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# INTERACTION BETWEEN CHOLECYSTOKININ AND THE FIBROBLAST GROWTH FACTOR SYSTEM IN THE VENTRAL TEGMENTAL AREA OF SELECTIVELY BRED HIGH- AND LOW-RESPONDER RATS

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**Abstract—Individual differences in the locomotor response to novelty have been linked to basal differences in dopaminergic neurotransmission. Mesolimbic dopaminergic outputs are regulated by cholecystokinin (CCK), a neuropeptide implicated in anxiety. In turn, CCK expression is regulated by fibroblast growth factor-2 (FGF2), which has recently been identified as an endogenous regulator of anxiety. FGF2 binds to the high-affinity fibroblast growth factor receptor-1 (FGF-R1) to regulate the development and maintenance of dopamine neurons in the ventral tegmental area (VTA). However, the relationship between the FGF and CCK systems in the VTA is not well understood. Therefore, we utilized the selectively-bred low-responder (bLR; high-anxiety) and high-responder (bHR; low-anxiety) rats to examine the effects of repeated (21-day) FGF2 treatment on CCK and FGF-R1 mRNA in the rostral VTA (VTAr). In vehicle-treated controls, both CCK and FGF-R1 mRNA levels were increased in the VTAr of bLR rats relative to bHR rats. Following FGF2 treatment, however, bHR–bLR differences in CCK and FGF-R1 mRNA expression were eliminated, due to decreased CCK mRNA levels in the VTAr of bLR rats and increased FGF-R1 expression in bHR rats. Differences after FGF2 treatment may denote distinct interactions between the CCK and FGF systems in the VTAr of bHR vs. bLR rats. Indeed, significant correlations between CCK and FGF-R1 mRNA expression were found in bHR, but not bLR rats. Colocalization studies suggest that CCK and FGF-R1 are coexpressed in some VTAr neurons. Taken together, our findings suggest that the FGF system is poised to modulate both CCK and FGF-R1 expression in the VTAr, which may be associated with individual differences in mesolimbic pathways associated with anxiety-like behavior.** 

**Key words: fibroblast growth factor-2, fibroblast growth factor receptor-1, novelty response, *in situ* hybridization, colocalization, individual differences.**

## INTRODUCTION

Altered cholecystokinin (CCK)-mediated neurotransmission has traditionally been associated with anxiety in rodents (van Megen et al., 1996; Chen et al., 2006) and panic attacks in humans (Zwanzger et al., 2012). Unfortunately, CCK antagonism has not been effective in alleviating anxiety in clinical trials (Harro, 2006). Even so, CCK plays an important role in behavioral aspects associated with anxiety such as negative affect and stress responses (Harro et al., 1992; Panksepp et al., 2004; Benedetti et al., 2006; Becker et al., 2008). CCK is widely expressed in the brain (Beinfeld, 1983), specifically within some dopaminergic neurons located in the ventral tegmental area (VTA) (Hökfelt et al., 1980; Seroogy et al., 1989). Given that dopamine has been implicated in anxiety-like behaviors (Cooper et al., 1973; Bertolucci-D'Angio et al., 1990; Cabib and Puglisi-Allegra, 2012) the VTA is of particular interest, since this region directly modulates areas involved in the expression of reward (Crespi et al., 2000; Olson et al., 2005) and anxiety (Gelowitz and Kokkinidis, 1999; de Oliveira et al., 2009; Beiderbeck et al., 2012), including the hippocampus, amygdala, and medial prefrontal cortex (Corral-Frias et al., 2013). The neurobiological factors that regulate CCK gene expression in the VTA are not well understood. In neuroblastoma models, CCK gene transcription is regulated by fibroblast growth factor-2 (FGF2), also known as basic FGF (Hansen et al., 1999; Hansen and Nielsen, 2001). FGF2 is an important regulator of anxiety-like behavior (Turner et al., 2012), with some conflicting findings such as increased FGF2 gene expression reported in the brain of hooded PVG (anxious) rats compared to their Sprague–Dawley counterparts after exposure to the Cat-freezing test (Wang et al., 2003) and increased fear extinction following acute exogenous FGF2 administration (Graham and Richardson, 2011). To date, no study has evaluated if the CCK system interacts with the FGF system in the VTA to regulate anxiety.

Differences in locomotor response to novelty are of interest as they predict individual differences in drug self-administration (Piazza et al., 1989) and responsiveness to environmental stress (Kabbaj et al., 2000), as well as differences in the expression of CCK (Ballaz et al.,

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*Abbreviations:* ANOVA, analysis of variance; bHR, bred high-responders; bLR, bred low-responders; CCK, cholecystokinin; Dig, digoxigenin; EPM, elevated plus-maze; FGF-R1, fibroblast growth factor receptor-1; FGF2, fibroblast growth factor-2 (basic fibroblast growth factor); IOD, integrated optical density; VTA, ventral tegmental area; VTAr, rostral VTA.

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2008). Thus, distinct CCK-ergic function may contribute to promoting individual differences in the adaptation to environmental novelty (Ballaz et al., 2007). Interestingly, selectively-bred lines of high-responder (bHR; low-anxiety) and low-responder (bLR; high-anxiety) rats show differences in tyrosine hydroxylase (Clinton et al., 2012), and dopamine-mediated transmission in the mesoaccumbal system (Flagel et al., 2011) which may be associated with differences in anxiety between these two lines (Beiderbeck et al., 2012). Compared to bLRs, bHRs are more active when exposed to an inescapable, novel environment (Stead et al., 2006) and exhibit less anxiety-like behavior in tests such as the elevated plus-maze (EPM) (Perez et al., 2009). Interestingly, repeated FGF2 treatment blunts differences in anxiety-like behavior in bLR and bHR rats by reducing active-avoidance responding specifically in bLR rats, which was associated with increased neurogenesis in the hippocampus of bLR rats (Perez et al., 2009). Because hippocampal neurogenesis is, at least in part, under dopaminergic control of the VTA (Suzuki et al., 2010), and since neonatal FGF2 treatment decreased tyrosine hydroxylase in the VTA of bLR rats (Clinton et al., 2012), the impact of repeated FGF2 treatment on gene expression in the VTA of bLR and bHR is of interest.

In the present study, we examined the relationship between the FGF and CCK systems in the VTA. Because the actions of FGF2 in the VTA occur through binding to high-affinity tyrosine kinase receptor-1 (fibroblast growth factor receptor-1 (FGF-R1)) (Guillonneau et al., 1996; Szebenyi and Fallon, 1999), FGF-R1 and CCK gene expression were examined in the rostral pole of the VTA (VTAr) of bHR and bLR rats following repeated treatment with either FGF2 or vehicle (Perez et al., 2009). We believed that the CCK system in the VTA may play an important role in the individual differences in environmental novelty-induced anxiety-like behavior that occur in response to repeated FGF2 treatment.

## EXPERIMENTAL PROCEDURES

### Subjects

Rats were obtained from our in house-breeding colony at the Molecular and Behavioral Neuroscience Institute at the University of Michigan. The bLR and bHR lines were selectively bred based on differences in locomotor activity when placed into a novel, inescapable environment (Stead et al., 2006). The gene expression data presented here correspond to a cohort of animals (Generation 19) in which novelty-driven anxiety was examined in the EPM test, a reliable index of anxiety-like behavior in rodents, following repeated FGF2 treatment, published previously by our group (Perez et al., 2009).

Briefly, the bHR–bLR phenotype was confirmed in adult male rats by assessing locomotor activity (between 9:00–11:30 am) in activity boxes (43 × 21.5 × 24.5 cm) similar to the home cages placed in a different room from where rats had been housed (Stead et al., 2006). The activity boxes were flanked by photo-beam cells to track both horizontal and vertical movements of the rat which were summed to generate an overall locomotor activity score. Animals were treated in accordance with the *Guidelines for the Care and Use of Mammals in Neuroscience and Behavioral Research* (National Research Council) and in accordance with the University of Michigan Committee on the Use and Care of Animals.

### Repeated FGF-2 treatment regimen and evaluation of anxiety-like behavior

Repeated injections of either FGF2 (5 ng/g, IP) or vehicle (0.1 M phosphate-buffered saline (PBS) with 0.1% bovine seroalbumin) were administered once per day at 4:00 pm for 21 consecutive days. On the next day (Day 22), EPM tests were conducted in a separate behavioral testing room between 8:00 and 11:30 am, less than 24 h following the last injection. FGF2 injections were administered to six bHR and seven bLR rats, while six bHR and six bLR rats received vehicle injections.

### Single-labeling *in situ* hybridization histochemistry

Rats were killed 24 h after the conclusion of the EPM test and brains quickly removed, snap frozen in *n*-methylbutane (−30 °C), and stored at −80 °C. Brains were sectioned on a cryostat at 10 µm (coronal sections), and thaw-mounted on Super Frost Plus slides (Fisher Scientific, Houston, TX, USA) at 100-µm intervals. Tissue sections were fixed in 4% paraformaldehyde phosphate buffer for 1 h, washed in 2× SSC (300 mM sodium chloride, and 30 mM sodium citrate buffer pH = 7.2), acetylated in 0.1 M triethanolamine buffer (0.1 M pH = 8.0) supplemented with acetic anhydride (0.25%) for 10 min, rinsed in distilled water, dehydrated in graded alcohol solutions (50–100%, 30 s) and subsequently air-dried. Thereafter, tissues were hybridized with $^{35}$S-labeled cRNA probes.

The following probes were cloned in house: CCK, (Gene Bank access # M10353) a 260-bp sequence coding for the exon III sequence of preproCCK mRNA (nucleotides 210–470) which included the sequence for the translation of the CCK octapeptide c-terminal; and FGF-R1 (Gene Bank access # NN_024146) a 657-bp sequence in the coding region (nucleotides 320–977). $^{35}$S-labeled cRNA probe was diluted in 50% hybridization buffer (50% formamide, 10% dextran sulfate, 2× SSC, 50 mM sodium phosphate buffer (pH = 7.4), 1× Denhardt's solution, 0.1 mg/ml yeast tRNA and 30 mM dithiothreitol), 70 µl of diluted probe placed on each slide ($2 \times 10^6$ dpm per six hemi-sections/slide) and cover slipped. Slides were placed in plastic trays moistened with 50% formamide and incubated in a 55 °C oven overnight. The following day, slides were rinsed in 2× SSC buffer to remove $^{35}$S-labeled probe and then incubated in RNAase A solution (200 µg/ml) for 1 h at 37 °C. Slides were then rinsed in increasingly stringent solutions (2× SSC, 1× SSC, 0.5× SSC) and placed in 0.1× SSC at 70 °C for 1 h. Slides were rinsed in distilled water, dehydrated through a graded alcohol series and exposed to Kodak XAR film (Eastman Kodak, Rochester, NY, USA) for a period ranging from 2 days for CCK to 2 weeks for FGF-R1.