Behavioral/Systems/Cognitive

Endocannabinoids in Amygdala and Nucleus Accumbens Mediate Social Play Reward in Adolescent Rats

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The brain endocannabinoid system plays a crucial role in emotional processes. We have previously identified an important role for endocannabinoids in social play behavior, a highly rewarding form of social interaction in adolescent rats. Here, we tested the hypothesis that endocannabinoid modulation of social play behavior occurs in brain regions implicated in emotion and motivation. Social play increased levels of the endocannabinoid anandamide in the amygdala and nucleus accumbens (NAc), but not in prefrontal cortex or hippocampus of 4- to 5-week-old male Wistar rats. Furthermore, social play increased phosphorylation of CB1 cannabinoid receptors in the amygdala. Systemic administration of the anandamide hydrolysis inhibitor URB597 increased social play behavior, and augmented the associated elevation in anandamide levels in the amygdala, but not the NAc. Infusion of URB597 into the basolateral amygdala (BLA) increased social play behavior, and blockade of BLA CB1 cannabinoid receptors with the antagonist/inverse agonist SR141716A prevented the play-enhancing effects of systemic administration of URB597. Infusion of URB597 into the NAc also increased social play, but blockade of NAc CB1 cannabinoid receptors did not antagonize the play-enhancing effects of systemic URB597 treatment. Last, SR141716A did not affect social play after infusion into the core and shell subregions of the NAc, while it reduced social play when infused into the BLA. These data show that increased anandamide signaling in the amygdala and NAc augments social play, and identify the BLA as a prominent site of action for endocannabinoids to modulate the rewarding properties of social interactions in adolescent rats.

Introduction

The endocannabinoid system is a unique neuromodulatory system in mammalian physiology. It consists of cannabinoid receptors (CB1 and CB2, mainly expressed in the brain and periphery, respectively), their endogenous ligands (endocannabinoids, including anandamide and 2-arachidonoylglycerol; 2-AG), and the enzymes for ligand synthesis and degradation (Freund et al., 2003; Piomelli, 2003; Di Marzo, 2006; Pacher et al., 2006). Endocannabinoids are key modulators of emotions, and altered endocannabinoid signaling has been implicated in several psychiatric disorders (Wotjak, 2005; Laviolette and Grace, 2006; Pacher et al., 2006; Di Marzo, 2008; Leweke and Koethe, 2008; Lutz, 2009; Marco et al., 2011).

Cannabinoids have been implicated in aspects of emotion, motivation and learning (Wotjak, 2005; Viveros et al., 2007; Solinas et al., 2008; Berridge et al., 2010; Fattore et al., 2010; Zanettini et al., 2011). Therefore, we have investigated their role in social play behavior. Social play, a characteristic social behavior in young mammals, is essential for the development of physical, cognitive and social capacities (Panksepp et al., 1984; Vanderschuren et al., 1997; Pellis and Pellis, 2009; Trezza et al., 2010). Social play is highly rewarding; it is an incentive for maze learning, lever pressing, and place conditioning in rats and primates (Falk, 1958; Mason et al., 1963; Humphreys and Einon, 1981; Normansell and Panksepp, 1990; Calcagnetti and Schechter, 1992; Ikemoto and Panksepp, 1992; Douglas et al., 2004; Thiel et al., 2008, 2009; Trezza et al., 2009, 2011a; for review, see Trezza et al., 2011b). Furthermore, it is modulated through neurotransmitters (Vanderschuren et al., 1997; Trezza et al., 2010; Siviy and Panksepp, 2011) implicated in the motivational properties of food and drugs, such as dopamine, or their pleasurable characteristics, such as endogenous opioids and endocannabinoids (Berridge and Robinson, 1998; Peciña and Berridge, 2005; Salamone et al., 2005; Barbano and Cador, 2007; Mahler et al., 2007; Solinas et al., 2008). We have previously shown that systemic

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administration of drugs that increase endocannabinoid signaling by blocking endocannabinoid deactivation enhances social play, through interaction with opioid and dopaminergic neurotransmission (Trezza and Vanderschuren, 2008a,b, 2009). This suggests that during social play, endocannabinoids are released in brain areas that mediate this behavior and that increased endocannabinoid activity facilitates social play. However, the brain areas within which endocannabinoids exert their effects on social play are unknown. CB1 cannabinoid receptors are abundant in brain areas involved in emotion and motivation, such as the nucleus accumbens (NAc) and amygdala (Tsou et al., 1998; Katona et al., 2001). Indeed, endocannabinoids in the NAc modulate the rewarding properties of food and drugs (van der Stelt and Di Marzo, 2003; Gardner, 2005; Mahler et al., 2007; Soria-Gómez et al., 2007; Orio et al., 2009; Shinohara et al., 2009; Berridge et al., 2010). Furthermore, endocannabinoids in the amygdala regulate affective states, stress responses, and emotional learning (Marsicano et al., 2002; Laviolette and Grace, 2006; Campolongo et al., 2009; Hill et al., 2010; McLaughlin and Gobbi, 2012). Therefore, we hypothesized that the stimulatory effects of endocannabinoids on social play are mediated within the NAc and the amygdala.

Materials and Methods

Animals. Male Wistar rats (Charles River) arrived in our animal facility at 21 d of age and were housed in groups of four in 40 × 26 × 20 (l × w × h) Macrolon cages under controlled conditions (temperature 20–21°C, 60–65% relative humidity, and 12 h light/dark cycle with lights on at 7:00 A.M.). Food and water were available *ad libitum*.

All animals used were experimentally naive. The experiments were approved either by the Animal Ethics Committee of Utrecht University and conducted in agreement with Dutch laws (Wet op de Dierproeven, 1996), or were in accordance with the guidelines released by the Italian Ministry of Health (D.L. 116/92). All experiments were in accordance with the European Communities Council Directive of November 24, 1986 (86/609/EEC).

Surgery. At 28 d of age, rats were anesthetized with 0.08 ml/100 g subcutaneous Hypnorm (fentanyl citrate 0.315 mg/ml and fluanison 10 mg/ml, Janssen) and positioned into a stereotaxic frame (David Kopf Instruments). Guide cannulae, consisting of 24 gauge thin-walled stainless steel tubing (Cooper's Needleworks), were implanted bilaterally, aimed 1.0 mm above the NAc [border between the shell and core subregions; coordinates: anteroposterior (AP), +1.5 mm from bregma; mediolateral (ML), ±1.9 mm from the midline; dorsoventral (DV), −7.0 mm from skull surface]. Other groups of rats were implanted with bilateral guide cannulae (24 gauge) aimed 1.0 mm above the NAc core (coordinates: AP, +1.5 mm; ML, ±1.9 mm; DV, −6.5 mm), NAc shell (coordinates: AP, +1.8 mm; ML, ±2.8 mm; DV, −7.5 mm, 10° angle), or the basolateral amygdala (BLA) (coordinates: AP, −1.9 mm; ML, ±4.6 mm; DV, −7.5 mm). Coordinates for each brain region were based on our previous studies (Trezza et al., 2011a) or determined by pilot placement experiments in 28-d-old rats using the atlas of Paxinos and Watson (2007). Cannulae were secured with stainless steel screws and dental acrylic; 29 gauge wire stylets (Cooper's Needleworks) were inserted into the guide cannulae to maintain patency. After surgery, rats were individually housed and allowed to recover for 4 d. On day 5, they were rehoused in groups of four with their original cage mates. Behavioral testing began 1 week after surgery.

Drug and infusion procedures. The indirect cannabinoid agonist URB597 (Cayman Chemical), which inhibits fatty acid amide hydrolase (FAAH), the enzyme that degrades the endocannabinoid anandamide (Kathuria et al., 2003), and the CB1 cannabinoid receptor antagonist/inverse agonist SR141716A (National Institute of Mental Health's Chemical Synthesis and Drug Supply Program, Bethesda, MD) were dissolved in 5% Tween 80/5% polyethylene glycol/saline. Bilateral infusions of drugs or an equivalent volume of the corresponding vehicle were made using 30-gauge injection needles (Bilaney) connected to 10 μl Hamilton microsyringes by polyethylene (PE-20) tubing. The injection needles protruded 1.0 mm beyond the cannula tips, and a 0.3 (for NAc) or 0.2 μl (for BLA) injection volume per hemisphere was infused over 60 s using a syringe pump (model 975A; Harvard Apparatus). The injection needles remained within the guide cannulae for 60 s following drug infusion to facilitate diffusion and to prevent backflow of drug along the cannula track. After infusions, stylets were replaced, and the animals were left in a holding cage for 5 min before testing.

Histological confirmation of injection sites. Injection sites were verified as previously described (Mahler et al., 2007; Simmons and Self, 2009; Trezza et al., 2011a). After testing, animals were killed by carbon dioxide inhalation and microinjected with 0.3 μl of black ink over 60 s through the guide cannulae. Animals were immediately decapitated and their brains removed. Slices (20 μm thick) were collected throughout the forebrain and analyzed under a dissecting microscope for the location of the infusion sites according to the atlas of Paxinos and Watson (2007). Only pairs in which both animals had bilateral needle tracks terminating into the target area and no damage to the target tissues were included in the final analysis.

Behavioral testing

Social play behavior. All 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 ~2 cm of wood shavings covering the floor. The behaviors of the animals were recorded using a camera with zoom lens, video tape recorder, and television monitor. The animals in a test pair did not differ by >10 g in body weight and had no previous common social experience.

To measure play-induced and/or URB597-induced changes in endocannabinoid levels (see Fig. 1), 28-d-old rats were individually habituated to the test cage for 10 min on each of the 2 d before testing, as previously described (Trezza and Vanderschuren, 2008a,b, 2009). On the test day, the animals were isolated for 3.5 h before testing, to enhance their social motivation and thus facilitate the expression of social play behavior during testing (Niesink and Van Ree, 1989; Vanderschuren et al., 1995, 2008). Two hours before testing, the rats were treated with URB597 (0.1 mg/kg, i.p.) or vehicle. The test consisted of placing the animals, either alone or together with a similarly treated partner, into the test cage for 15 min. Thus, the following four groups of animals were tested: (1) animals treated with vehicle and placed alone in the test cage, (2) animals treated with vehicle and placed in the test cage together with a vehicle-treated partner, (3) animals treated with URB597 and placed alone in the test cage, and (4) animals treated with URB597 and placed in the test cage together with a URB597-treated partner.

To measure play-induced changes in the expression of phosphorylated and total CB1 cannabinoid receptors and FAAH and *N*-acyl phosphatidylethanolamines-phospholipid D (NAPE-PLD) expression (see Fig. 2), 28-d-old rats were individually habituated to the test cage for 10 min on each of the 2 d before testing. On the test day, the animals were isolated for 3.5 h before testing, treated with vehicle, and placed in the test cage either alone or together with a vehicle-treated partner for 15 min.

To investigate the role of the NAc and the BLA in the play-enhancing effect of URB597, the following procedure was used. One week after surgery (35 d of age), rats equipped with bilateral guide cannulae were habituated to the experimental procedures on 2 consecutive days, as previously described (Trezza et al., 2011a). On the first habituation day, they were individually placed into the test cage for 10 min, and on the second habituation day, they were isolated for 2 h. Pairs of rats were then infused with a vehicle solution and placed into the test cage for 15 min to habituate them to the infusion procedures and determine baseline levels of social play behavior. On the test day, the animals were isolated for 2 h before testing. Pairs of rats were then infused simultaneously with either vehicle or drug solutions and placed into the test cage for 15 min. In the experiments shown in Figures 3, *a* and *b*, and 5, *a* and *b*, animals received intra-NAc or intra-BLA infusions of URB597 (0.005–0.01 μg/hemisphere), respectively. In the experiments shown in Figures 3, *c* and *d*, and 5, *c* and *d*, animals were treated intraperitoneally with URB597 (0.1 mg/kg) or its vehicle 2 h before infusion of SR141716A (1 and 0.1 μg/hemisphere in the NAc and BLA, respectively) or its vehicle into either NAc

(Fig. 3*c*,*d*) or BLA (Fig. 5*c*,*d*). In the experiments shown in Figure 4, *a–f*, and 5, *e–f*, animals received intra-NAc (border core/shell, core, or shell) or intra-BLA infusions of SR14171A (0.03–3.0 μg/hemisphere), respectively.

Behavior was assessed per pair of animals using the Observer 3.0 software (Noldus Information Technology). The following behavioral elements were scored per 15 min (Panksepp et al., 1984; Vanderschuren et al., 1997; Pellis and Pellis, 2009; Trezza et al., 2010). The frequency of pinning: one animal lying with its dorsal surface on the floor with the other animal standing over it. This is the most characteristic posture in social play in rats; it occurs when one animal is solicited to play by its test partner and rotates to its dorsal surface to prolong the playful interaction. Frequency of pouncing is an index of play solicitation, i.e., one animal is soliciting the other to play, by attempting to nose or rub the nape of the neck of the test partner. Time spent in social exploration behavior means sniffing any part of the body of the test partner, including the anogenital area.

Neurochemical experiments. Changes in endocannabinoid levels after social play were assessed using *ex vivo* isotope dilution liquid chromatography–atmospheric pressure chemical ionization–mass spectrometric analysis (Guidali et al., 2011). Endocannabinoids act as local mediators and retrograde neuromodulators in the brain, being biosynthesized and immediately released from cells. This means that there are no preformed endocannabinoids stored in vesicles. Therefore, what is measured in small amounts of tissue reflects what is biosynthesized and what is needed to stimulate local cannabinoid receptors. Differences in endocannabinoid concentrations before and after certain events then reflect stimulus-induced phasic cannabinoid receptor stimulation. Although *in vivo* microdialysis has the advantage of allowing for repeated measures in the same animal and direct correlations of endocannabinoid levels with behavioral parameters, it likely produces results that are comparable to *ex vivo* tissue measurements in terms of stimulus-induced endocannabinoid release. In addition, the energetic nature of social play behavior, with animals vigorously chasing, pouncing, and rolling onto their backs, is hardly compatible with being connected to tubing and a swivel, which is necessary for *in vivo* microdialysis.

Immediately after testing for social play behavior, rats were rapidly decapitated and their brains quickly removed and rinsed in ice-cold distilled water for 10 s. The brains were then cut into coronal slices on a cold plate, and the NAc, amygdala, hippocampus, and prefrontal cortex were dissected by hand under microscopic control within 2 min. Tissues were stored at −80°C until use.

Measurement of endocannabinoid levels. The extraction, purification, and quantification of anandamide, 2-AG, oleoylethanolamide (OEA), and palmitoylethanolamide (PEA) in serum were performed as described previously (Marsicano et al., 2002; Guidali et al., 2011). After addition of 5 pmol of the respective deuterated internal standards, repeated (three times) lipid extraction of the latter with chloroform:methanol (2:1 by volume), and prepurification of the lipid extracts on silica mini-columns eluted with chloroform:methanol (9:1 by volume), chromatographic fractions containing the compounds were subjected to isotope dilution liquid chromatography–atmospheric pressure chemical ionization–mass spectrometric analysis as described previously (Guidali et al., 2011). Tissue concentrations of endocannabinoids or PEA and OEA are reported in pmol/g wet tissue weight.

Western blot analysis of phosphorylated and total CB1 cannabinoid receptor. Rat brain punches were grinded in ice-cold lysis buffer (50 mM Tris-HCl, pH 7.4, 10% w/v sucrose, 5 mM EDTA, 1 mM DTT, 1% w/v SDS, protease inhibitors (Roche) and phosphatase inhibitors (Sigma)) (Orio et al., 2009). After centrifugation at 14,000 rpm at 4°C for 30 min, supernatant was collected and total protein concentration was measured using a detergent-compatible protein assay (Bio-Rad). The protein separation and Western blotting were performed as described previously with minor modifications (Zhou et al., 2011). Briefly, 20 μg of total protein from each sample was loaded on a 10% SDS-PAGE gel. Following separation in gel, proteins were transferred onto nitrocellulose membrane (Hybond-C Extra; GE Healthcare). The membrane was blocked for 1 h in 5% nonfat milk in TBS-T (50 mM Tris pH 7.5, 150 mM NaCl, and 0.1% Tween 20), followed by overnight primary antibody incubation at 4°C. Anti-CB1 (1:500 dilution in TBS-T) and anti-α-tubulin (1:2000 dilution in TBS-T) were purchased from Sigma, and anti-pCB1 (Ser 316) (1:250 dilution in TBS-T) was from Santa Cruz Biotechnology. After three washes, the membrane was incubated with species-specific secondary antibodies (horseradish peroxidase (HRP)-conjugated) for 1 h at room temperature. Chemiluminescence from Supersignal substrate (Thermo Scientific) was detected by CL-XPosure Film (Thermo Scientific). The intensity of the protein bands was quantified using ImageJ software (National Institutes of Health).

Quantitative PCR analysis of FAAH and NAPE-PLD expression. Total RNA was extracted from the dissected brain regions using Trizol (Invitrogen) according to manufacturer's recommendations. DNase treatment (Roche) was performed to remove genomic DNA. Integrity and concentration of RNA were checked using Nanodrop spectrophotometer (Thermo Scientific) and gel electrophoresis. RNA (0.25 μg) was reverse transcribed with random primers using the Taqman reverse transcriptase reagents kit (Promega) according to manufacturer's protocol in a volume of 25 μl. Primers were designed using Primer3 (Rozen and Skaletsky, 2000). All primers were checked for gene specificity by BLAST searching. In addition, all PCRs were checked for specificity using the melting curve analysis (StepOne Software V2.0; Applied Biosystems).

The following primers were used: FAAH (GenBank: NM_024132.3) forward primer: TGCTGAAGCCTCTGTTTCCT, reverse primer: TCTCATGCTGCAGTTTCCAC; NAPE-PLD (GenBank: NM_199381.1) forward primer: TCGCTGGGGATACTGGTTAC, reverse primer: AAGCTCCAATGGGAATAGCC. Quantitative PCRs (qPCR) were performed in a reaction volume of 10 μl with SYBR green master mix (Applied Biosystems) and cDNA corresponding to ~ 4 ng RNA. Cycle settings were 60°C 2 min, 95°C 2 min, 40 cycles of 95°C 15 s, and 60°C 45 s, followed by dissociation steps of 0.3°C from 60 to 95°C. To correct for differences of input amounts of RNA a normalization factor was used. This normalization factor was calculated as the geometric mean of two stable housekeeping genes, glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and β-actin. The following primers were used: GAPDH (GenBank: NM_017008) forward primer: GCTACAGCTTCACCACCACA, reverse primer: GCCATCTCTTGCTCGAAGTC; β-actin (GenBank: NM_031144) forward primer: TGCACCACCAACTGCTTAGC, reverse primer: GGCATGGACTGTGGTCATGA.

Statistical analysis. Pinning and pouncing frequencies and time spent in social exploration were expressed as mean ± SEM. To assess the effects of single treatments on social play behavior, data were analyzed using either one-way ANOVA followed by Student–Newman–Keuls *post hoc* test, or Student's *t* test. To assess the effects of combined treatments on social play behavior, data were analyzed using two-way ANOVA, followed by Student–Newman–Keuls *post hoc* test. To assess the effects of social play behavior and URB597 administration on endocannabinoid levels, data were analyzed using two-way ANOVA, using treatment (URB597 or vehicle) and test condition (tested alone or together with a play partner) as between-subjects factors. Two-way ANOVA was followed by Student–Newman–Keuls *post hoc* test. To assess the effects of social play behavior on total and phosphorylated CB1 receptors, FAAH and NAPE-PLD expression, data were analyzed by Student's *t* test.

Results

Social play-induced and/or URB597-induced changes in brain endocannabinoid levels

To determine which brain regions mediate endocannabinoid modulation of social play, we measured endocannabinoid levels in NAc, amygdala, prefrontal cortex, and hippocampus in adolescent rats treated systemically with either URB597 or vehicle, and killed immediately after being placed either alone or together with a play partner in the test cage for 15 min.

In line with previous findings (Trezza and Vanderschuren, 2008a,b), systemic administration of URB597 increased social play (pinning: $t = -2.7$, $df = 8$, $p < 0.05$; Fig. 1*a*; pouncing: $t = -3.47$, $df = 8$, $p < 0.01$; Fig. 1*b*). A two-way ANOVA performed on NAc anandamide levels in animals treated with either URB597 or vehicle, and placed either alone or together with a play partner

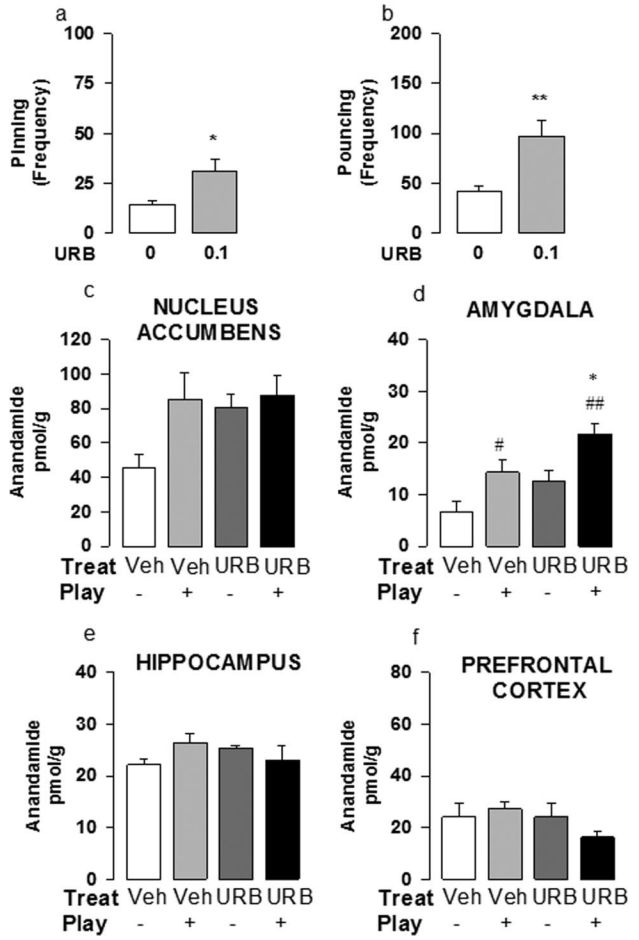

Figure 1. Endocannabinoid levels in NAc, amygdala, prefrontal cortex, and hippocampus of adolescent rats treated with either URB597 (URB) or vehicle (Veh), and placed in the test cage either alone or together with a play partner. Systemic administration of URB597 (0.1 mg/kg, i.p.) increased pinning (***a***) and pouncing (***b***). Increased NAc anandamide levels were observed in vehicle-treated rats allowed to interact with a play partner, and in all URB597-treated animals, regardless of whether they were allowed to interact with a play partner or not (***c***). Increased amygdala anandamide levels were observed after social play and after treatment with URB597, and the effects of social play and URB597 were additive (***d***). No changes in anandamide levels were observed in hippocampus (***e***) and prefrontal cortex (***f***). Data represent mean ± SEM frequency of pinning and pouncing (***a***, ***b***) and mean ± SEM anandamide levels (pmol/g, ***c–f***). $^{*}p < 0.05$ and $^{**}p < 0.01$ versus vehicle-treated rats allowed a play session (URB 0 or Veh/+); $^{\#}p < 0.05$ and $^{\#\#}p < 0.01$ versus vehicle-treated rats placed alone in the test cage (Veh/−) (Student–Newman–Keuls *post hoc* test, $n = 5$ per treatment group).

in the test cage, gave the following results: ($F_{(\text{test condition})1,16} = 4.53$, $p < 0.05$; $F_{(\text{treatment})\ 1,16} = 3$, n.s.; $F_{(\text{test condition} \times \text{treatment})\ 1,16} = 2.21$, n.s.). *Post hoc* analysis showed that the opportunity to engage in social play increased NAc anandamide levels in vehicle-treated rats (Fig. 1*c*). In addition, URB597 administration increased NAc anandamide levels regardless of whether animals were placed alone or together with a play partner in the test cage (Fig. 1*c*). A two-way ANOVA performed on anandamide levels in the amygdala gave the following results: ($F_{(\text{test condition})1,16} = 14.36$, $p < 0.01$; $F_{(\text{treatment})\ 1,16} = 8.81$, $p < 0.01$; $F_{(\text{test condition} \times \text{treatment})\ 1,16} = 0.12$, n.s.). *Post hoc* analysis showed that vehicle-treated rats allowed to interact with a play partner for 15 min had higher anandamide levels in the amygdala than vehicle-treated rats placed alone in the test cage (Fig. 1*d*). Animals treated with URB597 and placed alone in the test cage had slightly increased anandamide levels compared with vehicle-treated animals in the same experimental condition ($p = 0.08$). Importantly, anandamide levels in the amygdala further increased in animals treated with URB597 and allowed to interact with a play partner during the test session (Fig. 1*d*), showing that social play and URB597 administration had additive effects on anandamide levels in the amygdala.

Table 1. Social play and/or URB597 administration did not affect 2-AG, OEA, and PEA levels in NAc, amygdala, hippocampus, and prefrontal cortex

Brain area	Treatment	2-AG	OEA	PEA
NAc	Vehicle/no play	3.1 ± 0.2	663.9 ± 63	433.7 ± 58
	Vehicle/play	3.1 ± 0.3	573.4 ± 50	369.0 ± 26
	URB/no play	3.2 ± 0.1	657.0 ± 65	436.6 ± 36
	URB/play	3.2 ± 0.2	702.0 ± 65	462.8 ± 47
Amygdala	Vehicle/no play	3.4 ± 0.3	346.7 ± 26	199.9 ± 15
	Vehicle/play	4.1 ± 0.4	367.8 ± 10	201.4 ± 12
	URB/no play	4.0 ± 0.1	345.2 ± 32	218.8 ± 13
	URB/play	3.5 ± 0.4	379.8 ± 32	241.8 ± 27
Hippocampus	Vehicle/no play	1.0 ± 0.2	245.7 ± 15	116.8 ± 16
	Vehicle/play	1.1 ± 0.2	258.2 ± 8	114.8 ± 4
	URB/no play	1.3 ± 0.1	267.3 ± 10	128.4 ± 7
	URB/play	1.4 ± 0.2	266.4 ± 13	124.5 ± 5
Prefrontal cortex	Vehicle/no play	2.8 ± 0.3	157.2 ± 19	338.0 ± 30
	Vehicle/play	2.7 ± 0.2	156.1 ± 17	319.8 ± 24
	URB/no play	3.3 ± 0.2	154.1 ± 14	303.5 ± 31
	URB/play	3.2 ± 0.2	139.7 ± 17	281.1 ± 22

Data represent mean ± SEM of neurotransmitter levels (pmol/g or nmol/g for 2-AG). $N = 5$ per treatment group.

Social play and/or URB597 administration did not affect anandamide levels in the hippocampus and prefrontal cortex (hippocampus: $F_{(\text{test condition})1,16} = 0.32$, n.s.; $F_{(\text{treatment})\ 1,16} = 0.001$, n.s.; $F_{(\text{test condition} \times \text{treatment})\ 1,16} = 3.53$, n.s.; Figure 1*e*; prefrontal cortex: $F_{(\text{test condition})1,16} = 0.3$, n.s.; $F_{(\text{treatment})\ 1,16} = 1.57$, n.s.; $F_{(\text{test condition} \times \text{treatment})\ 1,16} = 1.62$, n.s.; Figure 1*f*).

Furthermore, in no brain region investigated did social play and/or URB597 administration affect levels of 2-AG or of two other bioactive fatty acid ethanolamides that are cleaved by FAAH but do not activate cannabinoid receptors, i.e., OEA and PEA (Table 1). This is not surprising since these compounds, unlike anandamide, are good substrates for other hydrolyzing enzymes in rodents (Di Marzo, 2008; De Petrocellis and Di Marzo, 2009). Together, these results suggest that, during social play, anandamide levels increase in both NAc and amygdala. Furthermore, treatment with URB597 augments the social play-induced increase in anandamide levels in the amygdala, but not the NAc.

Social play-induced changes in CB1 cannabinoid receptor phosphorylation and FAAH and NAPE-PLD expression

It has previously been shown that phosphorylated CB1 receptors, which may reflect the activation of this receptor (Garcia et al., 1998; Daigle et al., 2008), are abundant in reward-related brain areas such as the NAc and the amygdala (Orio et al., 2009). To further investigate the role of endocannabinoid activity in the NAc and amygdala in the modulation of social play behavior, we measured both phosphorylated and total CB1 receptor protein expression in these brain areas in adolescent rats tested for social play behavior. The ratio between phosphorylated and total CB1 receptor protein was increased in the amygdala ($t = 4.66$, $df = 4$, $p < 0.01$; Fig. 2*a*), but not the NAc ($t = 0.09$, $df = 4$, n.s.; Fig. 2*b*) in adolescent rats allowed to play compared with control animals.

Furthermore, qPCR experiments revealed no changes in the expression of NAPE-PLD, the enzyme that catalyzes the one-step conversion of NAPEs to anandamide (Okamoto et al., 2004), in amygdala and NAc of rats allowed to interact with a play partner (amygdala: $t = -0.38$, $df = 14$, n.s.; NAc: $t = 0.16$, $df = 14$, n.s.;

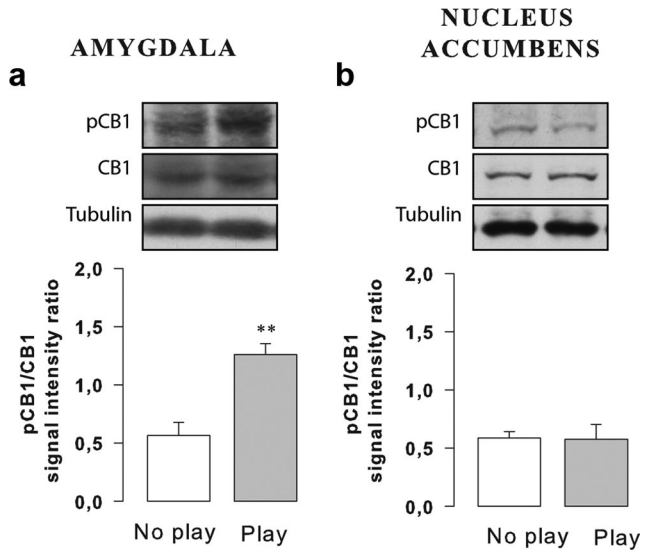

Figure 2. Western blot analysis of phosphorylated and total CB1 receptor protein expression in NAc and amygdala. The ratio between phosphorylated and total CB1 receptor protein was increased in the amygdala (***a***), but not the NAc (***b***) of adolescent rats allowed to interact with a play partner (Play) compared with rats placed alone in the test cage (No Play). Top shows representative immunoblots for phosphorylated and total CB1 receptor protein and the loading control α-tubulin. Data in graphs represent mean ± SEM signal intensity ratio between phosphorylated and total CB1 receptor protein. $^{**}p < 0.01$ versus No Play rats (Student's *t* test, $n = 5$ per treatment group).

Table 2. Social play did not affect the expression of NAPE-PLD and FAAH in NAc and amygdala

		Normalized Ct value	
Brain area	Condition	NAPE-PLD	FAAH
NAc	No play	8.59 ± 0.09	7.77 ± 0.10
	Play	8.61 ± 0.10	7.80 ± 0.15
Amygdala	No play	8.50 ± 0.09	6.85 ± 0.05
	Play	8.45 ± 0.07	6.89 ± 0.04

Data represent mean ± SEM of normalized cycles to threshold (Ct) values. $N = 8$ per treatment group.

Table 2). Thus, the increase in anandamide levels observed in the amygdala and NAc of adolescent rats after a play session might not depend on increased anandamide synthesis. We found no changes in the expression of FAAH in either amygdala or NAc after social play (amygdala: $t = 0.58$, $df = 14$, n.s.; NAc: $t = 0.2$, $df = 14$, n.s.; Table 2).

Role of the amygdala and NAc in the social play-enhancing effect of URB597

To further pinpoint the role of endocannabinoid neurotransmission in the NAc and amygdala in the modulation of social play, we tested the effects of intra-NAc and intra-BLA infusion of URB597 (NAc: 0.005–0.01 μg/0.3 μl; amygdala: 0.005–0.01 μg/0.2 μl) and the CB1 cannabinoid receptor antagonist/inverse agonist SR141716A (NAc: 0.3–1-3 μg/0.3 μl; BLA: 0.03–0.1–0.3–1-3 μg/0.2 μl) on social play behavior in adolescent rats. URB597, infused into the NAc at the dose of 0.01 μg, increased pinning ($F_{(2,23)} = 3.9$, $p < 0.05$; Fig. 3*a*) and pouncing ($F_{(2,23)} = 4.66$, $p < 0.05$; Fig. 3*b*), with no effect on social exploration ($F_{(2,23)} = 2.27$, n.s.; Table 3). We next determined whether the endocannabinoid-mediated increase in social play critically depended on activation of cannabinoid receptors in the NAc. To this aim, we tested whether blockade of NAc cannabinoid receptors by SR141716A antagonized the play-enhancing effects of systemic URB597 administration. We found that SR141716A, infused in the NAc at a dose (1 μg/0.3 μl) that did not affect social play by itself, did not antagonize the effects of systemic URB597 treatment (0.1 mg/kg, i.p.) on social play (pinning: $F_{(SR)1,50} = 0.14$, n.s.; $F_{(URB)1,50} = 17.61$, $p < 0.0001$; $F_{(SR \times URB)1,50} = 0.003$, n.s.; Figure 3*c*; pouncing: $F_{(SR)1,50} = 0.002$, n.s.; $F_{(URB)1,50} = 31.26$, $p < 0.0001$; $F_{(SR \times URB)1,50} = 0.23$, n.s.; Figure 3*d*). *Post hoc* analysis showed that URB597 increased social play both in rats that received intra-NAc vehicle and in animals that received intra-NAc SR141716A.

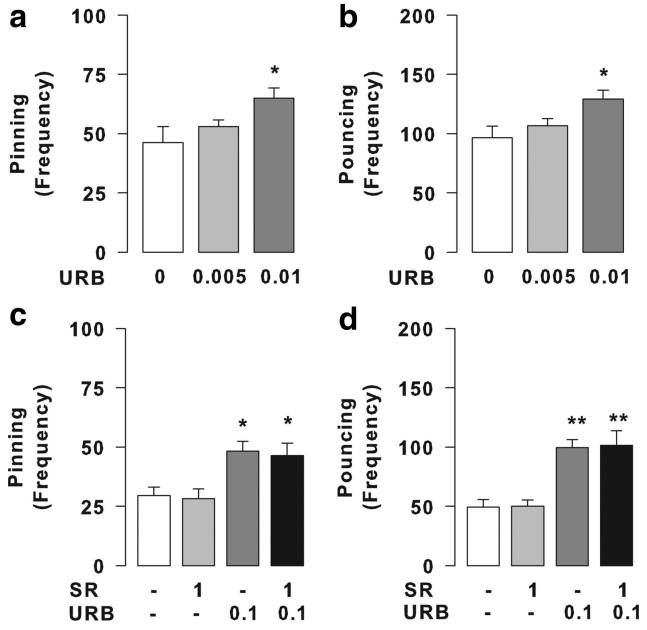

Figure 3. Infusion of the anandamide hydrolysis inhibitor URB597 into the NAc enhanced social play. However, blockade of NAc cannabinoid receptors did not antagonize the effects of systemic URB597 administration on social play. Intra-NAc infusion of URB597 (URB; 0.005–0.01 μg/0.3 μl) increased both pinning (***a***) and pouncing (***b***). Intra-NAc infusion of the cannabinoid receptor antagonist/inverse agonist SR141716A (SR; 1 μg/0.3 μl) did not antagonize the effects of systemic URB597 treatment (0.1 mg/kg, i.p.) on pinning (***c***) and pouncing (***d***). Data represent mean ± SEM frequency of pinning and pouncing. $^{*}p < 0.05$ and $^{**}p < 0.01$ versus vehicle/vehicle (Student–Newman–Keuls *post hoc* test, $n = 10–14$ per treatment group).

Table 3. Intra-NAc and intra-amygdala infusion of URB597 (URB; 0.005–0.01 μg/0.3 μl in the NAc and 0.005–0.01 μg/0.2 μl in the amygdala) had no effect on social exploration

Social exploration (s/15 min)	Time spent	*F*
NAc		
Vehicle	64 ± 8	2.27 (n.s.)
URB 0.005 μg	50 ± 5	
URB 0.01 μg	66 ± 5	
Amygdala		
Vehicle	55 ± 6	0.48 (n.s.)
URB 0.005 μg	40 ± 6	
URB 0.01 μg	45 ± 5	

Data represent mean ± SEM time spent in social exploration. $N = 6–14$ per treatment group.

The NAc can be divided into two main subregions, i.e., core and shell, which differ in their connectivity and function (Cardinal et al., 2002; Kelley, 2004; Voorn et al., 2004). In the previous experiments, it was not possible to differentiate between these two subregions, because cannula placements were located at the border between the core and the shell. To investigate the relative contributions of the different subregions of the NAc in the modulation of social play behavior under physiological conditions, we implanted groups of

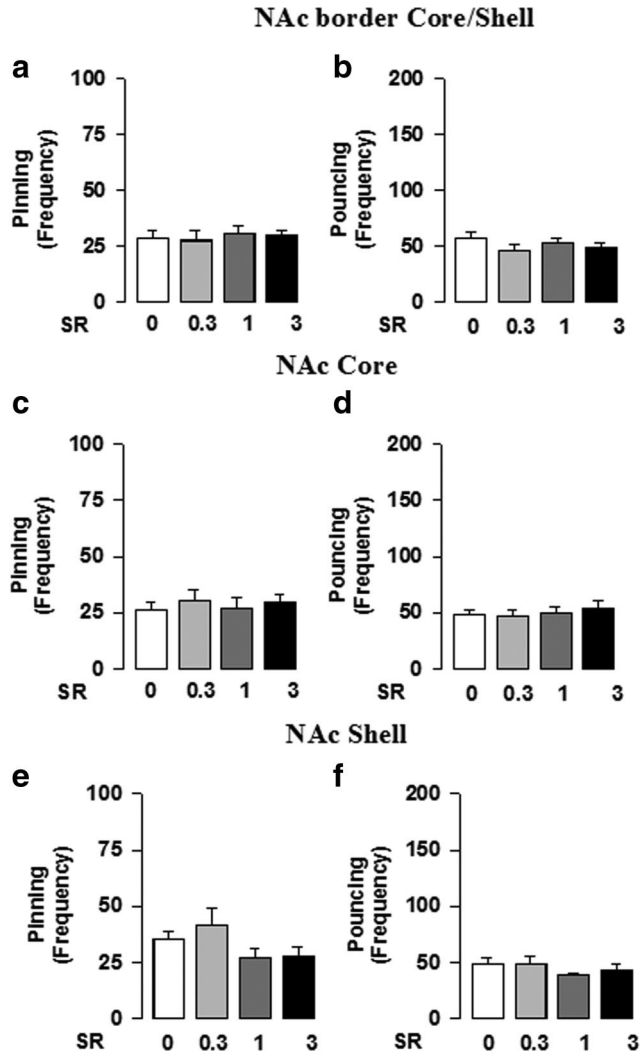

Figure 4. Blockade of NAc cannabinoid receptors did not affect social play. The cannabinoid receptor antagonist/inverse agonist SR141716A (0.3–3 μg/0.3 μl) did not affect pinning and pouncing when infused at the border between the core and the shell subregions of the NAc (***a***, ***b***). Furthermore, it did not affect social play behavior when infused either into the core (***c***, ***d***) or into the shell (***e***, ***f***). Data represent mean ± SEM frequency of pinning and pouncing. (n = 10–12 per treatment group).

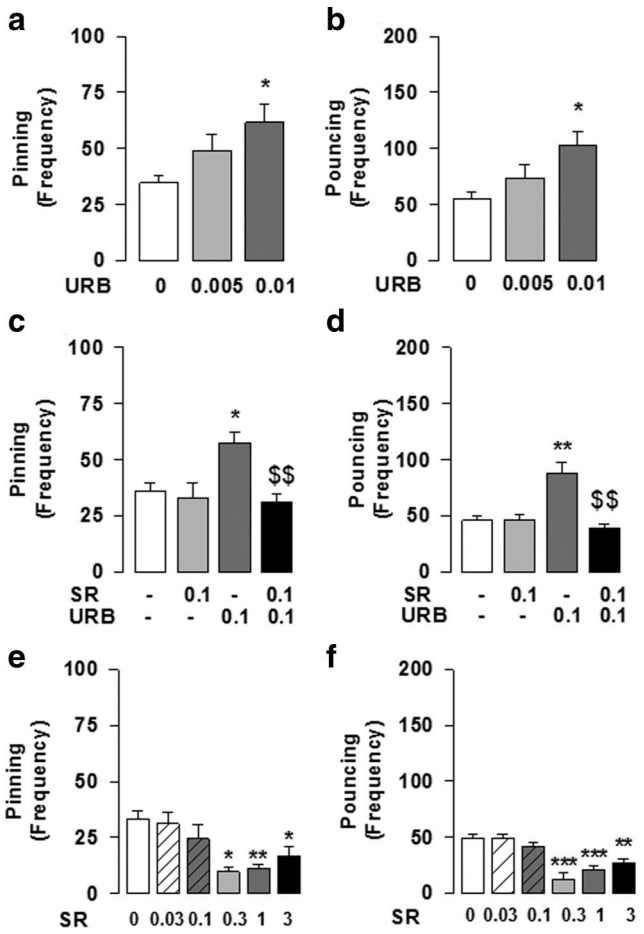

Figure 5. Infusion of URB597 into the amygdala enhanced social play. Blockade of amygdala cannabinoid receptors antagonized the effects of systemic URB597 administration and reduced social play. Intra-amygdala infusion of URB597 (URB; 0.005–0.01 μg/0.2 μl) increased both pinning (***a***) and pouncing (***b***). Intra-NAc infusion of SR141716A (SR; 0.1 μg/0.2 μl), at a dose that did not affect social play by itself, antagonized the effects of systemic URB597 treatment (0.1 mg/kg, i.p.) on pinning (***c***) and pouncing (***d***). Intra-amygdala infusion of SR141716A (0.03–3 μg/0.2 μl) reduced both pinning (***e***) and pouncing (***f***). Data represent mean ± SEM frequency of pinning and pouncing. *$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$ versus vehicle/vehicle; $^{\$\$}p < 0.01$ versus vehicle/URB (Student–Newman–Keuls *post hoc* test, n = 10–14 per treatment group).

rats with bilateral guide cannulae targeted at the border between the core and the shell, at the NAc core or at the NAc shell, and infused the cannabinoid receptor antagonist/inverse agonist SR141716A (0.3–1-3 μg/0.3 μl) into each of these NAc subregions. The drug did not affect pinning and pouncing when infused at the border between the core and the shell subregions of the NAc (pinning: $F_{(3,24)} = 0.13$, n.s.; Fig. 4*a*; pouncing: $F_{(3,24)} = 0.93$, n.s.; Fig. 4*b*). Furthermore, SR141716A did not affect social play behavior when infused either into the core (pinning: $F_{(3,37)} = 0.24$, n.s.; Fig. 4*c*; pouncing: $F_{(3,37)} = 0.23$, n.s.; Fig. 4*d*) or into the shell (pinning: $F_{(3,29)} = 1.61$, n.s.; Fig. 4*e*; pouncing: $F_{(3,29)} = 0.98$, n.s.; Fig. 4*f*). SR141716A, infused in any NAc subregion, did not alter social exploration (data not shown). Collectively, these results indicate that increased anandamide levels within the NAc facilitate social play, but that endocannabinoid signaling in the NAc is not essential for the proper expression of social play. Therefore, endocannabinoids likely also act outside the NAc to modulate this behavior.

Concerning the role of the amygdala in the modulation of social play, we implanted rats with bilateral guide cannulae aimed at the BLA, where CB1 cannabinoid receptors are highly abundant (Tsou et al., 1998; Katona et al., 2001). We found that URB597, infused into the BLA at the dose of 0.01 μg/0.2 μl, increased pinning ($F_{(2,19)} = 3.609$, $p < 0.05$; Fig. 5*a*) and pouncing ($F_{(2,19)} = 4.56$, $p < 0.05$; Fig. 5*b*), with no effect on social exploration ($F_{(2,19)} = 0.48$, n.s.; Table 3). Importantly, intra-BLA infusion of SR141716A (0.1 μg/0.2 μl) antagonized the effects of systemic URB597 treatment (0.1 mg/kg, i.p.) on social play (pinning: $F_{(SR)1,44} = 9.11$, $p < 0.01$; $F_{(URB)1,44} = 4.31$, $p < 0.05$; $F_{(SR \times URB)1,44} = 5.97$, $p < 0.05$; Figure 5*c*; pouncing: $F_{(SR)1,44} = 11.54$, $p < 0.01$; $F_{(URB)1,44} = 11.68$, $p < 0.01$; $F_{(SR \times URB)1,44} = 13.60$, $p < 0.001$; Figure 5*d*). *Post hoc* analysis showed that URB597 increased social play in rats that received intra-BLA vehicle but not in animals that received intra-BLA SR141716A. These results show that anandamide-mediated stimulation of cannabinoid receptors within the BLA is necessary and sufficient for URB597 to increase social play behavior. Furthermore, we found that intra-BLA infusion of SR141716A at doses of 0.3–3 μg reduced both pinning ($F_{(5,48)} = 5.28$, $p < 0.001$; Fig. 5*e*) and pouncing ($F_{(5,48)} = 12.63$, $p < 0.001$; Fig. 5*f*), without affect-

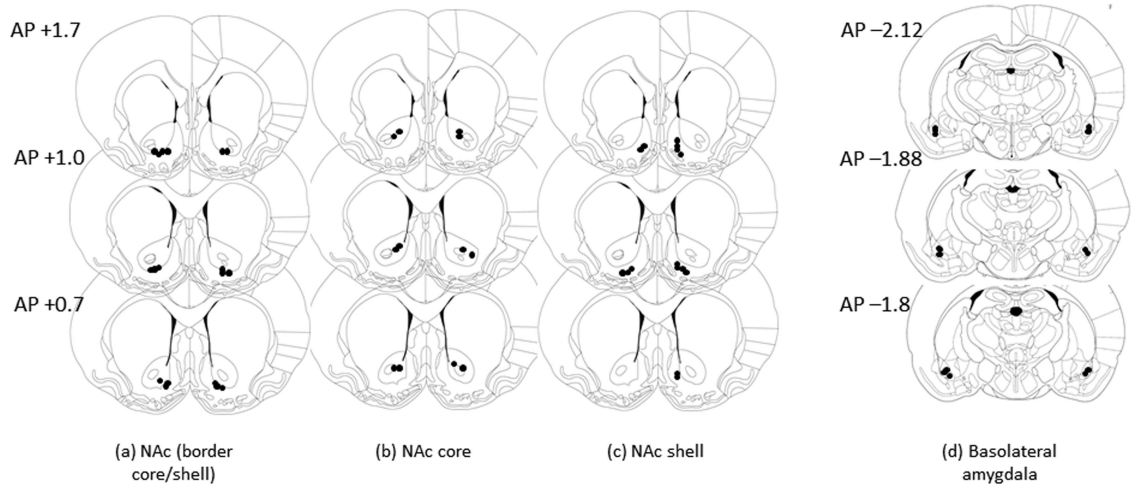

Figure 6. Diagrams of rat brain sections showing representative microinjection sites (filled circles) at the border between core and shell subregions of the NAc (***a***), NAc core (***b***), NAc shell (***c***), and BLA (***d***). Only data from test pairs in which both animals had bilateral needle tracks terminating in the target area and no damage to the target tissues were included in the final analyses.

ing social exploration ($F_{(5,48)} = 2.21$, n.s.; data not shown). This indicates that endocannabinoid activity in the BLA critically modulates social play.

For histological assessment of representative experiments see Figure 6.

Discussion

Endocannabinoids in the limbic forebrain are known to regulate affective states and to modulate the rewarding properties of food and drugs (van der Stelt and Di Marzo, 2003; Gardner, 2005; Caillé et al., 2007; Mahler et al., 2007; Soria-Gómez et al., 2007; Di Marzo et al., 2009; Orio et al., 2009; Shinohara et al., 2009; Berridge et al., 2010; Fattore et al., 2010; Spano et al., 2010; McLaughlin and Gobbi, 2012). Consistent with this notion, our findings provide new evidence that endocannabinoid signaling in the limbic forebrain mediates rewarding social interactions in adolescent rats.

Our previous studies using systemic drug injections have shown that drugs that prolong ongoing endocannabinoid activity by inhibiting endocannabinoid deactivation enhance social play, suggesting that during social play, endocannabinoids are released in brain areas that mediate this behavior (Trezza and Vanderschuren, 2008a,b, 2009). Given the role of endocannabinoid activity in the NAc and amygdala in emotion and motivation (van der Stelt and Di Marzo, 2003; Gardner, 2005; Laviolette and Grace, 2006; Mahler et al., 2007; Orio et al., 2009; Shinohara et al., 2009; Berridge et al., 2010; Hill et al., 2010; McLaughlin and Gobbi, 2012), we hypothesized that these regions were also involved in endocannabinoid modulation of social play. To test this hypothesis, we measured endocannabinoid levels in NAc, amygdala, as well as hippocampus and prefrontal cortex after social play. Vehicle-treated adolescent rats allowed to play showed higher anandamide levels in the NAc and amygdala compared with vehicle-treated animals placed alone in the test cage. This suggests that, during social play, anandamide signaling is increased in these areas. Interestingly, systemic administration of the anandamide hydrolysis inhibitor URB597 further increased the social play-induced elevation in anandamide levels in the amygdala, suggesting that URB597-enhanced anandamide signaling in the amygdala mediates its stimulatory effect on social play. Anandamide levels were unaffected in the hippocampus and prefrontal cortex. The increase in anandamide levels observed in the amygdala and NAc of adolescent rats after social play could depend on increased anandamide synthesis, or reduced anandamide hydrolysis. We found no changes in the expression of NAPE-PLD and FAAH in amygdala and NAc after social play. Therefore, the higher anandamide levels observed in the amygdala and NAc after social play might be the result of changes in: (1) expression of other anandamide biosynthetic enzymes (Di Marzo, 2008), (2) protein levels and/or activities of NAPE-PLD and FAAH, or (3) levels of the anandamide biosynthetic precursor, *N*-arachidonoyl-phosphatidylethanolamine. Identification of the molecular mechanisms underlying the social play-associated changes in brain anandamide levels was beyond the scope of this study, and should be the subject of future investigations.

URB597 selectively inhibits FAAH activity, being 7500–25,000 times more selective for FAAH than any other cannabinoid-related target, including endocannabinoid transport proteins and monoacylglycerol lipase, the enzyme that degrades the endocannabinoid 2-AG (Kathuria et al., 2003). In addition to anandamide, FAAH also cleaves other bioactive fatty acid ethanolamides such as OEA and PEA. However, social play and/or URB597 administration did not affect 2-AG, PEA, and OEA levels in any brain region investigated. Together, these results suggest that URB597 increases social play by selectively increasing anandamide signaling in the amygdala. In support of this notion, we found that social play enhanced levels of phosphorylated CB1 receptor in the amygdala, but not in the NAc of adolescent rats. Although the role of CB1 receptor phosphorylation in endocannabinoid signaling has not been studied extensively, it has been suggested that phosphorylation of the distal carboxy terminus of the CB1 receptor promotes internalization of agonist-activated full-length receptors (Daigle et al., 2008). Thus, increased CB1 receptor phosphorylation may reflect a compensatory response to reduce CB1 receptor-mediated signaling after endocannabinoid system activation (Garcia et al., 1998). Interestingly, high levels of phosphorylated CB1 receptor protein were also observed in the NAc and the amygdala of rats given extended access to cocaine (Orio et al., 2009), showing that both natural and drug rewards can induce upregulation of CB1 receptor signaling in brain areas implicated in emotion and motivation.

To further investigate the relative role of NAc and amygdala in endocannabinoid modulation of social play, we performed be-

havioral experiments where we tested the effects of intra-NAc and intra-amygdala infusion of URB597 or the CB1 cannabinoid receptor antagonist/inverse agonist SR141716A. We also determined whether blockade of CB1 cannabinoid receptors in the NAc or amygdala with SR141716A antagonized the increase in social play induced by systemic URB597 administration. Infusion of URB597 into the NAc enhanced social play but not social exploratory behavior, indicating that cannabinoid neurotransmission in the NAc modulates playful aspects of social interaction in adolescent rats, rather than social behavior in general. However, intra-NAc infusion of SR141716A, at a dose that reduced social play when infused into the BLA (see below), did not affect social play, or antagonize the play-enhancing effects of systemic URB597 administration. The NAc can be divided into two subregions, i.e., core and shell, which differ in terms of connectivity and function (Cardinal et al., 2002; Kelley, 2004; Voorn et al., 2004; Ikemoto, 2007). Of these two subregions, especially the shell is thought to be involved in positive emotions (Kelley, 2004; Ikemoto, 2007; Berridge et al., 2010), although both core and shell have previously been implicated in opioid modulation of social play (Trezza et al., 2011a). To exclude the possibility that the involvement of endocannabinoids in the modulation of social play was underestimated when microinfusions were aimed at the core/shell border, we infused SR141716A selectively into either the core or shell. However, SR14716A did not affect social play in either region. It is known that endocannabinoid activity in the NAc increases the rewarding properties of food and drugs (Gardner, 2005; Caillé et al., 2007; Mahler et al., 2007; Soria-Gómez et al., 2007; Di Marzo et al., 2009; Orio et al., 2009; Shinohara et al., 2009; Berridge et al., 2010). Our present results of increased social play after intra-NAc infusion of URB597 extend these findings, suggesting that endocannabinoids in the NAc also modulate social play reward. However, our finding that infusion of SR141716A into the NAc did not affect social play or antagonize the play-enhancing effects of systemic treatment with URB597 indicates that other regions are critical for endocannabinoid modulation of social play.

The amygdala is essential for the attribution of emotional value to salient cues and events (Baxter and Murray, 2002; Cardinal et al., 2002; Balleine and Killcross, 2006). Within the amygdala, CB1 cannabinoid receptors are highly abundant in the BLA, whereas their expression and functional relevance in the central amygdala (CeA) is less clear (Tsou et al., 1998; Katona et al., 2001; Cota et al., 2007). Endocannabinoids in the BLA play a prominent role in the encoding of emotionally salient stimuli, but this has so far only been shown for negatively valenced stimuli. Thus, endocannabinoids in the BLA modulate acquisition, consolidation, and extinction of fear memories and regulate anxiety as well as stress responses (Marsicano et al., 2002; Laviolette and Grace, 2006; Campolongo et al., 2009; Hill et al., 2010; Karst et al., 2010; McLaughlin and Gobbi, 2012). Although in our biochemical experiments it was not possible to differentiate between the BLA and CeA, our observations of increased anandamide levels and phosphorylated CB1 receptor protein after social play indicate enhanced amygdala endocannabinoid signaling in adolescent rats during a playful social interaction. In our behavioral experiments, cannula placements were specifically aimed at the BLA. These experiments confirmed that the BLA plays a critical role in endocannabinoid modulation of social play. Intra-BLA infusion of URB597 increased, and intra-BLA infusion of SR141716A reduced social play, without affecting social exploratory behavior. This suggests that endocannabinoid activity in the BLA critically modulates social play. Importantly, intra-BLA infusion of SR141716A antagonized the effects of systemic URB597 treatment on social play, demonstrating that stimulation of CB1 cannabinoid receptors in the amygdala is necessary and sufficient for anandamide to facilitate social play behavior. Together, our findings extend the knowledge about the physiological function of endocannabinoid signaling in the amygdala by demonstrating that stimulation of cannabinoid receptors in the BLA modulates rewarding social interactions in adolescent rats. Thus, BLA endocannabinoids are not exclusively involved in the processing of negative emotions. Given the role of the amygdala in the assignment of emotional value to behaviorally meaningful stimuli (Baxter and Murray, 2002; Cardinal et al., 2002; Balleine and Killcross, 2006), our findings suggest that anandamide signaling within the BLA enhances the rewarding properties of confrontation with a playful conspecific, or of the playful social interaction itself.

Our results are consistent with human studies that have demonstrated a role of endocannabinoids in social behavior. Thus, variations in the CB1 cannabinoid receptor gene have been shown to modulate social reward responsivity in reward-related forebrain areas (Chakrabarti et al., 2006; Chakrabarti and Baron-Cohen, 2011). In addition, the endocannabinoid system modulates amygdala reactivity to social threat signals (Phan et al., 2008). In view of the role of the endocannabinoid system in positive social interactions, altered function of this system may be involved in neuropsychiatric diseases characterized by aberrant socio-emotional responses.

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