FIBROBLAST GROWTH FACTOR SIGNALING IN THE DEVELOPING SEROTONERGIC SYSTEM AND ANXIETY-RELATED BEHAVIOR

BY

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FIBROBLAST GROWTH FACTOR SIGNALING IN THE DEVELOPING SEROTONERGIC SYSTEM AND ANXIETY-RELATED BEHAVIOR

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ABSTRACT

Anxiety disorders are some of the most commonly diagnosed psychopathologies in both pediatric and adult populations and have been linked to disrupted brain serotonergic systems. The risk for adult anxiety disorders increases in people with a history of childhood or adolescent anxiety, and the average age of onset for anxiety is eleven years old. These data suggest that anxiety has neurodevelopmental origins, yet our understanding of how anxiety-related serotonergic neurocircuits are formed and how their malformation contributes to the etiology of anxiety disorders is far from complete. This dissertation examined the role of fibroblast growth factor 8 (Fgf8) signaling in the development of midbrain serotonergic neurons and anxiety-like behavior. Fgf8 is a signaling molecule that coordinates the genesis of the putative

midbrain region. My hypothesis is that moderately reduced Fgf8 signaling during development impacts the structure and function of anxiety-related serotonergic neuronal subpopulations, thereby leading to anxiety-related behavior. Using adult male mice genetically altered to produce one-third less Fgf8, we observed defects specifically in anxiety- and panic-related serotonergic subpopulations. These defects included 1) fewer serotonergic neurons, 2) abnormal activation and functional responses of serotonergic neurons following a stressful stimulus, and 3) increased baseline anxiety-like behaviors. The results from this dissertation expand our knowledge on how developmental disruption of specific subpopulations of serotonergic neurons can affect their structural and functional integrity, thereby contributing to the persistent manifestation of anxiety behaviors. Overall, this dissertation suggests a role of Fgf signaling in the neurodevelopment of circuits that are disrupted in anxiety and affective disorders.

DEDICATION

I lovingly dedicate this dissertation to my brother, Chris Brooks, who has always been by my side and lifts me up when things get rough. I would not be the person I am today without his selflessness and unwavering support, encouragement, and love.

Composure, Focus, Execute

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-Ir	Immunoreactive
5-HIAA	5-hydroxyindoleacetic acid
5-HT	5-hydroxytryptamine (serotonin)
5-HT_{1A}	5-HT receptor 1A
5-HT_{2A}	5-HT receptor 2A
ABC	Avidin-biotin complex
ANOVA	Analysis of variance
ASD	Autism spectrum disorder
BLA	Basolateral nucleus of the amygdala
CA1d	CA1 region of the dorsal hippocampus
CA1v	CA1 region of the ventral hippocampus
CeA	Central nucleus of the amygdala
DAB	3,3′-diaminobenzidine tetrahydrochloride
DLPAG	Dorsolateral periaqueductal gray
DPAG	Dorsal periaqueductal gray
DR	Dorsal raphe nucleus
DRC	Dorsal raphe nucleus, caudal part
DRD	Dorsal raphe nucleus, dorsal part
DRI	Dorsal raphe nucleus, interfascicular part
DRV	Dorsal raphe nucleus, ventral part
DRVL/VLPAG	Dorsal raphe nucleus, ventrolateral part/ventrolateral periaqueductal gray
E12.5	Embryonic day 12.5
EPM	Elevated plus-maze
Fgf	Fibroblast growth factor

Fgf8-deficient	Fgf8-hypomorphic heterozygous
Fgfr	Fibroblast growth factor receptor
Fgfr1-deficient	Fgfr1-hypomorphic heterozygous
Fgfrl1	Fgf-like receptor 1
GABA	Gamma-aminobutyric acid
HET	Heterozygous
HPLC-ED	High-performance liquid chromatography with electrochemical detection
Ig	Immunoglobulin
IHC	Immunohistochemistry
IL	Infralimbic cortex
LD	Light-dark exploration
mlf	Medial longitudinal fasciculus
MnR	Median raphe nucleus
NS	Non-stress
OF	Open-field
Pet-1	Pheochromocytoma 12 E26 transformation-specific (ETS)
PrL	Prelimbic cortex
PTSD	Post-traumatic stress disorder
R1	Rhombomere 1
S	Stress
Tph	Tryptophan hydroxylase
vmPFC	Ventral medial prefrontal cortex
WT	Wild-type

CHAPTER I: GENERAL INTRODUCTION

1. INTRODUCTION

Many forms of psychopathology like mood, anxiety, and autism spectrum disorders are characterized by affective dysfunction (Davidson, Jackson et al. 2000). Advancements in basic and human research have made identification of the neural substrates and circuitry of emotion and disorders of emotion feasible (Davidson, Abercrombie et al. 1999; Cryan and Holmes 2005). Anxiety is a psychological and physiological state that is elicited in anticipation of threat or potential threat and is a normal part of human experience that helps us cope with stressors. However, excessive or inappropriate anxiety can become an illness (Gross and Hen 2004). Cortico-limbic structures such as the prefrontal cortex, hippocampus and the amygdala as well as the midbrain structure, the dorsal raphe nucleus (DR), have emerged as critical components of affective circuitry that are dysfunctional in anxiety disorders (Davidson, Abercrombie et al. 1999; Etkin and Wager 2007; Woon, Sood et al. 2010; Kroes, Rugg et al. 2011; Lesch and Waider 2012).

The majority of anxiety disorder neuroimaging research in humans to date has focused on adult pathology. However, the risk for adult pathology increases in people with a history of childhood or adolescent anxiety, and the median age of onset for anxiety is 11 years old (Kessler, Berglund et al. 2005; Pine 2007). These data suggest that anxiety may have neurodevelopmental origins (Leonardo and Hen 2008). One of the most common manifestations of pediatric anxiety is separation anxiety disorder (Ehrenreich, Santucci et al. 2008). Some evidence suggests a higher risk for adult panic disorder in this population, however, pediatric neuroimaging studies have not

been conducted thus limiting our ability to compare brain structure and function between the two populations (Blackford and Pine 2012; Kossowsky, Pfaltz et al. 2013). One manifestation of pediatric anxiety that is supported by structural neuroimaging studies is pediatric post-traumatic stress disorder (PTSD). Childhood PTSD is associated with volume reductions in prefrontal areas, the temporal lobe, and regions of the corpus callosum (De Bellis, Keshavan et al. 2002; Karl, Schaefer et al. 2006). Morphological changes in adult PTSD include consistent reductions in prefrontal, amygdalar, hippocampal, and insular volumes (Karl, Schaefer et al. 2006; Woon, Sood et al. 2010; Kroes, Rugg et al. 2011). These reports demonstrate overlap of prefrontal structural abnormalities in pediatric and adult subjects, which suggest early developmental deficiencies in this region that persist into adulthood. It is difficult, however, to examine whether perturbations in prenatal developmental programming precede the structural and functional changes seen in postnatal human brains. Models where prenatal development of affective brain circuits is specifically disrupted are needed to answer this question. Transgenic mice with deficiencies in various fibroblast growth factor signaling components are promising candidates in that regard.

Fibroblast growth factors (Fgfs) are morphogens essential to the development of brain regions associated with affect (Ye, Shimamura et al. 1998; Storm, Garel et al. 2006). Fgfs orchestrate the formation and organization of cortical, hippocampal and midbrain structures and recent advances in transgenic mouse technology have enabled researchers to more finely manipulate their expression *in vivo* (Nery, Fishell et al. 2002; Jukkola, Lahti et al. 2006; Storm, Garel et al. 2006). The results of these manipulations have provided a greater understanding of how alterations in Fgf signaling during

prenatal development not only impact the structure and function of affective circuitries, but also associated behaviors. Thus, Fgfs provide a unique opportunity to study the links between developmental molecular events, brain structure and function, and behavior.

2. FIBROBLAST GROWTH FACTORS AND AFFECTIVE DISORDERS

There is increasing evidence in both rodents and humans that dysregulated Fgf signaling is associated with affective disorders (Turner, Akil, Watson, & Evans, 2006). Based on brain imaging studies, children with autism spectrum disorder (ASD) (Schepers, Teasdale, & Koopman) have altered cerebral developmental trajectories and neuroanatomical abnormalities, which may be mediated by disrupted FGF signaling (Giedd et al., 2008; Rubenstein, 2010). Human *FGF8* (10q24) and *FGFR2* (10q26) genes are also near autism linkage sites on chromosome 10q, supporting the idea that these genes may be contributing risks for ASD (Vaccarino, Grigorenko, Smith, & Stevens, 2009). Associative studies have also shown that FGFR mutations increase the risk for schizophrenia (O'Donovan et al., 2009; Terwisscha van Scheltinga, Bakker, & Kahn, 2009), a finding that has been supported by Fgf mutant mouse models (Klejbor et al., 2006). In postmortem brain tissue of humans diagnosed with major depression, there is reduced FGF tone compared to controls (Evans et al., 2004). In mice, loss of Fgf2 creates an imbalance between excitatory and inhibitory neuron number in rostral cortical regions, which results in altered behavioral responses to a GABA receptor agonist (Korada, Zheng, Basilico, Schwartz, & Vaccarino, 2002). There are also a number of social deficits in mice whose prefrontal cortical patterning was altered by loss

of Fgf17 (Scearce-Levie et al., 2008). Lastly, studies in adult rats have shown that Fgf2 is anxiolytic (Perez, Clinton, Turner, Watson, & Akil, 2009). These studies suggest that Fgf signaling insufficiency contributes to abnormal brain development and psychopathologies. However, the human studies are associative and do not reveal temporal information on deficits in brain development. Further, little is known about how midbrain structures involved in modulating stress- and anxiety-related behaviors, such as the DR, are affected by prenatal loss of Fgf signaling.

3. FIBROBLAST GROWTH FACTORS: LIGANDS AND RECEPTORS

Fgf ligands

The human and murine Fgf families of peptides feature 22 known signaling molecules involved in developmental events such as cell proliferation, differentiation, survival, and patterning as well as in organogenesis, tissue repair and cancer pathology (Dono 2003). Structurally, Fgfs share a highly conserved core of about 120 amino acids and are grouped into subfamilies based on sequence similarity (Itoh and Ornitz 2011). Fgfs generally act in an autocrine or paracrine fashion, with the exception of Fgfs 11-14 which are intracellular molecules that do not bind Fgf receptors and Fgfs 15 (murine), 19 (human), 21, and 23 which have endocrine actions (Itoh 2007). Secreted Fgfs have a cleavable N-terminal signal sequence and are processed via a traditional endoplasmic reticulum-Golgi pathway (Mason 2007). However, some secreted Fgfs either lack a signal peptide (Fgfs 1, 2) or have a non-cleavable N-terminal signal sequence (Fgfs 9, 16, 20), but are nevertheless secreted (Revest, DeMoerlooze et al. 2000; Mohan, Rani

et al. 2010; Nickel 2011). Fgf ligand splice variants increase the functional diversity of this signaling family.

Fgf receptors

Fgf signaling is mediated through seven Fgf receptor (Fgfr) proteins encoded by four genes (Fgfr1-4) due to alternate splicing (Mason 2007). These receptors are membrane bound tyrosine kinase receptors consisting of three main regions: 1) extracellular domain which contains ligand and heparan-sulfate binding sites and three immunoglobulin (Ig)-like domains, 2) transmembrane domain, and 3) intracellular tyrosine kinase domain. The Ig-like domain III (IgIII) in Fgfrs 1-3 can be alternatively spliced into two forms which results in seven possible Fgf receptor variants with variable affinities for Fgf ligands (Itoh and Ornitz 2011). Heparan-sulfate acts as a cofactor and is required for stable binding of Fgf ligands to Fgfrs (Krejci, Prochazkova et al. 2009). Upon Fgf and heparan-sulfate binding, the receptors form homodimers or heterodimers resulting in transphosphorylation of intracellular tyrosine residues (Krejci, Prochazkova et al. 2009; Itoh and Ornitz 2011). The main signal transduction pathways activated by Fgfrs are the Ras-Raf-mitogen-activated protein kinase and phosphatidylinositol 3-kinase-Akt pathways (Itoh 2007). Some Fgfrs have been visualized in the nucleus and likely have different downstream effects than typical membrane bound receptors (Wiedlocha and Sorensen 2004; Bryant and Stow 2005; Beenken and Mohammadi 2009). There is also evidence for a fifth Fgf-like receptor (Fgfrl1) that can bind Fgf ligands but lacks a tyrosine kinase domain (Wiedemann and Trueb 2000). When considered in the context of development, where the Fgf ligands, receptors and splice

variants are expressed in temporally and spatially distinct patterns, one comes to appreciate the complexity of this functionally diverse family.

4. DORSAL RAPHE NUCLEUS: GENESIS AND FUNCTIONAL ANATOMY

Genesis

The genesis and organization of DR serotonergic neuronal populations are orchestrated by a number of signaling molecules and transcriptional networks during development (Cordes 2005; Kiyasova and Gaspar 2011; Deneris and Wyler 2012). Of these, Fgf8 and one of its cognate receptors, Fgfr1, represent morphogenic signals most critical to the early genesis and organization of the DR serotonergic neurons (Partanen 2007). These neurons are derived from the most anterior portion of the developing hindbrain known as the isthmus and rhombomere 1 (isthmus-r1) (Saarimaki-Vire, Peltopuro et al. 2007; Alonso, Merchan et al. 2013). During development, *Fgf8* is expressed in a temporally and spatially restricted fashion within this region (Martinez, Crossley et al. 1999; Liu and Joyner 2001; Wurst and Bally-Cuif 2001), and the secreted Fgf8 protein creates a diffusion gradient essential for the anterior-posterior patterning of isthmus-r1 and specification of serotonergic cell fate (Ye, Shimamura et al. 1998; Liu and Joyner 2001; Chi, Martinez et al. 2003; Echevarria, Vieira et al. 2003; Toyoda, Assimacopoulos et al. 2010). A conditional loss of Fgfr1 signaling in the isthmus-r1 region results in a substantial loss of serotonergic cells (Jukkola, Lahti et al. 2006; Partanen 2007). Interestingly, not all DR neurons are lost in the conditional Fgfr1 knockout mice, suggesting that Fgf signaling has differential effects on subpopulations of serotonergic neurons.

Functional anatomy of DR

DR serotonergic neurons are heterogeneous and are organized into five functional and topographically organized subregions (dorsal, ventral, ventrolateral DR/ventrolateral periaqueductal gray, interfascicular, and caudal) with distinct anatomical locations, afferent inputs, efferent targets, and physiological properties (Hale and Lowry 2011). For example, subpopulations of serotonergic neurons located within the mid- to caudal dorsal DR (DRD, DRC) connect with brain structures involved in the control of emotional behavior such as the basolateral amygdala and medial prefrontal cortex and have anxiety-facilitating actions. Serotonergic neurons in the ventrolateral DR/ventrolateral periaqueductal gray (DRVL/VLPAG) project to brain structures involved in physiological and behavioral responses to panic-inducing stimuli. In contrast to the DRD, however, DRVL/VLPAG serotonergic neurons play a panic- and anxiety-inhibiting role in the modulation of emotional behavior (Paul and Lowry 2013). Panicogenic agents such as CO_2 and sodium lactate strongly activate DRVL/VLPAG serotonergic neurons in control rats in the absence of panic-like physiological responses, consistent with the hypothesis that these neurons normally function to inhibit panic-like responses (Johnson, Lowry et al. 2008). Conversely, panic-susceptible rats challenged with panicogenic stimuli fail to activate DRVL/VLPAG neurons and display anxiety- and panic-like responses such as decreased social interaction and increased heart and respiratory rates (Johnson and Shekhar 2006; Johnson, Lowry et al. 2008). Structural or functional disruption of serotonergic systems modulating emotional behavior may lead to dysregulated anxiety-like behavior.

5. SUMMARY

Although previous studies have reported defects of the developing DR in association with Fgf signaling deficiencies (Jukkola, Lahti et al. 2006; Blak, Naserke et al. 2007; Saarimaki-Vire, Peltopuro et al. 2007; Sato and Joyner 2009), they have not addressed whether serotonergic neuronal loss under Fgf signaling deficiency was uniform across the DR or specific to certain subpopulations, and whether there were functional and behavioral consequences.

This dissertation focuses on the role of fibroblast growth factor (Fgf) signaling in the development of DR serotonergic neurons and anxiety-like behavior. My hypothesis is that reduced Fgf signaling during prenatal development 1) reduces specific DR serotonergic neuronal subpopulations and 2) compromises the functional integrity of DR serotonergic systems, thereby leading to dysregulated stress- and anxiety responses in adult male mice. Chapter II investigates whether Fgf signaling during development is important for the structural integrity of DR subregions, and Chapter III examines whether the developmental loss of Fgf8 impacts the functional integrity of stress- and anxiety-related DR serotonergic circuitries. The results from this dissertation expand our knowledge on how developmental disruptions of DR serotonergic subpopulations can affect their structural and functional integrity and ultimately impact the manifestation of stress- and anxiety-related behaviors.

CHAPTER II: FIBROBLAST GROWTH FACTOR DEFICIENCIES IMPACT ANXIETY-LIKE BEHAVIOR AND THE SEROTONERGIC SYSTEM

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1. ABSTRACT

Serotonergic neurons in the dorsal raphe nucleus (DR) are organized in anatomically distinct subregions that form connections with specific brain structures to modulate diverse behaviors, including anxiety-like behavior. It is unclear if the functional heterogeneity of these neurons is coupled to their developmental heterogeneity, and if abnormal development of specific DR serotonergic subregions can permanently impact anxiety circuits and behavior. The goal of this study was to examine if deficiencies in different components of fibroblast growth factor (Fgf) signaling could preferentially impact the development of specific populations of DR serotonergic neurons to alter anxiety-like behavior in adulthood. Wild-type and heterozygous male mice globally hypomorphic for *Fgf8*, *Fgfr1*, or both (*Fgfr1/Fgf8*) were tested in an anxiety-related behavioral battery. Both *Fgf8*- and *Fgfr1/Fgf8*-deficient mice display increased anxiety-like behavior as measured in the elevated plus-maze and the open-field tests. Immunohistochemical staining of a serotonergic marker, tryptophan hydroxylase (Tph), revealed reductions in specific populations of serotonergic neurons in the ventral, interfascicular, and ventrolateral/ventrolateral periaqueductal gray subregions of the DR in all Fgf-deficient mice, suggesting a neuroanatomical basis for increased anxiety-like behavior. Overall, this study suggests Fgf signaling selectively modulates the development of different serotonergic neuron subpopulations. Further, it suggests anxiety-like behavior may stem from developmental disruption of these neurons, and individuals with inactivating mutations in Fgf signaling genes may be predisposed to anxiety disorders.

2. BACKGROUND

Serotonergic neurons in the dorsal raphe nucleus (DR) modulate diverse physiological and behavioral outputs, including anxiety-like behavior (Lowry, Johnson et al. 2005). DR serotonergic neurons are functionally heterogeneous and are organized into five functional topographically organized subregions (dorsal (DRD), ventral (DRV), ventrolateral DR/ventrolateral periaqueductal gray (DRVL/VLPAG), interfascicular (DRI), and caudal (DRC)) with distinct anatomical locations, afferent inputs, efferent targets, and physiological properties (Jacobs and Azmitia 1992; Lowry, Johnson et al. 2005; Calizo, Akanwa et al. 2011; Hale and Lowry 2011). Two different serotonergic subsystems that modulate anxiety-like states emerge from this functional topography (Hale, Shekhar et al. 2012; Paul and Lowry 2013). One facilitates anxiety-like responses and includes subpopulations of serotonergic neurons in the DRD and DRC. Another system that includes DRVL/VLPAG, DRV, and DRI are co-activated in conditions associated with the inhibition of panic-like responses and thought to promote stress-resistance. Therefore, loss of and/or failure to activate subpopulations of these panic-reducing serotonergic cells can lead to increased vulnerability to panic- and anxiety-like responses (Hale, Shekhar et al. 2012; Paul and Lowry 2013). Thus, data suggest that while some subpopulations of DR serotonergic neurons facilitate anxiety-like responses, others inhibit anxiety- or panic-like responses.

The genesis and organization of the DR serotonergic neuronal populations are orchestrated by a number of signaling molecules and transcriptional networks during development (Cordes 2005; Kiyasova and Gaspar 2011; Deneris and Wyler 2012). Of these, fibroblast growth factor 8 (Fgf8) and one of its cognate receptors, Fgf receptor 1

(Fgfr1), represent morphogenic signals most critical to the early genesis and organization of the DR serotonergic neurons (Partanen 2007). During development, *Fgf8* is expressed in a temporally and spatially restricted fashion (Martinez, Crossley et al. 1999; Liu and Joyner 2001; Wurst and Bally-Cuif 2001), and the secreted Fgf8 protein creates a diffusion gradient essential for the anterior-posterior patterning of the developing hindbrain region and specification of serotonergic cell fate (Ye, Shimamura et al. 1998; Liu and Joyner 2001; Wurst and Bally-Cuif 2001; Echevarria, Vieira et al. 2003). In this regard, developmental deficiencies of Fgf8 and Fgfr1 may lead to abnormally formed DR serotonergic neuron populations and impact anxiety-related behaviors modulated by these neurons.

A complication associated with the study of DR serotonergic neurons is their heterogeneity. Not only are these neurons functionally heterogeneous (Hale and Lowry 2011), they are also developmentally heterogeneous (Deneris 2011; Gaspar and Lillesaar 2012). For example, in the hindbrain, the transcription factor Pet-1 is found exclusively in serotonergic neurons and is critical for the differentiation, maturation and maintenance of serotonergic neuronal phenotype (Hendricks, Fyodorov et al. 2003). Despite this critical role, about 20-30% of serotonergic neurons do not require Pet-1 for differentiation (Hendricks, Fyodorov et al. 2003; Kiyasova, Fernandez et al. 2011). Further analysis revealed that all DR serotonergic neurons in this Pet-1-independent population project to the same functionally related forebrain regions that modulate affective behavior (Kiyasova, Fernandez et al. 2011), suggesting DR serotonergic neurons with similar developmental requirements are also similar in function. Although previous studies have reported malformations of the developing DR in association with

Fgf signaling deficiencies (Jukkola, Lahti et al. 2006; Blak, Naserke et al. 2007; Saarimaki-Vire, Peltopuro et al. 2007; Sato and Joyner 2009), the differential impacts of Fgf signaling disruption on serotonergic neurons in DR subregions have not been described in detail and lack topographical resolution. The behavioral outcome of these differential impacts has also not been examined.

The goal of the present study is to use transgenic mouse models deficient in Fgf8, Fgfr1, or both to understand the differential impact of these deficiencies on the topographically organized DR serotonergic neurons and anxiety-related behavior. These mouse models may also provide clinically useful insights into the phenotypic manifestations, including any anxiety disorders, in humans harboring loss-of-function mutations on *Fgfr1* and *Fgf8* genes (Dode, Levilliers et al. 2003; Falardeau, Chung et al. 2008). Our results suggest that serotonergic neurons in some DR subregions are more dependent on Fgf signaling than others, and their disruption was associated with increased anxiety-like behavior. Overall, these data expand our knowledge on developmental heterogeneity of serotonergic neurons and correlate the disruption of specific DR serotonergic subpopulations to specific behavioral outcomes.

3. MATERIALS AND METHODS

3.1 Animals

All experiments were conducted using 8-10 week-old offspring from crosses of *Fgfr1* (129sv/CD-1; Canadian Mutant Mouse Repository, Toronto, ON) and *Fgf8* heterozygous hypomorphic mice (129p2/OlaHsd* CD-1; obtained from Mouse Regional Resource Centers, Davis, CA) (Meyers, Lewandoski et al. 1998; Partanen, Schwartz et

al. 1998). *Fgfr1 and Fgf8* hypomorphic mice contain a neomycin-resistance element inserted into non-coding regions of the *Fgfr1 or Fgf8* genes. This element contains false splice sites which lead to about a 66-80% and 55% reduction in functional *Fgfr1* and *Fgf8* transcript levels, respectively (Meyers, Lewandoski et al. 1998; Partanen, Schwartz et al. 1998), under homozygous condition. Both *Fgfr1* and *Fgf8* homozygous hypomorphic mice die within 24 h of birth but heterozygous (HET) mice survive normally and have no obvious health problems. The four offspring genotypes used in these studies were: wild-type (WT), Fgfr1 HET, Fgf8 HET, and Fgfr1/Fgf8 double HET (Fgfr1/Fgf8 HET). Male mice were housed in same-sex littermate groups of 2-5 at weaning and genotyped using DNA isolated from tail clips and polymerase chain reaction. All mice were bred at the University of Colorado Boulder in the Integrative Physiology department animal facility under a 12L:12D photoperiod with free access to water and rodent chow. All animal procedures complied with the protocols approved by the Institutional Animal Care and Use Committee at the University of Colorado Boulder.

3.2 Battery of behavioral tests

3.2.1 General procedures

Two cohorts of male mice (Cohort 1: n = 3 WT, n = 4 Fgfr1 HET, n = 10 Fgf8 HET, n = 4 Fgfr1/Fgf8 HET; Cohort 2: n = 12 WT, n = 12 Fgfr1 HET, n = 12 Fgf8 HET, n = 15 Fgfr1/Fgf8 HET) were used to test anxiety-related behavior in a test battery. Both cohorts of mice experienced the exact same behavioral testing procedures, except the second cohort of mice were also tested for motor ability following the completion of the behavioral battery. Other than the handling associated with cage changes, mice were

not handled prior to behavioral testing. Behavioral testing commenced 2 h and was completed within 6 h of light phase onset. The interval between different anxiety-related behavioral tests in the test battery was 2 days (Paylor, Spencer et al. 2006) and was conducted in the following order: (1) elevated plus-maze, (2) open-field, and (3) light-dark exploration. Despite the anxiogenic nature of the elevated plus-maze test, it was performed first as it has been shown to be sensitive to prior testing experience (Voikar, Vasar et al. 2004; Crawley 2008). Due to this design, we cannot rule out the possibility that exposure to the elevated plus-maze test interacted with Fgf deficiencies to influence behavior on subsequent tests. Two additional motor tasks were performed in the second cohort of mice immediately after the light-dark exploration test: vertical pole, and wire grip tests. Table 1 outlines the testing order and interval for the mice. Room lighting was approximately 480 lx. Behavioral testing equipment was cleaned with 70% ethanol before testing and in between each test subject. A video camera was mounted above the behavioral test apparatus and behavior was recorded for later scoring by an observer blinded to the genotypes. For each behavioral test, the entries or total duration within an area began when all four paws crossed into the area of interest.

3.2.2 Elevated plus-maze (EPM)

The brown acrylic EPM consisted of a center area (5.5 cm x 5.5 cm) from which two opposing open arms (30 cm x 5.5 cm) and two opposing closed arms with the same dimensions and walls (15 cm high) were extended. The maze was elevated 60 cm off the ground. Mice were placed in the center area of the EPM facing an open arm to start the 5 min test (Voikar, Vasar et al. 2004; Bailey, Pavlova et al. 2007). Mice that fell off the maze were excluded from analysis (n = 1 WT, n = 1 Fgfr1 HET, n = 5 Fgf8 HET, n =

5 Fgfr1/Fgf8 HET). The time spent in the open, closed and center areas and number of entries into each arm were scored manually. For analysis, the time spent on the arms and number of entries were expressed as a percentage of the total test duration and number of arm entries, respectively.

3.2.3 Open-field (OF)

The OF test measures both locomotion and anxiety-related behaviors. Mice were placed in the center of a white 40 cm x 40 cm x 30 cm-high white acrylic box with an open top and recorded for 15 min (McIlwain, Merriweather et al. 2001; Bailey, Pavlova et al. 2007). Sixteen 10 cm x 10 cm squares were drawn onto the OF floor to visually divide the box into an outer perimeter zone surrounding an inner zone (20 cm x 20 cm) for analysis by EthoVision XT software (version 6.0; Noldus Information Technologies). The time spent (expressed as percent time for analysis) in each zone and total distance traveled (locomotor activity) were scored.

3.2.4 Light-dark exploration (LD)

The final anxiety-related behavioral test in the battery was the LD test (Crawley and Goodwin 1980; Bourin and Hascoet 2003). An acrylic box was divided into two unequal-sized compartments. The larger “light” compartment was white with an open top (25 cm x 20 cm x 30 cm) and was connected to the “dark” smaller enclosed black compartment (15 cm x 20 cm x 30 cm) by a floor-level 7.5 cm x 7.5 cm opening centered in the partition separating the two compartments. Mice were placed in the middle of the light compartment facing away from the dark compartment, and the time spent (expressed as percent time for analysis) and total distance traveled in the light

compartment during the 10 min test were scored using EthoVision XT software (version 6.0; Noldus Information Technologies). The latency to enter the dark compartment and total number of transitions were scored manually.

3.2.5 Vertical pole and wire grip tests

The vertical pole and wire grip tests were included in Cohort 2 to measure motor coordination, balance, and strength (Crawley 2000). Both tests were performed as described by (Crawley 2000) immediately after the LD test. Briefly, the vertical pole test consisted of placing a mouse on the center of a wooden dowel (2 cm x 40 cm) wrapped in masking tape that is elevated above a cage filled with bedding. The dowel is lifted from a horizontal to vertical position over the course of 45 s. Mice that remained on the pole throughout the test were considered to have passed the test. The wire grip test was performed 15-30 s after the vertical pole test. As described (Crawley 2000), mice were placed on a wire cage top that was tapped three times to cause the mouse to grip and subsequently turned upside down. The cage top was held about 20 cm above a cage filled with bedding for 60 s. The latency to fall was recorded. Mice that fell while the cage top was being inverted were excluded from analysis (n = 5 WT, n = 4 Fgfr1 HET, n = 4 Fgf8 HET, n = 1 Fgfr1/Fgf8 HET).

3.3 Tissue collection and preparation

For immunohistochemistry, behaviorally naive male mice (n = 8 WT, n = 8 Fgfr1 HET, n = 7 Fgf8 HET, n = 10 Fgfr1/Fgf8 HET) were terminally anesthetized with pentobarbital sodium and perfused transcardially with 15 mL of heparinized saline and 50 mL of 4% paraformaldehyde in 0.1 M sodium phosphate buffer. Brains were

removed and post-fixed in 4% paraformaldehyde for 24 h at 4°C then cryoprotected in 30% sucrose until sectioning. Before sectioning, brains were blocked at the caudal border of the mammillary body using a mouse brain matrix (RBM 2000C, ASI Instruments). The tissue block posterior to the mammillary body containing the midbrain raphe complex was immediately sectioned using a cryostat into 30 µm frozen coronal floating sections that were collected into a series of six microcentrifuge tubes filled with a cryoprotectant (30% sucrose, 30% ethylene glycol, 1% polyvinylpyrolidone in 0.2 M sodium phosphate buffer).

3.4 Immunohistochemistry (IHC)

The IHC used tryptophan hydroxylase (Tph; the rate-limiting enzyme for serotonin biosynthesis) as a marker of serotonergic neurons. Briefly, one third of the sections were taken through a series of rinses and sequential incubations on an orbital shaker with a sheep anti-tryptophan hydroxylase antibody that has been previously characterized and has been shown to bind specifically to both isoforms of Tph (Hale, Dady et al. 2011; Hale, Shekhar et al. 2011) (T8575, Sigma-Aldrich), a biotinylated donkey anti-sheep secondary antibody (713-065-147, Jackson ImmunoResearch Laboratories), avidin-biotin complex (ABC; NeutrAvidin® biotin-binding protein, A2666, Life Technologies; Peroxidase-biotinamidocaproyl conjugate, P-9568, Sigma-Aldrich), and reacted with 3,3′-diaminobenzidine (DAB; D5637, Sigma-Aldrich) for color detection (Brooks, Chung et al. 2010; Hale, Dady et al. 2011; Hale, Shekhar et al. 2011). After the color reaction, sections were rinsed, mounted on gelatin-coated glass slides, dehydrated through increasing concentrations of ethanol (70- 100%), cleared in Histo-Clear (National Diagnostics), and coverslipped with Permount (Fisher Scientific).

3.5 Quantification of Tph neurons

The numbers of Tph-immunoreactive (ir) neurons were counted by an investigator blind to the treatment groups at five rostrocaudal levels (−4.36, −4.54, −4.72, −4.90, and −5.08 mm bregma, Figure 1A) under a brightfield microscope. Tph-ir neurons were quantified in the dorsal (DRD; −4.36, −4.54, −4.72, and −4.90 mm bregma), ventral (DRV; −4.36, −4.54, −4.72, and −4.90 mm bregma), ventrolateral part/ventrolateral periaqueductal gray (DRVL/VLPAG; −4.54, −4.72, and −4.90 mm bregma), interfascicular (DRI; −4.72, −4.90, and −5.08 bregma), and caudal (DRC; −5.08 mm bregma) subregions of the DR. Representative photomicrographs for each genotype at each rostrocaudal level of the DR are shown in Figure 1B.

3.6 Statistical analysis

All statistical analyses were completed using SPSS Statistics (version 21.0 for Mac; IBM). Cohorts 1 and 2 were combined for the anxiety-related behavioral test analyses. The behavioral and wire grip tests were analyzed using one-way ANOVA with Welsh's correction for unequal variance when necessary, followed by planned pairwise contrasts corrected for unequal variance when appropriate. All mice passed the vertical pole test; hence no data analysis was performed. Data for the number of Tph-ir neurons were analyzed using a linear mixed model analysis using *genotype* as the between-subjects factor and *subregion* as the repeated-measure. Planned pairwise contrasts corrected for unequal variance when appropriate were applied for each of the five subregions of the DR to reveal subregion-specific genotype effects on Tph-ir neuron number. Statistical outliers were determined using the Grubbs' test and were removed

(Grubbs 1969). For the EPM, 2 out of 72 data points for percent time in open arms were excluded (2.8% of total data), and 1 out of 72 data points for each the percent time in closed arms and center area were excluded (1.4% of total data for each); for the OF, 1 out of 72 data points for percent time in outer zone were excluded (1.4% of total data) and 4 out of 72 data points for percent time in inner zone were excluded (5.6% of total data). There were no outliers for the LD. Values are shown as the mean ± the standard error of the mean (SEM). Data were significant when $p < 0.05$.

4. RESULTS

4.1 EPM

As shown in Figure 2, there was a statistically significant genotype effect on the percentage of time spent in the open [*Welsh's* $F(3, 24.56) = 3.45$, $p = 0.032$], closed [*Welsh's* $F(3, 26.97) = 8.57$, $p = 0.001$], and center area [*Welsh's* $F(3, 27.66) = 5.92$, $p = 0.003$] of the EPM. Post hoc planned contrasts revealed that Fgf8 HET mice spent significantly less time on the open arms [$t(18.37) = 3.89$, $p = 0.001$] and center area [$t(18.57) = -3.11$, $p = 0.006$] and more time in the closed arms [$t(15.43) = -2.16$, $p = 0.047$] than WT mice. There were no significant genotype differences in the total number of closed arm entries (a measure of exploratory behavior and motor function; data not shown) or percentage of open or closed entries (Table 2).

4.2 OF

Locomotor activity and anxiety-like behavior were measured during the OF test. There were no genotype differences in the total distance traveled (Table 2), indicating that motor function is not impacted by Fgf deficiency. There was a significant effect of

genotype on the percentage of time spent in the inner zone of the OF [$F(3, 64) = 3.04$, $p = 0.035$] and a corresponding trend towards differences in time spent in the outer zone that did not reach statistical significance [$F(3, 67) = 2.53$, $p = 0.065$]. Post hoc planned contrasts revealed that both Fgf8 HET [$t(64) = -2.55$, $p = 0.013$] and Fgfr1/Fgf8 HET mice [$t(64) = -2.58$, $p = 0.012$] displayed increased anxiety-like behavior by spending significantly less time in the inner zone compared to WT controls (Figure 2).

4.3 LD, vertical pole, and wire grip tests

There was no significant genotype effect on any LD behavior or latency to fall during the wire grip motor task (Table 2). All mice passed the vertical pole test.

4.4 Tph-ir neuron counts

Linear mixed model analysis of the number of Tph-ir neurons within specific subregions of the DR revealed a significant interaction between subregion and genotype [$F(42, 29.12) = 1.84$, $p = 0.044$]. As Figure 3 illustrates, post hoc planned contrasts revealed significant reductions in the number of Tph-ir neurons mainly in the mid- to caudal DR between WT and Fgf8 HET in the DRV and DRVL [−4.72 mm bregma; $t(28) = -3.35$, $p = 0.002$, $t(11.99) = -2.89$, $p = 0.014$, respectively] and DRI [−5.08 mm bregma; $t(7.70) = -4.29$, $p = 0.003$], between WT and Fgfr1 HET in the DRI [−4.72 mm bregma; $t(9.73) = -2.93$, $p = 0.016$], and between WT and Fgfr1/Fgf8 HET in the DRVL [−4.72 mm bregma; $t(14.97) = -2.76$, $p = 0.015$]. There were also significant main effects for both genotype [$F(3, 28.12) = 4.01$, $p = 0.017$] and DR subregion $F(14, 27.87) = 172.96$, $p = 0.001$]. Post hoc analyses indicated that Fgf8 HET [$t(22) = -3.79$, $p = 0.001$] and Fgfr1/Fgf8 HET [$t(22) = -2.68$, $p = 0.014$] mice had significantly fewer total

DR Tph-ir neurons than WT controls (1166 ± 68.74, 1264 ± 53.36, 1479 ± 56.65, mean ± SEM for Fgf8 HET, Fgfr1/Fgf8 HET, and WT, respectively).

5. DISCUSSION

Fgf signaling deficiencies differentially reduced subpopulations of DR serotonergic neurons, and these reductions were associated with elevated anxiety-like behavior as measured by the EPM and OF tests in adult male mice. Decreases in serotonergic neurons were restricted to specific subregions within the DR. Specifically, Fgf8 deficiency increased anxiety-like behavior and decreased serotonergic cell numbers in the DRVL/VLPAG, caudal DRV, and DRI. Due to the unique projections to and from these serotonergic cell groups, they have collectively been implicated in multiple animal models of chronic anxiety-like states and increased susceptibility to panic- and anxiety-like behaviors, including models of early life adverse experience (Hale and Lowry 2011; Paul and Lowry 2013). The effects of compound Fgfr1 and Fgf8 deficiencies were somewhat similar to Fgf8 deficiency alone but less severe. These data support the documented necessity of Fgf signaling in the formation of DR serotonergic neurons (Ye, Shimamura et al. 1998; Chi, Martinez et al. 2003; Jukkola, Lahti et al. 2006; Saarimaki-Vire, Peltopuro et al. 2007). Importantly, they highlight the subregional specificity of Fgf signaling in the developing DR and behavioral consequences associated with Fgf8 or Fgfr1 deficits.

Serotonin-modulated anxiety-like behaviors depend on the unique afferent and efferent connections between the DR and selective brain structures involved in emotional regulation. There are two DR serotonergic subsystems that modulate

anxiety-like states: the anxiety-promoting DRD/DRC and the anxiety-reducing DRVL/VLPAG, caudal DRV, and DRI systems (for in depth reviews see (Hale, Shekhar et al. 2012; Paul and Lowry 2013)). DRD/DRC connect with forebrain structures involved in emotional regulation and anxiety-related behavior such as the infralimbic and prelimbic cortices, lateral habenula, central and basolateral nucleus of the amygdala, and bed nucleus of the stria terminalis (Hale and Lowry 2011; Hale, Shekhar et al. 2012). Together these circuits facilitate anxiety-like responses to anxiogenic drugs, inescapable shock, and behavioral tests such as social defeat and fear-potentiated startle (Abrams, Johnson et al. 2005; Gardner, Thrivikraman et al. 2005; Rozeske, Evans et al. 2011; Spannuth, Hale et al. 2011). On the other hand, the DRVL/VLPAG connect with brain structures involved in both the autonomic and behavioral components of emotional states including the rostral ventrolateral medulla, dorsal periaqueductal gray, lateral hypothalamus, lateral parabrachial nucleus, nucleus of the solitary tract, central nucleus of the amygdala, lateral and perifornical hypothalamic nuclei, median preoptic area, and the infralimbic cortex (Hale and Lowry 2011; Hale, Shekhar et al. 2012). The DRI has afferent and efferent connections with several forebrain structures involved in emotional control including the infralimbic and prelimbic cortices, dorsal and ventral hippocampus, median preoptic nucleus and lateral parabrachial nucleus and is thought to be co-activated with the DRVL/VLPAG and caudal DRV to inhibit panic-like responses and promote stress resistance (Hale, Shekhar et al. 2012; Paul and Lowry 2013). Our data suggest Fgf8 deficiency disrupts a subpopulation of “stress-resistant” serotonergic neurons and possibly the associated connectivity, leading to elevated anxiety-like behavior. Indeed, reduced activity in these

anxiolytic serotonergic subregions has been implicated in multiple animal models of chronic anxiety-like states and increased susceptibility to panic- and anxiety-like behaviors, including models of early life adverse experience (Hale and Lowry 2011; Hale, Shekhar et al. 2012).

Fgf signaling deficiency was associated with decreased serotonergic cell numbers in specific subregions of the DR, including the DRVL/VLPAG (Fgf8 HET and Fgfr1/Fgf8 HET), caudal DRV (Fgf8 HET), and DRI (Fgf8 and Fgfr1 HET). Based on the modulatory roles of serotonin in these circuitries, these affected DR subregions have been implicated in rodent models of panic- and anxiety-like behavior. These models include amygdala priming, adolescent social isolation, and disinhibition of the dorsomedial hypothalamus (Johnson, Lowry et al. 2008; Donner, Johnson et al. 2012; Lukkes, Kopelman et al. 2013). For example, adolescent social isolation in rats, which led to increased vigilance behaviors following treatment with an anxiogenic drug, was associated with lower baseline *tph* expression in the DRVL/VLPAG and caudal DRV. Similarly, in a model of panic-like anxiety, serotonergic neurons in the DRVL/VLPAG and caudal DRV and DRI became dysregulated in panic-prone rats and could not be activated by sodium lactate (a panicogenic agent) (Johnson, Lowry et al. 2008). Given their role in reducing panic-like responses, loss of neurons in these subregions may lead to increased panic-vulnerability. Together these data suggest that loss of function of subsets of serotonergic neurons in the DRVL/VLPAG, caudal DRV, and DRI may contribute to increased vulnerability to panic- and anxiety-like behaviors. Despite this evidence in postnatal models, detailed subregional analyses of DR have not been described in animal models where serotonergic neurons are disrupted prenatally

(Brodski, Weisenhorn et al. 2003; Hendricks, Fyodorov et al. 2003; Blaess, Corrales et al. 2006; Jukkola, Lahti et al. 2006; Saarimaki-Vire, Peltopuro et al. 2007; Kiyasova, Fernandez et al. 2011). To our knowledge, this study is the first to demonstrate developmental disruption of specific serotonergic subregions and a correlation between neuroanatomical and behavioral disruptions.

The mechanisms underlying the topographical specificity of Fgf8 deficiency on serotonergic neuron development are unclear. One possibility is related to the spatial pattern of Fgf8 distribution during development. Peak *Fgf8* expression in the developing hindbrain occurs around embryonic day (E) 9-9.5 and is restricted to a tight band in the rostral-most portion of the anterior hindbrain known as the isthmus (Crossley and Martin 1995). This peak expression coincides with the birth of DR serotonergic neurons (E9.5-12.5) (Cordes 2005). At E12.5, isthmic *Fgf8* expression is nearly gone, but serotonergic neurons continue to differentiate (Xu, Liu et al. 2000; Deneris and Wyler 2012). Between E9-12.5, secreted Fgf8 peptide forms a diffusion gradient that diminishes in strength as it diffuses further away from the isthmus (Trokovic, Jukkola et al. 2005; Vieira, Pombero et al. 2010). DR serotonergic neurons arise from the entire rostral to caudal extent of the anterior portion of the developing hindbrain, an area known as rhombomere 1 (Jensen, Farago et al. 2008). Hence, serotonergic neurons that are derived further away from the isthmus may be more vulnerable to loss of Fgf8 and fail to develop properly when there is inadequate Fgf8. This may explain the selective reduction of the mid- to caudal serotonergic neurons in the Fgf8-deficient mice. In addition to a spatial element, the temporal pattern of Fgf8 production may also contribute to the selective reduction of the more ventral and lateral

serotonergic neuron populations. For example, serotonergic neurons that arise earlier generally form the more ventral and lateral DR subregions (i.e. DRV, DRI, DRVL) (Wallace and Lauder 1983; Hawthorne, Wylie et al. 2010), whereas the more dorsal DRD is composed of cells that arise slightly later (Hawthorne, Wylie et al. 2010). The late-arising population may be more resilient to loss of Fgf8 because those neurons normally form during a time when Fgf8 signaling is diminished.

A surprising outcome is that compound deficiencies of Fgfr1 and Fgf8 do not result in a more severe phenotype than either Fgfr1 or Fgf8 deficiency alone. In fact, the compound hypomorphy abrogates the serotonergic neuron phenotype seen in Fgf8 HET. We believe that the redundancy in Fgf signaling may contribute to this phenomenon. Although Fgfr1 is the only Fgf receptor that continuously overlaps *Fgf8* expression in the isthmus during the time when serotonergic neurons are forming (Blak, Naserke et al. 2005), Fgfr2 has also been implicated in the development of this region. Supporting this notion is that conditional *Fgfr2* deletion, when compounded with *Fgfr1* deletion, led to a more deleterious impact on the DR than *Fgfr1* loss alone (Trokovic, Trokovic et al. 2003; Jukkola, Lahti et al. 2006; Saarimaki-Vire, Peltopuro et al. 2007). This suggests that the Fgf8 signal can be conveyed through redundant Fgfrs in rhombomere 1 (Saarimaki-Vire, Peltopuro et al. 2007). Further, Fgfrs form both hetero- and homodimers upon ligand binding (Dono 2003; Blak, Naserke et al. 2005), thus reductions in Fgfr1 may force it to heterodimerize in a configuration that is more favorable to Fgf8 binding, thereby preventing neuronal loss when compared to Fgf8 deficiency alone. In fact, it has been shown that Fgf8 binds with higher affinity to both Fgfr2 and Fgfr3 than to Fgfr1 (Olsen, Li et al. 2006; Zhang, Ibrahimi et al. 2006), which

are dynamically expressed in rhombomere 1 and are also found in E12.5 serotonergic neuronal cells (Wylie, Hendricks et al. 2010). There may also be functional redundancy with other isthmic Fgf ligands such as Fgf17 and Fgf18 (Xu, Liu et al. 2000) during this period. In sum, compensatory changes in other Fgf ligands and receptors may occur in compound hypomorphs to lessen their phenotype.

Two caveats are associated with our data interpretation. First, we cannot exclude the possibility that loss of Fgf signaling in other brain structures involved in emotional regulation contribute to the observed behavioral deficits. For example, loss of Fgf8 signaling results in cortical patterning defects, whereas deletion of Fgfr1 in the cortex and hippocampus results in dysgenesis of the corpus callosum and hippocampal atrophy (Garel, Huffman et al. 2003; Ohkubo, Uchida et al. 2004). Conditional knockout of Fgfr1 in dopamine neurons results in fewer dopamine neurons and decreased social interaction (Klejbor, Kucinski et al. 2009). Hence, it is possible that Fgf deficits in brain structures other than the DR may contribute to the behavioral phenotype observed in this study. Additional studies are needed to explore the interdependence of Fgf-related anatomical abnormalities and the anxiety-like behavior identified in this study. Second, because we used only three behavioral tests of anxiety, there may be missed opportunity for detecting additional anxiety-like behaviors. That said, we would not anticipate behavioral changes in tests that specifically activate the DRD/DRC system, like social defeat or learned helplessness, because the DRD and DRC are intact in our mice (Gardner, Thrivikraman et al. 2005; Rozeske, Evans et al. 2011). In contrast, we would expect increased panic-susceptibility and anxiety-like behavior following manipulations like adolescent social isolation or administration of panicogenic agents

(i.e. sodium lactate) that specifically involve the DRVL/VLPAG, caudal DRV and DRI (Johnson, Lowry et al. 2008; Lukkes, Kopelman et al. 2013).

In this study, we show that reduced Fgf signaling, particularly Fgf8, is correlated with increased anxiety-like behavior and specific reductions in serotonergic neuron numbers in the DRVL/VLPAG, caudal DRV, and DRI. Although the mechanisms underlying the regional specificity of serotonergic neuronal loss and how this manifests as anxiety-related behavior are unclear, it is likely that the dynamic spatio-temporal expression patterns of Fgf signaling components in the developing midbrain/hindbrain region contribute to this selectivity. Unraveling these mechanisms and exploring functional changes in serotonergic neurons associated with Fgf signaling defects will be important future objectives. Overall, this study adds to our understanding of the developmental heterogeneity of serotonergic neurons and how disruptions to this developmental programming can ultimately impact the manifestation of anxiety-related behavior.

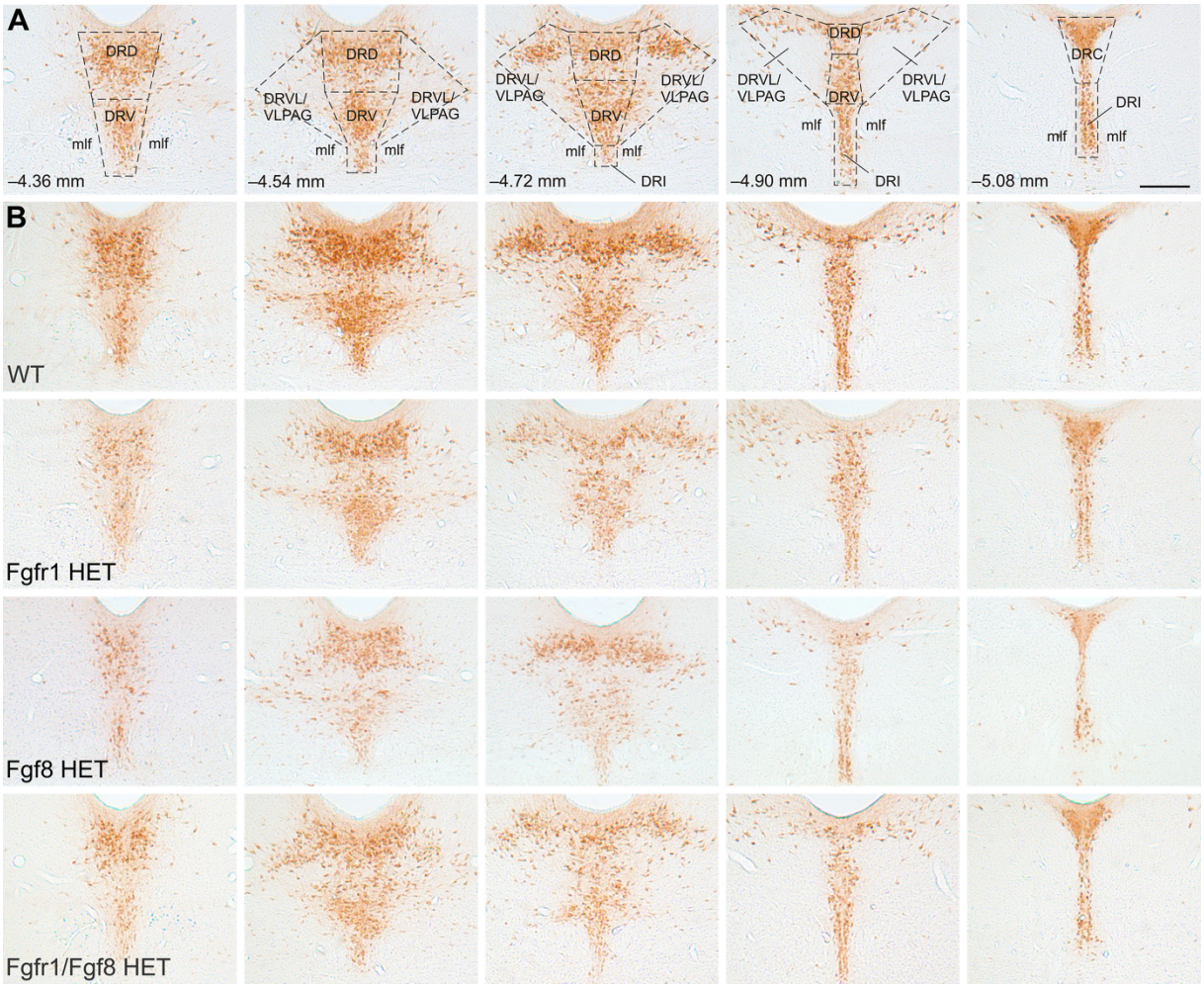

Figure 1. Representative photomicrographs of Tph-ir neurons for each genotype. (A) Schematic overlay outlining each DR subregion where Tph-ir neurons were quantified at five rostrocaudal levels. Each column represents an anatomical level organized from rostral (left) to caudal (right). Distance from bregma (mm) is indicated in lower left hand corner. Scale bar, 250 μm for all images in figure. (B) Representative photomicrographs for each genotype (organized by row) at each rostrocaudal level.

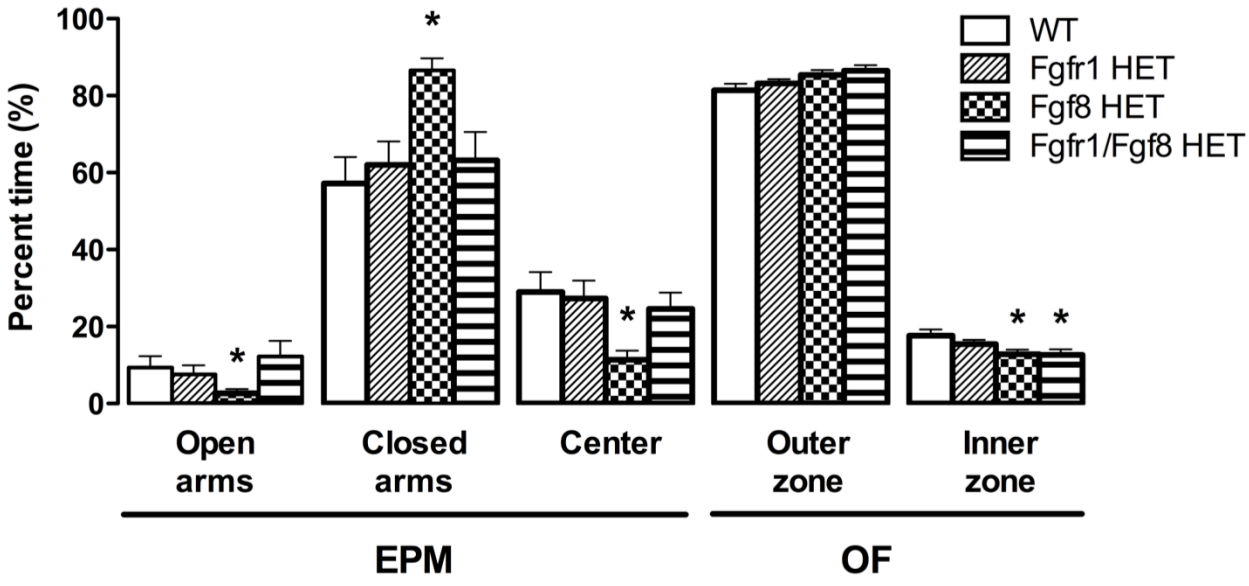

Figure 2. The elevated plus-maze (EPM) and open-field (OF) tests were used to detect anxiety-like behavior in Fgf-deficient mice. Only Fgf8 HET mice exhibited increased anxiety-like behavior in the EPM compared to WT controls as measured by a lower percentage of time spent in the open arms (n = 13-14 WT, n = 14-15 Fgfr1 HET, n = 16-17 Fgf8 HET, n = 13 Fgfr1/Fgf8 HET). However, both Fgf8 HET and Fgfr1/Fgf8 HET mice spent significantly less time in the inner zone of the OF (an indication of increased anxiety-like behavior) compared to WT controls (n = 15 WT, n = 14-15 Fgfr1 HET, n = 21-22 Fgf8 HET, n = 18-19 Fgfr1/Fgf8 HET). *$p < 0.05$ vs. WT; bars represent the mean ± SEM.

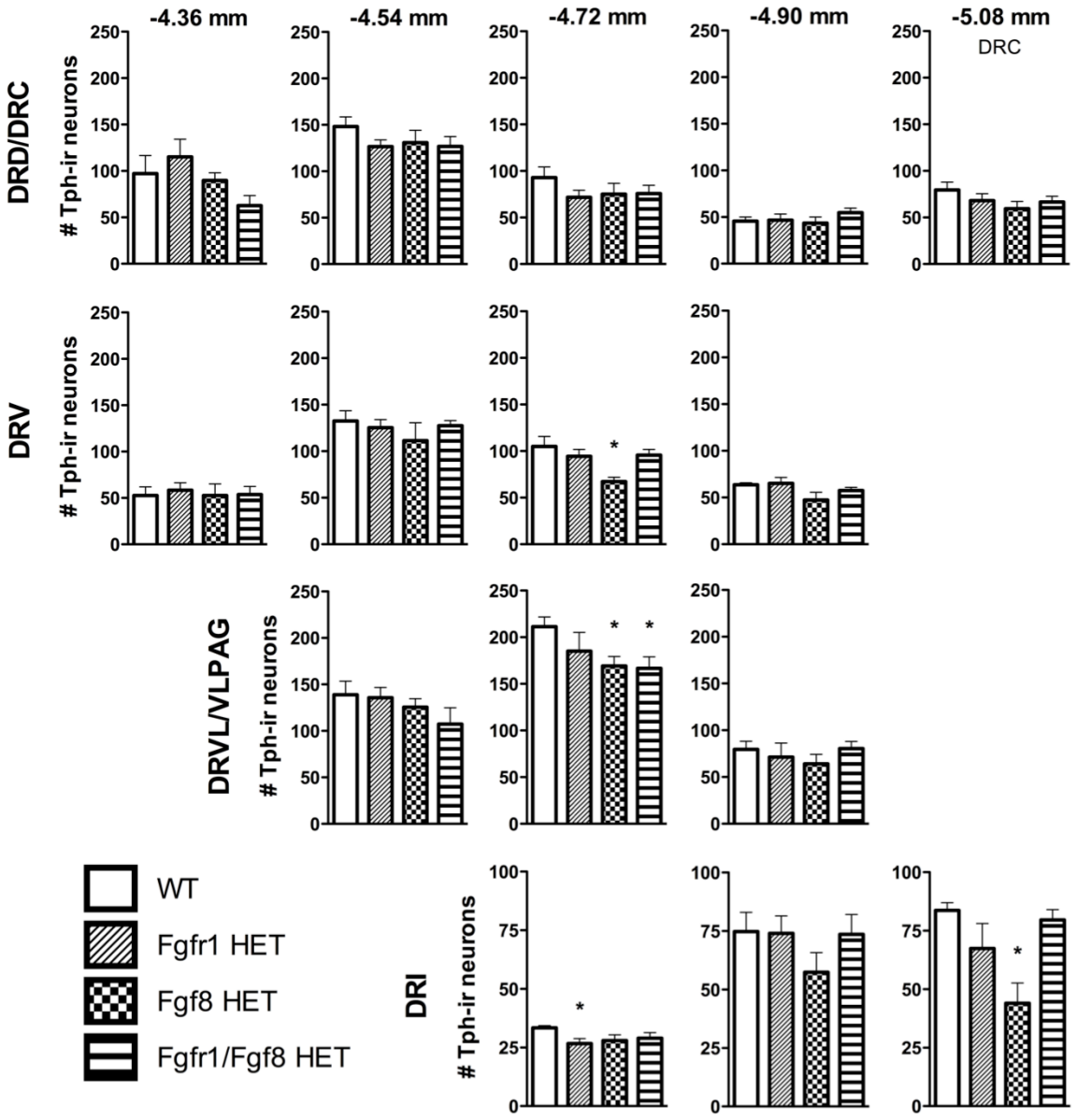

Figure 3. The number of Tph-ir neurons for each genotype within different subregions of the DR at five rostrocaudal levels. DR subregions are organized by row, and anatomical levels are organized in columns from rostral (left) to caudal (right). Distance from bregma is indicated above each column in millimeters. N = 7-8 WT, n = 6-8 Fgfr1 HET, n = 7 Fgf8 HET, n = 9-10 Fgfr1/Fgf8 HET; *p < 0.05 vs. WT; bars represent the mean ± SEM.

Table 1. Sequence of anxiety-related behavioral and motor tests

Day 1	Day 3	Day 5
Elevated plus-maze	Open-field	Light-dark exploration Vertical pole (Cohort 2) Wire grip (Cohort 2)

Table 2. Summary of behavioral and motor tests

		WT	Fgfr1 HET	Fgf8 HET	Fgfr1/Fgf8 HET
EPM	% Open entries	19.93 ± 5.82	15.51 ± 4.06	8.77 ± 3.61	24.80 ± 7.33
	% Closed entries	80.07 ± 5.82	81.78 ± 4.65	91.23 ± 3.61	75.20 ± 7.33
OF	Total distance traveled (cm)	4982 ± 185	4756 ± 284	4691 ± 179	4467 ± 178
LD	Total distance traveled in light compartment (cm)	1886 ± 92	1787 ± 107	1684 ± 117	1763 ± 119
	Latency to enter dark compartment (s)	9.00 ± 1.68	7.19 ± 1.24	5.90 ± 0.81	8.33 ± 1.40
	Total number transitions (#)	28.8 ± 1.17	25.44 ± 1.87	25.18 ± 1.28	27.68 ± 1.46
	% Time in light compartment	52.71 ± 3.55	48.31 ± 3.62	47.18 ± 3.11	49.09 ± 2.98
Grip Test	Latency to fall (s)	54.29 ± 3.48	49.25 ± 5.47	58.75 ± 0.996	52.00 ± 3.36

No significant differences between genotypes were found for any behavior or motor task listed in this table. EPM, OF, LD (n = 14-15 WT, n = 14-16 Fgfr1 HET, n = 16-22 Fgf8 HET, n = 13-19 Fgfr1/Fgf8 HET). Grip test (n = 7 WT, n = 8 Fgfr1 HET, n = 8 Fgf8 HET, n = 14 Fgfr1/Fgf8 HET). Values represent the mean ± SEM.

CHAPTER III: FIBROBLAST GROWTH FACTOR 8 DEFICIENCY COMPROMISES THE FUNCTIONAL RESPONSE OF THE SEROTONERGIC SYSTEM TO STRESS

This chapter has been submitted to PLOS ONE.

Leah R Brooks, Heide L Pals, Courtney L Enix, Rachel A Woolaver, Evan D Paul, Christopher A Lowry and Pei-San Tsai. Fibroblast growth factor 8 deficiency compromises the functional response of the serotonergic system to stress.

Submitted to PLOS ONE (2014).

1. ABSTRACT

Functionally heterogeneous populations of serotonergic neurons, located within the dorsal raphe nucleus (DR), play a role in stress-related behaviors and neuropsychiatric illnesses such as anxiety and depression. Abnormal development of these neurons may permanently alter their structure and connections, making the organism more susceptible to anxiety-related disorders. A factor that critically regulates the development of serotonergic neurons is fibroblast growth factor 8 (Fgf8). In this study, we used acute restraint stress followed by behavioral testing to examine whether Fgf8 signaling during development is important for establishing functional stress- and anxiety-related DR neurocircuits in adulthood. Wild-type and heterozygous male mice globally hypomorphic for Fgf8 were exposed to acute restraint stress and then tested for anxiety-like behavior on the elevated plus-maze. Further, we measured c-Fos immunostaining as a marker of serotonergic neuronal activation and tissue 5-hydroxyindoleacetic acid concentrations as a marker of serotonin functional output. Results showed that Fgf8 hypomorphs exhibited 1) an exaggerated response of DR anxiety-promoting circuits and 2) a blunted response of a DR panic-inhibiting circuit to stress, effects that together were associated with increased baseline anxiety-like behavior. Overall, our results provide a neural substrate upon which Fgf8 deficiency could affect stress response and support the hypothesis that developmental disruptions of serotonergic neurons affect their postnatal functional integrity.

2. BACKGROUND

Many psychopathologies, including anxiety and affective disorders, may be neurodevelopmental in origin (Leonardo and Hen 2008). The serotonergic system is a key component of the anxiety circuitries. Ample evidence suggests environmental stressors can affect the serotonergic system to precipitate anxiety or affective disorders (Gross, Zhuang et al. 2002; Sapolsky 2003; Gardner, Hale et al. 2009; Lupien, McEwen et al. 2009). A conspicuous gap in our knowledge is how the abnormal development of serotonergic neurons may permanently alter their structure and connections to affect emotional behaviors. In this regard, it is important to investigate the actions of factors critical for the formation of the serotonergic system, since deficiencies in these factors can contribute to permanent and serious neurochemical and behavioral consequences.

The serotonergic system, spanning from the midbrain to the medulla, consists of functionally heterogeneous neurons that project to diverse forebrain and brainstem targets. The dorsal population of serotonergic neurons, or the dorsal raphe nucleus (DR), is located in the caudal midbrain and rostral pons (Gaspar, Cases et al. 2003). The DR is further subdivided into dorsal (DRD), ventral (DRV), ventrolateral DR/ventrolateral periaqueductal gray (DRVL/VLPAG), interfascicular (DRI), and caudal (DRC) regions. These regions are functionally and topographically organized and project to regions responsible for modulating emotional and stress-related behavior, such as limbic and basal ganglia structures (Hale, Shekhar et al. 2012). DRD serotonergic neurons project to forebrain regions that respond to anxiogenic drugs and stressors, such as the prelimbic (PrL) and infralimbic (IL) cortices, and the basolateral

amygdala (BLA) (Singewald, Salchner et al. 2003; Lowry, Johnson et al. 2005), suggesting a role in modulating anxiety-related circuitries. Further, surgical and pharmacological manipulations of the DR result in altered anxiety states (Sommer, Moller et al. 2001; Maier and Watkins 2005; Greenwood, Strong et al. 2008). There is also evidence that serotonergic neurons in the DRVL/VLPAG provide inhibitory input to the dorsal periaqueductal gray (DPAG) to attenuate panic-like responses to mild to moderate stressors (Johnson, Lightman et al. 2004; Johnson, Lowry et al. 2008). Therefore, developmental disruption of subpopulations of “anxiogenic” or “panic-inhibiting” serotonergic neurons and the associated connectivity may profoundly impact stress-related anxiety- and panic-like responses.

Fibroblast growth factors (Fgfs) and their receptors (Fgfrs) are critical for DR development (Partanen 2007). *Fgf8* is expressed in the prenatal hindbrain (Lein, Hawrylycz et al. 2007). Deficient Fgf signaling, in particular Fgf8 and Fgf receptor (Fgfr) 1, results in fate specification failure and a loss of serotonergic neurons during development (Crossley and Martin 1995; Meyers, Lewandoski et al. 1998; Ye, Shimamura et al. 1998; Chi, Martinez et al. 2003; Jukkola, Lahti et al. 2006; Saarimaki-Vire, Peltopuro et al. 2007). Importantly, we recently showed that Fgf8 deficiency is associated with a loss of specific DR serotonergic neurons in the mid- to caudal DRV, DRVL/VLPAG, and DRI subregions and increased anxiety-like behavior (Brooks, Enix et al. 2014). However, it is unclear if the function and connectivity of anxiety- and panic-related DR subregions are also disrupted in these mice.

In this study, we tested the functionality of the developmentally compromised DR in Fgf8-deficient mice using acute restraint stress followed by behavioral testing. The

goal of this study is to extend our previous findings by examining if Fgf8 deficiency disrupts the activation of DR serotonergic neurons, their functional output to anxiety- and panic-related projection regions, and anxiety-like behavior following stress (Brooks, Enix et al. 2014). Indeed, we found dysregulated responses to stress in both anxiety-promoting and panic-inhibiting circuits of Fgf8-deficient mice, which were associated with increased baseline anxiety-like behavior. Together these data expand our knowledge on the developmental factors needed to establish functional serotonergic circuits and related behavioral responses. Further, they raise the possibility that some abnormal anxiety- and stress-related responses in humans may stem from developmental deficiencies in Fgf signaling, such as those resulting from inactivating mutations on the *Fgf8* gene (Falardeau, Chung et al. 2008).

3. MATERIALS AND METHODS

3.1 Animals

All experiments were conducted using 8-10 week-old male wild-type (WT) or *Fgf8* heterozygous (HET) hypomorphic mice (129p2/OlaHsd* CD-1; obtained from Mouse Regional Resource Centers) (Meyers, Lewandoski et al. 1998). *Fgf8* hypomorphic mice contain a neomycin-resistance element inserted into the non-coding region of the *Fgf8* gene. This element contains false splice sites that lead to about a 55% reduction in functional *Fgf8* transcript levels under homozygous condition (Meyers, Lewandoski et al. 1998). *Fgf8* homozygous hypomorphic mice die within 24 h of birth but Fgf8 HET mice survive normally and have no obvious health problems. WT and Fgf8 HET mice were housed in same-sex littermate groups of 2-5 at weaning and

genotyped by polymerase chain reaction of genomic DNA isolated from tail clips. All mice were bred at the University of Colorado Boulder in the Integrative Physiology department animal facility under a 12L: 12D photoperiod with free access to water and rodent chow. Prior to experiments, mice were left undisturbed except during routine cage changes. Experimental mice were moved to an adjacent room immediately prior to testing.

3.1.1 Ethics Statement

All animal procedures complied with the protocols approved by the Institutional Animal Care and Use Committee at the University of Colorado Boulder (Protocol # 1106.05) and adhered to the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. All efforts were made to minimize animal discomfort.

3.2 Restraint stress

Male mice were assigned to either non-stress (NS; n = 15 WT, n = 17 Fgf8 HET) or stress (S; n = 17 WT, n = 14 Fgf8 HET) groups. Each group experienced the exact same testing procedures, except the non-stress mice were left in their cages and the stress group were removed and restrained in ventilated 50 mL conical tubes for 1 h.

3.3 Elevated plus-maze (EPM)

Immediately after 1 h of non-stress or restraint stress, mice were placed on a black acrylic EPM for 5 min. The EPM consisted of a center area (5 cm × 5 cm) from which two opposing open arms (29 cm × 5 cm) and two opposing closed arms with the

same dimensions and walls (15 cm high) were extended. The maze was elevated 60 cm off the ground. Mice were placed in the center area of the EPM facing an open arm to start the test as previously described (Voikar, Vasar et al. 2004; Bailey, Pavlova et al. 2007). Mice that fell off the maze were excluded from analysis (n = 4 WT, n = 2 Fgf8 HET). After the test was completed, mice were returned to their cages. Behavioral testing commenced within 2 h and was completed within 6 h of light phase onset. Room lighting was approximately 480 lx. The EPM was cleaned with 70% ethanol before testing and between each test subject. A video camera was mounted above the EPM to record behavior for later scoring by an observer blinded to the genotypes and groups. The entries or total duration within an area began when all four paws crossed into the area of interest. The time spent in the open, closed and center areas, and number of entries into each arm were scored manually. For analysis, the time spent on the arms and number of entries were expressed as a percentage of the total test duration and number of arm entries, respectively.

3.4 Motor coordination

Baseline balance and motor coordination were tested using an accelerating rotarod (Ugo Basile) in a separate cohort of WT (n = 12) and Fgf8 HET (n = 16) mice. The test consisted of three trials separated by 15 min inter-trial intervals. Four animals were tested together in separate compartments on a rod 3 cm in diameter. Initial velocity was 4 rpm and the rod was gradually accelerated to a maximum of 40 rpm over 5 min. The latency to fall off of the rod during a 5 min test period was recorded. Passive rotations were considered a failure in performance, and the latency to the first full passive rotation was recorded as the latency to fall.

3.5 Tissue collection

Two hours after the onset of the non-stress or stress conditions, mice were deeply anesthetized with isoflurane and decapitated. Brains were removed and either immersion-fixed in 4% paraformaldehyde for 24 h at 4°C then stored in a 30% sucrose cryoprotectant at 4°C or flash-frozen in isopentane and stored at −70°C until processing for immunohistochemistry (IHC) and high-performance liquid chromatography with electrochemical detection (HPLC-ED), respectively.

3.6 Immunohistochemistry (IHC)

Double immunohistochemical staining for c-Fos (the protein product of the immediate-early gene *c-fos*) and tryptophan hydroxylase (Tph; the rate-limiting enzyme for serotonin biosynthesis) was performed (WT: n = 5 NS, n = 5 S; Fgf8 HET: n = 6 NS, n = 6 S). Before sectioning for IHC, brains were blocked at the caudal border of the mammillary body using a mouse brain matrix (RBM 2000C, ASI Instruments). The tissue block posterior to the mammillary body containing the raphe nuclei was immediately sectioned using a cryostat into 30 µm frozen coronal floating sections that were collected into a series of six microcentrifuge tubes filled with a cryoprotectant (30% sucrose, 30% ethylene glycol, 1% polyvinylpyrolidone in 0.2 M sodium phosphate buffer). For IHC, one third of the sections were taken through a series of rinses and sequential incubations on an orbital shaker using a rabbit anti-c-Fos antibody that has been previously characterized (Ferrer, Olive et al. 1996; Bailey, Centers et al. 2006) (SC-253, Santa Cruz Biotechnology), a biotinylated donkey anti-rabbit secondary antibody (711-065-152, Jackson ImmunoResearch Laboratories), a validated avidin-

biotin complex (NeutrAvidin®, A2666, Life Technologies; Peroxidase-biotinamidocaproyl conjugate, P-9568, Sigma-Aldrich), and reacted with nickel enhanced 3,3′-diaminobenzidine tetrahydrochloride (DAB; D5637, Sigma-Aldrich) for color detection (Brooks, Chung et al. 2010; Hale, Dady et al. 2011; Hale, Shekhar et al. 2011; Brooks, Enix et al. 2014). This was immediately followed by a second IHC using a sheep anti-tryptophan hydroxylase antibody that has been previously characterized and shown to bind specifically to both isoforms of Tph (Hale, Dady et al. 2011; Hale, Shekhar et al. 2011) (T8575, Sigma-Aldrich), a biotinylated donkey anti-sheep secondary antibody (713-065-147, Jackson ImmunoResearch Laboratories), and reacted with DAB for color detection as previously described (Brooks, Chung et al. 2010; Hale, Dady et al. 2011; Hale, Shekhar et al. 2011; Brooks, Enix et al. 2014). After the color reaction, sections were rinsed, mounted on gelatin-coated slides, dehydrated through increasing concentrations of ethanol (70- 100%), cleared in Histo-Clear (National Diagnostics), and coverslipped with Permount (Fisher Scientific).

3.7 Neuronal quantification

Neurons that were immunoreactive (ir) for c-Fos had a dark blue/black colored nucleus (c-Fos-ir), while Tph-ir neurons were characterized by a brown cytoplasm. Cells that were double-labeled with both c-Fos and Tph and those labeled with only Tph were counted by an investigator blind to the treatment groups at five rostrocaudal levels (−4.36, −4.54, −4.72, −4.90, and −5.08 mm bregma) under a brightfield microscope. Neurons were quantified in the dorsal (DRD; −4.36, −4.54, −4.72, and −4.90 mm bregma), ventral (DRV; −4.36, −4.54, −4.72, and −4.90 mm bregma), ventrolateral part/ventrolateral periaqueductal gray (DRVL/VLPAG; −4.54, −4.72, and −4.90 mm

bregma), interfascicular (DRI; −4.72, −4.90, and −5.08 bregma), and caudal (DRC; −5.08 mm bregma) subregions of the DR. For analysis, c-Fos/Tph-ir neurons were expressed as a percentage of the total number of Tph-ir neurons within each subregion at each rostrocaudal level. Representative photomicrographs for each genotype and stress condition at −4.72 mm bregma are shown in Figure 1.

3.8 Brain microdissection and high-performance liquid chromatography with electrochemical detection (HPLC-ED)

To investigate the functional output of DR circuits in WT and Fgf8 HET mice, the concentrations of 5-hydroxyindoleacetic acid (5-HIAA) and serotonin (5-HT) were measured in the DR and select stress-, anxiety-, and panic-related projection sites. Methods for microdissection and HPLC-ED have been previously described (Evans, Reinders et al. 2008; Neufeld-Cohen, Evans et al. 2010). Briefly, brains were cryosectioned into 300 µm-thick slices, placed on slides, and stored at −70°C (WT: n = 5 NS, n = 6 S; Fgf8 HET: n = 9 NS, n = 6 S). The regions selected for microdissection (Table 1) were micropunched under a stereomicroscope using a standard mouse brain stereotaxic atlas as a guide (Paxinos and Franklin 2001). These regions were chosen because they represent projection sites of anxiety- and stress-related subregions of the DR. Following extraction of micropunched tissues in acetate buffer, 45 µl of the supernatant from each sample was placed in an ESA 542 autosampler (ESA Analytical) and the pellet reconstituted in 0.2 M sodium hydroxide for protein quantification (23235, Thermo Scientific). A total of 25 µl of each sample was injected into the chromatographic system. Chromatographic separation and electrochemical detection were accomplished using a previously described method [13]. Data were normalized by

protein content and represented as pg/µg protein. The ratio of 5-HIAA to 5-HT was calculated (5-HIAA/5-HT ratio) and serves as an indication of 5-HT turnover in the DR and projection sites.

3.9 Corticosterone enzyme-linked immunoassay

To investigate the effects of non-stress or restraint stress conditions on corticosterone levels in WT and Fgf8-deficient mice, a separate cohort of mice (WT: n = 7 NS, n = 7 S; Fgf8 HET: n = 7 NS, n = 7 S) was sacrificed immediately after 1 h of control or restraint stress and their trunk blood collected into heparinized tubes. Plasma samples were isolated by centrifugation and stored at −20°C until the measurement of corticosterone by a commercial enzyme-linked immunoassay kit (ADI-900-097, Enzo Life Sciences) according to manufacturer's instructions. The intra- and inter-assay coefficients of variation were 6.6−8.4% and 7.8−13.1%, respectively, and the limit of detection was 26.99 pg/ml.

3.10 Statistical analysis

All statistical analyses were completed using SPSS Statistics (version 21.0 for Mac; IBM). All data were analyzed using two-way analysis of variance (ANOVA) for genotype and stress except the rotarod data, which were analyzed using repeated measures ANOVA. Post-hoc analysis was performed using Fisher's Protected LSD. Data that failed the homoscedasticity test were ln (n+1)-transformed. Statistical outliers were determined using the Grubbs' test and were removed (Grubbs 1969). For the EPM, 1 out of 57 data points for each the percent time in open, closed and center area were excluded (1.8% of total data for each), and 2 out of 57 data points for the

percentage of open entries were excluded (3.5%). For the rotarod, 4 out of 84 data points (4.8%) were excluded. Missing rotarod data were replaced using the Peterson method for the repeated measures ANOVA (Petersen 1985). For the percent c-Fos/Tph-ir and total number of Tph-ir neurons in each DR subregion, 15 and 5 out of 330 data points each (4.5% and 1.5%, respectively) were excluded. For the concentrations of 5-HIAA and 5-HT, 14 and 12 out of 310 data points each (4.5% and 3.9%, respectively) were excluded. For the 5-HIAA/5-HT ratio 22 out of 310 data points (7.1%) were excluded. For all of the HPLC data combined, 48 out of 930 data points (5.2%) were excluded. There were no outliers for the corticosterone data. Values were shown as the mean ± SEM. Data were considered significant when $p < 0.05$.

4. RESULTS

4.1 Anxiety-like behavior and motor coordination

As shown in Figure 2, there was a significant main effect of genotype on the percentage of time spent in the open and closed arms of the EPM [$F(1, 52) = 6.3$, $p = 0.015$, $F(1, 52) = 7.4$, $p = 0.009$, respectively]. There was also a significant interaction between genotype and stress on the percentage of open entries, and percent time spent in the open and closed arms of the EPM [$F(1, 52) = 7.9$, $p = 0.007$, $F(1, 52) = 9.4$, $p = 0.003$, $F(1,52) = 7.7$, $p = 0.008$, respectively]. The mean percentage of open entries were 23 ± 6, 10 ± 2, 8 ± 2, and 16 ± 4 for WT non-stress, WT stress, Fgf8 HET non-stress, and Fgf8 HET stress mice, respectively (mean ± SEM). Post hoc analysis revealed that restraint stress reduced the percentage of open entries ($p = 0.018$) and percentage of time WT mice spent on the open arms ($p = 0.007$; Figure 2) compared to

non-stress WT mice. Similarly, non-stress Fgf8 HET mice had a lower percentage of entries into the open arms ($p = 0.006$) and less overall time on the open arms ($p < 0.001$; Figure 2) compared to WT non-stress mice. Non-stress Fgf8 HET mice spent a larger percentage of time in the closed arms ($p < 0.001$) versus non-stress WT and stress Fgf8 HET mice ($p = 0.031$). There were no significant differences between genotypes or stress conditions in the percent time spent in the center area, the total number of closed arm entries (a measure of exploratory behavior and motor function, data not shown), or latency to fall from the rotarod (indicating that motor function was intact in Fgf8 HET mice, data not shown).

4.2 Neuron counts

To investigate the functional integrity of stress-, anxiety-, and panic-related serotonergic circuitries in Fgf8 HET mice, we quantified the percentage of serotonergic cells that co-expressed c-Fos and Tph (c-Fos/Tph-ir) following restraint stress (Figure 3, Table 2). Two-way ANOVA revealed a significant effect of stress on the percentage of c-Fos/Tph-ir neurons in several mid- and caudal subregions of the DR (Figure 3, Table 2). These included the DRD, DRVL/VLPAG (Figure 3), and DRV (Table 2) at −4.54 mm and −4.72 mm bregma, and the DRI at −4.90 mm bregma (Table 2). In general, post hoc analysis revealed that restraint stress increased the percentage of c-Fos/Tph-ir neurons in WT mice (−4.54 mm bregma, DRD $p = 0.03$, DRV $p = 0.003$, DRVL/VLPAG $p = 0.015$; −4.72 mm bregma, DRD $p = 0.01$, DRVL/VLPAG $p = 0.008$) except in the caudal DRI where stress was associated with a lower percentage of c-Fos/Tph-ir neurons ($p = 0.01$). There was a significant genotype x stress interaction in the percentage of c-Fos/Tph-ir neurons in the DRV (Table 2) and DRVL/VLPAG at −4.54

mm bregma (Figure 3). Non-stress Fgf8 HET mice had a higher percentage of c-Fos/Tph-ir than WT non-stress mice in the DRD (p = 0.041) at −4.72 mm bregma (Figure 3). Fgf8 HET mice also had significantly fewer Tph-ir neurons in the DRV at −4.90 mm bregma compared to WT mice [F(1, 17) = 5.8, p = 0.027; Supporting Information, Table S1].

4.3 5-HIAA tissue content

To investigate whether Fgf8 deficiency impacted serotonergic functional output in anxiety-, panic- and stress-related circuits, tissue content of 5-HIAA (Figure 4, Table 3), 5-HT (Table S2) and the 5-HIAA/5-HT ratio (Table S3) were analyzed. Stress elevated these parameters in a number of the brain regions analyzed, but here we focus on 5-HIAA content as a marker of serotonergic output in the anxiety-promoting DRD and panic-inhibiting DRVL/VLPAG circuits. Two-way ANOVA revealed a significant effect of stress on 5-HIAA content in both the anxiety-promoting DRD and the panic-inhibiting DRVL/VLPAG projection sites [BLA: F(1, 22) = 18.0, p < 0.001, PrL: F(1, 20) = 4.6, p = 0.045, IL: F(1, 20) = 9.5, p = 0.006, DLPAG: F(1, 20) = 10.2, p = 0.005; Figure 4]. There was also a significant genotype x stress interaction in 5-HIAA content in the DLPAG [F(1, 20) = 9.2, p = 0.007]. Post hoc analysis revealed that in the panic-inhibiting DRVL/VLPAG circuit, stress increased both DRVL/VLPAG (Table 3) and DLPAG 5-HIAA content (Figure 4) in WT mice (p = 0.044 and p = 0.001, respectively) but only the former in Fgf8-deficient mice (p = 0.001, Figure 4 and Table 3). In the anxiety-promoting DR projection sites (BLA, PrL, IL), post hoc analysis revealed stress increased 5-HIAA content in Fgf8 HET mice but not WT mice (p = 0.017, p = 0.010, and p = 0.001, respectively, Figure 4).

5. DISCUSSION

In this study, we used acute restraint stress and behavioral testing to examine the functionality of the DR serotonergic system in Fgf8-deficient mice. We found both anxiety-promoting and panic-inhibiting serotonergic neurocircuits were altered in these mice, and these changes were associated with elevated baseline anxiety-like behavior in Fgf8-deficient mice. Consistent with previous reports (Tanaka, Kohno et al. 1983; Stone, Rhee et al. 1996; Kirby, Chou-Green et al. 1997; Hale, Shekhar et al. 2012), stress was generally associated with increased DR serotonergic neuronal activation and metabolism in widespread DR projection sites. Differences in DR serotonergic neuronal activation and 5-HT metabolism between genotypes were limited to mid-rostrocaudal to caudal DR subregions and related projection sites. For example, stress increased DRD and DRVL/VLPAG serotonergic neuronal activation in WT mice. Interestingly, similar baseline activation of serotonergic DRD neurons was found in non-stress Fgf8-deficient mice, and their DRVL/VLPAG serotonergic neuronal activation was blunted following stress. This blunted DRVL/VLPAG serotonergic activation was associated with diminished 5-HT metabolism in the DLPAG. Disruption of these subregions and their concomitant connectivity has previously been correlated with chronic anxiety-like states and vulnerability to panic- and anxiety-like behavior (Paul and Lowry 2013). Together these data highlight the importance of developmental Fgf8 signaling in establishing functional anxiety- and panic-related DR serotonergic neurocircuits.

Elevated anxiety-like behavior in Fgf8-deficient mice

Exposure of WT mice to restraint stress increased anxiety-like behavior as measured by the EPM immediately following stress, whereas anxiety-like behavior was equally elevated in both the non-stress and stress Fgf8-deficient mice. This is consistent with previous studies reporting that stress can precipitate anxiety-like behavior (Heinrichs, Menzaghi et al. 1994; Korte and De Boer 2003) as well our finding that Fgf8 deficiency is associated with increased baseline anxiety-like behavior (Brooks, Enix et al. 2014). These data suggest that Fgf8-deficient mice are more susceptible to stress- and anxiety-related stimuli as they have exaggerated behavioral responses to mild aversive stimuli (such as EPM exposure) compared to WT controls (Belzung and Griebel 2001). This anxiogenic phenotype is not related to differences in the magnitude of the neuroendocrine stress response (Text S1), and likely reflects a functional disruption of central anxiety-related circuits resulting from Fgf8 deficiency.

Altered neuronal activation in anxiety- and panic-related DR subregions of Fgf8-deficient mice

Stress increased c-Fos expression in the mid-rostrocaudal DRD and DRVL/VLPAG serotonergic neurons in WT mice, which is consistent with previous studies examining the effects of stress on the activation of DR serotonergic neurons (Hale, Shekhar et al. 2012). In Fgf8-deficient mice, however, there was activation of the DRD in the non-stress group and blunted DRVL/VLPAG activation in response to stress compared to WT mice. The DRD and DRVL/VLPAG have been implicated in responses to stress-, anxiety- and panic-related stimuli, suggesting that these subregions of the DR may be especially sensitive to stress- or anxiogenic challenges (Hale, Shekhar et al. 2012). In particular, the DRD has been associated with anxiety-promoting responses

while the DRVL/VLPAG is related to inhibiting panic-like responses (Johnson, Lightman et al. 2004; Lowry, Hale et al. 2008). In support of this functional assignment, inescapable stress, anxiogenic drugs, fear-potentiated startle, and the avoidance task in the elevated T-maze activate serotonergic DRD neurons (Grahn, Will et al. 1999; Abrams, Johnson et al. 2005; Spannuth, Hale et al. 2011; Spiacci, Coimbra et al. 2012), and panicogenic stimuli such as hypercapnia or sodium lactate infusions activate DRVL/VLPAG serotonergic neurons that are associated with inhibition of panic-like behavior (Johnson, Hollis et al. 2005; Johnson, Lowry et al. 2008). Increased activation of the DRD in non-stress Fgf8-deficient mice compared to WT non-stress mice is consistent with their anxiety-like phenotype. Similarly, blunted activation of DRVL/VLPAG neurons in response to stress is consistent with a failure to activate the panic- and anxiety-suppressing serotonergic system, a circuit normally recruited in response to moderate stressors (Johnson, Lightman et al. 2004). Together these data suggest that disruptions in DRD and DRVL/VLPAG neuronal activation may contribute to increased vulnerability to panic- and anxiety-like behaviors in Fgf8-deficient mice.

Activation of serotonergic neurons in the DRD and DRVL/VLPAG following stress is likely related to afferent input from neural circuits regulating stress and emotional responses. DRD afferents include forebrain regions such as the IL, PrL, and bed nucleus of the stria terminalis, and some of these connections are reciprocal. The DRVL/VLPAG receives input from both forebrain and brainstem regions such as the IL, CE, lateral parabrachial nucleus, and the nucleus of the solitary tract (see reviews (Hale and Lowry 2011; Hale, Shekhar et al. 2012)). The activational perturbations in Fgf8-deficient mice may represent a primary defect in serotonergic neurons (i.e. 5-HT

synthesis, firing rate, release, uptake, receptor expression and sensitivity) or may reflect abnormal afferent input as a result of Fgf8 deficiency. There is evidence that Fgf signaling modulates neurite outgrowth and internal cellular calcium concentration following N-methyl-D-aspartate receptor activation (Mattson, Murrain et al. 1989; Boxer, Moreno et al. 1999). Hence it is possible that loss of Fgf8 signaling impacts the ability of serotonergic neurons to form proper synaptic connections due to abnormal dendritic growth or branching and to respond appropriately to excitatory input. It has also been shown that Fgfs act as target-derived organizers that guide differentiation of both excitatory and inhibitory presynaptic terminals (Terauchi, Johnson-Venkatesh et al.). In this regard, loss of Fgf8 in the developing DR may result in a subregional imbalance of excitatory and inhibitory synapses, and subsequently abnormal responses to stress-related and anxiogenic stimuli.

Altered 5-HIAA levels in anxiety- and panic- related neural circuits of Fgf8-deficient mice

Fgf8-deficient mice had greater stress-induced increases in 5-HIAA in anxiety-promoting DR target regions as well as decreased 5-HIAA in a panic-suppressing DR target region (Figure 4, Table 3). Together these effects promote an anxiety-like phenotype and may be a consequence of abnormalities within serotonergic neurons, serotonergic DR target regions or with afferent input to the DR due to Fgf8 deficiency. For example, DR has reciprocal connections with the ventral medial prefrontal cortex (vmPFC: IL, PrL) (Hale and Lowry 2011). 5-HT released in the vmPFC can bind postsynaptic 5-HT inhibitory receptors (5-HT_{1A}) or excitatory 5-HT_{2A} receptors located on excitatory and inhibitory neurons (Celada, Puig et al. 2004). The vmPFC in turn

sends glutamatergic projections to the DR that largely inhibit serotonergic neurons via activation of local γ-aminobutyric acid synthesizing neurons (Wang, Ochiai et al. 1992; Celada, Puig et al. 2001; Jankowski and Sesack 2004) or activation of the 5-HT_{1A} autoreceptors due to intra-DR 5-HT release (Celada, Puig et al. 2001). Fgf8 is expressed in the developing vmPFC and a loss of Fgf signaling in this region results in overall telencephalic hypoplasia, loss of glutamatergic neurons, and axon targeting defects (Huffman, Garel et al. 2004; Storm, Garel et al. 2006; Iwata and Hevner 2009; Toyoda, Assimacopoulos et al. 2010). These data, combined with the role of Fgfs in neurite outgrowth and synapse formation, raise the possibility that defects in serotonergic projection neurons, vmPFC afferents, or both, may result in elevated stress-induced tissue 5-HIAA content in the vmPFC. Although less is known about the developmental roles of Fgf8 in the DLPAG and BLA, similar mechanisms may account for altered 5-HIAA content in those regions. Additional studies are needed to explore these possibilities and whether Fgf8-related structural abnormalities in other brain regions contribute to vulnerability to stress- and anxiety-related behaviors.

In conclusion, this study demonstrates that reduced Fgf8 signaling is correlated with increased anxiety-like behavior and dysregulated serotonergic neuron activation and functional output in response to stress- and anxiogenic stimuli. The functional disruption is particularly prominent in DRD and DRVL/VLPAG serotonergic neurocircuits. Our results provide strong support for an early role of Fgf8 signaling in programming stress- and anxiety-related serotonergic neurocircuits responsible for proper behavioral response to stress. Specifically, these data suggest that Fgf8 signaling is not only critical for the early formation and positioning of DR neurons but

also the integration of serotonergic neurons into functional stress- and anxiety-related circuitries.

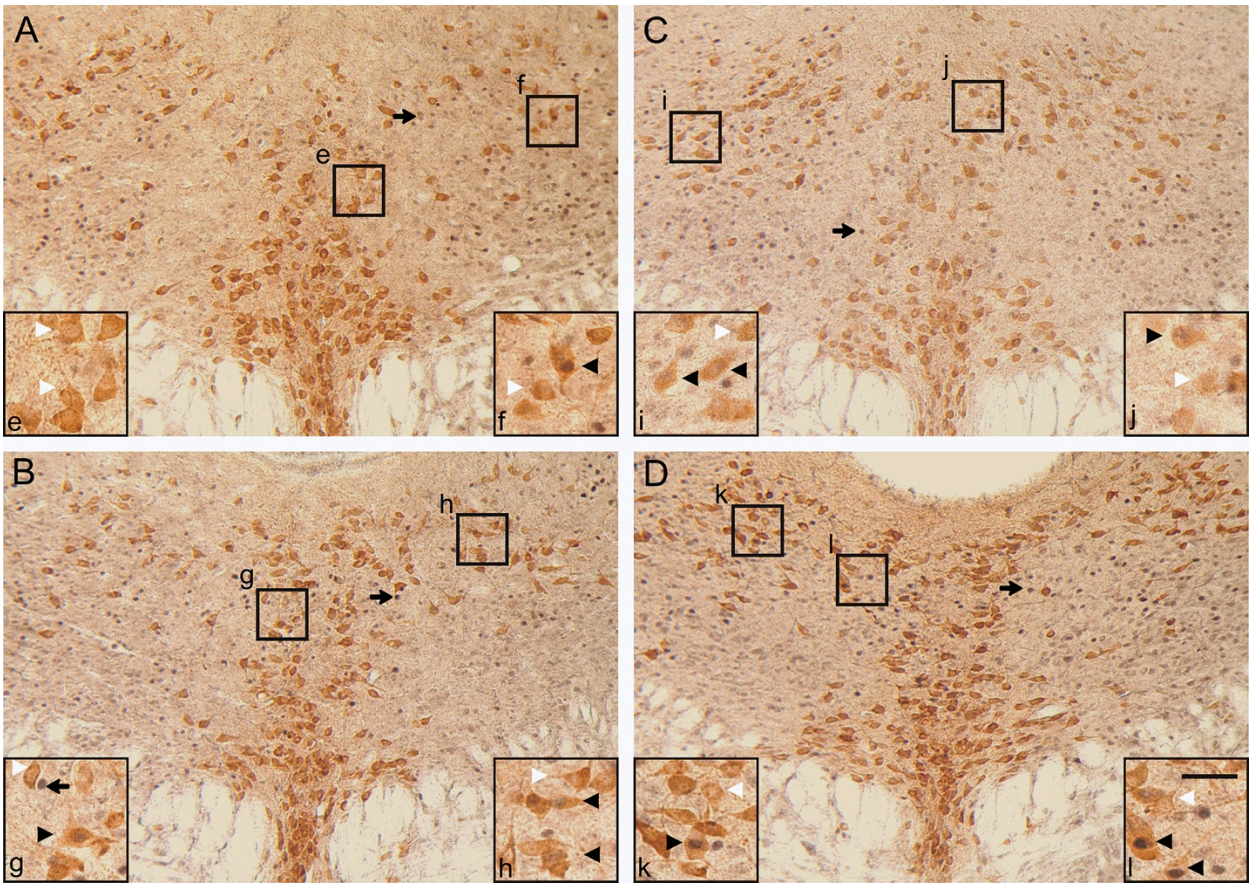

Figure 1. Representative photomicrographs illustrating c-Fos-ir and Tph-ir neurons in the DR at −4.72 mm bregma. WT (A, B) and Fgf8-deficient (C, D) mice exposed to non-stress (A, C) or restraint stress (B, D) conditions. Black boxes with lower case letters, e-l, correspond to higher magnification insets found in the corners of panels A-D. Arrows indicate c-Fos-ir nuclei (blue/black nuclear staining); white arrowheads indicate Tph-ir neurons (brown cytoplasmic staining); black arrowheads indicate c-Fos/Tph-ir double-immunostained neurons. Scale bar represents 100 μm for A-D and 40 μm for insets e-l.

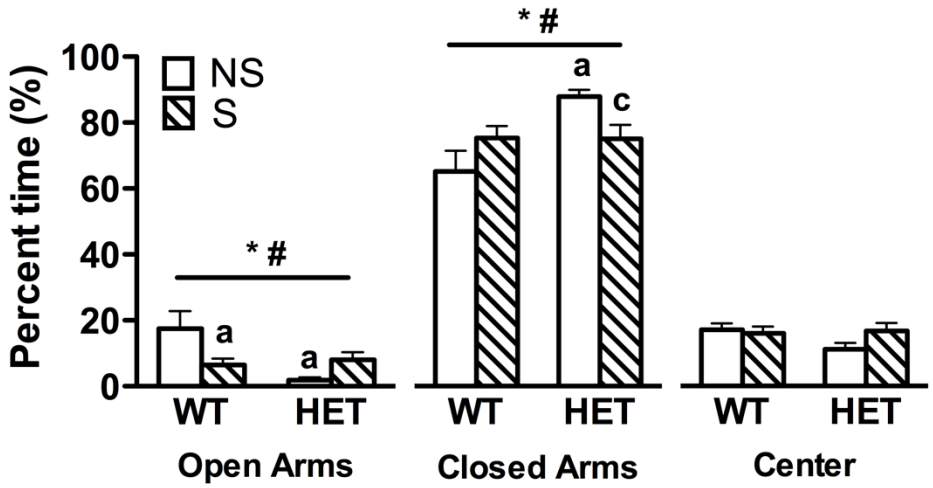

Figure 2. Fgf8-deficient mice have elevated anxiety-like behavior. Anxiety-like behavior was tested using the elevated plus-maze following non-stress (NS) or restraint stress (S) in WT and Fgf8-deficient (HET) mice. Restraint stress significantly increased anxiety-like behavior in WT mice as measured by a lower percentage of time spent in the open arms. Non-stress Fgf8 HET mice spent significantly less time in the open arms and more time in the closed arms than WT non-stress mice. No differences were found for the percent time spent in the center area. N = 12−16/group. Data are presented as mean ± SEM, $^{a}p < 0.01$ vs. WT NS, $^{c}p < 0.05$ vs. HET NS, *main effect of genotype, $^{\#}$genotype x stress interaction.

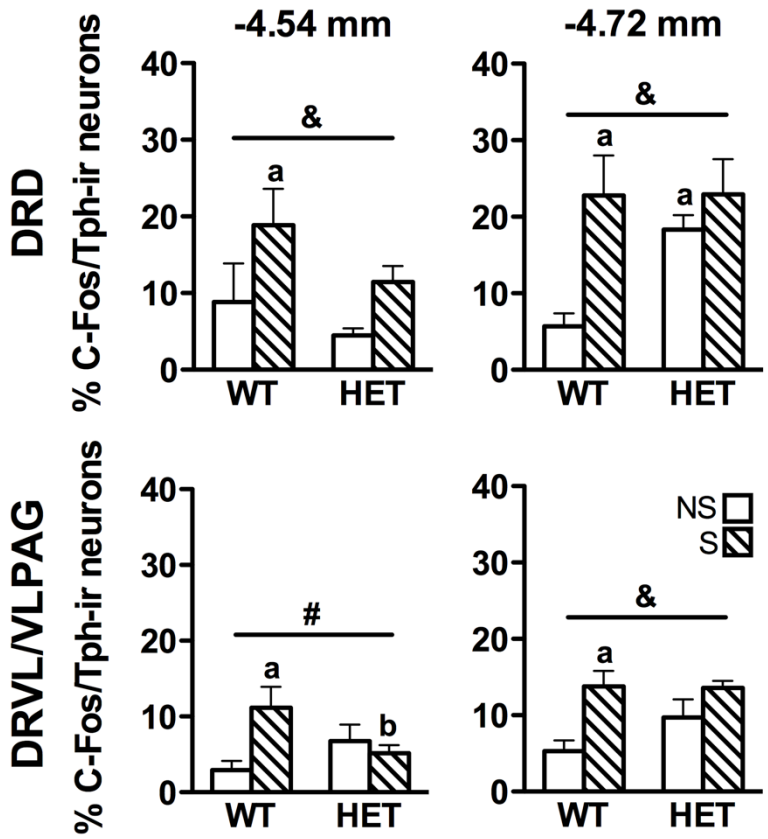

Figure 3. Serotonergic neuronal activation is dysregulated in Fgf8-deficient mice. The percentage of c-Fos/Tph-ir neurons in the mid-rostrocaudal DRD and DRVL/VLPAG in WT and Fgf8-deficient (HET) mice following non-stress (NS) or stress (S) conditions. Distance from bregma (in mm) is shown above each column of graphs. Restraint stress increased serotonergic neuronal activation (% c-Fos/Tph-ir) in the anxiety-related DRD and panic-inhibiting DRVL/VLPAG of WT mice compared to non-stress WT mice. In contrast, activation of serotonergic neurons was similar between the non-stress and stress conditions in Fgf8 HET mice. Importantly, non-stress Fgf8 HET mice had increased basal activation of the anxiety-related DRD at −4.72 mm bregma compared to non-stress WT mice and blunted stress-induced activation of the panic-inhibiting DRVL/VLPAG at −4.54 mm bregma compared to WT mice. N = 5−6/group. Data are presented as mean ± SEM, $p < 0.05$, [a]vs. WT NS, [b]vs. WT S, [&]main effect of stress, [#]genotype x stress interaction. See abbreviations in Table 1.

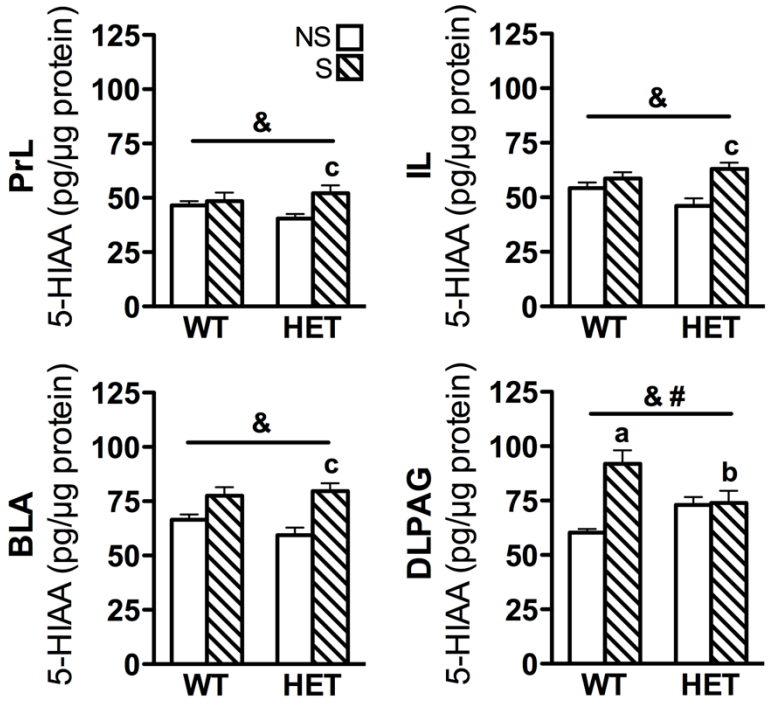

Figure 4. Serotonin metabolism is dysregulated in Fgf8-deficient mice. The 5-HIAA tissue content in anxiety-related and panic-related DR circuits of WT and Fgf8-deficient (HET) mice following non-stress (NS) or restraint stress (S) conditions. Stress increased 5-HIAA content in select anxiety-related projection sites of the DRD (BLA, PrL and IL) as well as in the panic-inhibiting DRVL/VLPAG and DLPAG projection. In WT mice, restraint stress increased 5-HIAA content in the panic-inhibiting DRVL/VLPAG and DLPAG circuit but not in anxiety-related DR projection sites (BLA, PrL, IL). In Fgf8 HET mice, converse effects were observed. In the DLPAG, Fgf8 HET mice also had lower 5-HIAA content following stress compared to WT mice. N = 4−9/group. Data are presented as mean ± SEM, $p < 0.05$, [a]vs. WT NS, [b]vs. WT S, [c]vs. HET NS, [&]main effect of stress, [#]genotype x stress interaction. See abbreviations in Table 1.

Table 1. Details of tissue sample collection for HPLC-ED analysis of 5-HIAA and 5-HT concentrations

Brain region	**Rostrocaudal level (mm bregma)**	**Micropunched sections (number, [diameter μm])**
Infralimbic cortex (IL)	1.70, 2.00	4, [410]
Prelimbic cortex (PrL)	1.70, 2.00	4, [410]
Central amygdaloid nucleus (CE)	−1.20, −1.50	4, [410]
Basolateral amygdaloid nucleus (BLA)	−1.50, −1.80, −2.10	6, [410]
Cornu ammonis 1 of dorsal hippocampus (CA1d)	−2.20, −2.50	12, [410]
Cornu ammonis 1 of ventral hippocampus (CA1v)	−3.00, −3.30	12, [410]
Dorsolateral periaqueductal gray (DLPAG)	−4.20, −4.50; −4.80	4, [410]; 2, [310]
Dorsal raphe nucleus, dorsal part (DRD)	−4.20, −4.50, −4.80	3, [310]
Dorsal raphe nucleus, ventral part (DRV)	−4.50, −4.80	2, [310]
Dorsal raphe nucleus, interfascicular part (DRI)	−4.50, −4.80, −5.10	3, [310]
Dorsal raphe nucleus, ventrolateral part/ventrolateral periaqueductal gray (DRVL/VLPAG)	−4.50, −4.80	4, [410]
Dorsal raphe nucleus, caudal part (DRC)	−5.10	1, [410]

Table 2. Percent of DR c-Fos/Tph-ir neurons in WT and Fgf8 HET mice

Sub-region	Rostro-caudal level (mm bregma)	WT NS Mean (%)	WT NS SEM	WT S Mean (%)	WT S SEM	HET NS Mean (%)	HET NS SEM	HET S Mean (%)	HET S SEM	F-statistic, p-value
DRD	−4.36	13.4	7.3	11.2	3.0	9.5	2.9	16.1	4.2	n.s.
DRV	−4.36	17.6	11	10.4	2.5	8.9	3.3	15.6	5.7	n.s.
DRD	−4.54	8.8	5.1	18.9	4.8	4.5	0.9	11.5	2.1	$^{\&}F(1, 17) = 8.4$, $p = 0.01$
DRV	−4.54	1.1	0.4	9.8	3.3	4.1	0.8	4.4	1.1	$^{\&}F(1, 17) = 6.1$, $p = 0.03$, $^{\#}F(1, 17) = 7.7$, $p = 0.01$
DRVL/ VLPAG	−4.54	2.9	1.2	11.2	2.8	6.7	2.2	5.2	1.1	$^{\&}F(1, 17) = 8.4$, $p = 0.01$, $^{\#}F(1, 17) = 5.9$, $p = 0.03$
DRD	−4.72	5.7	1.7	22.8	5.2	18.3	1.9	22.9	4.6	$^{\&}F(1, 17) = 7.6$, $p = 0.01$
DRV	−4.72	3.0	0.9	7.4	1.9	4.6	0.9	8.0	2.2	$^{\&}F(1, 17) = 5.3$, $p = 0.03$
DRVL/ VLPAG	−4.72	5.3	1.4	13.8	2.0	9.7	2.3	13.6	0.9	$^{\&}F(1, 16) = 11$, $p = 0.01$
DRI	−4.72	5.3	0.6	12.9	4.9	7.3	1.8	8.7	2.8	n.s.
DRD	−4.90	21.1	6.2	31.7	7.1	17.3	4.4	17.4	5.8	n.s.
DRV	−4.90	20.5	5.1	15.6	3.8	16.3	2.4	19.7	4.4	n.s.
DRVL/ VLPAG	−4.90	11.7	5.7	15.6	4.7	5.9	1.2	16.3	4.2	n.s.
DRI	−4.90	10.1	2.7	6.7	0.8	11.9	1.4	14.7	2.9	$^{*}F(1, 17) = 5.6$, $p = 0.03$
DRC	−5.08	16.3	6.6	11.8	2.6	17.7	4.2	21.2	3.9	n.s.
DRI	−5.08	33.0	6.8	14.5	3.0	19.7	4.0	16.3	3.1	$^{\&}F(1, 18) = 6.3$, $p = 0.02$

Data are presented as mean ± SEM, $^{\&}$main effect of stress, *main effect of genotype, $^{\#}$genotype x stress interaction, n.s. = not significant. See abbreviations in Table 1.

Table 3. 5-HIAA concentrations (pg/µg protein) across brain regions in WT and Fgf8 HET mice following non-stress or stress conditions

Brain region	WT				HET				F-statistic, p-value
	NS		S		NS		S		
	Mean (pg/µg)	SEM	Mean (pg/µg)	SEM	Mean (pg/µg)	SEM	Mean (pg/µg)	SEM	
CE	74	5	83	7	80	2	83	5	n.s.
CA1d	77	5	89	5	65	3	89	6	$^{\&}F(1, 22) = 17, p = 0.001$
CA1v	104	11	112	2	102	6	116	5	n.s.
DRD	134	11	164	16	128	6	150	14	$^{\&}F(1, 22) = 4.8, p = 0.04$
DRV	179	13	198	12	169	11	195	13	n.s.
DRVL/ VLPAG	118	11	163	15	118	9	175	17	$^{\&}F(1, 19) = 14, p = 0.001$
DRC	163	9	187	13	144	21	230	34	$^{\&}F(1, 21) = 5.3, p = 0.03$
DRI	242	22	264	11	206	8	282	19	$^{\&}F(1, 20) = 12, p = 0.003$

Data are presented as mean ± SEM, $^{\&}$main effect of stress, n.s. = not significant. See abbreviations in Table 1.

Table S1. Number of Tph-ir neurons for each subregion of the DR at different rostrocaudal levels in WT and Fgf8 HET mice

Subregion	Rostrocaudal level (mm bregma)	WT		HET	
		Mean (#)	SEM	Mean (#)	SEM
DRD	−4.36	78	11	82	9
DRV	−4.36	47	6	66	11
DRD	−4.54	103	8	93	10
DRV	−4.54	134	11	147	11
DRVL/VLPAG	−4.54	125	21	125	17
DRD	−4.72	67	9	65	8
DRV	−4.72	111	11	107	9
DRVL/VLPAG	−4.72	121	17	137	17
DRI	−4.72	49	4	48	5
DRD	−4.90	50	3	49	4
DRV	−4.90	56	7	39*	2
DRVL/VLPAG	−4.90	41	7	47	6
DRI	−4.90	75	7	73	6
DRC	−5.08	61	9	55	7
DRI	−5.08	62	6	60	5

Non-stress and stress groups were combined for analysis. Data are presented as mean ± SEM, *p< 0.05 vs. WT. See abbreviations in Table 1.

Table S2. Number of DR c-Fos/Tph-ir neurons in WT and Fgf8 HET mice

		WT				HET				
	Rostro-caudal	NS		S		NS		S		
Sub-region	**level (mm bregma)**	Mean (#)	SEM	Mean (#)	SEM	Mean (#)	SEM	Mean (#)	SEM	**F-statistic, p-value**
DRD	−4.36	7.3	4.3	14.2	5.2	8.6	3.2	12.3	3.9	n.s.
DRV	−4.36	8.8	4.7	7.2	2.4	6.0	2.7	7.0	1.8	n.s.
DRD	−4.54	9.0	5.3	18.0	3.7	4.0	1.0	12.0	3.3	$^{\&}$F(1, 17) = 5.3, p = 0.03
DRV	−4.54	1.8	0.8	13.8	4.5	6.0	1.4	7.2	2.0	$^{\&}$F(1, 17) = 4.8, p = 0.04, $^{\#}$F(1, 17) = 5.2, p = 0.04
DRVL/ VLPAG	−4.54	7.6	3.7	16.0	3.6	8.7	4.0	6.3	1.7	n.s.
DRD	−4.72	9.0	4.9	9.3	1.5	11.0	2.0	15.5	4.0	n.s.
DRV	−4.72	3.8	1.2	8.8	3.6	5.2	1.2	8.2	2.4	n.s.
DRVL/ VLPAG	−4.72	5.5	1.9	17.8	5.7	13.0	2.9	15.8	4.1	n.s.
DRI	−4.72	3.0	0.4	4.8	1.5	3.8	1.2	4.2	1.8	n.s.
DRD	−4.90	11.8	3.8	14.6	3.0	8.8	2.4	8.7	2.9	n.s.
DRV	−4.90	10.0	2.3	8.6	2.6	6.2	1.3	9.7	2.5	n.s.
DRVL/ VLPAG	−4.90	5.2	2.8	6.4	1.7	3.2	0.7	5.8	1.4	n.s.
DRI	−4.90	8.2	2.2	6.4	2.1	10.3	1.9	10.8	2.3	n.s.
DRC	−5.08	3.8	1.5	9.0	3.2	8.0	0.6	8.5	2.2	n.s.
DRI	−5.08	19.4	4.3	9.2	3.0	10.2	1.6	10.0	1.8	n.s.

Data are presented as mean ± SEM, $^{\&}$main effect of stress, $^{\#}$genotype x stress interaction, n.s. = not significant. See abbreviations in Table 1.

Table S3. 5-HT concentrations (pg/µg protein) across brain regions in WT and Fgf8 HET mice following non-stress or stress conditions

Brain region	WT				HET				F-statistic, p-value
	NS		S		NS		S		
	Mean (pg/µg)	SEM	Mean (pg/µg)	SEM	Mean (pg/µg)	SEM	Mean (pg/µg)	SEM	
BLA	16.1	0.78	17.1	2.1	14.6	0.41	18.0	1.7	n.s.
CE	16.9	1.6	14.4	1.7	18.6	0.92	18.8	2.2	n.s.
CA1d	9.9	0.10	10.0	0.44	9.7	0.66	10.1	0.41	n.s.
CA1v	17.5	1.9	17.1	0.29	18.5	0.87	18.7	1.5	n.s.
PrL	7.3	1.4	5.8	0.66	6.7	0.29	6.8	0.71	n.s.
IL	9.6	0.45	7.6	0.64	8.5	0.39	9.2	0.56	$^{\#}F(1, 19) = 6.5, p = 0.02$
DLPAG	13.9	1.2	19.2	1.1	20.1	1.5	17.0	1.3	$^{\#}F(1, 22) = 8.4, p = 0.01$
DRD	54.2	5.0	65.9	5.2	57.5	3.2	62.7	5.9	n.s.
DRV	69.9	5.5	69.2	6.9	73.6	4.0	77.2	3.1	n.s.
DRVL/ VLPAG	33.1	3.1	47.9	5.5	37.7	3.0	52.1	6.4	$^{\&}F(1, 19) = 9.0, p = 0.01$
DRC	47.7	4.3	49.8	4.6	52.1	6.6	62.2	6.5	n.s.
DRI	65.7	7.2	53.1	6.5	63.0	2.4	59.5	6.4	n.s.

Data are presented as mean ± SEM, $^{\&}$main effect of stress, $^{\#}$genotype x stress interaction, n.s. = not significant. See abbreviations in Table 1.

Table S4. 5-HIAA/5-HT ratios across brain regions in WT and Fgf8 HET mice following non-stress or stress conditions

Brain region	WT				HET				F statistic, p-value
	NS		S		NS		S		
	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM	
BLA	4.2	0.50	4.3	0.22	4.1	0.26	4.6	0.50	n.s.
CE	4.5	0.25	5.9	0.40	4.3	0.14	5.5	0.60	$^{\&}F(1, 19) = 15.3, p = 0.001$
CA1d	7.7	0.55	8.9	0.43	6.9	0.37	8.8	0.36	$^{\&}F(1, 22) = 13.1, p = 0.002$
CA1v	6.0	0.37	6.5	0.08	5.6	0.49	5.8	0.08	n.s.
PrL	7.0	1.1	8.6	0.78	6.1	0.32	7.7	0.63	$^{\&}F(1, 20) = 6.4, p = 0.02$
IL	5.7	0.24	7.8	0.51	5.5	0.24	6.9	0.31	$^{\&}F(1, 19) = 24.1, p < 0.001$
DLPAG	4.8	0.33	4.8	0.09	3.9	0.24	4.4	0.10	$^{*}F(1, 20) = 9.2, p = 0.01$
DRD	2.5	0.11	2.5	0.11	2.3	0.11	2.4	0.14	n.s.
DRV	2.6	0.12	2.9	0.17	2.3	0.08	2.7	0.19	$^{\&}F(1, 21) = 7.8, p = 0.01$
DRVL/ VLPAG	3.6	0.07	3.5	0.08	3.2	0.13	3.5	0.21	n.s.
DRC	3.5	0.15	3.9	0.42	3.5	0.43	3.3	0.18	n.s.
DRI	3.8	0.23	5.2	0.39	3.3	0.07	4.5	0.54	$^{\&}F(1, 20) = 19.7, p < 0.001$

Data are presented as mean ± SEM, $^{\&}$main effect of stress, *main effect of genotype, n.s. = not significant. See abbreviations in Table 1.

Text S1. Plasma corticosterone

Compared to non-stress animals, plasma corticosterone (ng/mL) was significantly elevated in the stress group in both genotypes [$F(1, 24) = 108.9$, $p < 0.001$, mean ± SEM, WT: 138 ± 11 NS; 383 ± 27 S; Fgf8 HET: 142 ± 16 NS; 361 ± 29 S]. There was no main effect of genotype or a genotype x stress interaction.

CHAPTER IV: CONCLUSIONS

Fgf signaling plays diverse roles during embryonic brain development including cell fate specification, survival, migration, axon targeting, neurite outgrowth, and patterning (Dono 2003; Reuss and von Bohlen und Halbach 2003; Mason 2007). The aim of this dissertation was to examine how dysregulation of Fgf signaling impacts the development of anxiety-related DR serotonergic systems. Chapter II established that global reductions of Fgf8 and Fgfr1 led to subregional decreases in serotonergic neuronal numbers and increased anxiety-like behavior. The key message from this chapter is that developmental signaling perturbations can impact DR structural integrity and can influence emotional behavior later in life. In Chapter III, we discovered that prenatal Fgf8 deficiency was associated with functional alterations in stress- and anxiety-related serotonergic DR neurons in adulthood, and these functional changes were correlated with increased anxiety-like behavior. The key message from this chapter is that the disruption of early signaling events impacts the integration of multiple circuitry components relevant to emotional behavior, resulting in functional consequences. This work contributes to our understanding of how Fgf signaling is involved in the development of neurocircuits governing emotional behaviors.

The mechanisms through which Fgf signaling mediates the observed effects in stress-, panic- and anxiety-related DR serotonergic neurocircuits are presently unclear and necessitate specific manipulation of DR circuitry to establish the precise role of Fgfs. The global nature of Fgf8 or Fgfr1 deficiencies in our models preclude causal conclusions as other brain structures implicated in control of emotional behavior and neuronal types may also be impacted by loss of Fgf signaling. Another consideration is that defects or compensatory changes in serotonergic neurons themselves contribute to

the functional and behavioral effects observed. This is a possibility because serotonin has been implicated in developmental events like neurogenesis, apoptosis, and neurite outgrowth, and studies where serotonergic function during development was disrupted have documented effects on anxiety-like behavior (Gaspar, Cases et al. 2003; Daubert and Condron 2010; Lesch and Waider 2012). Nonetheless, results from these models reflect the potency of mild perturbation of key morphogens during development and how this perturbation can manifest as abnormal emotional behavior later in life. Global deficiency of Fgf signaling also mirrors conditions in humans that harbor global loss of function mutations and not tissue-specific or inducible deficiencies. These studies serve as a starting point for future experiments that allow for greater tissue specificity.

This dissertation has revealed structural, functional and behavioral abnormalities in stress-, panic-, and anxiety-related serotonergic DR circuitries. Structurally, Fgf deficiency is associated with loss of specific subpopulations of DR serotonergic neurons in anxiety- and panic-related subregions. Functionally, Fgf signaling is associated with alterations in activation and output in anxiety-promoting and panic-inhibiting DR circuitries. Behaviorally, loss of Fgf signaling is associated with increased baseline anxiety-like behavior. Overall, this dissertation suggests an exquisite sensitivity of DR circuits implicated in control of emotional behavior to subtle reductions of Fgf signaling.

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