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Deficient Adolescent Social Behavior Following Early-Life Inflammation is Ameliorated by Augmentation of Anandamide Signaling

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Abstract

Early-life inflammation has been shown to exert profound effects on brain development and behavior, including altered emotional behavior, stress responsivity and neurochemical/ neuropeptide receptor expression and function. The current study extends this research by examining the impact of inflammation, triggered with the bacterial compound lipopolysaccharide (LPS) on postnatal day (P) 14, on social behavior during adolescence. We investigate the role that the endocannabinoid (eCB) system plays in sociability after early-life LPS. To test this, multiple cohorts of Sprague Dawley rats were injected with LPS on P14. In adolescence, rats were subjected to behavioral testing in a reciprocal social interaction paradigm as well as the open field. We quantified eCB levels in the amygdala of P14 and adolescent animals (anandamide and 2-arachidonoylglycerol) as well as adolescent amygdaloid cannabinoid receptor 1 (CB1) binding site density and the hydrolytic activity of the enzyme fatty acid amide hydrolase (FAAH), which metabolizes the eCB anandamide. Additionally, we examined the impact of FAAH inhibition on alterations in social behavior. Our results indicate that P14 LPS decreases adolescent social behavior (play and social non-play) in males and females at P40. This behavioral alteration is accompanied by decreased CB1 binding, increased anandamide levels and increased FAAH activity. Systemic administration of the FAAH inhibitor PF-04457845 (1mg/kg) prior to the social interaction task normalizes LPS-induced alterations in social behavior, while not affecting social behavior in the control group. Infusion of 10ng PF-04457845 into the basolateral amygdala normalized social behavior in LPS injected females. These data suggest that alterations in eCB signaling following postnatal inflammation contribute to impairments in social behavior during adolescence and that FAAH could be a novel target for disorders involving social deficits such as social anxiety disorders or autism.

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Keywords

Early-life inflammation; social behavior; endocannabinoid; LPS; FAAH; amygdala; anxiety; autism

1. Introduction

Inflammation, particularly during the prenatal period, has been associated with increased incidence of disorders such as autism (Patterson, 2012) and schizophrenia (reviewed in Green and Nolan, 2014; Meyer et al., 2011; Miller et al., 2013) in the offspring. This phenomenon has been intensively investigated using a variety of animal models where inflammatory agents such as lipopolysaccharide (LPS) and Poly inositol:cytosine (Poly I:C) have been administered to pregnant dams. However, in the protected fetal environment, it is unlikely that these substances enter the fetal circulation and the effects on the offspring are most likely secondary to inflammatory changes occurring in the placenta (Hsiao and Patterson, 2012). Cultivable bacteria in the amniotic fluid are present in less than 1% of normal births (Romero et al., 2002), whereas after birth infants may be exposed directly to a variety of pathogens. Nevertheless, developmental windows beyond prenatal and very early postnatal stages have been less well studied in the context of inflammation.

Inflammatory insults with live bacteria on postnatal day (P) 4 have been associated with altered fear memory (Bilbo et al., 2005) and motor coordination (Lieblein-Boff et al., 2013), as well as potentiation of glial and cytokine responses in the adult rat (reviewed in Bilbo and Schwarz, 2012), particularly in response to a second inflammatory challenge (Bilbo et al., 2006). In a similar time frame, rats that were given LPS at P3 and P5 also show increased anxiety and hypersensitivity to stressful stimuli as adults (Sominsky et al., 2013), with hyper-responsiveness of the hypothalamus-pituitary-adrenal axis and decreased glucocorticoid receptor expression and abundance (Shanks et al., 2000, 1995; Sominsky et al., 2013). These changes are complemented by increases in corticotropin releasing hormone (CRH) mRNA expression in the hypothalamic paraventricular nucleus, but not the amygdala (Amath et al., 2012) as well as decreases in cannabinoid type 1 receptor (CB1) density in the hippocampus (Amath et al., 2012) and amygdala (Zavitsanou et al., 2013).

Inflammation and the endocannabinoid (eCB) system share a variety of interconnections. First, cyclooxygenase-2 (COX-2) is an important enzyme in the response to inflammation. This enzyme can degrade both anandamide (AEA) and 2-arachindoyl glycerol (2-AG) and convert them to the pro-inflammatory prostaglandins (Kozak et al., 2000; Yu et al., 1997). Secondly, the cytokines interleukin (IL)-1 and tumor necrosis factor (TNF)-α play an important role in synaptic transmission; IL-1 has been identified as a regulator of the effects of synaptic CB1, while CB1 in turn regulates the TNF-α mediated potentiation of excitatory synapses (reviewed by Rossi et al., 2014). In turn, inflammation has been shown to affect cannabinoid function. TNF-α has been suggested to increase AEA synthesis in hypothalamic fragments (Fernandez-Solari et al., 2006) and activation of toll-like receptor 4, the main signaling target of LPS, has been shown to increase AEA production in peripheral macrophages (Liu et al., 2006).

Long-term effects of LPS-induced inflammation at P14 in rodents have been extensively investigated in the past (e.g. Galic et al., 2008; Mouihate et al., 2010; Spencer et al., 2005). P14 is comparable to a human infant of approximately 0.5–2 years of age (Gottlieb *et al.,* 1977; Avishai-Eliner *et al.,* 2002). Inflammatory insults experienced at P14 are more comparable to infections a young child would experience, while earlier ages likely mirror insults experienced during the late intrauterine period. As such, it is important to determine if the effects that occur during a 'fetal' developmental stage are also seen postnatally, at a time when gene expression, electrophysiological and synaptic properties are undergoing experience-dependent maturation (Dehorter et al., 2012, reviewed in Semple et al., 2013). P14 represents a time of regional differentiation in gene expression (Stead et al., 2006) and high synaptic turnover, when microglia engulf synaptic elements actively (Paolicelli et al., 2011; Zhan et al., 2014). Within the first 14 postnatal days microglia are proliferating and migrate into the grey matter. Alterations in microglial function during the early postnatal period results in deficits in synaptic pruning, and ultimately less efficient neural transmission (Zhan et al., 2014). Behaviorally, this is associated with deficits in social interaction and increased repetitive-behavior phenotypes (Zhan et al., 2014). In adult rodents (Madore et al., 2013) and on P14 (Dinel et al., 2014), LPS administration leads to an upregulation of cytokines in the circulation and the brain. P14 further characterizes the end of the stress hypo-responsive period (Levine, 1994) and around this time, the brain reacts to LPS with an increase in paraventricular nucleus Fos, CRH mRNA and subsequent HPA axis activation (Dent et al., 1999). There is evidence that a variety of neuroinflammatory responses to LPS can be quite different around the second week of age, when compared to perinatal time points (P1) (Brochu et al., 2011). Animals that have experienced early-life inflammation show alterations in anxiety (novelty induced suppression of feeding) and depression related tests (Dinel et al., 2014).

Amongst possible regions mediating changes in response to early-life inflammation, the amygdala stands out due to its involvement in emotional regulation. This structure is known to be affected by LPS (Frenois et al., 2007; Prager et al., 2013); on P14 the amygdala shows increased concentrations in proinflammatory (TNF-α, IL-1, IL-6) but not anti-inflammatory (IL-10) cytokines, which distinguishes it remarkably from other structures such as hypothalamus, hippocampus or prefrontal cortex (Dinel et al., 2014).

Altered excitability, activation and structure of the amygdala have been implicated in a variety of disorders including post traumatic stress disorder (PTSD) (Weston, 2014), schizophrenia (van Erp et al., 2014) and autism spectrum disorder (ASD) (reviewed by Zalla and Sperduti, 2013). Interestingly, in recent years a growing body of work emerged suggesting the eCB system to be altered in ASD. In particular CB1 appears decreased in post-mortem brains of autistic individuals, and polymorphisms in the CB1 coding gene CNR1 appears to be a predictor for an autistic phenotype. Furthermore, individuals with ASD show altered FAAH activity and AEA (reviewed by Chakrabarti et al., 2015).

Social behavior and reward are strongly dependent on eCB signaling across many developmental epochs (Manduca et al., 2015; Wei et al., 2015). In adolescent rats, social experiences are associated with increased AEA levels within the amygdala and striatum (Marco et al., 2011; Trezza et al., 2012). During adolescence, and into adulthood, inhibition

of eCB signaling can induce social withdrawal and social anxiety (Trezza et al., 2012; Sellier et al., 2013; Litvin et al., 2013), while systemic or intra-amygdala amplification of AEA signaling can enhance social interaction and contact (Sellier et al., 2013; Trezza et al., 2012).

Given the relationship between eCB signaling and social behavior, as well as the fact that early-life inflammation has been associated with alterations in the eCB system within the amygdala (Zavitsanou et al., 2013), we hypothesize that P14 LPS will decrease adolescent social behavior and that these changes will be sensitive to enhancement of AEA signaling through the inhibition of its degradative enzyme fatty acid amide hydrolase (FAAH).

2. Methods

2.1. Animals

Sprague Dawley (SD) rats obtained from Charles River Laboratories were maintained under standard specific pathogen-free environmental conditions and bred locally. For all behavioral experiments, litters were culled to no more than 12 pups and efforts were made to obtain roughly equal numbers of males and females. In all studies, no more than 2 pups per litter/gender, per treatment group were used for any measure. Endocannabinoid levels were determined at 2 different time points (P14 and P40) and behavioral testing (social and anxiety testing) was executed during adolescence. For an overview of experiments see figure 1.

2.2. Early Immune Activation

Animals of each litter were assigned to either treatment or control group (~50% split). Each group contained males and females and was injected intraperitoneally on P14 with LPS (*Escherichia coli*, serotype O26: 100μg/kg) or with pyrogen-free saline. Injections were given between 1100h and 1300h and ear notches were used to mark treatment. It was previously established that a dosage of 100μg/kg LPS generates a mild inflammatory response in the host that lasts for roughly 6–8h (Heida et al., 2004)

Upon weaning on P21, rats were housed with a same-sex littermate. For cohorts tested for social behavior, rats were housed in pairs; for all other experiments, rats were housed in groups of 2–3 in clear Plexiglas cages (43 × 21 × 22 cm) with wood chip bedding. At least 1 LPS treated animal and 1 control treated animal was housed in each cage to assure equal conditions during adolescence. They were maintained under a 12:12h illumination cycle (lights on 0700h), at a room temperature of 20–22°C, and received *ad lib* access to lab chow and water. All procedures were approved by the University of Calgary Animal Care Committee in accordance with the guidelines of the Canadian Council on Animal Care.

2.3. Social Behavior

Adolescent rats (P40 +/− 2 days, n=48, 24 male, 24 female; 6 litters) were habituated to the test arena for 15 minutes (clear standard cage without water, food, enrichment and grid, illumination identical to housing conditions) and then separated from their cage mate overnight. Every animal experienced two social encounters, one with a treatment matched

and one with a differently treated counterpart (see figure 2 for an illustration). Animals were paired for 10 minutes with a litter- (but not cage-) mate, matched for sex. After the social encounters, animals were returned to their homecage and their previous cagemate. The following day animals were separated over night and then exposed to another social encounter. The order in which animals encountered treatment matched or non-treatment matched counterparts was counterbalanced. The experiment was taped and scored offline by a blinded investigator for social play and social non-play behaviors. Social play behaviors were defined as pouncing, chasing, wrestling and pinning and social non-play behaviors were defined as grooming, anogenital sniffing and crawling (over/under) (Meaney and Stewart, 1981; Vanderschuren et al., 1997).

2.4. Open Field

To confirm that differences in social behavior were not due to differences in general behavioral patterns within an arena, animals received early immune activation on P14 (as described in section 2.1). 32 adolescent (P40 +/− 2 days) rats (16 male/16 female) were habituated for two consecutive days to handling and tested in an open field paradigm. Briefly, animals were placed in an open arena (50×30×30cm) at very low red light intensity (<1 lux) illumination during the beginning of the wake period (19:00 – 21:00h) and recorded for 5 minutes using a Canon camera system with high light sensitivity (F 2.8, ISO 28 000). Time in the center quadrant as well as grooming and rearing behavior were scored offline.

2.5. Analysis of the Endocannabinoid System

For the characterization of the eCB system in the amygdala, animals received P14 LPS or saline injections. At two different timepoints, 6h after LPS administration on P14 or on P40, animals were decapitated quickly and brain tissue from the amygdaloid region was dissected as previously described (Gray et al., 2015). Tissue was rapidly frozen on dry ice and subsequently stored at −80°C until further analysis of CB1 receptor binding, eCB concentrations and FAAH enzymatic activity. All assays are explained briefly in the following sections.

2.5.1. CB1 receptor Binding—Membrane preparations from amygdala tissue from 20 P40 (+/− 2 days) animals (10 male/10 female, 50% LPS/control; 6 litters) were generated as previously described (Lee and Hill, 2013). In brief, tissue samples were homogenized in TME buffer (50mM Tris-HCl, 1mM EDTA, 3mM $MgCl_2$, pH 7.4), using a Dounce homogenizer, centrifuged at 18,000 × *g* and the resulting pellet was resuspended in 20 volumes of TME buffer. Protein content was determined using the BCA method using a commercially available kit (Pierce Biotechnology, Rockville, IL, USA). Samples were then aliquoted and stored at −80°C until further testing for measures of CB1 receptor binding and FAAH activity (see below).

CB1 receptor agonist binding parameters were determined using a previously detailed radioligand binding assay (Lee and Hill, 2013) using a Multiscreen Filtration System with Durapore 1.2-μM filters (Millipore, Bedford, MA). In brief, membranes (10μg protein) were added to wells in triplicate containing different concentrations of radioactively labeled CB1 agonist ([^{3}H]CP 55,940). Addition of the CB1 antagonist AM251 (10μM) was used to

determine non-specific binding. Dissociation constant (K_D) and maximum binding (B_{max}) were determined by nonlinear curve fitting of specific binding data to the single site binding equation using GraphPad Prism (GraphPad 7, La Jolla, CA, USA).

2.5.2. Endocannabinoid Extraction and Analysis—Amygdala samples from 39 perinatal (P14) animals (20 male/19 female; 6 litters) and 34 adolescent (P40 +/− 2) animals (17 male/7 female; 6 litters) as well as 20 animals that were exposed to either no treatment or received vehicle or a FAAH inhibitor (4 naïve, 8 vehicle and 8 FAAH inhibitor of which 50% were females/males and LPS/SAL) and were subjected to a lipid extraction process as described previously (Gray et al., 2015). The content of the two primary eCBs, AEA and 2-AG, within lipid extracts in methanol from brain tissue was determined using isotope-dilution, liquid chromatography–mass spectrometry as described previously (Hermanson et al., 2013).

2.5.3. Fatty Acid Amide Hydrolase (FAAH) Activity Assay—FAAH activity within the amygdala was measured in 17 animals (P40 +/− 2; 10 male/7 female; 6 litters). Membrane samples were processed for FAAH activity by examining the conversion of AEA to ethanolamine as previously described (Hill et al., 2009). In brief, membrane samples were exposed to 0.2 nM 3H-AEA and varying concentrations of cold AEA (0.05–1.5 M) to determine the maximal hydrolytic activity (V_{max}) that FAAH displayed for AEA (Hillard et al., 1995). The binding affinity of AEA for FAAH (K_m) and maximal activity of FAAH (V_{max}) was determined by fitting the data to the Michaelis-Menten equation.

2.6. Pharmacological Reversal of the Phenotype with oral FAAH inhibitor

The cohort for pharmacological manipulation consisted of 32 animals (16 male/16 female; 4 litters) that were given LPS or saline on P14 (see section 2.2). To determine the impact of FAAH inhibition on social behavior in saline and LPS administered animals, we used a previously validated method of oral administration of the FAAH inhibitor PF-04457845, which results in significant and selective elevations in AEA, but not 2-AG in central tissue 4h following administration (Qi et al., 2015). Specifically, on P35 (+/− 1) animals were habituated to handling and the consumption of a 100mg of peanut butter pellet for 4 consecutive days. On day 4 the animals were habituated in pairs to the testing arena for 15 minutes and single housed over night. The following day, test subjects were given a peanut butter pellet containing 1 mg/kg of the selective FAAH inhibitor PF-04457845 or a control peanut butter pellet with no drug. Consistent with our previous work demonstrating elevations in central AEA levels (Qi et al., 2015), we chose a testing window of 4–6h after oral administration of PF-04457845. At that time point, animals were placed in the arena where they encountered a novel rat, matched for treatment, age and sex. Social play and non-play behaviors were scored offline for each rat, according to the same criteria as described in section 2.2. Rats were returned to their former cage mates for 48h and then single housed for another 24h. Subsequently, testing was repeated with a different novel animal following the same procedure as on testing day 1. All testing was done within subjects and treatments were counterbalanced so that animals received vehicle or FAAH inhibitor on either the 1st or 2nd testing day to prevent any order effects. To verify the effect

of the FAAH inhibitor, 20 animals were sacrificed after testing and eCB concentrations were determined (see section 2.5.2).

2.7. Pharmacological Reversal with FAAH Inhibitor Injected into the Amygdala

55 animals (28 males/27 female; 7 litters) were successfully cannulated on P30 (+/−2 days) in the basolateral amygdala (BLA) as described previously (Trezza et al., 2012). Briefly, animals were anesthetized with isoflorane and positioned into a stereotaxic frame. 16mm long 24 gauge stainless steel tubing (coordinates: AP, −1.9 mm; ML, +/− 4.6 mm; DV, −7.5 mm) was implanted bilaterally. After surgery, rats were housed with their previous cagemates to reduce post-operative stress and social isolation. Rats were allowed to recover for 7–10 days. PF-04457845 was dissolved in 5% Tween 80 5% polyethylene glycol and 80% saline and sonicated before use. Animals were handled for 3 consecutive days and separated over night before testing. The drug was infused 2 hours before testing, bilaterally into the BLA (0.2ul per hemisphere over 60 seconds, 10ng/hemisphere), the injector protruding 1mm beyond the guiding cannula. The vehicle served as equivolumetric control. Reciprocal social behavior was recorded over 10 minutes and scored offline by a blinded evaluator. After testing rats were perfused with saline and brains were extracted and preserved in 4% paraformaldehyde. Brains were cryoprotected (20% sucrose) and sliced on the cryostat at 40μm thickness to confirm cannula placements.

2.8. Statistical analysis

GraphPad Prism® (7th edition, San Diego) was used for statistical analysis and curve fitting. Samples were tested for equality of variances using an F-test, considering a p value of $< .05$ as statistically significant. Bonferroni's post-hoc test for multiple comparisons was used to localize significant differences within an analysis of variance (ANOVA). Student's t-tests were applied under a two-tailed assumption ($\mu \neq \mu_0$) unless the null hypothesis clearly stated that a one-sided effect ($\mu > \mu_0$ or $\mu < \mu_0$) was expected.

3. Results

3.1. Social Behavior

P14 LPS injected adolescent animals displayed significantly less combined social behavior (figure 3A), when interacting reciprocally with a non-cagemate of equal age, treatment and sex (n=48, F (1,44) =20.55, $p < .0001$). Post-hoc testing localized showed this effect of early-life inflammation in males (t (22) = 3.328, $p < .01$) and females (t (22) = 3.038, $p < .01$). No significant effect of sex was found (n=48, F (1,44) = 1.064, $p = .3079$). When distinguishing social play and social non-play behaviors (figure 3B and C), P14 LPS exposure was associated with a strong reduction in social non-play behaviors (grooming, sniffing and crawling, combined) as a function of treatment (n=48, F (1,44) = 13.17, $p = .0007$). This was evident in males (n=24, t (22) = 2.348, $p < .05$) as well as females (n = 24, t (22) = 2.784, $p < .05$) without a significant difference in sex (n=48, F (1,44) = 2.520, $p = .6182$). Social play behavior showed larger variability between subjects, yet P14 LPS significantly decreased the time engaged with social play (n=48, F (1,44) = 5.623, $p = .0222$). No effect of sex on time engaged in play was detected (n=48, F (1,44) =1.130, $p = .2935$).

A treatment effect can also be seen when animals encountered a non-treatment matched conspecific. Animals who were exposed to P14 LPS showed decreased combined social behavior (n=46, F (1,42) = 13.17, $p = .0007$) in males (t (44) = 2.348, $p < .05$) and females (t (44) = 2.784, $p < .05$) and social non-play behavior (n=46, F (1,42) = 7.247, $p = .0102$). Play behavior decreased to an average of less than 4 seconds in all groups, making comparison difficult (n=46, F (1,42) = .1242, $p = .7262$). In general, it appears that the saline injected population attempted to initiate contact, while the LPS injected animals avoided these attempts.

3.2. Anxiety-Like Behavior

Analysis of behavior in the open field paradigm (figure 4) indicated no significant differences between treated and control group in time spent in the open (n=32, F (1,28) = .3914, $p = .5366$), engaged with grooming (n=32, F (1,28) = .1778, $p = .6765$), rearing (n=32, F (1,28) = .01010, $p = .9207$), or defecation (n=32, F (1,28) = .1951, $p = .6621$). Additionally, we observed that sexes appeared to behave differently when placed in an open field; females engaged significantly more in rearing than males (n=32, F (1,28) = 10.21, $p = .0034$). We also gathered open field data during the light phase at higher light intensity (~350 lux) on an independent cohort and found, similarly to the dark phase, no difference of LPS on time spent in the open (t (14) = 0.04058, $p = .9682$), grooming (t (14) = 1.145, $p = .2714$) or rearing (t (14) = 0.5878, $p = .5661$) during those conditions (data not presented).

3.3. Endocannabinoid System

AEA and 2-AG were measured in the amygdala of P14 animals that had been exposed to LPS 6h previous. A two-way ANOVA was used to identify differences in treatment, sex effect and possible interactions. P14 LPS exposure leads to a significant decrease in AEA 6h after LPS injection (n=39, F (1,37) = 4.395, $p = .0433$.) No significant treatment effects were seen in 2-AG concentration at the 6h time point. Sex as well as the interaction between treatment and sex, did not significantly contribute to the variation in AEA and 2-AG (figure 5).

In adolescence, LPS mediated changes in social behavior were accompanied by significant changes within the eCB of the amygdala. LPS injected animals showed a decrease in CB1 binding site density (n=18, F (1,16) = 7.28, $p = .0158$), while the CB1 dissociation constant remained statistically non-significant for a treatment effect (n=18, F (1,16) =3.446, $p = .0819$) (figure 6A and B). AEA concentration increased significantly in animals that experienced early-life inflammation (n=32, F (1,30) = 4.927, $p = .0341$) (figure 6C). When analyzing the eCB system, 2-AG (figure 6D) was the only component that showed a significant sex effect (n=32, F (1,30) =8.850, $p = .0057$) as well as a significant interaction between sex and treatment (n=32, F (1,30) =6.910, $p = .0134$), while a main effect of treatment could be detected (n=32, F (1,30) =.3817, $p = .5413$). Remarkably, the maximal velocity of the AEA degrading enzyme FAAH was significantly increased in P14 LPS injected animals (n=15, F (1,13) =8.089, $p = .0138$) (figure 6E), while the binding affinity of AEA for FAAH (Km) was not statistically significant for a treatment effect (n=15, F (1,13) =2.275, $p = .1554$) (Figure 6F).

3.4. Pharmacological Reversal of the Phenotype with Oral FAAH Inhibitor

To determine if alterations in social behavior induced by P14 LPS were reversible by reducing the amplified FAAH activity, thereby further augmenting AEA signaling, we examined the impact of a pharmacological intervention with an oral dose of the FAAH inhibitor PF-04457845. No effect of sex on the treatment could be detected; therefore data was collapsed to increase the statistical power. First, we confirmed that the FAAH inhibitor indeed increased AEA concentration in the amygdala (figure 7). PF-04457845 significantly elevated AEA concentrations (n=20, F (2,17) = 37.34, $p < .0001$) 6h after oral administration of the drug when compared to naïve (t (17) = 6.917, $p < .0001$) and vehicle (t (17) = 7.554, $p < .0001$). Administration of the vehicle alone did not significantly affect AEA concentration (t (17) = .7493, $p > .99$) when compared to naïve animals. No main effect of PF-04457845 on 2-AG could be detected 6h after drug administration within the amygdala (F (2,17) = .4881, $p = .6221$). As expected, there was no significant effect of sex or perinatal treatment on the drug-mediated elevation of AEA, therefore data of both sexes and perinatal treatments were collapsed to allow for better comparison of the drug effect.

Next, we confirmed the predicted decrease in social behavior using a one-tailed t-test ($\mu > \mu_0$, t (30) = 2.005, $p = .027$) (figure 8A). Analysis of the combined social interaction time (figure 8B) in a repeated measures ANOVA design showed a significant interaction between early-life treatment and the drug response (n=32, F (1, 30) = 6.222, $p = .0086$). Bonferroni's test for multiple comparisons further revealed that PF-04457845 significantly increased total social interaction time of P14 LPS injected animals (n=32, t (30) = 2.510, $p < .05$), while no significant effect on saline injected animals could be detected (n=32, t (30) = 1.467, $p > .05$). We conclude that the FAAH inhibitor PF-04457845 rescues the deficient social behaviour of rats that experienced P14 LPS. In combined social behavior, no differences between sexes could be observed and the data were collapse (tested in a three-way ANOVA, Sex: F (1,1) = 0.3838, $p = .5381$; Drug x Sex: F (1,1) = 0.1047, $p = .7475$; Sex x Perinatal Treatment: F (1,1) = 0.08907, $p = .7665$; Sex x Perinatal Treatment x Drug: F (1,1) = 0.06673, $p = .7971$). However, when analyzing the different modalities of social behaviour (social play and social non-play), we were able to replicate in our control group the FAAH inhibitor-mediated increase in male rat play behaviour shown by Trezza et al. (2012) (tested in a paired t-test, $\mu < \mu_0$, t (7) = 1.912, $p < .0488$, data not shown). Female play behaviour, however, does not change significantly in response to a FAAH inhibitor when compared to control (Wilcoxon ranked sum test, W = −10, $p = .1250$). After P14 LPS injection, oral administration of a FAAH inhibitor to adolescent animals does not significantly affect play behaviour in males or females (tested in a paired Wilcoxon ranked sum test, W=−15, $p = .3281$ and a paired t-test, t (7) = 1.378, $p = 2107$, respectively; data not shown).

3.5. Administration of a FAAH Inhibitor into the Basolateral Amygdala

Administration of a FAAH inhibitor (10ng) bilaterally into the BLA produces a variety of effects (figure 9). A significant interaction between the effect of the FAAH inhibitor and perinatal exposure to LPS can be seen in female combined social behavior (n=27, F (1, 23) = 14.43, $p = .0009$). In males, no main effect of the drug (n=28, F (1, 24) = .2656, $p = .611$) or interaction between drug and prenatal treatment (n=28, F (1, 24) = .00001, $p = .99$) could be found on combined social behaviour. Adolescent females that experienced a perinatal LPS

injection interacted significantly less with conspecifics than those who were injected with vehicle (t (23) = 2.486, $p < .05$). Furthermore, LPS females who received a FAAH inhibitor into their BLA, showed significantly increased social behaviour (t (23) = 3.537, $p < .01$) when compared to LPS animals that received vehicle, as well as compared to saline animals that received the FAAH inhibitor (t (23) = 2.956, $p < .05$). Males showed a significant interaction between perinatal treatment and drug response (F (1, 24) = 5.43, $p = .0285$) specifically in play behaviour, which increased in response to the FAAH inhibitors in control animals but decreased in LPS injected animals.

4. Discussion

With this research, we have shown that a single immune challenge during a sensitive developmental window can affect adolescent social behavior. Furthermore, early-life inflammation acutely altered AEA levels and evoked a multi-level reprogramming of the amygdalar eCB system. In adolescent animals, P14 LPS injections were associated with reduced CB1 receptor binding site densities, elevated levels of AEA and, surprisingly, increased levels of AEA hydrolysis by FAAH. Interestingly, pharmacological inhibition of FAAH was able to reverse the behavioral changes caused by postnatal LPS to control levels, while control animals were unaffected. This would suggest that despite increased levels of AEA, increased levels of FAAH activity are involved in the deficits in social behavior following LPS exposure and that suppression of FAAH activity can ameliorate these changes. In females, administration of 10ng PF-04457845 directly into the BLA was sufficient to increase social interaction to control levels. Remarkably, again in control animals, the drug has little effect on male and female combined reciprocal social interaction, but appears to specifically increase male play behavior.

To date we lack a comprehensive understanding of how inflammation affects the developing brain. Early-life inflammation at P14 appears to create a long-term shift in eCB signaling: in adolescence CB1 receptor binding is decreased, AEA levels are increased and, somewhat paradoxically, FAAH activity is increased. These data are consistent with previous reports that early-life inflammation can downregulate CB1 receptors in the amygdala (Zavitsanou et al., 2013) and supports the hypothesis that early-life LPS may affect and potentially disturb the function of the adolescent eCB system. It is surprising that 6 hours after LPS exposure AEA is decreased in LPS injected animals, as previous reports indicate that the cytokine TNF-α as well as activation of Toll-like receptor 4, both downstream targets of LPS, increase AEA synthesis in hypothalamic fragments (Fernandez-Solari et al., 2006) and in peripheral macrophages (Liu et al., 2006). However, given that CRH is known to increase AEA hydrolysis and reduce AEA levels in the amygdala (Gray et al., 2015), we hypothesize that a potential upregulation of CRH within the amygdala may be a contributor to such change. CRH signaling is enhanced by inflammatory stimuli, and CRH mRNA upregulation in response to LPS has been shown, for example in the paraventricular nucleus of the hypothalamus (Dent et al., 1999).

It remains to be identified if the residual changes in the eCB system are dependent upon each other, e.g. that upregulation of FAAH is related to the down-regulation of CB1 receptors, or if components are altered relatively independent from one another. It has

recently been demonstrated that AEA interacting with membrane cholesterol can directly increase FAAH activity (Dainese et al., 2014). It is possible that despite the initial decline AEA levels increase as a consequence of the inflammatory event, and that this constitutive elevation in AEA levels could in turn drive the increase in FAAH activity that persists long after resolution of the inflammation. Alternatively, a recent study in humans has demonstrated a similar inverse relationship between AEA content and CB1 receptors, such that low levels of circulating AEA correlated to increased levels of CB1 receptor binding density (Neumeister et al., 2013). As such, the increase in AEA could be involved in CB1 receptor downregulation, although this seems unlikely as FAAH-deficient mice, which have dramatic elevations in AEA far beyond what is seen here, have normal CB1 receptor densities (Lichtman et al., 2004). Accordingly, a third possibility is that P14 LPS results in a downregulation of CB1 receptors, through an unknown mechanism, to which a compensatory increase in AEA evolves in an attempt to maintain homeostatic levels of AEA-CB1 receptor signaling. This compensatory increase in AEA could in turn trigger the increase in FAAH activity described above, thereby preventing AEA to compensate for reduced CB1 signaling leading to the emergence of deficits in social behavior. This hypothesis would be consistent with our data demonstrating that FAAH inhibition normalized the deficits in social behavior as it removed the increased FAAH activity and allowed a greater increase in AEA signaling to occur and compensate for the deficiency in CB1 receptors in the amygdala. While speculative, this model would account for the biochemical and behavioral data generated herein, and will be the subject of further investigation to determine the causal sequence of events determining the trajectory of these changes.

Social behavior is a unique and complex process that is dependent upon hedonic aspects as well as anxiety levels. The amygdala is a crucial component in regulating social behavior, especially in reciprocal interactions with novel animals. Amygdala lesions have been shown to specifically increase social interactions with non-familiar animals (shown in primates by Amaral, 2003). Consistently, increasing output from the BLA is known to decrease social behavior and increase anxiety-like phenotypes (Felix-Ortiz and Tye, 2014). With respect to the eCB system, CB1 receptor signaling in the BLA can regulate both, glutamatergic and GABAergic transmission. Based on the aforementioned data regarding amygdala activity and social behavior, the most parsimonious hypothesis is that inhibition of FAAH and amplification of AEA signaling is suppressing the excitability and outflow of BLA neurons, which consequently increases social behavior. Interestingly, inhibiting FAAH only restores the combined social behavior of P14 LPS rats, but does not increase combined social behavior in control rats. A potential explanation is that AEA signaling may not normally impact these social behavior-regulating amygdala neurons, but that early-life inflammation changes eCB regulation of these neurons and makes them them sensitive to increased AEA signaling following FAAH inhibition. The systemic route of administration of our drug, while demonstrating potential translational benefit of FAAH inhibitors for deficits in social behavior, limits us in determining the exact location of the effect. However, since intra-amygdalar administration is also effective (at least in females) we can conclude that the likely site of action is localized in the amygdala. This is in keeping with previous findings that decreasing FAAH activity in the amygdala alone can cause increases in social (play)

behavior through a CB1 receptor dependent mechanism (Trezza et al., 2012). We see a similar increase in play with oral FAAH inhibition in males as well as amygdalar FAAH-inhibition, yet combined social behavior (which is mostly composed of social non-play behavior) appears only affected by a FAAH inhibitor in animals that have been pre-exposed to LPS.

In conclusion, our results suggest that FAAH inhibitors may provide a novel approach for the treatment of social disorders. Particularly, in disorders with high amygdala output and altered eCB system components (e.g. ASD), FAAH inhibition could stabilize the eCB system and decrease symptoms. FAAH inhibitors have been tested previously in the fragile X mouse model of autism, and showed promising effects in alleviating symptoms (Qin et al., 2015). Thorough testing of FAAH inhibition in a variety of ASD models will hopefully provide more insight in the intricate mechanisms of ASD and may lead to novel approaches to its treatment.

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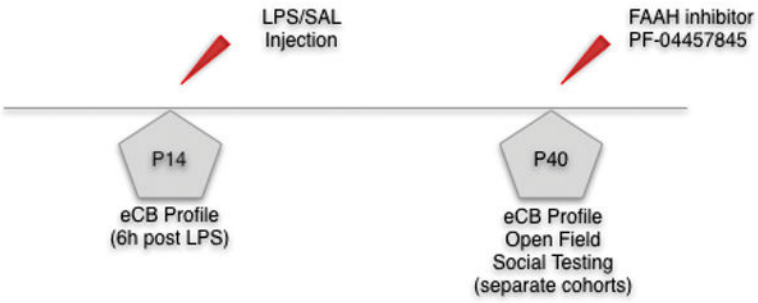

FIGURE 1. Timeline of experimental procedures and testing

Animals were injected on P14 with LPS or Saline. Components of the eCB system were measured 6h after LPS injection on P14 as well as in adolescence (24 days after LPS injection). In adolescence, animals underwent testing in the open field as well as a social behavioral challenge. Interventions with a FAAH inhibitor were also performed during adolescence to reverse the effect of LPS on social behavior. Every experiment was performed on a separate cohort in both males and females.

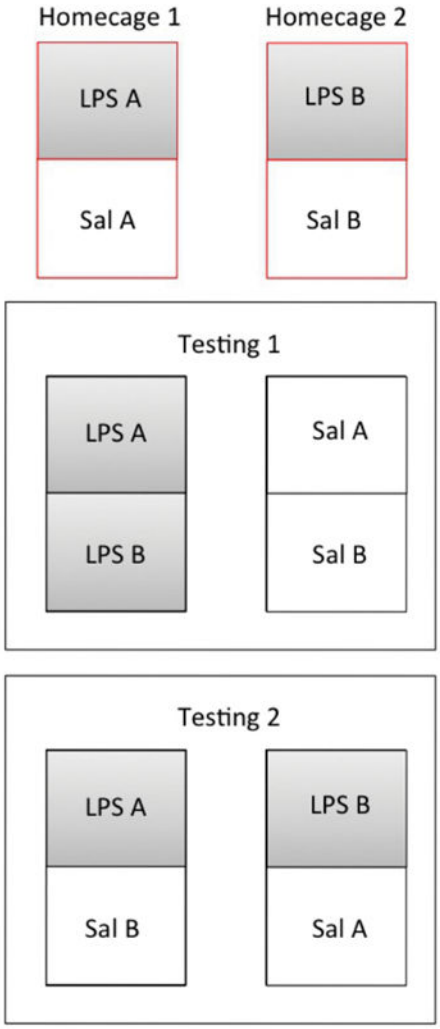

FIGURE 2. Experimental design to assess reciprocal social behavior

Animals at ~P40 were separated overnight and subsequently exposed to either a treatment matched or differently treated partner and social interaction was recorded. Following this encounter, animals returned to their home cage for 24h; they were then separated overnight and subsequently encountered another unfamiliar counterpart.

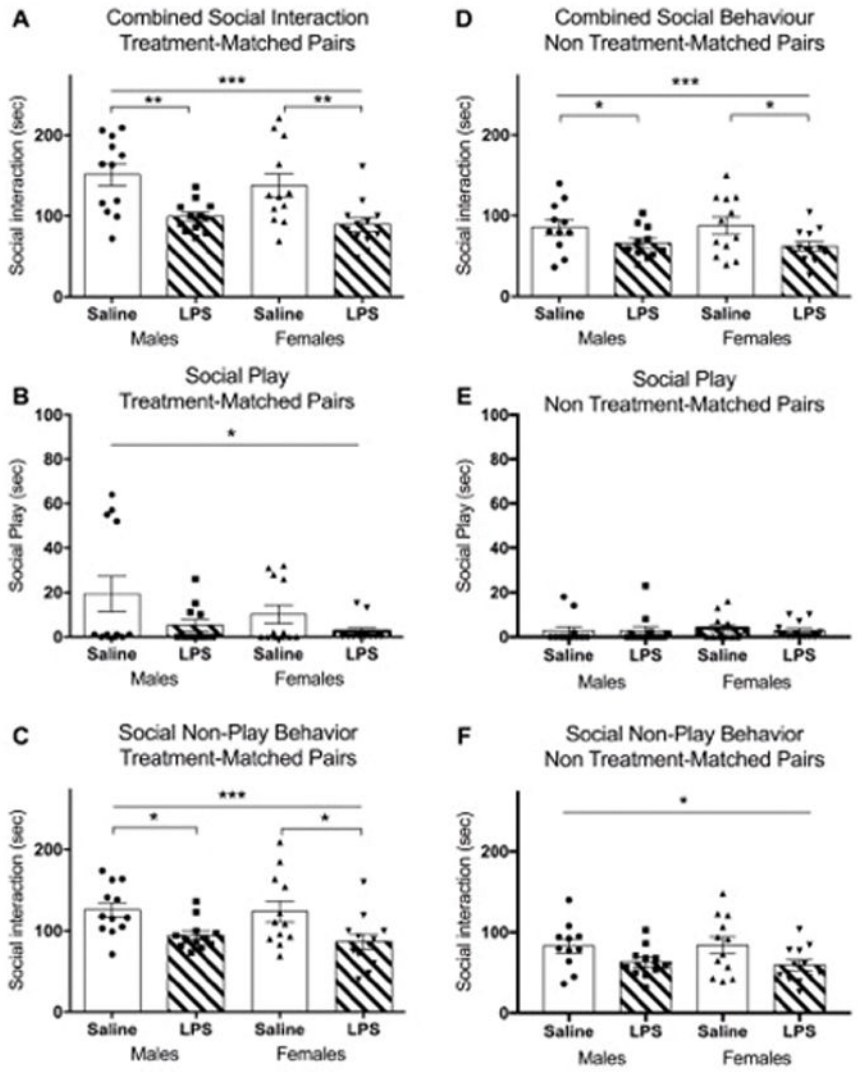

FIGURE 3. Social play and social non-play behavior

(A & D) Combined social behavior (play and social non-play) **(B & E)**, social play and **(C & F)** social non-play behavior (grooming, crawling over/under and sniffing) on P40 (+/− 2) in LPS and control treated animal. Figures **A–C** show pairings matched for treatment, while **D–F** show non-treatment matched pairings. No significant differences in sex could be detected. In this and subsequent figures, bars indicate mean (+SEM) and individual animal values are plotted to clarify spread or the samples. Two-way analysis of variance Bonferroni post hoc testing was used to analyze the data. * $p < .05$, *** $p < .001$

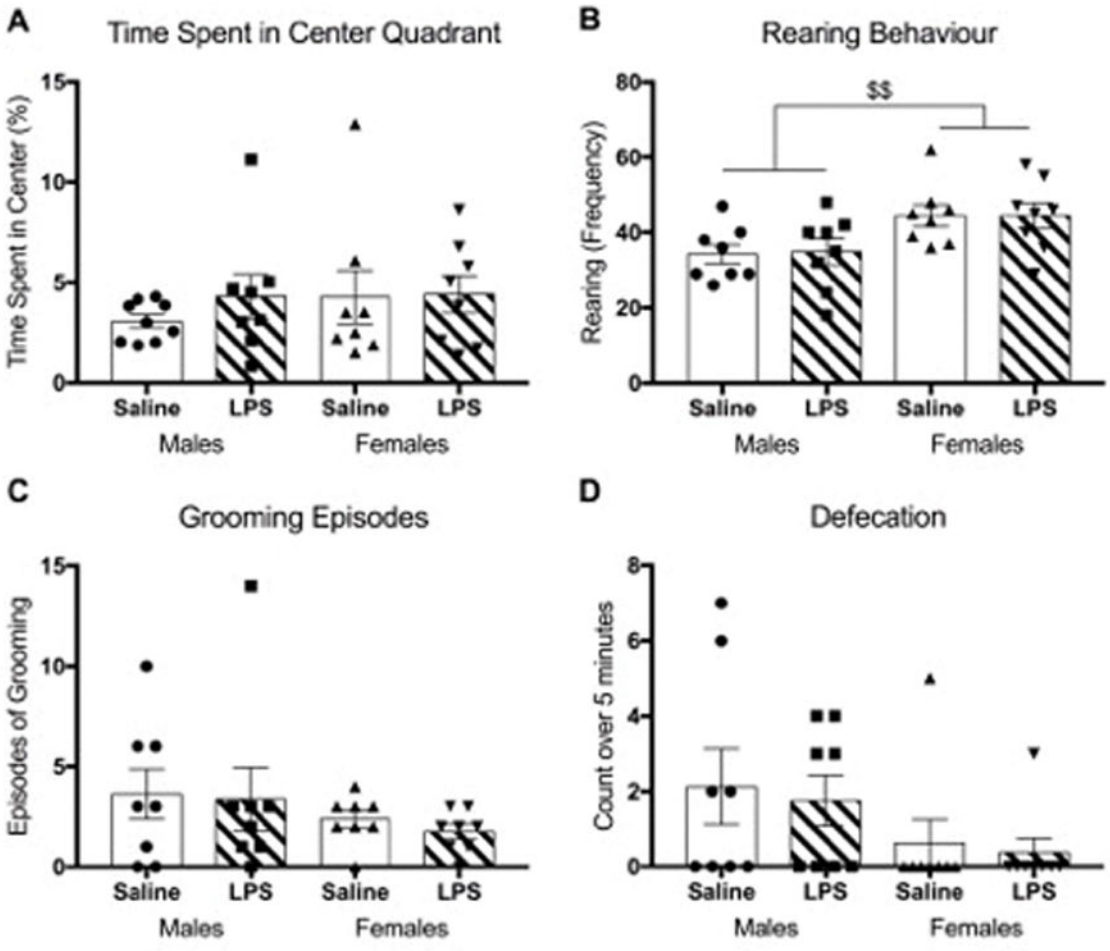

FIGURE 4. Open field behavior

Open field behavior of P40 rats over 5 minute that received LPS or saline on P14. **A)** Percentage of time spent in the open quadrant, **B)** time spent with grooming behavior, **C)** mean rearing frequency and **D)** amount of defecation during the 5 minute trial period. Two-way analysis of variance was used to analyze the data. $$ significant main effect of sex, $p <.01$

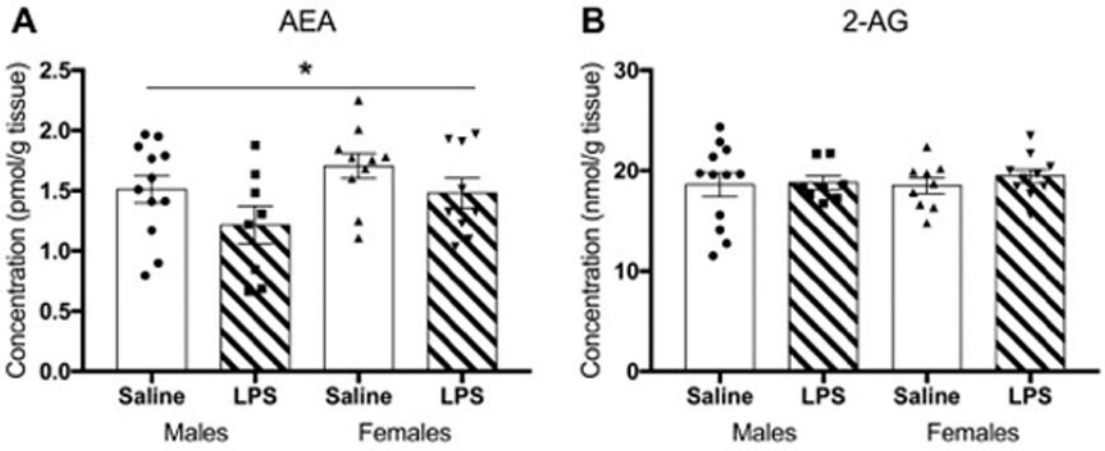

FIGURE 5. eCB concentrations in the amygdala on P14 6h following LPS

A) Anandamide (AEA) and **B)** 2-arachidnoyl glycerol (2-AG) concentration subsequent to an LPS challenge on P14. LPS significantly decreases AEA 6h after LPS exposure in males and females. A two-way analysis of variance was used to analyze the data. * significant main effect of LPS $p < .05$

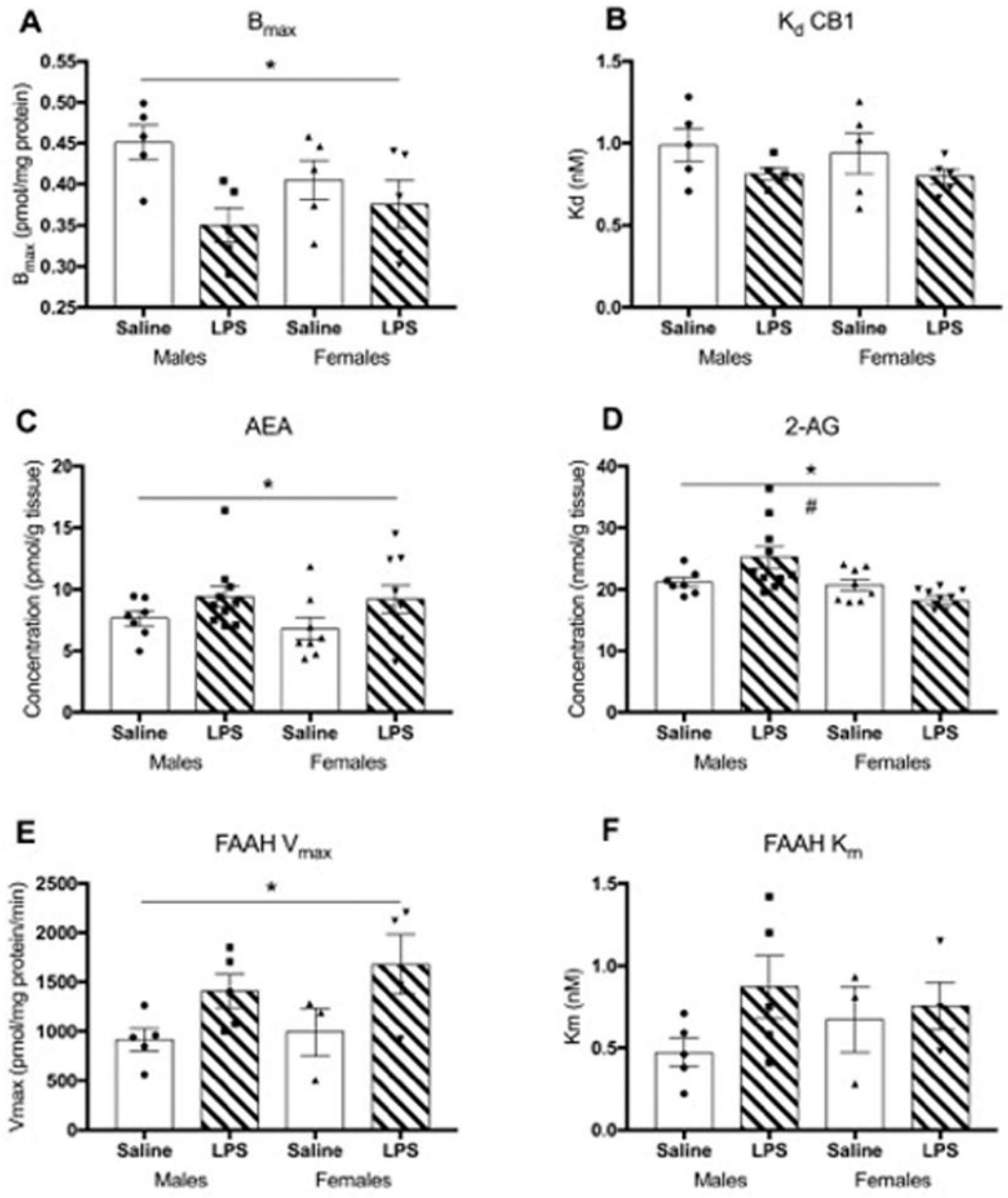

FIGURE 6. The adolescent (P40) eCB system in the amygdala following P14 LPS

Average (+SEM) in **A)** maximal cannabinoid receptor 1 (CB1) binding, **B)** CB1 binding site density, **C)** anandamide (AEA) and **D)** 2-arachidonoylglycerol (2-AG) content, **E)** maximal fatty acid amide hydrolase (FAAH) velocity (Vmax) and **F)** Michelis-Menten constant (Km). A two-way analysis of variance was used to analyze the data. * significant main effect of LPS $p < .05$, # significant interaction $p < .05$, $$ significant sex effect $p < .01$.

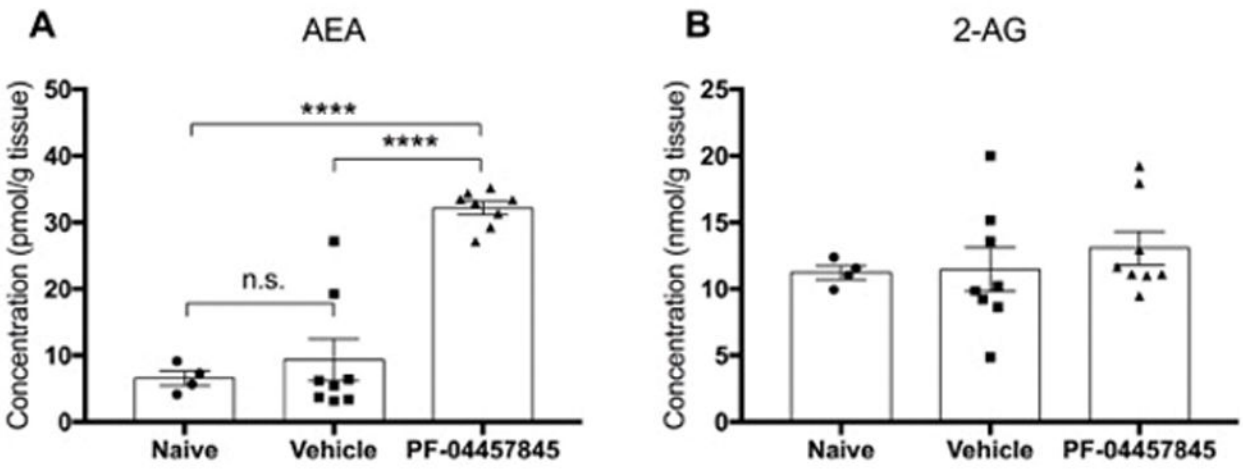

FIGURE 7. AEA and 2-AG concentrations in the amygdala of naïve, vehicle or FAAH inhibitor treated animals

A) Anandamide (AEA) and **B)** 2-arachidonoylglycerol (2-AG) in the amygdala after oral administration of a FAAH inhibitor. Naïve animals never received vehicle or drug before. Vehicle animals received vehicle (peanut butter) 6–8h before they were sacrificed and received a FAAH inhibitor in a previous testing session (>48h before). The PF-04457845 group received the drug 6h before they were sacrificed. A two-way analysis of variance was used to analyze the data. **** $p < .0001$ in Bonferroni's test for multiple comparisons.

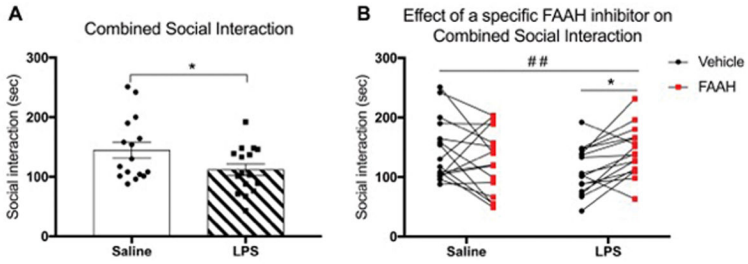

FIGURE 8. Total time spent in social interactions with and without FAAH inhibitor

A) P14 LPS decreases adolescent social behavior. * $p < .05$ in one-sided t-test **B)** Social behavior of P14 LPS/Saline treated rats (50% male and female) that have received the FAAH inhibitor PF-04457845. Repeated measures ANOVA revealed a significant interaction between early-life LPS and drug treatment. LPS animals increase significantly in social interaction time when treated with the FAAH inhibitor, however, no significant effect on the saline treated controls can be observed. A repeated measures analysis of variance was used to analyze the data. ## early-life treatment X drug treatment interaction, $p < .01$; * $p < .05$ in Bonferroni's test for multiple comparisons.

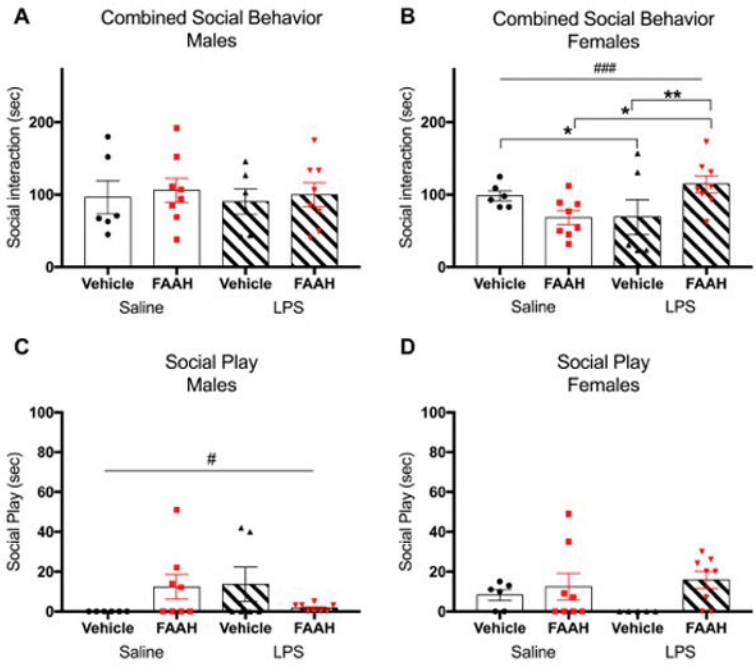

FIGURE 9. Social behavior and play behavior after injection of PF-04457845 into the basolateral amygdala

A) and **B)** Combined social interaction (social play and non-play behaviors) increases in females who were exposed to LPS on P14 (### early-life treatment X drug treatment interaction $p < .001$; * $p < .05$ and ** $p < .01$ in Bonferroni's test for multiple comparisons) **C)** Social play and drug treatment show a significant interaction in males (# $p < .05$). **D)** Females show a trend of increased social play with administration of a FAAH inhibitor.