Behavioural Brain Research 347 (2018) 158–166

Contents lists available at ScienceDirect

Behavioural Brain Research

journal homepage: www.elsevier.com/locate/bbr

Research report

# On the central noradrenergic mechanism underlying the social play-suppressant effect of methylphenidate in rats

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ARTICLE INFO

*Keywords:*
Social play behaviour
Methylphenidate
Alpha-adrenoceptor
Prefrontal cortex
Amygdala
Habenula

ABSTRACT

Social play behaviour is a vigorous, highly rewarding social activity abundant in the young of most mammalian species, including humans. Social play is thought to be important for social, emotional and cognitive development, yet its neural underpinnings are incompletely understood. We have previously shown that low doses of methylphenidate suppress social play behaviour through a noradrenergic mechanism of action, and that methylphenidate exerts its effect within the prefrontal cortex, amygdala and habenula. In the present study, we sought to reveal whether these regions work in parallel or in series to mediate the play-suppressant effect of methylphenidate. To that aim, we tested whether infusion of the α2-adrenoceptor antagonist RX821002 into the anterior cingulate cortex, infralimbic cortex, basolateral amygdala or habenula prevents the effect of methylphenidate on social play behaviour, or the psychomotor stimulant effect of methylphenidate. We found that the social play-suppressant effect of methylphenidate was not prevented by infusion of the α2-adrenoceptor antagonist into either region, or by infusion of RX821002 into both the anterior cingulate and infralimbic cortex. By contrast, RX821002 infusion into the anterior cingulate modestly enhanced social play, and infusion of the antagonist into the infralimbic cortex attenuated the psychomotor stimulant effect of methylphenidate. We conclude that there is redundancy in the neural circuitry that mediates the play-suppressant effect of methylphenidate, whereby prefrontal cortical and subcortical limbic mechanisms act in parallel. Moreover, our data support the notion that prefrontal noradrenergic mechanisms contribute to the locomotor enhancing effect of psychostimulant drugs.

## 1. Introduction

Social play behaviour is a highly energetic form of social interaction that is abundantly expressed in the young of most mammalian species, including humans. Social play behaviour is thought to be of major importance for the development of appropriate social behavioural patterns, as well as emotional and cognitive capacities [1–10]. However, the neuro-circuitry underlying social play behaviour remains incompletely understood, even though our understanding of the neural mechanisms mediating social play behaviour in rats has grown considerably in recent years (for review, see [10]).

Of particular interest is the finding that methylphenidate specifically suppresses social play behaviour without affecting general social interest [11–14]. Methylphenidate (Ritalin®, Concerta®), which enhances extracellular levels of the monoamines noradrenaline (NA) and dopamine (DA) by inhibiting their reuptake [15,16] is the standard treatment for attention-deficit/hyperactivity disorder (ADHD) in children and adolescents [17–19]. Although its effectivity in the treatment of ADHD is widely acknowledged, its therapeutic mechanism of action remains elusive. Given the importance of social play for social, emotional and cognitive development, it is of high relevance to understand the mechanisms by which methylphenidate affects this behaviour. That is, suppression of social play behaviour by methylphenidate could be an indirect expression of its therapeutic effect, a side-effect or a dose-dependent combination of both.

It has been demonstrated that the play-suppressant effects of methylphenidate rely on stimulation of α2-adrenoceptors [12–13]. Recently, we have identified key brain regions in which methylphenidate acts to suppress social play behaviour, i.e. the basolateral amygdala, the habenula, and the infralimbic and anterior cingulate cortices [11]. The noradrenergic nature of this effect was supported by the finding that infusion of the noradrenaline reuptake inhibitor atomoxetine into these same brain regions also reduced social play behaviour. Importantly, the basolateral amygdala, habenula, infralimbic and anterior cingulate cortex share reciprocal connections with the locus coeruleus, the main source of noradrenaline in the forebrain [20–28], and these four regions

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https://doi.org/10.1016/j.bbr.2018.03.004
Received 13 October 2017; Received in revised form 28 February 2018; Accepted 2 March 2018
Available online 08 March 2018
0166-4328/ 

are also interconnected [21,23,25,28–30].

Thus, methylphenidate likely acts in a distributed network of brain regions, possibly affecting different emotional and cognitive aspects of social play behaviour at the same time, which results in the inhibition of this behaviour [11,13]. Therefore, in the present study we investigated the possible interconnectivity of this network of brain regions in the mediation of the play-suppressant effect of methylphenidate. We hypothesized that if this circuit is connected in parallel, redundancy in the system is to be expected, which means that the play-suppressing effect of systemically administered methylphenidate cannot be counteracted by infusion of an α2-adrenoceptor antagonist into one of the involved brain regions: i.e. the basolateral amygdala, habenula, infalimbic and anterior cingulate cortex. In contrast, if the circuit is connected in series, we expect that the suppression of play can be counteracted by infusion of the α2-adrenoceptor antagonist into at least one of the four brain regions.

## 2. Experimental procedures

### 2.1. 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 40 × 26 × 20 (l × w × h) cm Macrolon cages under controlled conditions (i.e. temperature 20–21 °C, 55–65% relative humidity and 12/12 h light cycle with lights on at 7.00 a.m.). Food and water were available ad libitum. During the first 6 days after arrival, the 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 (Directive 2010/63/EU).

### 2.2. Surgical procedures

The surgical procedures were performed as described before [11,31–34]. At 27–28 days of age, the rats were anesthetised with 0.08 mL/100 g (s.c.) Hypnorm (fentanylcitrate 0.315 mg/mL and fluanison 10 mg/mL; Janssen, Belgium) and positioned into a stereotaxic frame (David Kopf Instruments, USA). Guide cannulae (24 gauge microblasted thin-walled 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), infralimbic cortex (coordinates: AP + 2.6 mm; ML ± 0.8 mm; DV −4.1 mm), habenula (coordinates: AP −3.0 mm; ML ± 0.8 mm; DV −4.7 mm), or basolateral amygdala (coordinates: AP −1.9 mm; ML ± 4.4 mm; DV −7.8 mm). Cannulae were secured with stainless steel screws and dental acrylic. Stainless steel stylets (29 gauge) were inserted into the guide cannulae to maintain patency. After surgery, rats were individually housed for 4 days to recover, after which they were housed with their original cage mates.

### 2.3. Drugs and infusion procedures

Methylphenidate-HCl (1.0 mg/kg, s.c.; Sigma, St. Louis, USA), and the α2-noradrenergic receptor antagonist RX821002-HCl (0.1 μg/0.3 μL; Tocris Bioscience, Avonmouth, UK) were dissolved in saline. Drug doses were based on previous studies [12,13,35–38] and pilot experiments. As for the dose of RX821002, we aimed to use a dose that provided sufficient antagonism of alpha2-adrenoceptors, without affecting social play behaviour by itself. Therefore, on the basis of the affinity of this drug for alpha2-adrenoceptors (which is in the nanomolar range, see [39]), and previously published studies using intracranial administration of this drug (that reported effects on behaviour at doses of 0.08 μg and higher [36–38] we selected two doses that are on the low end of this dose range (i.e. 0.1 and 0.2 μg) for a dose-finding experiment. In this experiment, we tested the effect of these doses of RX821002 after infusion into the habenula, since to our knowledge, the behavioural effects of intra-habenula RX821002 have so far not been tested. Infusion procedures were as previously described [11,31–34]. In short, bilateral infusions 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. In one experiment, animals received an infusion into the infralimbic cortex, followed by an infusion into the anterior cingulate cortex 5 min later. This was accomplished by using the same guide cannulae with injectors of different lengths, targeting either the anterior cingulate or the infralimbic cortex.

### 2.4. Behavioural testing

Experiments were performed as previously described [11,31–34], in a sound attenuated chamber under red light conditions. The testing arena was a Plexiglas cage (40 × 40 × 60 cm; l × w × h) with approximately 2 cm of wood shavings covering the floor. Animals were paired with an unfamiliar partner (i.e., not a cage mate). Animals in a test pair did not differ more than 10 g in body weight. Prior to testing, the rats were habituated to the experimental procedures on 2 consecutive days. On the first habituation day (PND 32), rats were individually placed into the test cage for 10 min. On the second habituation day (PND 33), the animals were socially isolated for 2.5 h. Pairs of rats were then injected and infused with saline solutions and placed into the test cage for 15 min, to habituate them to the injection, infusion and testing procedures. On the third day (PND 34), which was the first test day, rats were isolated for 2.5 h. Thirty minutes before the test, both animals in a pair were injected either with methylphenidate or saline. Both rats in a pair were then simultaneously infused with RX821002 or vehicle before testing. Importantly, since social play behaviour in rats strongly depends on the playfulness of its partner [40,41], both animals in a play pair were similarly treated and a pair of animals was considered as one experimental unit. On the second test day (PND 36), the animals were also isolated for 2.5 h, and infusion treatments but not injection treatments were reversed, so that animals that received intracranial treatment with RX821002 on the first test day now received intracranial treatment with saline and vice versa. The first and second test day were separated by a wash-out day (PND 35) during which the animals received no treatment and were not tested. On PND 37, animals were tested for horizontal locomotor activity and sacrificed directly after the test. A combined between and within-subjects design (between: saline or methylphenidate, within: RX821002 or saline) was used in all experiments.

Testing consisted of placing a pair of animals into the arena for 15 min. Behaviour of the animals was recorded using a camera with zoom lens, video tape recorder and television monitor. The behaviour of the rats was assessed using the Observer 5.1 software (Noldus Information Technology B.V., Wageningen, The Netherlands). The structure of social play behaviour in rats has been previously described in detail [42–47; for reviews see 4,8,48,49]. In rats, a bout of social play behaviour 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 [44–47]. Pinning and pouncing frequencies can be easily quantified and they are considered the most characteristic parameters of social play behaviour in rats [44]. During the social encounter, animals may also display social behaviours not directly associated with play, such as sniffing or grooming the partner's body [44,50]. The following parameters were therefore scored per pair of animals:

Social behaviours related to play:

- Frequency of pinning
- Frequency of pouncing

Social behaviours unrelated to play:

- Time spent in social exploration: the amount of time spent in non-playful social interaction (i.e., sniffing or grooming).

Pinning, pouncing and other playful behaviours 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 found that pinning and pouncing are very reliable play parameters, that occur consistently and with considerable frequency during playful encounters (see also [44,50]), whereas the occurrence of chasing and boxing can be quite variable.

To assess whether effects of the drug treatment on social play were associated with changes in locomotor activity, the rats were tested for horizontal locomotor activity as previously described [11,51]. Animals were randomly assigned to one of the four treatment groups (i.e., s.c. saline/intracranial saline; s.c. methylphenidate/intracranial saline; s.c. saline/intracranial RX821002; s.c. methylphenidate/intracranial RX821002). The infusion protocol was similar to the one described above. After the infusion procedure, rats were transferred to a plastic cage (50 × 33 × 40 cm, l × w × h) and their position was tracked five times per second for 30 min using a video-tracking system (EthoVision, Noldus Information Technology, Wageningen, The Netherlands).

### 2.5. Histological confirmation of injection sites

Animals were sacrificed using carbon dioxide inhalation and microinjected with 0.3 μL of black ink over 1 min through the guide cannulae, comparable to the drug infusion procedure. After the infusion, animals were decapitated, their brains removed and immediately frozen. Cryostat sections (20 μm) were collected and stained with cresyl violet. Placement of the microinjection sides was determined using a light microscope according to Paxinos and Watson [52]. Only pairs in which both animals had bilateral needle tracks terminating into the target area were included in the final analysis of play behaviour (see Fig. 1). However, horizontal locomotor activity was assessed per individual animal. When cannulae placements of one animal of an excluded pair were correct, it was included in the analysis for locomotor activity.

### 2.6. Statistical analysis

Pinning and pouncing frequencies and time spent on social exploration (s) were scored per pair of animals and expressed as mean + SEM. To assess the effects of methylphenidate and RX821002 on social play behaviour, data were analysed using a repeated measures ANOVA with RX821002/saline infusion as within-subjects factor and methylphenidate/saline injection as between-subjects factor. Horizontal locomotor activity was assessed per individual animal and expressed as mean ± SEM travelled distance (cm) in 5 min bins. The effects of the treatments on locomotor activity were analysed using a repeated measures ANOVA with time as within-subjects factor and methylphenidate and RX821002 treatment as between-subjects factors.

## 3. Results

### 3.1. Effect of infusion of the α2-adrenoreceptor antagonist RX821002 into the habenula on social play behaviour

To determine an appropriate subeffective dose of RX821002, two different doses (0.1 μg/0.3 μL and 0.2 μg/0.3 μL) were infused into the habenula (n = 7). Both doses did not affect pinning ($F_{dose}(2,19) = 2.33$, $p = 0.13$), pouncing ($F_{dose}(2,19) = 2.45$, $p = 0.26$) or social exploration ($F_{dose}(2,19) = 2.31$, $p = 0.14$) (Fig. 2A–C). In addition, locomotor activity was not altered by RX821002 infusion ($F_{dose}(2,19) = 0.42$, $p = 0.67$; $F_{time}(5,95) = 37.83$, $p < 0.001$; $F_{dose \times time}(10,95) = 0.42$, $p = 0.46$) (Fig. 2D). However, visual inspection of the graphs revealed a slight increase in pinning and pouncing and decrease in social exploration after infusion of the 0.2 μg/0.3 μL dose. Therefore, in the remaining experiments we used 0.1 μg/0.3 μL RX821002.

### 3.2. Infralimbic cortex

Systemic injection of methylphenidate reduced pinning ($F_{methylphenidate}(1,12) = 9.01$, $p = 0.01$) and pouncing ($F_{methylphenidate}(1,12) = 12.14$, $p = 0.01$) but had no effect on social exploration ($F_{methylphenidate}(1,12) = 0.16$, $p = 0.69$) (Fig. 3A–C; methylphenidate n = 6, saline n = 8). No effect of RX821002 infusion into the infralimbic cortex nor an interaction with the effect of methylphenidate was found for pinning ($F_{RX821002}(1,12) = 0.34$, $p = 0.84$; $F_{methylphenidate \times RX821002}(1,12) = 0.34$, $p = 0.84$), pouncing ($F_{RX821002}(1,12) = 0.02$, $p = 0.98$; $F_{methylphenidate \times RX821002}(1,12) = 0.51$, $p = 0.49$) or social exploration ($F_{RX821002}(1,12) = 0.76$, $p = 0.40$; $F_{methylphenidate \times RX821002}(1,12) = 0.96$, $p = 0.35$).

Locomotor activity decreased over the session and it was increased by methylphenidate treatment ($F_{time}(5,120) = 63.23$, $p < 0.001$; $F_{methylphenidate}(1,24) = 18.53$, $p < 0.001$; $F_{time \times methylphenidate}(5,120) = 0.84$, $p = 0.54$; saline-saline n = 8, saline-RX821002 n = 8, methylphenidate-saline n = 6, methylphenidate-RX821002 n = 6) (Fig. 3D). Furthermore, RX821002 infusion attenuated the effect of methylphenidate on locomotion ($F_{methylphenidate \times RX821002}(1,24) = 4.21$, $p = 0.05$). Post-hoc analysis revealed that methylphenidate injected-saline infused animals were significantly more active than saline injected-saline infused and saline injected-RX821002 infused animals, (saline-saline vs methylphenidate-saline: $t(12) - 4.57$, $p = 0.001$; saline-RX821002 vs methylphenidate-saline: $t(12) = -4.65$, $p = 0.001$) but not the methylphenidate injected-RX821002 infused animals (methylphenidate-saline vs methylphenidate-RX821002: $t(12) = 1,78$, $p = 0.11$; Fig. 3E). No further between-group differences in locomotor activity (saline-saline vs saline-RX821002: $t(12) = -1.05$, $p = 0.32$; saline-saline vs methylphenidate-RX821002: $t(12) = -2.06$, $p = 0.06$; saline-RX821002 vs methylphenidate-RX821002: $t(12) = -1.57$, $p = 0.16$; methylphenidate-saline vs sal-RX821002: $t(12) = 1.77$, $p = 0.10$), or effects of the RX821002 infusion were found ($F_{RX821002}(1,24) = 0.79$, $p = 0.38$; $F_{time \times RX821002}(5,120) = 1.82$, $p = 0.12$; $F_{time \times methylphenidate \times RX821002}(5,120) = 1.37$, $p = 0.24$).

### 3.3. Anterior cingulate cortex

After systemic methylphenidate treatment, a reduction in pinning ($F_{methylphenidate}(1,9) = 39.38$, $p < 0.001$) and pouncing ($F_{methylphenidate}(1,9) = 51.06$, $p < 0.001$) but no effect on social exploration was found ($F_{methylphenidate}(1,9) = 2.92$, $p = 0.12$) (Fig. 4A–C, methylphenidate n = 6, saline n = 5). An increase in pinning ($F_{RX821002}(1,9) = 5.26$, $p = 0.05$) and a tendency to increase pouncing ($F_{RX821002}(1,9) = 4.23$, $p = 0.07$) was found after RX821002 infusion into the anterior cingulate cortex. The RX821002 infusion did not affect social exploration ($F_{RX821002}(1,9) = 0.10$,

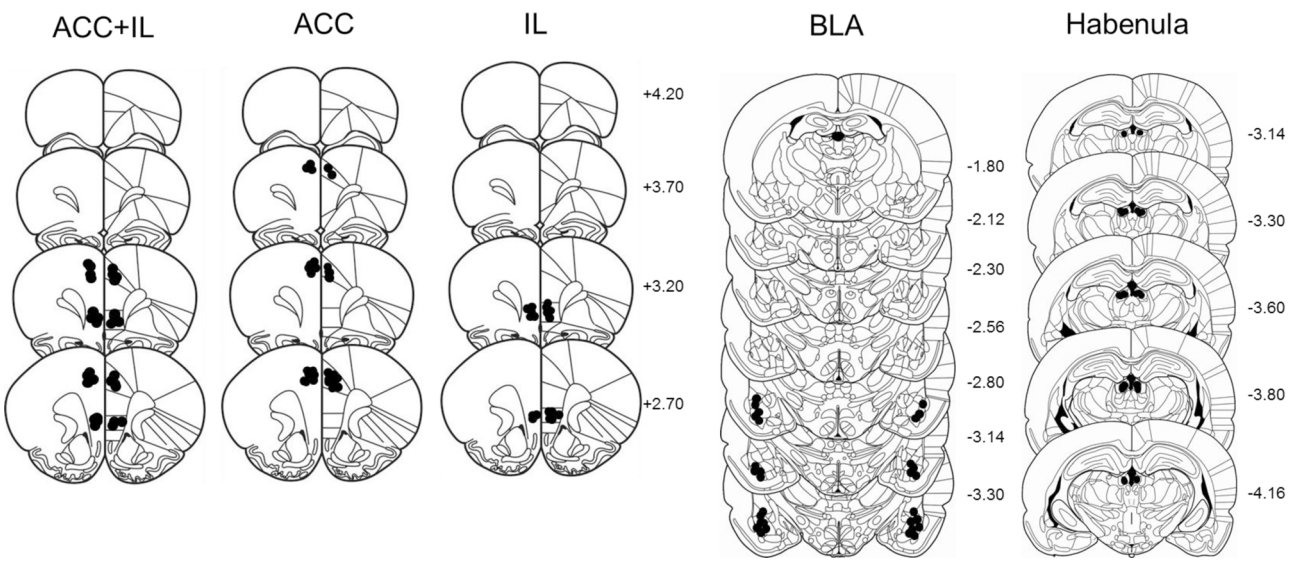


**Fig. 1.** Schematic representation of brain sections with microinjection placements. Microinjections were performed in the anterior cingulate cortex (ACC), infralimbic cortex (IL), combined ACC-IL infusions (ACC + IL), basolateral amygdala (BLA) and habenula. AP = anterior-posterior level in mm from Bregma. Adapted from [52].

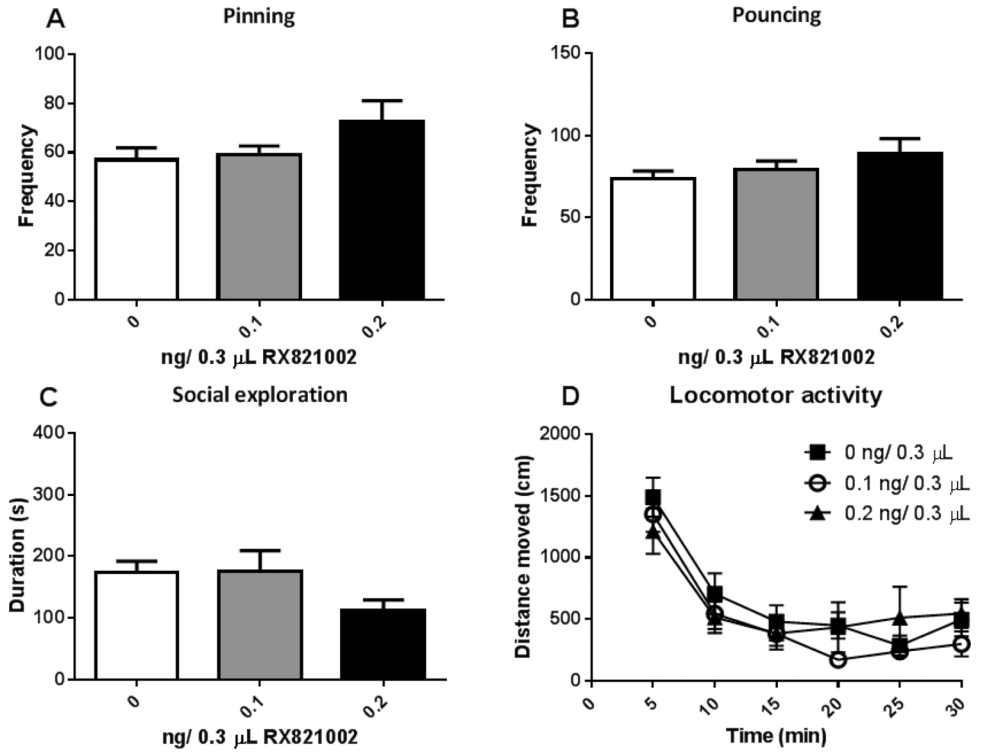


**Fig. 2.** Intra-habenula α2-adrenoreceptor antagonist. The effect of infusion of the α2-adrenoreceptor antagonist RX821002 (RX: 0.1 µg/0.3 µL, grey bar; 0.2 µg/0.3 µL, black bar) into the habenula on social play behaviour. Data are presented as mean + SEM. Both doses of RX did not affect pinning (A), pouncing (B), social exploration (C) or locomotor activity (D) (n = 7).

p = 0.76). Furthermore, no interactions between treatment with methylphenidate and RX821002 were found for pinning ($F_{methylphenidate \times RX821002}(1,9) = 1.79$, p = 0.21), pouncing ($F_{methylphenidate \times RX821002}(1,9) = 0.57$, p = 0.47) or social exploration $F_{methylphenidate \times RX821002}(1,9 = 0.61$, p = 0.45). Locomotor activity decreased as time progressed and it was increased by treatment with in methylphenidate ($F_{time}(5,105) = 58.89$, $p < 0.001$; $F_{methylphenidate}(1,21) = 17.38$, $p < 0.001$; $F_{time \times methylphenidate}(5,105) = 1.61$, p = 0.16), but RX821002 did not influence locomotor activity ($F_{RX821002}(1,21) = 1.27$, p = 0.27; $F_{time \times RX821002}(5,105) = 1.94$, p = 0.09; $F_{methylphenidate \times RX821002}(1,20) = 0.33$, p = 0.57; $F_{time \times methylphenidate \times RX821002}(5,105) = 0.82$, p = 0.54; saline-saline n = 8, saline-RX821002 n = 5, methylphenidate-saline n = 4, methylphenidate-RX821002 n = 8) (Fig. 4D and E).

### 3.4. Combined RX821002 infusion into the infralimbic and anterior cingulate cortex

Systemic methylphenidate treatment reduced pinning ($F_{methylphenidate}(1,9) = 22.74$, p = 0.001) and pouncing ($F_{methylphenidate}(1,9) = 12.57$, p = 0.01) but had no effect on social exploration ($F_{methylphenidate}(1,9) = 2.80$, p = 0.13) (Fig. 5A – C, n(methylphenidate) = 5, n(sal) = 4). RX821002 infusion into both the infralimbic and anterior cingulate cortex did not affect pinning

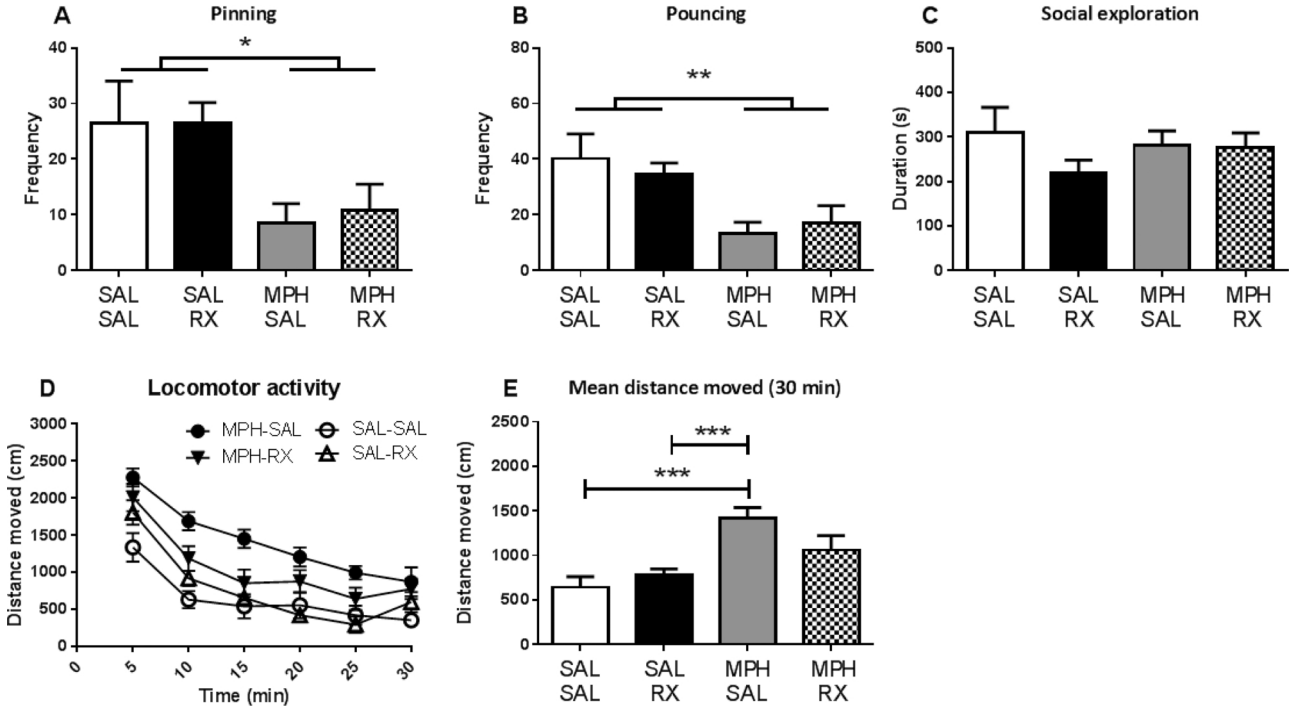


**Fig. 3.** The α2-adrenoreceptor antagonist in the infralimbic cortex. The effect of infusion of the α2-adrenoreceptor antagonist RX821002 (RX; 0.1 μg/0.3 μL) into the infralimbic cortex on the social play-suppressant effect of systemic methylphenidate (MPH; 1.0 mg/kg) treatment. Data are presented as mean + SEM. Methylphenidate reduced pinning (A) and pouncing (B) but had no effect on social exploration (C). RX821002 infusion into the infralimbic cortex did not affect these behaviours in rats pretreated with saline (SAL) or methylphenidate (A–C). Methylphenidate increased locomotor activity, which was attenuated in the RX821002-treated rats (D and E). * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, 2-way ANOVA (pinning, pouncing and social exploration); 2-way repeated measures ANOVA (locomotor activity) with post hoc paired t-tests.

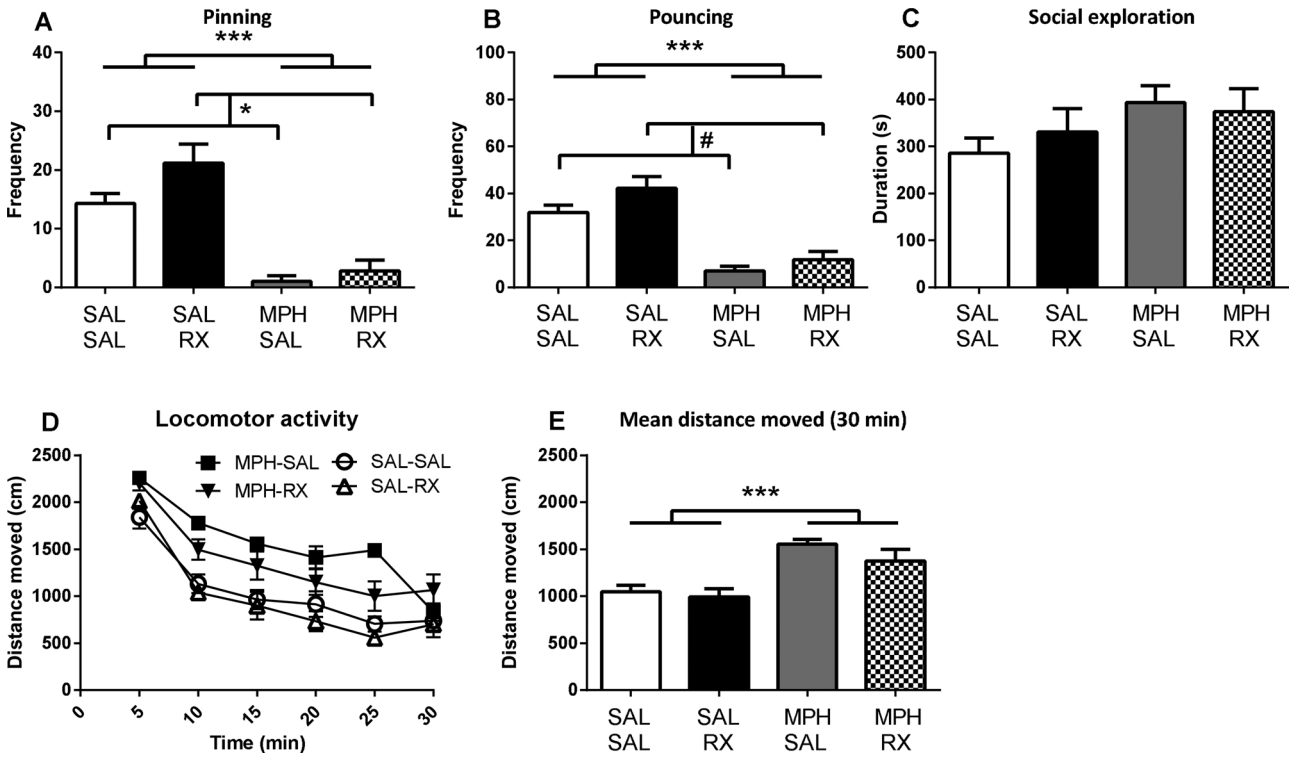


**Fig. 4.** Intra-anterior cingulate cortex α2-adrenoreceptor antagonist. The effect of infusion of the α2-adrenoreceptor antagonist RX821002 (RX; 0.1 μg/0.3 μL) into the anterior cingulate cortex on the social play-suppressant effect of systemic methylphenidate (MPH; 1.0 mg/kg) treatment. Data are presented as mean + SEM. Methylphenidate reduced pinning (A) and pouncing (B) but had no effect on social exploration (C). RX821002 infusion increased pinning and tended to increase pouncing in both saline (SAL) or methylphenidate pre-treated animals (A and B). Locomotor activity was increased in methylphenidate treated rats, but RX821002 infusion did not affect locomotor activity (D and E). *$p = 0.05$, ***$p < 0.001$, #$p = 0.07$, 2-way ANOVA (pinning, pouncing and social exploration), 2-way repeated measures ANOVA (locomotor activity).

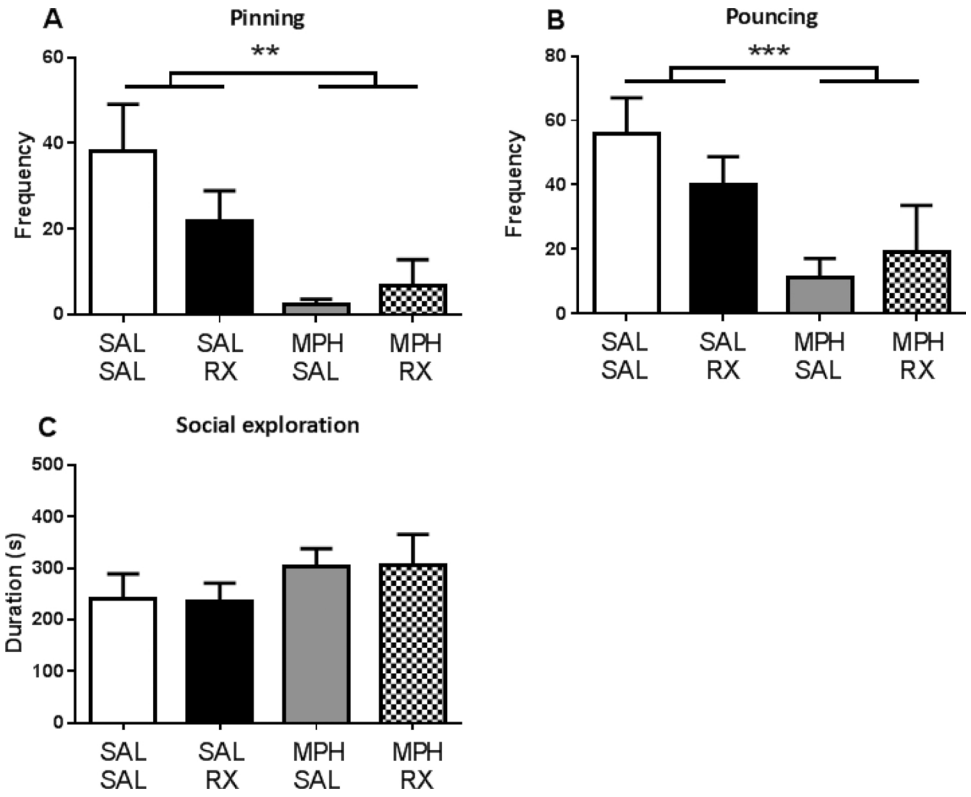


**Fig. 5.** The α2-adrenoreceptor antagonist in both the infralimbic and anterior cingulate cortex. The effect of infusion of the α2-adrenoreceptor antagonist RX821002 (RX; 0.1 μg/0.3 μL) into both the anterior cingulate cortex and infralimbic cortex on the social play-suppressant effect of systemic methylphenidate (MPH; 1.0 mg/kg) treatment. Data are presented as mean + SEM. Methylphenidate reduced pinning (A) and pouncing (B) but had no effect on social exploration (C). RX821002 infusion did not affect either of these parameters **$p < 0.01$, ***$p < 0.001$, 2-way ANOVA.

($F_{RX821002}(1,9) = 1.39$, $p = 0.27$; $F_{methylphenidate \times RX821002}(1,9) = 2.63$, $p = 0.14$), pouncing ($F_{RX821002}(1,9) = 0.43$, $p = 0.53$; $F_{methylphenidate \times RX821002}(1,9) = 0.60$, $p = 0.24$) and social exploration ($F_{RX821002}(1,9) = 0.23$, $p = 0.65$; $F_{methylphenidate \times RX821002}(1,9) = 0.44$, $p = 0.52$).

### 3.5. Habenula

Systemic treatment with methylphenidate reduced pinning ($F_{methylphenidate}(1,9) = 43.40$, $p < 0.001$) and pouncing ($F_{methylphenidate}(1,9) = 179.00$, $p < 0.001$), and increased social exploration ($F_{methylphenidate}(1,9) = 11.61$, $p = 0.01$) (Fig. 6A–C, methylphenidate n = 5, saline n = 6). No effect of RX821002 infusion into the habenula was found for pinning ($F_{RX821002}(1,9) = 1.59$, $p = 0.24$; $F_{methylphenidate \times RX821002}(1,9) = 0.93$, $p = 0.36$), pouncing ($F_{RX821002}(1,9) = 1.55$, $p = 0.25$; $F_{methylphenidate \times RX821002}(1,9) = 0.43$, $p = 0.53$) and social exploration ($F_{RX821002}(1,9) = 2.19$, $p = 0.17$; $F_{methylphenidate \times RX821002}(1,9) = 0.05$, $p = 0.83$). Locomotor activity decreased over the test session and it was increased in methylphenidate-treated rats ($F_{time}(5,110) = 44.10$, $p < 0.001$; $F_{methylphenidate}(1,22) = 34.72$, $p < 0.001$; $F_{time \times methylphenidate}(5,110) = 1.05$, $p = 0.39$) but no effects of the RX821002 infusion were found ($F_{RX821002}(1,22) = 1.54$, $p = 0.23$; $F_{time \times RX821002}(5,110) = 0.50$, $p = 0.49$; $F_{methylphenidate \times RX821002}(1,22) = 0.02$, $p = 0.91$; $F_{time \times methylphenidate \times RX821002}(5,110) = 1.98$, $p = 0.09$; saline-saline n = 7, saline-RX821002 n = 7, methylphenidate-saline n = 6, methylphenidate-RX821002 n = 6) (Fig. 6D and E).

### 3.6. Basolateral amygdala

After systemic methylphenidate treatment, a reduction in pinning ($F_{methylphenidate}(1,10) = 46.76$, $p < 0.001$) and pouncing ($F_{methylphenidate}(1,10) = 47.00$, $p < 0.001$) and an increase in social exploration was found ($F_{methylphenidate}(1,10) = 12.65$, $p = 0.005$) (Fig. 7A–C, methylphenidate n = 6, saline n = 6). No effect of RX821002 infusion into the basolateral amygdala was found for pinning ($F_{RX821002}(1,10) = 0.01$, $p = 0.92$; $F_{methylphenidate \times RX821002}(1,10) = 0.14$, $p = 0.72$), pouncing ($F_{RX821002}(1,10) = 0.003$, $p = 0.96$; $F_{methylphenidate \times RX821002}(1,10) = 0.06$, $p = 0.81$) and social exploration ($F_{RX821002}(1,10) = 2.29$, $p = 0.16$; $F_{methylphenidate \times RX821002}(1,10) = 0.52$, $p = 0.49$). Locomotor activity decreased during the test session and it was increased by systemic methylphenidate treatment ($F_{time}(5,100) = 74.96$, $p < 0.001$; $F_{methylphenidate}(1,20) = 29.35$, $p < 0.001$; $F_{time \times methylphenidate}(5,100) = 1.17$, $p = 0.33$), but locomotor activity was not altered by the infusion of RX821002 ($F_{RX821002}(1,20) = 0.45$, $p = 0.51$; $F_{time \times RX821002}(5,100) = 1.23$, $p = 0.30$; $F_{methylphenidate \times RX821002}(1,20) = 2.16$, $p = 0.16$; $F_{time \times methylphenidate \times RX821002}(5,100) = 1.29$, $p = 0.27$; saline-saline n = 6, saline-RX821002 n = 5, methylphenidate-saline n = 8, methylphenidate-RX821002 n = 5) (Fig. 7D and E).

## 4. Discussion

In the present study, we found that the social play-suppressant effect of systemic methylphenidate treatment was not altered after infusion of the α2-noradrenergic antagonist RX821002 into brain regions that we previously found to be important for the effect of methylphenidate on social play behaviour, i.e. the anterior cingulate cortex, the infralimbic cortex, the basolateral amygdala and the habenula. In addition, infusion of RX821002 into both the anterior cingulate and infralimbic cortex did not influence the play suppressant effect of systemic methylphenidate. Infusion of RX821002 into the anterior cingulate cortex modestly increased social play behaviour. Methylphenidate treatment enhanced horizontal locomotor activity, which was attenuated by RX821002 infusion into the infralimbic cortex.

### 4.1. Circuitry differences: parallel versus serial connections

The inability to counteract the play-suppressive effect of methylphenidate with brain region-specific α2-adrenoreceptor antagonism is likely due to characteristics of the underlying circuitry. The infralimbic cortex reciprocally innervates the anterior cingulate cortex, and both

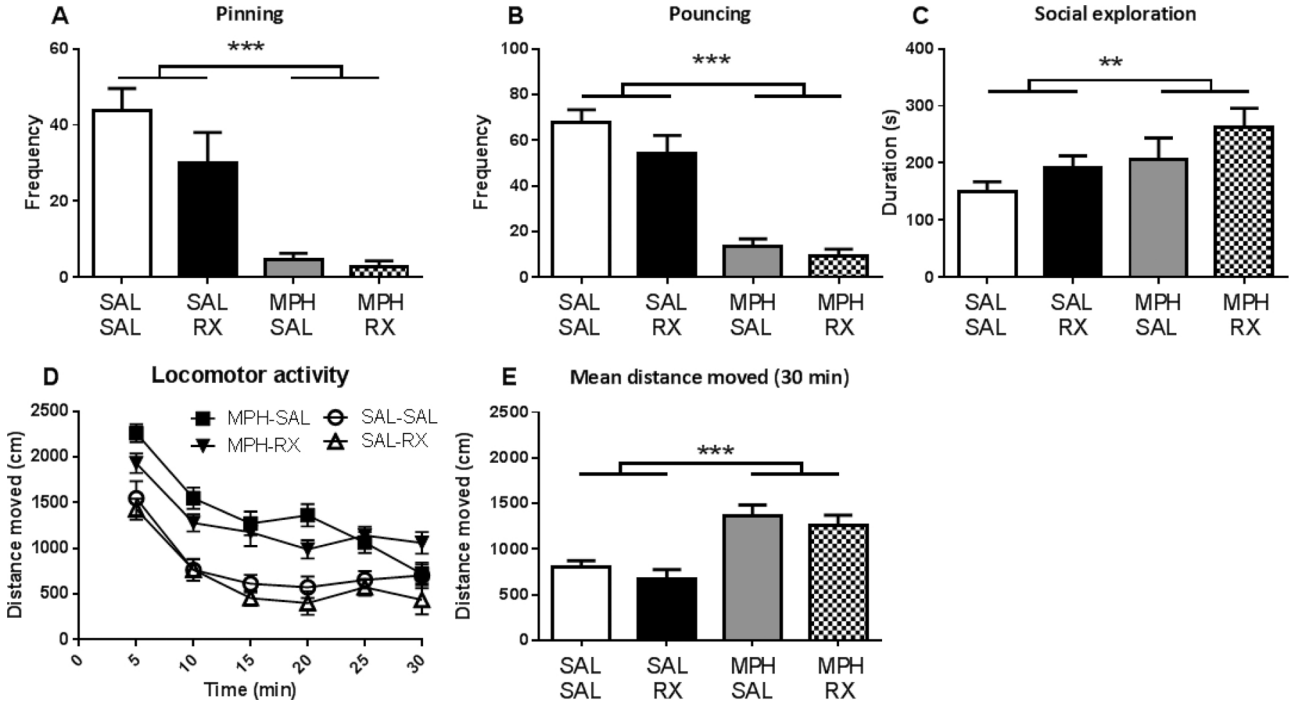


**Fig. 6.** Intra-habenula α2-adrenoreceptor antagonist. The effect of infusion of the α2-adrenoreceptor antagonist RX821002 (RX; 0.1 μg/0.3 μL) into the habenula on the social play-suppressant effect of systemic methylphenidate (MPH; 1.0 mg/kg) treatment. Data are presented as mean + SEM. Methylphenidate reduced pinning (A) and pouncing (B) and increased social exploration (C); RX821002 infusion did not affect these behaviours (A–C). Locomotor activity was increased in methylphenidate treated rats, and no effect of RX821002 infusion was found (D and E). $^{**}p < 0.01$, $^{***}p < 0.001$, 2-way ANOVA (pinning, pouncing and social exploration), 2-way repeated measures ANOVA (locomotor activity).

have reciprocal connections with the basolateral amygdala [21,25,28]. The infralimbic cortex also sends a, somewhat less dense, innervation to the habenula. Last, both the basolateral amygdala and the habenula innervate the prefrontal cortex via the mediodorsal thalamus [23,29,30]. We hypothesized that if the circuit is connected in series, the methylphenidate-induced suppression of play can be counteracted by infusion of RX821002 in at least one of the four brain regions. However, the data points to a circuit connected in parallel, as infusion of RX821002 into a single brain region was not sufficient to counteract the play-suppressive effect of systemic methylphenidate treatment.

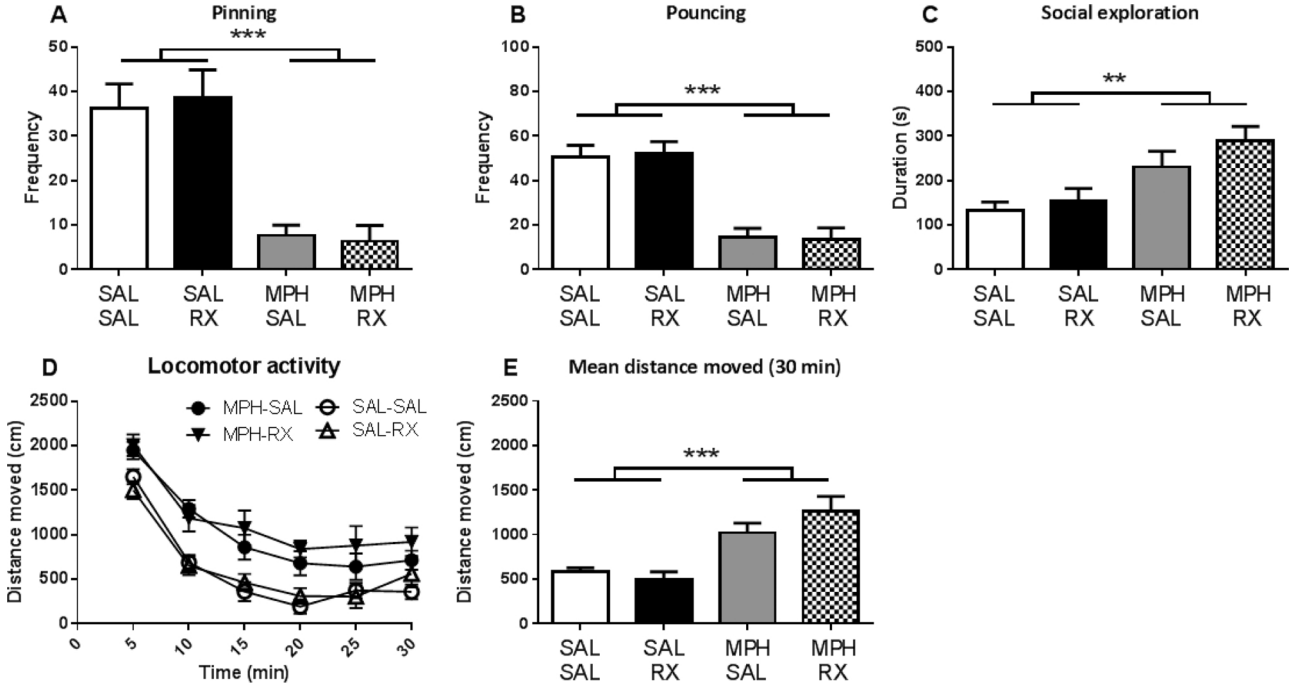


**Fig. 7.** The α2-adrenoreceptor antagonist in the basolateral amygdala. The effect of infusion of the α2-adrenoreceptor antagonist RX821002 (RX; 0.1 μg/0.3 μL) into the basolateral amygdala on the social play-suppressant effect of systemic methylphenidate (MPH; 1.0 mg/kg) treatment. Data are presented as mean + SEM. Methylphenidate reduced pinning (A) and pouncing (B) and increased social exploration (C); RX821002 infusion did not affect these behaviours (A–C). Locomotor activity was increased in methylphenidate treated rats, and RX821002 infusion did not alter locomotor activity (D and E). $^{**}p < 0.01$, $^{***}p < 0.001$, 2-way ANOVA (pinning, pouncing and social exploration), 2-way repeated measures ANOVA (locomotor activity).

Moreover, infusion of RX821002 into both the anterior cingulate and infralimbic cortex did not counteract the effect of methylphenidate either. An infusion with RX821002 into the anterior cingulate cortex modestly increased social play irrespective of saline or methylphenidate pretreatment. This effect was not observed in the other regions, suggesting that the anterior cingulate cortex is an important brain region for the noradrenergic modulation of social play. The double infusion of RX821002 into both the anterior cingulate and infralimbic cortex did not produce an increase in social play. However, the possibility remains that effects of RX821002 in the infralimbic and anterior cingulate cortex influence each other, resulting in the absence of a change in social play behaviour. Of note, we have previously shown that infusion of methylphenidate into a single brain region only partially captures the effect of systemic methylphenidate, i.e. it results in a reduction, but not a complete suppression of social play [11], which we have found to occur after systemic methylphenidate treatment [12,13]. Although we cannot rule out that the difference between systemic and intracranial methylphenidate results from a difference in concentration of the drug in the target region, we think that these findings combined support the notion that multiple brain regions need to be functionally affected by methylphenidate in order to induce a full suppression of social play behaviour. Conversely, the action of methylphenidate in each one of the four brain regions involved is enough to induce a reduction in social play, even if its effects in one (or two) of the other regions are blocked by treatment with an α2-adrenoceptor antagonist.

### 4.2. Alpha-2 adrenoreceptors and locomotion in the infralimbic cortex

In all experiments, we found an enhancement of horizontal locomotor activity after methylphenidate treatment, consistent with previous findings that low doses of methylphenidate stimulate locomotor activity [53–55]. The locomotor enhancing effects of psychostimulants drugs, such as methylphenidate, are typically attributed to their effect on striatal dopaminergic neurotransmission [56–60]. Interestingly, our present findings show that α2-adrenoreceptors in the infralimbic cortex contribute to the locomotor enhancing effect of methylphenidate. That is, whereas treatment with RX821002 into the infralimbic cortex did not affect locomotor activity by itself, it reduced horizontal locomotor activity in methylphenidate-pretreated animals. These data add to the existing literature showing an involvement of prefrontal noradrenaline neurotransmission in the behavioural effects of psychostimulant drugs [61–63].

### 4.3. Methodological issues

It could be argued that our failure to counteract the effect of methylphenidate with intracranial infusions of RX821002 is that we used a too low dose of the α2-adrenoreceptor antagonist. We do, however, not think that this is the case. First, our dose response experiment showed a small increase in social play behaviour after intra-habenula infusion of a higher dose of RX821002. Thus, using a higher dose of RX821002 likely results in effects on social play, so that alterations in the effect of methylphenidate would be a matter of functional, rather than pharmacological antagonism. Second, we did observe an effect of this dose of RX821002 on social play after infusion into the anterior cingulate cortex, as well as on the psychomotor stimulant effect of methylphenidate after infusion into the infralimbic cortex. Therefore, using a higher dose of RX821002 than we did in the present study would likely make the results difficult to interpret.

One may also question our approach, as influencing noradrenergic signaling in a single brain area within a circuit may be unlikely to antagonize the effect of systemically administered methylphenidate. However, previous studies in our laboratory have shown that infusion of the mu-opioid receptor antagonist naloxone into the nucleus accumbens was sufficient to block the play-enhancing effect of systemic morphine treatment [31]. In addition, enhancing social play behaviour by systemic treatment with the indirect cannabinoid agonist URB597 could be counteracted by infusion of the cannabinoid-1 receptor antagonist SR141716A into the basolateral amygdala [32]. Together, this suggests that whereas opioids and endocannabinoids modulate social play behaviour through well-circumscribed brain regions, the effect of methylphenidate depends on an integrated neural circuit.

Another issue that needs to be addressed is the difference in baseline levels in the amount of pinning and pouncing found between experiments. One concern might be that high or low baseline levels of play may obscure any enhancing or reducing drug effects, respectively (i.e. as a result of ceiling or floor effects). This seems unlikely for two reasons. 1. The animals were isolated for 2.5 h prior to testing. Previous studies have shown that 2.5–3.5 h of social isolation induces half-maximal increases of social play [13,64], which should leave enough window to observe both increases and decreases in social play behavior. 2. Differences in baseline-levels of social play behaviour are observed regularly in our laboratory, but in animals isolated for several hours before the test, drug effects are consistently observed, irrespective of the amount of social play in vehicle-treated rats [11,31,32,65].

### 4.4. Neurocircuitry of social play behaviour

Our present data supports our previously postulated hypothesis that the suppression of social play behaviour by methylphenidate is not exerted through a single neural substrate and that the infralimbic cortex, anterior cingulate cortex, basolateral amygdala and habenula are part of a functional network involved in the modulation of social play behaviour [11]. We conclude that methylphenidate affects different behavioural, emotional and cognitive aspects of social play behaviour at the same time by acting in a distributed network of brain regions [11,13]. Future studies may provide more detailed insight into how this neural network is functionally affected by methylphenidate.

## Conflict of interest

All authors declare that they have no conflicts of interest.

## Contributors

EJMA and LJMJV designed the experiments. EJMA and RD performed the experiments and analysed the data. EJMA and LJMJV wrote the paper. All authors have approved the final version of the manuscript.

## Acknowledgement

This research was supported by internal funds from Utrecht University.

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