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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.

1. 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,b). 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

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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).

2. Material and methods

2.1. 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.

2.2. 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 h 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.

2.3. 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 (ddH_2O; $n = 8$) 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 (0.1 M PBS with 0.1% BSA, s.c.) on the day after birth (PND1).

2.4. Behavioral testing (see Fig. 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 min 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 min 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 5 min. 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.

2.5. 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 1 h and then rinsed in 2× SSC three times for 5 min each. The slides were then incubated in 1 M TEA with 0.25% acetic anhydride for 10 min. 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, 3× SSC, 50 mM sodium phosphate, pH 7.4, 1× denhardts, 0.1 mg/ml yeast tRNA and 10 mM dithiothreitol. The ^{35}S-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 2× SSC. The slides were then rinsed in RNAse A solution (10 mM Tris–HCl, 0.5 M NaCl, 200 μg/ml RNAse A, pH 8.0) at 37 °C for 1 h. The slides were then rinsed in 2× SSC, 1× SSC, and 0.5× SSC for 5 min each. The slides were then incubated in 0.1× SSC for 1 h 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 3.5× the standard deviation of individual pixel signal values above mean background signal, was

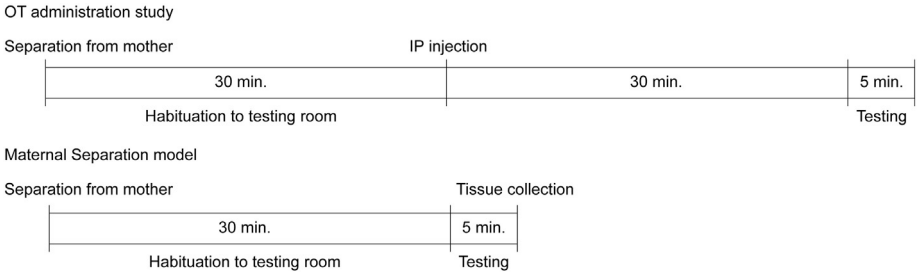

Fig. 1. Behavioral timelines for the various experiments.

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