Fibroblast Growth Factor 2 alters the oxytocin receptor in a developmental model of anxiety-like behavior in male rat pups

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Abstract

We aimed to determine the short-term effects of early-life stress in the form of maternal separation (MS) on anxiety-like behavior in male rat pups. In order to assess anxiety, we measured 40 kHz separation-induced ultrasonic vocalizations (USV) on postnatal day (PND) 11. We further aimed to evaluate the potential involvement of two neurochemical systems known to regulate social and anxiety-like behaviors throughout life: oxytocin (OT) and fibroblast growth factor 2 (FGF2). For these purposes, we tested the effects of neonatal administration (on PND1) of an acute dose of FGF2 on USV and its potential interaction with MS. In addition, we validated the anxiolytic effects of OT and measured oxytocin receptor (OTR) gene expression, binding and epigenetic regulation via histone acetylation. Our results show that MS potentiated USV while acute administration of OT and FGF2 attenuated them. Further, we found that both FGF2 and MS increased OTR gene expression and the association of acH3K14 with the OTR promoter in the bed nucleus of the stria terminalis (BNST). Comparable changes, though not as pronounced, were also found for the central amygdala (CeA). Our findings suggest that FGF2 may exert its anxiolytic effects in male MS rats by a compensatory increase in the acetylation of the OTR promoter to overcome reduced OT levels in the BNST.

Keywords:

Anxiety, Fear, Stress, FGF2, Oxytocin, Depression

Introduction

Chronic anxiety is a stress-related disorder determined by interacting genetic susceptibilities and experiential factors. There are early-life behavioral manipulations in rodents, such as maternal separation (MS), that promote anxiety-like behavior and are thus used as models to study this condition. In fact, a plethora of research supports the validity of this approach, as MS significantly increases anxiety-like behavior in adult rodents (Caldji et al., 2000; Lehmann and Feldon, 2000; Sanchez et al., 2001; Eiland and McEwen, 2012).

Research in animals and humans suggests that oxytocin (OT) is prosocial and facilitates trust and affiliation by inhibiting systems involved in fear and anxiety (Murgatroyd et al., 2004; Lim and Young, 2006; Carter, 2007; Heinrichs and Domes, 2008). Accordingly, OT and its receptor (OTR) are prevalent throughout neural circuits that mediate emotionality, stress and anxiety, such as the amygdala, hypothalamus, bed nucleus of the stria terminalis (BNST) and lateral septum. Similarly, fibroblast growth factor-2 (FGF2) is a key molecular regulator of anxiety-like behavior (Turner et al., 2012a, 2012b). Previous results show that an acute dose of FGF2 on postnatal day (PND) 1 alters hippocampal development and decreases anxiety-like behavior in highly anxious adult rats without changing other behaviors (Turner et al., 2011). Although mechanisms of FGF2 action on anxiety-like behavior are as of yet largely unknown, epigenetic mechanisms have recently been implicated (Chaudhury et al., 2014), such as effects on the glucocorticoid receptor (GR) (Chaudhury et al., 2014).

In this series of experiments, we hypothesized that experiential factors in the form of MS may interact with FGF2 to affect the OTR, which in turn regulates anxiety-like behavior in early-life in male rat pups. OTR may be downstream of FGF2 in the modulation of anxiety-like behavior and epigenetic mechanisms may play a role. In order to test our hypothesis, we first validated the ability of OT to modulate 40 kHz separation-induced ultrasonic vocalizations (USV), an index of anxiety-like behavior in rat pups separated from their dam (Hofer et al.,

2002), during their peak expression on postnatal day (PND) 11. Afterwards, we examined the effect of FGF2 in MS. Finally, we determined OTR gene expression, receptor binding levels and promoter acetylation in two regions that are rich in OTR, comprise the extended amygdala and are implicated in anxiety-like and social behaviors (Lukas et al., 2010), namely the BNST and the central amygdala (CeA).

Material and Methods

Animals

Pregnant Sprague Dawley rats (Charles-River) were received 15-18 days into gestation and individually housed on a 12:12 light-dark cycle under controlled temperature (21 ± 2 °C) with water and food ad libitum. On postnatal day 1 (PND1), litters were culled to a maximum of 10 pups each litter. Cages were changed twice, once when the groups were sexed on PND1 and an additional time on PND7 or 8. Only males were included in the analyses. Procedures were authorized by the Institutional Animal Care and Use Committee (IACUC) at The Rockefeller University and were performed in accordance with NIH guidelines of care and use of animals for experimental procedures.

Maternal Separation

Groups were divided into those that received MS and those that were left unseparated. Litters designated for MS were separated from their respective dams for 3 hours daily starting on PND2 until behavioral testing on PND11. Dams were placed into a new cage for the duration of the separation and litters were taken from the nesting cages and placed into new, smaller cages. Separated pups were placed in an area maintained at 34°C in a different room than the dams. After the separations, pups were returned to their home cages and then reunited with the dam. Controls remained in their home cages and were undisturbed, except for cage changes.

Drugs

To determine the effects of acute OT administration on USV, outbred animals were injected intraperitoneally (i.p.) with one of four doses of OT (Tocris Bioscience; 0.5 mg/kg (n=10), 1 mg/kg (n=11), 2 mg/kg (n=9), 5 mg/kg (n=10) or vehicle (ddH₂O; n=8) on the day of USV testing (PND11, see below).

To test the effects of early life FGF2 administration, a separate group of rats were injected with either FGF2 (20 ng/g in 50 µl, s.c.; Sigma-Aldrich) or vehicle (0.1M PBS with 0.1% BSA, s.c.) on the day after birth (PND1).

Behavioral testing (see Figure 1)

In all experiments male rat pups were tested for USV on PND11. The oxytocin dose-response study was aimed at validating previous findings showing behavioral changes following peripheral OT injections (Insel and Winslow, 1991; Hofer et al., 2002). Animals were tested 30 minutes after injections.

The goal of MS was to assess the effects of FGF2 on USV, an experimentally-induced anxiety-like behavior. For the MS study, the number of animals per group was as follows: No MS VEH (n=11), No MS FGF2 (n=9), MS VEH (n=10), and MS FGF2 (n=10). The dam was removed and remained in the housing room while pups were moved to the testing room for at least 30 minutes of acclimation in a 34°C incubator (Airshields C-100 incubator). Pups were tested on PND11 immediately after acclimation.

Each pup was tested for USV individually in a novel chamber (32°- 34°C) for five minutes. During this time, 40 kHz USV were counted using the UltraSoundGate condenser microphone NCMX-HD (Avisoft, Bioacoustics, Germany). The microphone was routed through an Ultrasoundgate amplifier (416H, gain set at 5.5 notches) connected by a USB Audio Device

to a computer that contained Avisoft-Recorder USGH Software (Avisoft Bioacoustics). For all recordings, USV were recorded within a range of 20-120 kHz, with a sample rate of 250 kHz. Some animals in the MS study were sacrificed immediately after testing for mRNA *in situ* hybridization and *in vitro* autoradiography.

mRNA *in situ* hybridization

For the *in-situ* hybridization analysis we used 5-7 animals per group. The oxytocin receptor transcript (OTR, NM_012871; pos. 379-1107) was generated and tested in-house. PND 11 brains were rapidly removed and snap frozen in isopentane (-30°C) and then stored at -80°C. The brains were then sectioned at 30 µm at -20°C on Superfrost Plus slides (Thermo Fisher Scientific, Waltham, MA) and stored at -80°C until processing. Briefly, slides were immersed in 4% paraformaldehyde for one hour and then rinsed in 2X SSC three times for five minutes each. The slides were then incubated in 1M TEA with 0.25% acetic anhydride for ten minutes. The sections were rinsed in water and then dehydrated through graded ethanols. Sections were then hybridized in a buffer consisting of 50% formamide, 10% dextran sulfate, 3X SSC, 50 mM sodium phosphate, pH 7.4, 1X denhardts, 0.1 mg/ml yeast tRNA and 10mM dithiothreitol. The ³⁵S-labeled riboprobe was added to the buffer for a final dpm of 2 million/slide. The slides were hybridized at 55°C overnight. The next day, the slides were rinsed three times in 2X SSC. The slides were then rinsed in RNase A solution (10mM Tris-HCL, 0.5M NaCl, 200 µg/ml RNase A, pH 8.0) at 37°C for one hour. The slides were then rinsed in 2X SSC, 1X SSC, 0.5X SSC for five minutes each. The slides were then incubated in 0.1X SSC for one hour at 65°C. Finally, the slides were rinsed in water, dehydrated and dried. The slides were then exposed to Kodak Biomax MR film for 19 days. The film was developed and scanned using a ScanMaker 1000XL Pro (Microtek, Carson, CA) with LaserSoft Imaging software (AG, Kiel, Germany). Digitized images were analyzed using ImageJ software (<http://rsbweb.nih.gov/ij/>).

Signal, as defined by 3.5X the standard deviation of individual pixel signal values above mean background signal, was converted to optical density. The background was subtracted from the signal for an optical density value averaged across samples per animal for each probe.

Templates were created to analyze brain areas associated with emotion and sociality including the BNST, medial amygdala, CeA (-1.8 to -2.8), CA1 region of the hippocampus and lateral septum according to Paxinos and Watson (1998). The BNST was analyzed with using data combined from two different templates (rostral: 0.2 to -0.26; caudal: -0.3 to -0.4).

In vitro autoradiography

For the *in vitro* autoradiography analysis we used 5-7 animals per group. Animals were sacrificed immediately after testing on PND11 via decapitation after which their brains were removed and quickly frozen in chilled (-40°C) methylbutane on dry ice. Brains were then stored at -80 °C. When ready (not more than 1 month post freezing) the brains were sliced coronally on a cryostat (-20°C) to 30 µm sections and mounted onto slides. Autoradiography was performed, per the procedure outlined in Lukas et al. (2010), with a linear OT-R antagonist [125I]-d(CH₂)₅[Tyr(Me)₂-Tyr-NH₂]₉-OVT (NEX254010UC, Perkin Elmer, USA). Slides were stored in an -80° freezer. When ready for autoradiography, slides were taken out of the freezer and placed in a desiccator for one hour to thaw prior to fixing in 0.1% paraformaldehyde for 2 min, followed by 2 x 10 min washes in Tris buffer (50mM Tris pH 7.4), after which slides incubated in tracer buffer (50 pM tracer, 50mM Tris, 10mM MgCl₂, 0.01% BSA) for 1 hour. Probe was washed off (3 x 7 min 50mM Tris, 10mM MgCl₂ buffer) and slides were set in fresh Tris MgCl₂ buffer for 30 min (stirring), dipped in distilled, de-ionized water, and then dried with a fan. Once dry, slides were placed into a cassette with BioMax MR Kodak film (Cedex, France) for 7 days. Films were developed using the Kodak M35A X-OMAT Processor. This protocol was adopted with guidance from Dr. Alexa Veenema at Boston College. The optical densities of OTR were

measured using MCID Elite software (Vision Systems Design, NH). Identical brain regions were analyzed here as with *in situ* hybridization.

Chromatin immunoprecipitation (ChIP) Assay

In a separate set of animals, the target brain regions (CeA and BNST) at P11 were dissected from MS/No MS (n=6/group) rat brains collected at PND11 by a punch method using a 19 gauge guided syringe (C310GA/TW/SP). Tissue was not pooled. The dissected regions were cross-linked in 1% paraformaldehyde for 15 min at 25°C. This cross-linking reaction was stopped by the addition of freshly prepared glycine to a final concentration of 0.125 mol/L followed by four washes in cold 0.1 M phosphate-buffered saline. The tissues were then homogenized in a cell lysis buffer (1X PBS, 0.4% NP-40, 10µl/5ml complete protease inhibitors cocktail, Abcam, USA) and kept for 15 min at 4°C. The homogenate was first centrifuged at 200Xg for 2 min to pellet extracellular debris, followed by centrifugation at 5500Xg for 5 min of the supernatant. The pellet were suspended in the nuclear lysis buffer (50 mM Tris–HCl pH 8.0, 10 mM EDTA, 1% sodium dodecyl sulfate, protease inhibitors cocktail), and using the Sonicator (Branson, USA), the extracted chromatin was sheared to 200– 300 bp for proper binding with antibody. The amount of DNA was quantified and a 1/5 part of lysate was aliquoted before immunoprecipitation (input), so as to compare the relative binding to the specific antibody in different samples before and after immunoprecipitation. The chromatin was then subjected to immunoprecipitation using antibodies against anti-ach3K14 (Abcam, USA) overnight at 4°C. As an experimental control for the specificity of antibody binding, non-immune rabbit IgG (Abcam, USA) and a no-antibody immunoprecipitation were performed for each chromatin sample. Protein-DNA-antibody complexes were precipitated with Dynabeads@protein A (Invitrogen, USA) for 2 h at 4°C, followed by two washes each in low salt, high salt, LiCl and 1X TE buffers. The precipitated protein-DNA complexes were eluted from the antibody with 1% sodium dodecyl

sulfate and 0.1 M NaHCO₃, and then reversed-cross-linking was done in 0.3 M NaCl at 65°C overnight. Proteins were digested with proteinase K for 1 hr at 45°C. The DNA were extracted and purified (Qiagen, USA). Primers directed to the gene promoter OXTR (F 5'-CTTGAAGCAGGAGGTGAAG-3' and R 5'-TTTCCCCAAGAACAAGAACG-3') were used for amplification. Following PCR, the 'Input' and immunoprecipitated DNA amplification reactions were run on 1% Agarose gels. Immunoprecipitated DNA normalized to 'Input' was obtained using Image J (NIH Software).

Statistical Analyses

A one-way ANOVA was used followed by Dunnett's post hoc test for the OT administration study. For all other studies, a two-way analysis of variance was used followed by Newman-Keuls post hoc test. Statistica (Statsoft) software was used to analyze all data. Data are presented as means and S.E.M. Effect size estimates were calculated using partial eta squared for ANOVAs.

Results

Oxytocin administration decreased USV

We first sought to determine whether peripheral OT administration had an effect on USV at PND11. As shown in Figure 2, vehicle-treated control rats emitted an average of 488 USV during the five-minute test, indicating they were responding to the separation procedure with an anxiety response. An acute administration of OT significantly decreased the number of USV emitted ($F_{(4,43)}=8.42$, $p<0.001$; eta squared=0.23) in a dose-dependent manner. All four doses tested were significantly different from vehicle.

MS increased USV and FGF2 decreased USV

MS significantly increased the number of USV emitted ($F_{(1,37)}=6.83$, $p=0.01$; eta squared=0.02; see Figure 3A), an index of increased anxiety-like behavior. Since FGF2 decreased anxiety-like behavior in adulthood in a genetic model of anxiety (Turner, 2011), we sought to determine if FGF2 would also reduce USV in an experiential model of anxiety in early-life. FGF2 significantly decreased the number of USV within a five minute testing duration ($F_{(1,37)}=18.98$, $p<0.001$; eta squared=0.06; see Figure 3B). There were no significant interactions.

FGF2 significantly increased OTR gene expression and receptor binding in the BNST

FGF2 significantly increased OTR gene expression in the BNST by mRNA *in situ* hybridization ($F_{(1,20)}=15.42$, $p<0.001$; eta squared=0.07; Figure 4). FGF2 also significantly increased OTR receptor binding in the BNST by *in vitro* autoradiography ($F_{(1,15)}=5.71$, $p=0.03$; eta squared=0.01). There was no main effect of MS in the BNST on either gene expression or receptor binding. However, a significant interaction was observed ($F_{(1,15)}=5.00$, $p=0.04$; eta squared=0.01) by *in vitro* autoradiography in the BNST, see Figure 5. Here, No MS animals treated with FGF2 exhibited more OTR receptor binding than No MS animals treated with vehicle. In summary, FGF2 increased OTR binding in No MS rats, suggesting that the effects of FGF2 on OTR binding depended on whether or not the animal experienced early life stress.

For comparison, we also assessed OTR receptor binding and gene expression in the CeA. As shown in Figure 6, MS significantly increased OTR gene expression in the CeA by mRNA *in situ* hybridization ($F_{(1,20)}=6.12$, $p=0.02$; eta squared=0.02). However, no effect was observed on receptor binding. There was no effect of FGF2 in the CeA for either gene expression or receptor binding, nor was there any interaction.

MS and FGF2 increased the association of acH3K14 with the OTR promoter in the BNST

Consistent with an epigenetic regulation of OTR, MS significantly increased the association of acH3K14 at the OTR promoter (i.e. acetylation) in the BNST ($F_{(1,28)}=147.88$, $p<0.001$; eta squared=0.01) compared to No MS animals. Moreover, FGF2 significantly increased the association of acH3K14 at the OTR promoter in the BNST ($F_{(1,28)}=96.67$, $p<0.001$; eta squared=0.01) compared to vehicle controls. There was also a significant interaction in the BNST ($F_{(1,28)}=19.74$, $p<0.001$; eta squared=0.00). As shown in Figure 7, MS animals treated with FGF2 had significantly more association of acH3K14 at the OTR promoter than No MS animals treated with FGF2 ($p<0.005$), and MS animals treated with vehicle ($p<0.001$). Moreover, No MS animals treated with FGF2 had more association of acH3K14 at the OTR promoter than No MS animals treated with vehicle ($p<0.001$). Finally, MS animals treated with vehicle had more association of acH3K14 at the OTR promoter than No MS animals treated with vehicle ($p<0.001$).

For comparison we also assessed association of acH3K14 at the OTR promoter in the CeA (not shown). MS significantly increased the association of acH3K14 at the OTR promoter ($F_{(1,28)}=85.17$, $p<0.001$; eta squared=0.01) compared to No MS animals while FGF2 significantly increased the association of acH3K14 at the OTR promoter in the CeA ($F_{(1,28)}=92.65$, $p<0.001$; eta squared=0.01) compared to control animals.

In summary, both MS and FGF2 increase binding of acH3K14 with OTR promoter in the BNST and CeA resulting in activation of OTR in these regions. Taken together, the results suggest that both MS and FGF2 increased acetylation of the OTR in the BNST to enhance expression of the OTR receptor. It is possible that this increase may be a compensatory response to an MS-induced deficiency, although this was not tested in the present study.

Discussion

This study showed that OT and FGF2 decreased, while MS increased separation-induced USV in an early-life model of anxiety, separation-induced USV in male rat pups. Behavioral differences were accompanied by increased levels of OTR expression and binding in the BNST, but not receptor binding in the CeA. Our findings suggest that early-life FGF2 regulates the developmental tone for OTR, which can then interact with experiential factors to regulate anxiety-like behavior.

Key role of oxytocin

Oxytocin has long been known to promote prosocial behavior, prevent social avoidance and induce anxiolysis (Murgatroyd et al., 2004; Lim and Young, 2006; Carter, 2007; Heinrichs and Domes, 2008; Lukas et al., 2011). In lieu of its prosocial and anxiolytic properties, OT has been suggested to mediate therapeutic effects of selective-serotonin-reuptake-inhibitors (SSRIs) (Uvnas-Moberg et al., 1999; Emiliano et al., 2007). Furthermore, evidence shows decreased sociality and enhanced aggression due to low OT levels (Winslow et al., 1993; Beery and Zucker, 2010; Olff et al., 2013; Calcagnoli et al., 2014b; Calcagnoli et al., 2014a). OT has also been shown to attenuate aggression (Ferris, 2005) and by so doing, enable social investigation. Accordingly, in our experiments, peripheral administration of OT significantly and dose-dependently reduced USV, in line with an anxiolytic role in this model of fear and anxiety-like behavior.

It is hypothesized that OT may affect anxiety by: 1) modulation of the HPA axis (Neumann, 2002) and; 2) facilitation of prosocial/ affiliative behaviors concurrent with attenuation of neural systems involved in fear and anxiety (Carter, 2007) by binding to various components of the extended amygdala, which modulate behavioral and autonomic fear reactivity (Huber et al., 2005). The CeA and BNST are key components of the extended amygdala (Alheid et al., 1998), a network that has been implicated in addiction, anxiety and

aggression (Davis et al., 2010; Jennings et al., 2013; Calcagnoli et al., 2014b; Zorrilla et al., 2014).

Role of FGF2

FGF2 is known to have various effects on behavior and brain function (for review, see Turner et al., 2012b). Increased FGF2 has been shown following chronic administration of an anxiolytic in adult animals (Gomez-Pinilla et al., 2000). However, only a few studies have looked at the effects of FGF2 on anxiety-like behavior in animal models. For example, FGF2 administered chronically in adulthood decreased anxiety-like behavior in animals that were naturally highly anxious (Perez et al., 2009). More recently, administration of FGF2 early in life had an anxiolytic effect on highly anxious animals in adulthood (Turner et al., 2011, 2012a, 2012b). In line with these findings in the present series of experiments, FGF2 attenuated USV at PND11, consistent with an early anxiolytic role for this growth factor.

Given that both FGF2 and OT can modulate anxiety-like behavior, we wondered whether FGF2 can also alter the OTR. Both OT and FGF2 are known to cross the blood-brain-barrier (Wagner et al., 1999). FGF2 and FGFR1 mRNA and protein are localized in the BNST and CeA (Gonzalez et al., 1995). Therefore, FGF2, FGFR1 and the OTR are likely present in the same cells allowing them to respond to FGF2 endogenously or exogenously. Here, our findings show that FGF2 elevated OTR gene expression, binding and promoter acetylation in the BNST with some effects in CeA in male rat pups.

Epigenetic findings

FGF2 has previously been found to alter histone methylation in a genetic model of anxiety-like behavior (Chaudhury et al., 2014). In addition, this same study provided evidence that FGF2 modulates receptors by epigenetic mechanisms, such as the glucocorticoid receptor

(GR). The literature suggests that the OTR gene associates with acH3K14, a transcriptional activator, in the promoter region (Wang et al., 2013). Therefore, the association of the OTR promoter with acH3K14 can explain our findings of increased expression of OTR in the BNST and CeA. Although we did not measure peptide levels, OT levels may be reduced by MS in male rat pups. Interestingly, others have shown that OTR binding following MS can be either increased or decreased depending on age and brain region (Lucas et al., 2010). Thus, FGF2 may then increase OTR transcription by increasing acH3K14 binding to the OTR promoter and hence increase mRNA expression.

The MS experiential model

We assessed the effects of an experiential manipulation on the earliest form of anxiety-like behavior recorded in the rat, namely USV, together with indices of OTR using several methodologies. Our results show that early-life stress in the form of MS produced changes in behavior and OTR expression, binding and epigenetic regulation in male rat pups. Our findings suggest that MS increased OTR, possibly to compensate for other as-yet-unknown alterations produced by MS, such as a potential reduction in OT release (Oreland et al., 2010). This hypothesis is supported by a previous study that showed changes in OTR binding in animals that underwent MS in various brain regions, including the extended amygdala (Lukas et al., 2010). Furthermore, animals characterized as “excessively aggressive” showed significantly lower OTR mRNA in the paraventricular nucleus of the hypothalamus (PVN) and higher OTR binding in the CeA and BNST when compared to low and highly aggressive groups (Calcagnoli et al., 2014b). The authors suggest that this may be due to low release from the PVN, in conjunction with a compensatory upregulation of OTR in the BNST and the CeA.

Conclusion

Consistent with the anxiolytic property of FGF2 in previous studies, FGF2 attenuated USV in male rat pups in a developmental model of anxiety-like behavior, MS. When examining the effects of FGF2 on OTR promotor histone acetylation, FGF2 increased OTR acH3K14 in the BNST in both unseparated and MS groups. These results suggest that FGF2 potentially modulates the OTR in an experiential model of anxiety. It is possible that this OTR upregulation in the BNST and, to a lesser extent the CeA, may be a compensatory response to low OT release. Future studies should seek to test this hypothesis.

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Figure Legends

Figure 1. Behavioral timelines for the various experiments.

A) Ultrasonic vocalization (USV) response to oxytocin (OT) administration.

B) Ultrasonic vocalization (USV) response to maternal separation.

Figure 2. Oxytocin administration decreased ultrasonic vocalizations (USV) thirty minutes after injection at postnatal day (PND)11. The dose-response curve for oxytocin administration shows that its administration significantly reduced USV compared to vehicle (VEH) and that higher doses were more effective.

****p<0.01; #p<0.001**

Figure 3. MS and FGF2 altered USV at PND11.

A) Maternal separation (MS) significantly increased ultrasonic vocalizations (USV) compared to NoMS animals.

B) Fibroblast growth factor 2 (FGF2) significantly decreased USV compared to vehicle controls.

Following a brief 5-minute MS, the animals that experienced the early life stress of MS showed increased USV compared to No MS animals. However, the animals that experienced one postnatal injection of FGF2 exhibited decreased USV compared to vehicle (VEH) animals.

***p<0.05**

Figure 4. Fibroblast growth factor 2 (FGF2) increased oxytocin receptor (OTR) gene expression (*in situ* hybridization) in the bed nucleus of the stria terminalis (BNST) at postnatal day (PND)11.

#p<0.001

Figure 5. Fibroblast growth factor 2 (FGF2) increased oxytocin receptor (OTR) binding (autoradiography) in No maternal separation (NoMS) animals in the bed nucleus of the stria terminalis (BNST) compared to vehicle (VEH) controls.

***p<0.05**

Figure 6. Maternal separation (MS) increased oxytocin receptor (OTR) gene expression (*in situ* hybridization) in the central amygdala (CeA) at postnatal day (PND)11 compared to No maternal separation (NoMS) animals.

* $p < 0.05$

Figure 7. Maternal separation (MS) and fibroblast growth factor 2 (FGF2) increased the association of acH3K14 with the oxytocin receptor (OTR) promoter in the bed nucleus of the stria terminalis (BNST) at postnatal day (PND)11. Here, MS animals treated with vehicle had more association than No MS animals treated with vehicle. No MS animals treated with FGF2 had more association than MS animals treated with vehicle. Finally, MS animals treated with FGF2 had more association compared to No MS animals treated with FGF2 or MS animals treated with vehicle. ** $p < 0.01$; # $p < 0.001$

On left: Representative gel image from ChIP assay showing internal control (input) of extracted chromatin, the IgG (negative control), and the immunoprecipitated chromatin with the anti-acH3K14 for OTR.

OT administration study

Separation from mother

IP injection

Maternal Separation model

Separation from mother

Tissue collection