omdat uit eerder onderzoek bekend is, dat blokkade van beta-noradrenerge receptoren in andere tests verschillende fasen van de geheugen-vorming kan verstoren. Om de rol van beta-noradrenerge receptoren tijdens de verschillende fasen in de geheugenvorming te onderzoeken, hebben we op verschillende momenten tijdens de geheugenvorming propranolol toegediend. Het bleek dat toediening van propranolol zowel vóór als direct na het terughalen van de herinnering de reconsolidatie van geheugen voor sociaal spelgedrag beïnvloedt. Dieren die behandeld waren met propranolol, die een dag na het terughalen van het geheugenspoor opnieuw werden getest, lieten geen plaats-preferentie meer zien. Deze uitkomst kan twee dingen betekenen: 1. het beïnvloeden van reconsolidatie of 2. Versnelde onderdrukking van het gedrag door de vorming van een nieuw geheugenspoor met een tegengestelde betekenis (nl. dat de bewuste zijde van de test-kooi niet langer geassocieerd is met spel; dit proces wordt extinctie genoemd). Om een onderscheid te maken tussen de twee hypotheses hebben we de associatie tussen

spel en de bewuste zijde van de test-kooi verzwakte door de dieren dagelijks het hele apparaat te laten exploreren (extinctie-training). Dit hebben we gedaan totdat dieren vier dagen achter elkaar geen plaats-preferentie meer lieten zien. Vervolgen hebben we dieren één spel-sessie gegeven om de plaats-preferentie opnieuw op te wekken. Wij namen aan dat als het reconsolidatie-proces beïnvloed is, het geheugenspoor zodanig aangetast is dat één spelsessie niet zal leiden tot plaats-preferentie, wanneer de dieren opnieuw getest worden. Wanneer er sprake is van versnelde extinctie kan één spel-sessie leiden tot hernieuwde plaats-preferentie. Na de extinctie-training en één spelsessie lieten dieren in de controle groep weer plaats-preferentie zien, terwijl dieren die vóór en direct na het terughalen van het geheugenspoor behandeld waren met propranolol dit niet lieten zien. De andere fasen in de geheugenvorming waren niet aangetast door toediening van propranolol. Dit patroon van effecten suggereert dat blokkade van de beta-noradrenerge receptor specifiek de reconsolidatie-fase van de geheugenvorming voor sociaal spelgedrag beïnvloedt. Deze resultaten geven aan dat geheugen voor sociale stimuli d.m.v. vergelijkbare mechanismen verwerkt wordt in het brein als andere stimuli zoals angst en andere typen beloningen.

In hoofdstuk 7 hebben we gekeken naar de effecten van het blokkeren van glucocorticoïde, mineralocorticoïde, NMDA glutamaterge en CB1 endocannabinoïde receptoren op twee specifieke fasen in de geheugenvorming, namelijk het terughalen van de herinnering en de reconsolidatie. Eerder studies hebben uitgewezen dat het blokkeren van deze receptoren zowel positieve als negatieve herinneringen kan verstoren. Daarom wilden we onderzoeken of dit ook van toepassing was voor de verwerking van geheugen voor sociale stimuli. We vonden dat het blokkeren van de glucocorticoïde receptor vóór het ophalen van het geheugenspoor, maar niet direct erna, de reconsolidatie van geheugen voor sociaal spelgedrag in de plaats-preferentie test remde. In tegenstelling wat er gevonden is geheugen voor andere beloningen, bleken mineralocorticoïde, NMDA glutamaterge en CB1 endocannabinoïde receptoren niet betrokken te zijn bij de reconsolidatie van de herinnering aan sociaal spel. We concluderen hieruit dat glucocorticoïde neurotransmissie de reconsolidatie moduleert van geheugen voor sociaal spelgedrag in de plaats-preferentie test. Ook geven deze resultaten aan dat de mechanismen in de hersenen van reconsolidatie van geheugen voor sociaal spelgedrag in de plaats-preferentie test bij jonge ratten slechts gedeeltelijk overeenkomt met de mechanismen voor andere typen positieve herinneringen.

De studies beschreven in dit proefschrift vergroten onze kennis over de hersengebieden en signaalstoffen die betrokken zijn bij de plezierige, motivationele en cognitieve aspecten van sociaal spelgedrag bij jonge ratten. De verrichte studies dragen bij aan het inzicht van hoe sociaal (spel)gedrag tot stand komt in het brein en welke signaalstoffen dit gedrag reguleren. Deze studies bieden ook aanknopingspunten voor het ontwikkelen van farmacologische therapieën voor ziektes die gekenmerkt worden door dysfuncties in sociaal gedrag. Bovendien geven ze inzicht in het werkingsmechanisme van medicijnen die op dit moment gebruikt worden voor de behandeling van ziektes waarbij afwijkingen in sociaal gedrag voorkomen, zoals ADHD.

Curriculum Vitae

Eefje Juliëtte Marijke Achterberg werd geboren op 15 januari 1986 te Ermelo. In 2004 behaalde zij haar VWO diploma aan RSG 't Slingerbos te Harderwijk. In datzelfde jaar begon zij haar studie Biologie aan de Universiteit van Utrecht. In 2007 startte zij haar master Neuroscience and Cognition, richting Behavioral Neuroscience, aan dezelfde universiteit. Tijdens deze master werden twee wetenschappelijke stages uitgevoerd. In haar eerste stage onderzocht zij variaties in 50 kHz ultrasone geluiden bij twee verschillende rattenstammen onder begeleiding van Dr. Bart Houx op de afdeling Dier in Wetenschap en Maatschappij aan de Faculteit Diergeneeskunde in Utrecht. Onder begeleiding van Dr. Viviana Trezza en Dr. Louk Vanderschuren van de afdeling Neurowetenschappen en Farmacologie van het Hersencentrum Rudolf Magnus te Utrecht onderzocht zij tijdens haar tweede stage de rol van beta-noradrenerge receptoren op specifieke fasen in de geheugen-vorming voor sociaal spelgedrag in jonge ratten. In 2009 behaalde zij haar diploma en startte haar promotie-onderzoek, eveneens onder begeleiding van Dr. Viviana Trezza en nu Prof. Dr. Louk Vanderschuren, naar neurotransmitters en hersengebieden die betrokken zijn bij de plezierige, motivationele en cognitieve aspecten van sociaal spelgedrag bij jonge ratten. De resultaten van dit onderzoek staan beschreven in dit proefschrift. Sinds augustus 2013 is zij werkzaam als post-doctoraal onderzoeker onder begeleiding van Prof. Dr. Vanderschuren en Dr. Barbara Biemans van de afdeling Developmental and Behavioral Neuroscience bij Hoffmann-La Roche Ltd. te Basel, Zwitserland. Haar onderzoek richt zich op de rol van neurexine 1 in sociale dysfunctie bij autisme en schizofrenie.

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