Coordinate and Region-Specific Roles for Fibroblast Growth Factors 2 and 9 as Molecular Organizers in Major Depression and Animal Models of Affective Disorders

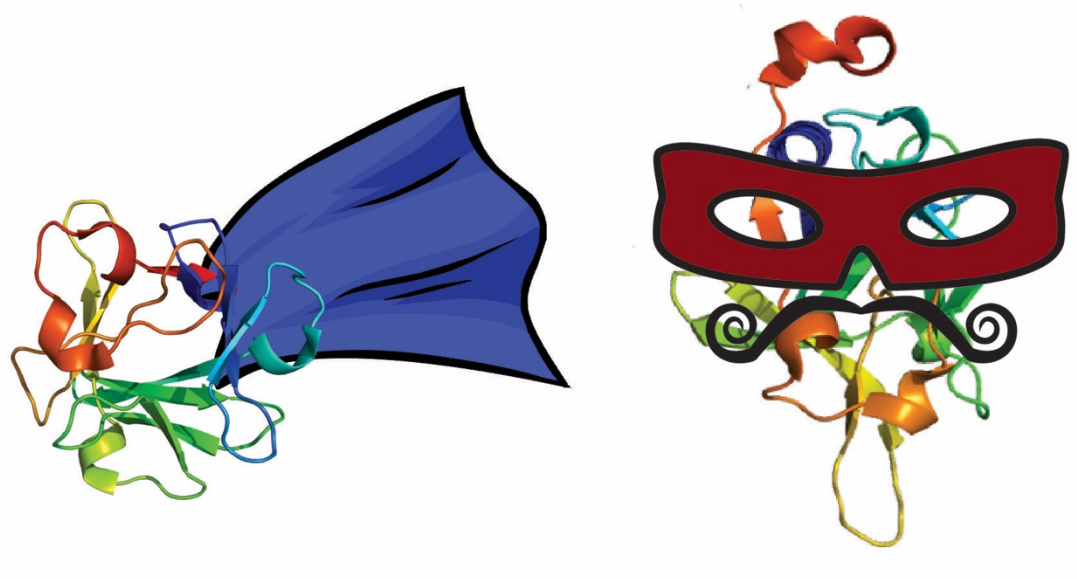
by

Elyse Louise Aurbach

A dissertation submitted in partial fulfillment
of the requirements for the degree of
Doctor of Philosophy
(Neuroscience)
in the University of Michigan
2016

Doctoral Committee:

Professor Huda Akil, Chair
Professor Jack M. Parent
Professor Audrey F. Seasholtz
Professor Delia M. Vazquez
Professor Stanley J. Watson Jr.



Dedication

For those who struggle with mental illness, for their support networks, for their treatment providers, for their advocates…

For the brave men and women who donated their brains to science to give us the opportunity to study psychiatric disease and work towards developing better treatments…

For the animals whose lives and deaths help us to deepen our understanding of brain function and dysfunction…

And for my own incredible network of support: you have lifted me up, sat with me in stillness, challenged me to strengthen me, taught me to set and maintain boundaries, and given me safe haven. I would not be here without you.

Acknowledgements

They say that it takes a village to raise a child. I think it has taken a village to get me here, and this small section is inadequate to express my gratitude.

Huda and Stan: Thank you for taking a chance on me. You gave me every opportunity to learn, exposure and access to an incredible group of minds and ideas, kind and nurturing support, and incredible flexibility to pursue my different passions. You helped me to find my role and my voice as a scientist, and you taught me how to tell my story. I feel so incredibly privileged and honored to have been your student.

My committee: Thank you for sharing your perspectives and feedback so freely over the last six years. Your guidance and expertise has been invaluable to shaping my thinking. To Audrey and Jack in particular: thank you for giving me access to space and resources in your labs; you helped keep me moving when I felt the most stuck.

The incomparable Wakils: Thank you for teaching, supporting, and collaborating with me. You have taught me basically all of the bench science that I know, commiserated confusing data, helped me troubleshoot when techniques just were not working, celebrated clear results, and kept me laughing. I could not ask for a better group of colleagues. To Cortney, Megan, and Linda in particular: thank you for helping me

develop different forms of expertise: your friendship, training, and continued guidance have been instrumental in shaping my thinking.

Leah, Paula, Andrew, Sonya, Brian, Kelli, Peter, Lori, David, Jim, and Don, my mentors outside of the Wakils: Thank you for supporting and enhancing my education. You've given me incredible opportunities and helped nurture me as a teacher, as a science communicator, as a mentor, as a researcher, and as a good science citizen. My professional life is enriched invaluably for your presence in it.

My family and my friends (the family of my heart): Thank you for being my roots, my support, my last and best failsafe. You are the first people I turn to when things get rough and those who come immediately to mind when there is good news to share. I love you, and I would not be the same person without you. Especially to my parents: thank you for helping me find my passions and for teaching me how to work to achieve them. Thank you for teaching me to be a good person, to value service, and to never underestimate the effects of enthusiasm and perseverance.

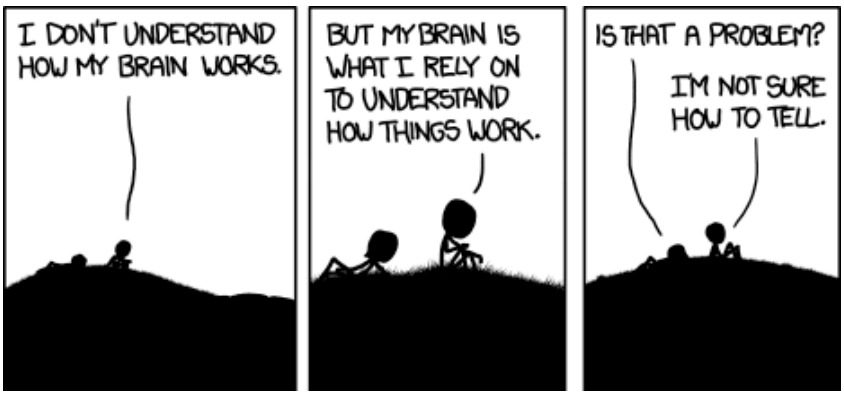
Patrick: Thank you for being the key to my lock. Thank you for helping me to grow. You simultaneously accept me wholeheartedly and inspire me to be my best self – you help me to open my eyes and see things differently. You let me be vulnerable, you make me feel safe. I respect your mind, your integrity, and your incredible heart more than words can express. I could not ask for a better or more kind partner. I am so proud of how far we have come, and I'm infinitely excited about the road ahead of us. I love you.

Katie: Thank you for your incredible partnership. Our collaboration and friendship – in lab, with RELATE, while teaching, in life – immeasurably transformed my path in graduate school and helped me to reach so much further than I thought that I could. I am so proud of what we have accomplished together. I am looking forward to many more years of thoughtful conversations, exciting and innovative projects, goofy Halloween costumes, and just the right brand of mutual support.

Sara, Amy, and Nick: Thank you for helping me to find – and keep finding – my way.

Rackham and the whole RELATE team: The ability to develop, launch, and grow RELATE is unequivocally at the top of the list of opportunities for which I am the most grateful. RELATE utterly transformed my graduate education, and it has shaped my trajectory in ways that I am still discovering. I could not ask for a better group of collaborators or a better environment in which to work. Thank you for helping me find my passion and for giving me the tools to follow it.

Preface



Comic courtesy of xkcd: http://xkcd.com/1163/

Table of Contents

List of Figures

List of Tables

Abstract

The neurotrophic hypothesis posits that changes in the expression and function of growth factors in the brain underlie the pathophysiology of Major Depressive Disorder (MDD). Previous work implicated the fibroblast growth factor (FGF) system, identifying FGF2 as an endogenous anxiolytic and antidepressant molecule whose expression is downregulated in the depressed brain. Notably, FGF9 showed a diagnosis-specific pattern of expression that was opposite to FGF2. Therefore, we investigated the hypotheses that FGF2 and FGF9 were critical to the regulation of affect and that their expression becomes disrupted in MDD. Because the literature supporting the role of FGF9 in affect regulation was small, we performed exploratory analyses and demonstrated that FGF9 expression is consistently upregulated in the hippocampus (but not the anterior cingulate cortex or dorsolateral prefrontal cortex) of individuals diagnosed with MDD. We also showed that reducing endogenous expression of FGF9 in the dentate gyrus is sufficient to reduce anxiety-like behavior, and hippocampal FGF9 levels differ in an animal model of affective dysregulation. Because they showed opposite effects in MDD and animal models, we hypothesized that FGF2 and FGF9 might act as physiological antagonists to mediate affect. We examined more complex questions regarding FGF2. We used animal models to demonstrate that altered hippocampal FGF2 expression predisposes individuals for affective dysregulation. Because we hypothesized that relative levels of FGF2 and FGF9 might be important to MDD pathophysiology, we examined diagnosis-specific relationships in expression between FGF2, FGF9, and FGF receptors, and we found

regional patterns of alteration with MDD. In the anterior cingulate cortex, correlations between FGF family members were lost in MDD, while in the hippocampus, new relationships emerged. These changes were related to alterations in correlated gene expression of transcripts related to fundamental biology and circuit function, supporting the hypothesis that FGF2 and FGF9 may influence affect by acting as molecular organizers whose effects become dysregulated during MDD. Future studies will examine the role of FGF2 and FGF9 in MDD, with a particular emphasis on understanding how neural circuitry is altered at the cellular level.

Chapter 1.

Introduction

Major Depressive Disorder: A debilitating illness with complicated and poorly-understood etiology

Approximately one in five Americans will develop a mood or anxiety disorder at some point during their lifetimes (American Psychiatric Association, 2013). Associated with high mortality (American Psychiatric Association, 2013), major depressive disorder (MDD) has a lifetime prevalence of ~17% (and ~30% lifetime morbid risk), with ~7% of individuals experiencing the disorder during a 12-month period (Kessler et al., 2003; Kessler, Petukhova, Sampson, Zaslavsky, & Wittchen, 2012). The World Health Organization estimates that approximately 350 million people are affected by MDD, and it identifies MDD as the leading cause of disability worldwide, making MDD a disease with an incredibly detrimental economic effect. Given the debilitating nature, high prevalence, and financial burden of the disease, developing a thorough understanding of the underlying biology of MDD will be critical to reducing its impact.

The symptomatology of MDD is highly complex, yet it forms the basis for diagnosis and treatment. Indeed, MDD diagnosis is based on the presence or absence and longevity of psychological and physical symptoms as opposed to biomarker-based objective measures that have been developed for some other illnesses and/or conditions (American Psychiatric Association, 2013). MDD is characterized by two core symptoms,

depressed mood and anhedonia, and at least one must be debilitating and persistently present for at least two weeks to diagnose a major depressive episode. In addition, a constellation of other psychological and physical symptoms co-occur, including feelings of worthlessness or inappropriate guilt, fatigue or loss of energy, psychomotor agitation or retardation, sleep disturbances including hypersomnia or insomnia, and significant weight loss or weight gain. In addition, MDD patients can experience suicidal thoughts or ideation, and many individuals attempt or commit suicide (American Psychiatric Association, 2013). Notably, MDD is a disease of extremes: all symptoms represent a substantial deviation from "normal" behavior, but both ends of the spectrum could be considered symptoms of MDD (e.g., hypersomnia vs. insomnia). As a result of this heterogeneity, two individuals with dramatically different constellations of symptoms can both be diagnosed with MDD. (e.g., Patient A may show signs of anhedonia, extreme fatigue, hypersomnia, psychomotor retardation, and weight gain, while Patient B could display depressed mood, psychomotor agitation, weight loss, insomnia, and have overwhelming feelings of guilt. While these patients experience very different symptoms, both could be diagnosed with MDD.)

While the etiology of MDD is not well understood, progress has been made by studying patients currently or recently experiencing the disease. Because many patients with MDD display different symptomatology, many clinicians and researchers classify subtypes of MDD (e.g., melancholic, psychotic, etc) in the hope that these classifications increase the odds of uncovering differential biological underpinnings associated with the subtypes. In addition, MDD frequently co-occurs with other medical conditions or mental disorders (American Psychiatric Association, 2013). An estimated 70% of individuals

diagnosed with MDD also carry at least one additional DSM diagnosis (Kessler et al., 2003). Anxiety disorders in particular are common comorbid diagnoses (American Psychiatric Association, 2013; T. A. Brown, Campbell, Lehman, Grisham, & Mancill, 2001; Grant et al., 2005; Hunt, Issakidis, & Andrews, 2002). Indeed, experiencing MDD may predispose individuals to developing clinical anxiety (especially panic disorders) or vice-versa (American Psychiatric Association, 2013). Furthermore, high reactivity to stress is a major vulnerability factor, and intensive stress frequently precipitates major depressive episodes (American Psychiatric Association, 2013). The complicated nature and high co-morbidity of MDD with other illnesses suggests that the underlying biology of MDD is complex and requires physiological systems that are critical to daily function.

There are a number of treatment avenues for MDD, including a variety of drugs and non-pharmacological therapeutic interventions (
Table 1.1). However, these approaches come with a number of limitations. Notably, there is high individual variability in many patients' response to different drugs, and the most common approach in clinical practice to determining a course of drug treatment is through trial-and-error. In addition, most commonly prescribed antidepressant drugs require weeks before symptoms alleviate. Together, these factors can mean that patients seeking relief from MDD frequently go months before their symptoms begin to resolve. Furthermore, despite the availability of a number of different effective treatments, almost 50% of patients never achieve full remission (Berton & Nestler, 2006). Even worse, almost one in three individuals are resistant to treatment and never helped by current approaches (Fava & Davidson, 1996), although recent studies indicate that the NMDA receptor antagonist ketamine (Berman et al., 2000; Zarate et al., 2006) and/or deep brain

stimulation to the subcallosal cingulate white matter (Holtzheimer & Mayberg, 2011; Ressler & Mayberg, 2007) may be effective for some of these individuals.

Table 1.1. Currently available antidepressant treatments. Adapted by permission from Macmillan Publishers Ltd: Nature Reviews Neuroscience. Berton & Nestler (2006). New approaches to antidepressant drug discovery: Beyond monoamines, 7, 137-151, © 2006.

Type of Treatment	Mode of Action	Examples
Medication		
Tricyclics	Inhibition of mixed noradrenaline and serotonin reuptake	Imipramine, desipramine
Selective serotonin reuptake inhibitors (SSRIs)	Inhibition of serotonin-selective reuptake	Fluoxetine, citalopram
Noradrenaline reuptake inhibitors (NRIs)	Inhibition of noradrenaline-selective reuptake	Atomoxetine, reboxetine
Serotonin and noradrenaline reuptake inhibitors (SNRIs)	Inhibition of mixed noradrenaline and serotonin reuptake	Venlafaxine, duloxetine
Monoamine oxidase inhibitors (MAOIs)	Inhibition of monoamine oxidase A (MAO_A). Inhibition of MAO_B does not have antidepressant effects	Tranylcypromine, phenelzine
Lithium	Lithium has many molecular actions (for example, inhibition of phosphatidylinositol phosphates, adenylyl cylclases, glycogen synthase kinase 3β and G proteins) but which of its actions is responsible for its antimanic and antidepressant effects is unknown	
Atypical antidepressants	Unknown. Although these drugs have purported monoamine-based mechanisms (for example, bupropion inhibits dopamine reuptake, mirtazapine is an α_2-adrenergic receptor antagonist and tianeptine is an activator of monoamine reuptake), these actions are not necessarily the mechanisms that underlie the drugs' therapeutic benefit	Bupropion, mirtazapine, tianeptine
Non-medication		
Electroconvulsive therapy (ECT) Magnetic stimulation	General brain stimulation General brain stimulation? A magnetic field is thought to affect the brain by inducing electric currents and neuronal depolarization	
Vagal nerve stimulation	Unknown	
Psychotherapies	Exact mechanism is uncertain, but is thought to involve learning new ways of coping with problems	Cognitive-behavioral therapy, interpersonal therapy
Deep brain stimulation	In severely ill patients, stimulation of a region of the cingulate cortex found to function abnormally in brain imaging scans reportedly has antidepressant effects	

!

The complexity in diagnosis and treatment is reflected in the heterogeneity of brain regions and circuits that are thought to underlie MDD pathophysiology. Many

regions and circuits likely interact in complex ways to give rise to cognitive, emotional, behavioral, and physiological states, and disruption of these functions can be connected to the symptoms of mood disorders (Drevets, Price, & Furey, 2008; Price & Drevets, 2012). There are many such regions implicated, including limbic areas such as the hippocampus, medial prefrontal cortex, and amygdala (Godsil, Kiss, Spedding, & Jay, 2013; Price & Drevets, 2012), thalamic regions (Tekin & Cummings, 2002), mesolimbic components including the ventral tegmental area, basal ganglia, and nucleus accumbens (Nestler & Carlezon, 2006; Drevets et al., 2008; Gunaydin & Kreitzer, 2016), hypothalamic areas (Plotsky, Owens, & Nemeroff, 1998; Swaab, Bao, & Lucassen, 2005; Drevets et al., 2008) and regions in the brainstem (Drevets et al., 2008; Ressler & Nemeroff, 2000; Bernard et al., 2011; Gold et al., 2015). These regions serve many functions and have been closely studied both in the context of their individual properties as microcircuits (e.g., the hippocampus) and as collective assemblies (e.g., the separation distress circuit; Panksepp, Nelson, & Bekkedal, 1997; Panksepp, Knutson, & Burgdorf, 2002; Panksepp, 2003). There are competing – though not mutually exclusive – theories about the relative importance and functional contributions of these various regions and circuits, and some assemblies are more relevant to particular MDD symptoms than others. Though it is beyond the scope of this thesis to extensively review every region implicated in MDD and the functions that they subserve, we will briefly highlight the prefrontal cortices and the hippocampus, as they are particularly relevant to the data we will present in the following chapters.

The hippocampus (HPC) and prefrontal cortices (PFC) are regions known to be particularly sensitive to disruption in MDD (Price & Drevets, 2012). Indeed, voxel-based

morphometry studies consistently show volumetric decreases in PFC and HPC (Bremner et al., 2000, 2002; MacQueen & Frodl, 2011; Sheline, Gado, & Kraemer, 2003). A recent meta-analysis highlighted the anterior cingulate cortex in particular as one of the most consistent sites for MDD-related primary volume loss (Bora, Fornito, Pantelis, & Yücel, 2012), and another showed substantial volume loss in the same region across DSM Axis I psychiatric disorders, including anxiety (Goodkind et al., 2015). The same study also highlighted volume loss in the anterior HPC and amygdala as being particularly important for internalizing disorders, including MDD and anxiety (Goodkind et al., 2015). While the molecular mechanisms underlying this volume loss are not well understood, these structural changes are thought to be related to chronic stress and may arise from cellular atrophy (Duric & Duman, 2012). It is an active area of research to determine if this volume loss is predisposing to or concomitant with disease pathophysiology and if volume loss is a good biomarker for MDD.

Historical perspective on MDD research and the search for novel targets

Until relatively recently, the primary approach that many researchers took to understand MDD biology was to examine the mechanisms underlying the therapeutics that were effective in alleviating depressive symptoms. Much of this research focused on understanding the molecular targets and mechanisms of serendipitously-discovered drugs with known antidepressant properties, most of which target monoamine systems (
Table 1.1; Berton & Nestler, 2006; Nestler & Hyman, 2010). The emergent "monoamine hypothesis of MDD" posited that dysregulation of monoaminergic neurotransmitters, including serotonin and noradrenaline, and their associated neural circuitry gave rise to MDD symptomology. This research was highly informative and yielded critical insights into some aspects of MDD, as well as insight into the serotonergic and noradrenergic systems in the brain. However, decades of research on these topics failed to conclusively provide a holistic understanding of MDD

pathophysiology, as highlighted by the inability for the monoaminergic hypothesis to fully account for complexity and variation in symptoms, diagnosis, and treatment.

Limitations in the monoaminergic hypothesis of MDD spurred researchers to search more broadly for molecular players capable of mediating the highly individualized, broad-scale dysregulation in the brain. Members of the Pritzker Neuropsychiatric Disorders Research Consortium, including our laboratory, approached this challenge strategically. Using a bank of postmortem human brain tissue donated by individuals without neuropsychiatric conditions as well as those diagnosed with MDD, bipolar disorder, or schizophrenia during life, they performed microarray studies to broadly profile transcriptional changes in different regions associated with psychiatric pathology in unbiased screens. Surprisingly, the fibroblast growth factor (FGF) family emerged among the top sets of genes whose expression was disrupted in MDD, and quantitative PCR assays validated the initial diagnosis-specific expression changes. Furthermore, additional evidence suggested that this dysregulation was partially ameliorated by a treatment history which included antidepressant drugs, suggesting that FGF dysregulation may be a component of the mechanism of action underlying some effective therapeutics (Evans et al., 2004).

This discovery dovetailed with an emerging literature primarily in animal models which implicated neural plasticity, including dysregulation of other growth factors, in affective dysregulation. To account for the long time-course required for MDD symptom amelioration, some depression researchers had begun to examine physical changes in the cells of different brain regions related to affect, hypothesizing that the observed neuroplastic changes were necessary for antidepressant action (Duman, Heninger, &

Nestler, 1997; Nestler et al., 2002). This hypothesis was supported by studies showing that stress promoted atrophy of neurons in the hippocampus and other regions while antidepressant treatment had the opposite effect (Berton & Nestler, 2006; E. S. Brown, Rush, & McEwen, 1999; McEwen, 1999). Brain-derived neurotrophic factor (BDNF) arose as an appealing molecular target because its expression was downregulated after stress but upregulated after treatment with antidepressant drugs. Reinforcing the appeal of BDNF as a mediator of plasticity and affect, these transcriptional changes co-occurred with changes in cellular circuitry, including alterations in adult hippocampal neurogenesis, and altering endogenous expression of BDNF induced changes in affective behavior (reviewed in Duman & Monteggia, 2006). However, BDNF's role in affective dysregulation was first examined as an *a priori* hypothesis; in contrast, the FGF family emerged from an unbiased screen of tissue from individuals diagnosed with MDD.

The Neurotrophic Hypothesis of MDD

This research into BDNF, FGFs, and other growth factors has given rise to the neurotrophic hypothesis, which posits that changes in growth factors may be related to the circuit abnormalities and changes in mood and physiological function that arise in MDD (Duman & Monteggia, 2006; Turner, Watson, & Akil, 2012). Growth factors impact function of many different physiological systems at different scales; some of these functions could include: tuning or influencing molecular networks, modulating neurotransmission and/or neurotransmitter reuptake/recycling, and regulating circuit assembly and maintenance. Different growth factors have been shown to impact all of these functions, and a wide variety of growth factors have been directly implicated in affective dysregulation and MDD. Among these, BDNF is the best-studied, though

others, including glial derived neurotrophic factor (GDNF), vascular endothelial growth factor (VEGF), insulin-like growth factor (IGF), and the previously-noted FGFs have all been implicated in MDD (for reviews, see Banasr, Dwyer, & Duman, 2011; Duman & Monteggia, 2006; Duric & Duman, 2012; Krishnan & Nestler, 2008; McClung & Nestler, 2007; Turner et al., 2012; Yu & Chen, 2011).

As described above, work from our laboratory and others indicates that the FGF system is dysregulated in the depressed human brain across a number of regions (Bernard et al., 2011; Evans et al., 2004; Gaughran, Payne, Sedgwick, Cotter, & Berry, 2006). Therefore, we will briefly review the members of the FGF family and their fundamental biological functions which are relevant to MDD.

FGF System Biology: Emphasis on FGF2 and FGF9

The FGF system is composed of 22 ligands and 4 receptors (Figure 1.1), though only 10 of the ligands and 3 of the receptors are expressed in the central nervous system (Guillemot & Zimmer, 2011; Reuss & von Bohlen und Halbach, 2003; Yazaki et al., 1994). The FGF family ligands have been organized into seven sub-families according to genetic synteny, structural homology, and receptor binding affinity profiles (Beenken & Mohammadi, 2009; Guillemot & Zimmer, 2011; Itoh & Ornitz, 2008, 2011). The canonical FGF ligands are secreted extracellularly and have local, spatially-limited paracrine functions, requiring heparin-sulfate proteoglycans (HSPG) to bind to receptors (Beenken & Mohammadi, 2009; Guillemot & Zimmer, 2011; Itoh & Ornitz, 2011; Turner, Eren-Koçak, Inui, Watson, & Akil, 2015; Turner et al., 2012). This HSPG binding serves several purposes, including contributing to the specificity of the FGF-FGF receptor interaction, protecting FGF ligands from degradation, limiting the diffusion of

FGF ligands, and bridging FGF receptor subunits to promote autophosphorylation (Guillemot & Zimmer, 2011).

LIGANDS			RECEPTORS	
	Family	Members	Binding FGFRs	Isoforms
Canonical FGFs	FGF4	FGF3	1, 2	IIIb
		FGF4	1, 2, 3, 4	IIIc
		FGF6	1, 2, 4	
	FGF5	FGF1	1, 2, 3, 4	IIIb, IIIc
		FGF2	**1, 2, 3, 4**	
		FGF5	1, 2	IIIc
	FGF8	FGF8	1, 2, 3, 4	IIIc
		FGF17	1, 2, 3, 4	
		FGF18	2, 3, 4	
	FGF9	**FGF9**	**1, 2, 3**	IIIb, IIIc
		FGF16	2, 3	IIIc
		FGF20	1, 2, 3, 4	IIIb, IIIc
	FGF10	FGF7	2, 4	IIIb
		FGF10	1, 2	
		FGF22	1, 2	
Hormone FGFs	FGF15/19	FGF15/19	1, 2, 3, 4	IIIb, IIIc
		FGF21	1, 2, 3, 4	
		FGF23	1, 2, 3, 4	
Intracellular FGFs	FGF11	FGF11		
		FGF12		
		FGF13		
		FGF14		

Figure 1.1. Phylogeny of FGF ligands and specificity of their interactions with FGF receptors. Adapted from Neuron, 7/4, Guillemot & Zimmer, From Cradle to Grave: The Multiple Roles of Fibroblast Growth Factors in Neural Development, 574-588, © 2011, with permission from Elsevier.

On the other hand, several subsets of FGF ligands act in other ways. FGFs ligands 11-14 are not secreted and remain in the nucleus of the cell, where they do not interact with FGF receptors (Mason, 2007). Hormone-like FGF ligands 15/19, 21, and 23 have low affinity for FGF receptors and HSPGs: they have long-range endocrine functions and mediate metabolism (Guillemot & Zimmer, 2011; Itoh & Ornitz, 2011). ***Two canonical, paracrine, FGF ligands, FGF2 and FGF9, are of particular interest because they are among those molecules whose expression is dysregulated in the depressed postmortem human brain*** (Bernard et al., 2011; Evans et al., 2004; Gaughran et al., 2006), ***and they will be the focus of this dissertation.***

FGF2 and FGF9 have differential patterns of expression in the central nervous system. FGF2 is primarily expressed at relatively low levels in astrocytes across the nervous system, although hippocampal CA2 pyramidal neurons express high levels of FGF2, which is thought to remain in the nucleus of the cell (Gonzalez, Berry, Maher, Logan, & Baird, 1995). In contrast, FGF9 is primarily expressed by neurons in the cortex, hippocampus, thalamus, cerebellum, and spinal cord (Garcès, Nishimune, Philippe, Pettmann, & deLapeyrière, 2000; Lin et al., 2009; Nakamura et al., 1997; Tagashira, Ozaki, Ohta, & Itoh, 1995). FGF9 is also expressed by glia in the hindbrain and spinal cord (Nakamura et al., 1999). Both FGF2 and FGF9 interact with all the FGF receptors that are expressed in the central nervous system, but they show different patterns of binding affinity.

The FGF receptors (FGFR) are receptor tyrosine kinases with a prototypical structure and variants that confer ligand specificity. FGFRs have an extracellular domain comprised of three immunoglobulin domains (IgI, IgII, and IgIII) and an acid box region between IgI and IgII, a transmembrane domain, and an intracellular domain consisting of a split tyrosine kinase domain that autophosphorylates to activate signaling cascades upon ligand binding (Figure 1.2).

All FGFRs are alternatively spliced, and splicing of the IgIII domain by alternative use of exon 8 or exon 9 into two variants (IIIb and IIIc) determines ligand binding specificity (Beenken & Mohammadi, 2009; Guillemot & Zimmer, 2011; Itoh & Ornitz, 2011; Mason, 2007; Turner et al., 2015, 2012). FGF2 and FGF9 display different patterns of binding affinity for FGFR1, FGFR2, and FGFR3 *in vitro*. FGF2 binds most strongly to FGFR1 and least strongly to FGFR3, while FGF9 binds most strongly to

FGFR3 and least strongly to FGFR1. Both FGF2 and FGF9 have stronger affinities for the IIIc variants than the IIIb variants of the receptors (Ornitz et al., 1996; Zhang et al., 2006). However, it is unknown whether these relationships are similar or how HSPGs might alter binding tendencies *in vivo* (Guillemot & Zimmer, 2011).

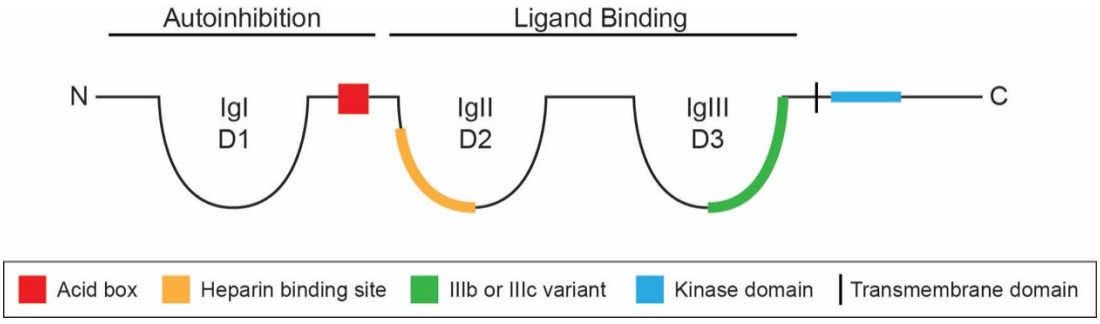


Figure 1.2. A schematic of the fibroblast growth factor receptor structure. Adapted by permission from Macmillan Publishers Ltd: Nature Reviews Drug Discovery. Beenken & Mohammadi (2009). The FGF Family: biology, pathophysiology, and therapy, 8, 235-253, © 2009.

As with the FGF ligands, the three FGFRs that are expressed in the central nervous system have different patterns of postnatal expression. FGFR1 is primarily expressed in neurons (though some studies report additional expression in astrocytes) in the hippocampus, cortex, thalamus, and many subcortical regions (Bansal, Lakhina, Remedios, & Tole, 2003; Belluardo et al., 1997; Gonzalez et al., 1995; Matsuo et al., 1994). FGFR2 is primarily expressed by glia across the brain, especially fiber-rich regions like the corpus callosum and internal capsule, though neuronal expression was also observed in the cerebellum (Bansal et al., 2003; Belluardo et al., 1997; Yazaki et al., 1994). FGFR2 is also highly expressed in the choroid plexus (Yazaki et al., 1994). Similarly, FGFR3 is almost exclusively expressed diffusely in astrocytes and oligodendrocytes, with moderate expression levels in cerebellum and hindbrain regions

including the superior colliculus (Bansal et al., 2003; Belluardo et al., 1997; Oh et al., 2003; Peters, Ornitz, Werner, & Williams, 1993; Pringle et al., 2003; Yazaki et al., 1994).

Ligand binding to FGFRs spurs autophosphorylation of the intracellular tyrosine kinase domains of the receptors, which activates three different signaling cascades in the brain to ultimately impact transcriptional activity, cytoskeletal targets, downstream intracellular function (including via transcriptional negative feedback on the pathways themselves), and FGF receptor activity. These cascades include: 1) the Ras pathway, which activates the Erk-MAPK pathway to mediate transcription, 2) the PI3K pathway, which activates Akt, and 3) the PLCγ pathway, which activates PKC to impact the cytoskeleton (Figure 1.3) (Beenken & Mohammadi, 2009; Duman & Monteggia, 2006; Guillemot & Zimmer, 2011; Itoh & Ornitz, 2011; Klint & Claesson-Welsh, 1999; Krejci, Prochazkova, Bryja, Kozubik, & Wilcox, 2009; Mason, 2007; Reuss & von Bohlen und Halbach, 2003; Wing, Chen, Chuang, Wu, & Tsai, 2005). Notably, some FGFs can themselves be transported across the plasma membrane and translocated to the nucleus where they interact with nuclear FGFRs, though the function that this underlies is not well understood (Mason, 2007).

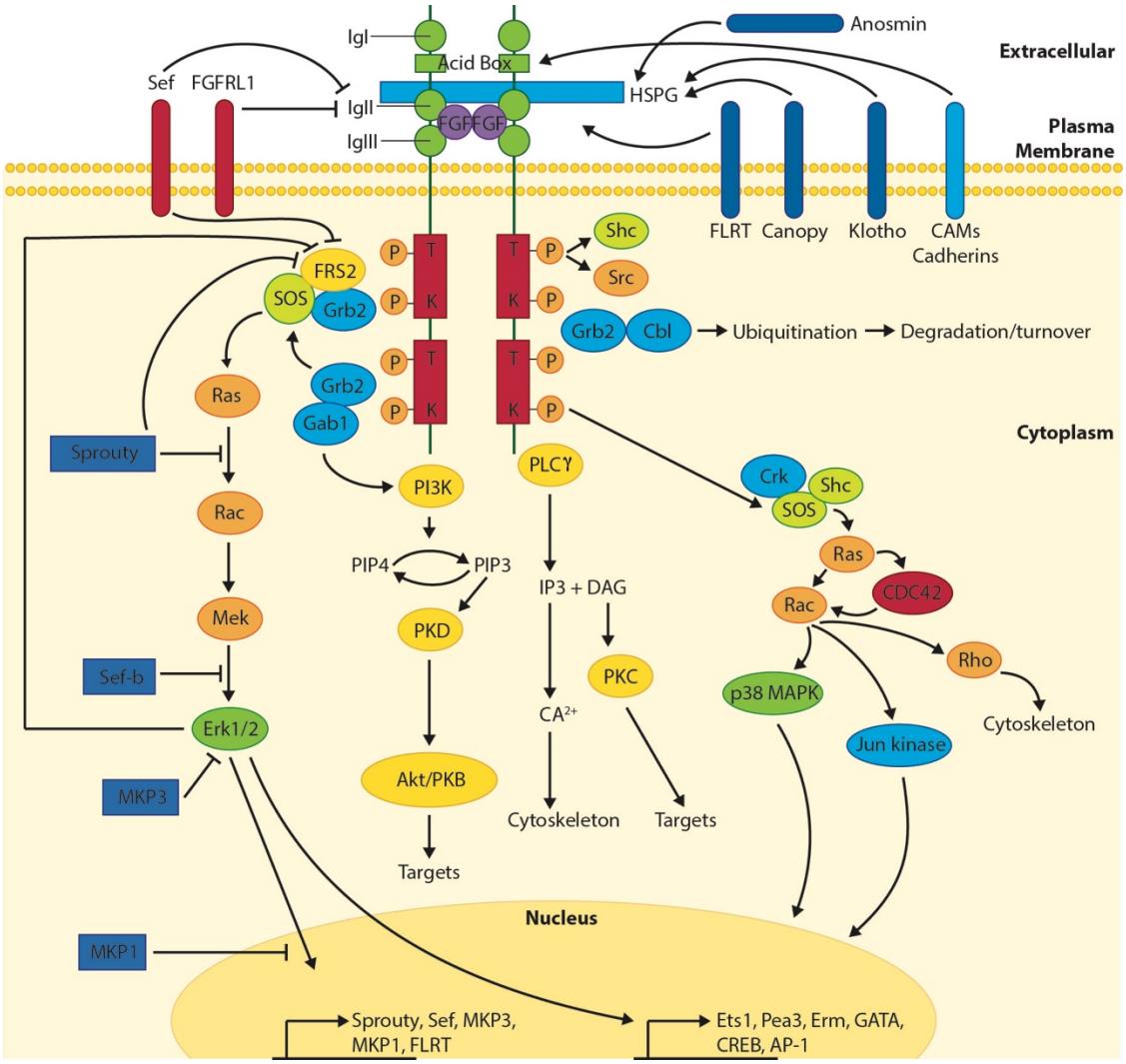


Figure 1.3. Signaling through fibroblast growth factor receptors. Adapted by permission from Macmillan Publishers Ltd: Nature Reviews Neuroscience. Mason (2007). Initiation to End Point: The Multiple Roles of Fibroblast Growth Factors in Neural Development, 8, 583-596, © 2007.

FGF System Influence on Circuit Development, Maintenance, and Plasticity During Development and Adulthood: Focus on FGF2 and FGF9

FGFs interact with many other systems and are responsible for molecular and cellular changes across a wide variety of brain functions. It is beyond the scope of this chapter to exhaustively review the demonstrated functions for all FGFs (though many

others have covered these topics extensively: Beenken & Mohammadi, 2009; Guillemot & Zimmer, 2011; Itoh & Ornitz, 2011; Vaccarino, Schwartz, Raballo, Rhee, & Lyn-Cook, 1999; Woodbury & Ikezu, 2013; Zechel, Werner, Unsicker, & Von Bohlen Und Halbach, 2010). *Since the neurotrophic hypothesis posits that disrupted growth factors influence the cellular circuitry and dysregulate functions underlying MDD symptoms, we will focus on functions influencing circuit formation and maintenance, with a particular emphasis on FGF2 and FGF9.* Previous research demonstrates that both FGF2 and FGF9 are responsible for cellular and molecular changes in the brain, though much less research has focused on the functions of FGF9.

Broadly speaking, FGF2 is expressed after neurulation and this expression is spatially and temporally regulated (Woodbury & Ikezu, 2013); it plays several roles important to circuit function in the nervous system, including: influencing cell proliferation and neurogenesis, promoting differentiation during development, mediating axonal growth, and regulating neurogenesis as well as maintenance and plasticity of specific circuits during adulthood (Zechel et al., 2010). The literature is rich in descriptions of FGF2-mediated effects in a number of disorders, including epilepsy/seizure, traumatic brain injury, and neurodegeneration (for reviews, see: Beenken & Mohammadi, 2009; Palmer, Markakis, Willhoite, Safar, & Gage, 1999; Paradiso, Zucchini, & Simonato, 2013; Turner et al., 2015; Woodbury & Ikezu, 2013; Yoshimura et al., 2001), though a full discussion of all these disorders is beyond the scope of this chapter.

While much work remains to be done on FGF9, early indications suggest that it acts as a survival factor across a number of brain regions (Garcès et al., 2000; Huang,

Hong, & Chuang, 2009; Kanda et al., 1999; Pataky, Borisoff, Fernandes, Tetzlaff, & Steeves, 2000) and that it is critical for circuit formation in the cerebellum (Lin et al., 2009; Meier et al., 2014). It was recently suggested that FGF9 signaling disrupts myelination in multiple sclerosis (Lindner et al., 2015), though its effects on other disorders of central nervous system are unknown.

Functions of FGF2 During Development

Knockout studies provided early evidence that FGF2 was necessary for cellular proliferation and cytoarchitectonic organization in the central nervous system: constitutive FGF2 knockout mice display reduced neuronal, astrocytic, and progenitor density as well as disorganization in several cortical regions both after birth and during adulthood (Chen et al., 2008; Dono, 1998; Ortega, Ittmann, Tsang, Ehrlich, & Basilico, 1998). Other studies demonstrated that FGF2 promoted proliferation in neurogenic zones, beginning during embryogenesis and persisting through adulthood (Wagner, Black, & DiCicco-Bloom, 1999; Zheng, Nowakowski, & Vaccarino, 2004), while administration of FGF2-neutralizing antibodies dramatically reduced neurogenesis (Tao, Black, & DiCicco-Bloom, 1997). Furthermore, FGF2 seems to be particularly important for developmental cellular proliferation in the cerebral cortex and hippocampus. In the anterior cingulate cortex, FGF2 knockout mice display a marked deficit in excitatory neurons (Korada, Zheng, Basilico, Schwartz, & Vaccarino, 2002). Additionally, *in vitro* culture studies indicate that FGF2 is mitogenic for hippocampal stem cells (Vicario-Abejón, Johe, Hazel, Collazo, & McKay, 1995). Collectively, these studies indicate that one of FGF2's primary roles in the brain is to mediate cellular proliferation.

FGF2 also impacts fate determination, and studies suggest that a number of factors, including the timing of FGF2 manipulations (either by augmentation or reduction) and/or expression has differential effects on the identity of affected cell populations (Qian et al., 2000; Vaccarino et al., 1999). This is supported by evidence from a wide variety of studies showing a diversity of cellular fates that are influenced by FGF2, and indeed, FGF2 is commonly used in culture studies to induce differentiation. For example, FGF2 supports astroglia and oligodendrocyte fates in cortical cultures (Morrow, Song, & Ghosh, 2001; Qian et al., 2000; Qian, Davis, Goderie, & Temple, 1997), as well as the development of immature dopamine neurons (Vescovi, Reynolds, Fraser, & Weiss, 1993). Furthermore, it seems that FGF2 has a particular impact in the cortex, where it supports glutamatergic excitatory neuronal development (Korada et al., 2002; Vaccarino, Schwartz, Hartigan, & Leckman, 1995), and in the hippocampus, where culture studies indicate that it may have selective impact on CA3 (Shetty, 2004) and calbindin-expressing neurons (Vicario-Abejón et al., 1995). This FGF2-mediated diversity strongly supports FGF2 as an organizer capable of influencing cellular fate determination, which likely impacts regional microcircuit function.

FGF2 may directly influence circuit formation by playing an organizational role on regional morphology, both by directing proliferation and/or cytoarchitectonic organization and by influencing axon growth and branching. Supporting this idea, augmentation of FGF2 during embryogenesis induced cortical gyrification in rodents, strongly suggesting that the proliferative effects of FGF2 also played an organizational role on developing regional morphology (Rash, Tomasi, Lim, Suh, & Vaccarino, 2013). In parallel, FGF2 is well-known to play a role in developmental axonal growth and

branching: FGF2 increases the size of growth cones and promotes axon branching in both cortical (Szebenyi et al., 2001) and hippocampal cultures (Patel & McNamara, 1995). Furthermore, in hippocampal cultures, FGF2 promoted microcircuit formation (Nakagami, Saito, & Matsuki, 1997). This organizational capacity supports the interpretation that FGF2 plays a role in circuit assembly.

Functions of FGF2 During Postnatal Development and Adulthood

FGF2 is known to modulate cellular proliferation and fate determination of adult progenitors in both neurogenic zones in the adult brain, and some have hypothesized that these effects may partially underlie the circuit dysregulation observed in MDD. Indeed, animals treated with systemic FGF2 showed increases in mitogenic activity in the subventricular zone and the dentate gyrus of the hippocampus both during the early postnatal period and into adulthood (Wagner et al., 1999). Additional studies have supported the action of FGF2 in the hippocampus in particular. Another group demonstrated that chronic intracerebroventricular infusions of FGF2 similarly stimulated adult neurogenesis and survival and increased growth and complexity of dendrites for newborn dentate granule cells (Rai, Hattiangady, & Shetty, 2007). Work from our laboratory supports these findings in a rodent model of selective vulnerability to affective disorders. In selectively bred animals vulnerable to higher levels of anxiety- and depression-like behavior, chronic FGF2 administration during adulthood increased survival of newborn cells (Perez, Clinton, Turner, Watson, & Akil, 2009). Similarly, a single administration of FGF2 on the day after birth increased cellular proliferation and survival during adolescence, though only the effects on survival persisted into adulthood. This results in FGF2-treated animals having denser, more neuron-rich dentate gyri, which

likely impacts hippocampal function (Turner, Clinton, Thompson, Watson, & Akil, 2011). Collectively, these findings strongly support the idea that FGF2 is an important mediator of adult neurogenesis, and therefore its effects on neural circuitry extend beyond development to impact circuit function throughout life.

In addition to the well-characterized effects on neurogenesis, there two studies have indicated that FGF2 is important for the dynamic regulation and tuning of neural circuits, especially in the hippocampus. FGF2 treatment increases functional excitatory synapses in hippocampal cultures (Li, Suzuki, Suzuki, Mizukoshi, & Imamura, 2002), and intracerebroventricular FGF2 administration facilitates long-term potentiation in the hippocampus (Abe, Ishiyama, & Saito, 1992; Ishiyama, Saito, & Abe, 1991). While much work remains to be done to fully elucidate the mechanisms by which FGF2 mediates these effects, these data support the interpretation that FGF2 mediates circuit maintenance and function throughout the lifespan.

Notably, all these experiments examining FGF2's impact on neural circuitry during adulthood have utilized administration paradigms. These exogenous administration effects likely induce or mimic endogenous mechanisms to influence functions like circuit tuning, which may provide the basis for environmental effects on FGF2 expression. Indeed, a number of studies have shown that manipulations with positive effects like environmental enrichment (Perez et al., 2009) and exercise (Gómez-Pinilla, Dao, & So, 1997) induce FGF2 expression, while manipulations with negative effects like chronic social defeat reduce FGF2 expression (Turner, Calvo, Frost, Akil, & Watson, 2008). Taken with the effects previously described, this supports the idea that

FGF2 is poised to exert effects on neural circuits as a result of behavioral interventions, especially in neurogenic-sensitive microcircuits like the hippocampus.

Functions of FGF9 During Development and Adulthood

The role of FGF9 in the central nervous system has been studied much less than FGF2. Knocking out FGF9 systemically is neonatally lethal, due to a dramatic effect on lung development (Colvin, White, Pratt, & Ornitz, 2001), and as such, much of the work to understand FGF9 function has been conducted *in vitro* or using models of FGF9 dysregulation which do not involve constitutive knockout.

In the central nervous system, FGF9 may promote cellular survival and progenitor fate determination across a number of regions and cell types, supporting the hypothesis that it can also act to modulate circuit formation. Indeed, *in vitro* FGF9 promoted survival of cortical neurons, nigrostriatal and mesencephalic dopamine neurons (Chuang et al., 2015; Huang et al., 2009), basal forebrain cholinergic neurons (Kanda et al., 2000), bulbospinal neurons (Pataky et al., 2000), and both medial thoracic and sacral motoneurons in the spinal cord (Garcès et al., 2000; Kanda et al., 1999). FGF9 also has been shown to have specific effects on glia *in vitro*. In cultured oligodendrocytes, FGF9 treatment induced expression of the FGF receptors and spurred activity of the MAPK pathway (Cohen & Chandross, 2000), and this effect may be related to astroglia proliferation. Indeed, downregulation of FGF9 dramatically reduced the pool of proliferating astroglia (Falcone, Filippis, Granzotto, & Mallamaci, 2015). Another recent study suggests that it may promote cellular proliferation and survival but reduce astrocyte differentiation in adult subventricular neural progenitor cells (Lum, Turbic, Mitrovic, & Turnley, 2009), while other studies suggest that FGF9 itself may influence or control the

timing of astrogliogenesis in the cortex (Seuntjens et al., 2009). While there are many open questions regarding the role of FGF9 in cellular proliferation, fate determination, and cell survival, this initial evidence supports the hypothesis that the endogenous functions of FGF9 could influence neural circuitry.

Indeed, two recent studies demonstrate that FGF9 has critical effects on circuit formation in the cerebellum. Lin and colleagues (2009) used a neural tube-specific ablation of FGF9 expression to demonstrate that the loss of FGF9 had profound effects on cerebellar circuitry: FGF9-null mice were ataxic, and this impairment arose from malformed Bergmann glia scaffold formation as well as deficits in granule neuron migration and maturation of Purkinje cells. They went on to investigate these effects further *in vitro*, demonstrating that neuron-derived FGF9 activated the MAPK pathway through FGFR1 and FGFR2 to mediate effects on Bergmann glia scaffolding and neuronal migration (Lin et al., 2009). Another group extended these findings, showing that FGFR2 may be particularly important to circuit formation in the cerebellum and that FGF9 could act as a positioning cue (Meier et al., 2014). While it is unknown if FGF9 has similar effects in regions like the hippocampus or cerebral cortex, these results provide impetus to better understand the region-specific roles that FGF9 plays in circuit formation.

There is much work to be done to elucidate the role of FGF9 during development and adulthood, both during health and in psychiatric disease. Relative to the relative wealth of data on FGF2's influence on neural circuitry, there is a particular need to develop a better understanding of FGF9's impacts on adult neurogenesis, circuit tuning and maintenance, and its response to behavioral manipulations.

Neurotrophic Factors Are Poised To Exert Effects on Affective Circuitry

Because of the patterns of expression, known interactions, and functions influencing cells and circuits, growth factors are poised to act as organizers to mediate effects on affective function and brain circuitry. In particular, the functions described above strongly indicate that FGF2 can exert effects on affective brain circuits, and while many fewer studies have implicated FGF9 in brain plasticity, those that have suggest that it could serve a similar function.

Other mechanisms by which the FGF family could mediate affective neural circuitry, including by modulating neurotransmission and/or affecting molecular networks, have been less studied, but preliminary data are promising. In particular, recent studies indicate that the FGF system can interact with the serotonin system. The researchers present evidence that FGF receptors can heterodimerize with serotonin (5-HT1A) receptors (Borroto-Escuela et al., 2015), and co-treatment with both FGF2 and the serotonin receptor agonist 8-OH-DPAT have very strong effects on behavioral despair (Borroto-Escuela et al., 2012). This indicates that the FGF family, including FGF2 and FGF9, has a point of direct convergence with the monoamine system and could directly influence neurotransmission to modulate affective behavior. It also reinforces the idea that the FGFs could play a major role in affective regulation through interaction with a system that is the target of known antidepressant drugs.

However, many questions remain. For example, it is unknown if the FGFs can modulate other monoamines like norepinephrine or affect excitatory and/or inhibitory neurotransmitters. Furthermore, it is not well-understood how the molecular networks that modulate ligand binding or activate cascades downstream of FGF receptors are

altered with perturbations to the FGF system. It is similarly unknown how disruptions in FGF system molecules interact with or unbalance other molecular networks, including those regulating immune and/or circadian function, both of which show alterations in MDD. Because the need is great to develop novel approaches for new therapeutics that effectively treat MDD, more emphasis should be given to these topics to better understand the impact of growth factor systems on many different levels of analysis, including molecular networks, cells and cellular interactions, microcircuits, and plasticity of macrocircuits.

FGF2 and FGF9 Are Dysregulated in MDD

As previous sections have described, the known patterns of expression and biological functions of FGF2 and FGF9 support the idea that they could act as organizers in the brain to mediate neural circuit function. In order to implicate their dysregulation in the pathophysiology underlying MDD, additional evidence would need to support their role in affective function and vulnerability to disruption in MDD. Ideally, this evidence would be multi-faceted and include demonstrations that: 1) FGF2 and FGF9 expression is altered in the postmortem depressed brain, especially in regions of selective vulnerability like the prefrontal cortices and hippocampus; 2) variability in endogenous expression or in genetic polymorphisms of FGF2 and FGF9 tracks with individual differences in vulnerability to affective disruption; 3) environmental manipulations, including stress, can alter FGF2 and FGF9 expression; 4) manipulation of endogenous expression of FGF2 and FGF9 is sufficient to alter affective behavior; and 5) drugs with known antidepressant or anxiolytic qualities alter expression of FGF2 and FGF9.

Our laboratory and others have demonstrated that FGF2 is downregulated in the frontal cortices (Evans et al., 2004), hippocampus (Gaughran et al., 2006), and locus coeruleus (Bernard et al., 2011) in depressed individuals. This consistent change in expression across regions is striking and underscores the likely importance of FGF2 and the control of affect, but additional studies – including validation in animal models – were necessary to convincingly implicate FGF2 in affective dysregulation and MDD.

Subsequent studies from individuals diagnosed with MDD and animal models support the idea that FGF2 expression varies across individuals and is associated with vulnerability to affective dysregulation. In humans, single-nucleotide polymorphisms in the FGF2 gene predict the response to selective serotonin reuptake inhibitor treatment over six weeks (Kato et al., 2009). Moreover, serum FGF2 levels were decreased in MDD patients (He et al., 2014), though this biomarker may be state-specific, as plasma FGF2 was not found to differ from controls if MDD patients were in remission (Takebayashi, Hashimoto, Hisaoka, Tsuchioka, & Kunugi, 2010). This association between FGF2 and vulnerable individuals has also been observed in animal models. In rats, FGF2 expression in the CA2 subfield of the hippocampus of outbred animals varied and significantly correlated with affective behavior on the elevated plus maze (Eren-Koçak, Turner, Watson, & Akil, 2011). A selective breeding strategy developed by our laboratory has further emphasized the importance of the link between FGF2 expression and individual variation in vulnerability to affective dysregulation. We have generated two lines of rats selectively bred for their locomotor response to a novel environment, one measure of novelty-seeking and anxiety-like behavior (Stead et al., 2006), and these rats have been shown to vary along a number of different traits, including affective

behavior, response to antidepressant treatment, aggression, novelty-seeking, attribution of incentive salience, and propensity to developing dependence to drugs of abuse (Flagel, Waselus, Clinton, Watson, & Akil, 2014; Jama, Cecchi, Calvo, Watson, & Akil, 2008; Stead et al., 2006; Stedenfeld et al., 2011). Bred High Responders (bHRs) show high levels of locomotion in a novel environment and low levels of spontaneous anxiety- and depression-like behavior, while Bred Low Responders (bLRs) show low levels of locomotion in a novel environment and high levels of spontaneous anxiety- and depression-like behavior (Jama et al., 2008; Perez et al., 2009; Stead et al., 2006; Stedenfeld et al., 2011; Turner et al., 2011). Notably, bHR and bLR rats differ in their hippocampal FGF2 expression: bHRs have higher expression of FGF2 in the dentate gyrus and CA2 subfields relative to bLRs (Perez et al., 2009). Moreover, this difference in phenotype and basal expression has been linked to the regulatory epigenetic marker H3K9me3, providing a mechanistic avenue whereby environmental manipulations could impact FGF2 expression (Chaudhury et al., 2014). Furthermore, these phenotypes are sensitive to stress, environmental enrichment, and administration of both antidepressants and FGF2, supporting FGF2's role in affect regulation (Jama et al., 2008; Perez et al., 2009; Stedenfeld et al., 2011; Turner et al., 2011). Collectively, this series of studies demonstrates that FGF2 expression varies across individuals and is associated with differences in affective behavior and dysregulation.

FGF2 has been shown to be responsive to stress, one environmental factor which is thought to be strongly associated with MDD. Acute restraint stress or corticosterone treatment elevated FGF2 levels in a number of regions including the prefrontal cortices and hippocampus (Molteni et al., 2001), and escapable foot-shock showed a similar

induction in the same regions (Bland et al., 2007). This FGF2 induction after acute manipulations is striking, and the researchers suggest that it may be related to neuroprotection. Supporting this interpretation, FGF2 infusions were sufficient to block the effects of chronic unpredictable stress in mice (Elsayed et al., 2012). However, chronic social defeat stress without a positive intervention, an animal model recapitulating some aspects of MDD, strongly decreased FGF2 expression in the hippocampus (Turner, Calvo, et al., 2008). Moreover, prenatal stress or corticosterone exposure, another factor known to increase vulnerability to MDD in humans, decreased FGF2 expression in the prefrontal cortex and hippocampus, and altered the brain's responses to stressful experiences during adulthood (Fumagalli, Bedogni, Slotkin, Racagni, & Riva, 2005; Molteni et al., 2001). Collectively, these studies strongly support FGF2 as a molecule tuned to environmental stress, supporting its possible role in the pathophysiology underlying MDD.

Administration of FGF2 is sufficient to alter anxiety- and depression-like behavior, further implicating this molecule in affective regulation. In outbred mice and rats, subchronic and chronic FGF2 administration had anxiolytic and antidepressant properties (Elsayed et al., 2012; Turner, Gula, Taylor, Watson, & Akil, 2008). In addition, chronic administration of FGF2 eliminated basal differences in spontaneous anxiety-like behavior between bHR and bLR animals, paralleling similar effects of environmental enrichment (Perez et al., 2009). Similarly, bHR and bLR animals who received a one-time administration of FGF2 on the day after birth showed no differences in anxiety- and depression-like behavior during adulthood (Turner et al., 2011). These changes were accompanied by alterations in hippocampal neurogenesis, with particular

effects on cell survival, supporting the notion that FGF2 can influence hippocampal microcircuitry during adulthood to modulate affective behavior (Perez et al., 2009; Turner et al., 2011). As a whole, these studies indicate that chronic administration of FGF2 reduces anxiety- and depression-like behavior in a rodent model, supporting the idea that perturbations in FGF2 levels can impact affect.

Effects of knocking down FGF2 expression further support the role of this molecule in affective behavior. Eren-Koçak and colleagues demonstrated that using a lentivirus containing a short-hairpin RNA targeted against FGF2 could reduce expression in the dentate gyrus of the hippocampus. Furthermore, animals treated with the FGF2 knockdown virus showed significantly more anxiety-like behavior on the elevated plus maze relative to controls, with no effect on depression-like behavior in the forced swim test (Eren-Koçak et al., 2011). These findings reinforce the observations in selectively-bred animals suggesting that bLR's high levels of anxiety and depressive-like behavior may be mediated through low levels of hippocampal FGF2. This observation further demonstrates that endogenous FGF2 expression is necessary for appropriate expression of affective behavior.

Moreover, administration of antidepressant and anxiolytic drugs induced FGF2 expression (Bachis, Mallei, Cruz, Wellstein, & Mocchetti, 2008; Gómez-Pinilla, Dao, Choi, & Ryba, 2000; Mallei, Shi, & Mocchetti, 2002), and recent studies have suggested that this induction is necessary for the effectiveness of the antidepressants imipramine and fluoxetine (Elsayed et al., 2012). Indeed, one possible mechanism underlying this observation is a convergence on the serotonergic system, which both antidepressant drugs target. Because an FGF receptor antagonist was sufficient to disrupt the behavioral

effects of antidepressants (Elsayed et al., 2012) and FGF receptors can heterodimerize with serotonin receptors (Borroto-Escuela et al., 2012, 2015), these studies provide evidence that there may be an interaction between the serotonin and FGF systems to mediate neurotransmission and affective behavior.

Collectively, these studies suggest that FGF2 is an endogenous anxiolytic and anti-depressant that plays both an organizational role during development and an ongoing role during adulthood to modulate emotional reactivity. In contrast, the same microarray datasets which highlight FGF2 reductions in MDD also show a concomitant increase in FGF9 expression across regions in the anterior cingulate, dorsolateral prefrontal cortex (Evans et al., 2004), and locus coeruleus (Bernard et al., 2011). However, very little is known about FGF9's role in affective regulation in animal models, and as noted above, much additional evidence is needed to convincingly implicate FGF9 as a disrupted factor in psychiatric disease. However, given that FGF2 and FGF9 were observed to be dysregulated in opposite directions, this also raises the interesting possibility that they have opposing effects on affective regulation, though there is much work to be done to demonstrate this due to the paucity of research in animal models.

As previous sections have discussed, FGF2 and FGF9 can impact both local microcircuitry between cells within the same region and regional macrocircuitry, especially by way of cellular proliferation, differentiation, and circuit formation. Taken together, these bodies of evidence support FGF2 and FGF9 as molecules capable of mediating the circuit dysregulation that forms the core of the neurotrophic hypothesis of MDD, though there are still many open questions that should be addressed to support this claim. Furthermore, in order to harness the possibility of targeting either FGF2 or FGF9

as possible candidates for therapeutic interventions, additional studies are needed to determine how FGF2 or FGF9 dysregulation impacts molecular networks, cellular functions, and circuity assembly and maintenance. Indeed, understanding the impact of FGF2 and FGF9 on different molecular networks will be of particular importance, since drugs that target FGF2 and FGF9 would also impact the function of these systems.

Goals for the Dissertation

We hypothesize that both FGF2 and FGF9 play opposite roles in the brain to mediate affect. Because we have observed preliminary evidence that FGF2 and FGF9 are disrupted in opposite directions, *we further hypothesize that FGF2 and FGF9 become coordinately dysregulated in MDD.* The data presented in this dissertation will address these hypotheses: we will present three data chapters, including data derived from both postmortem human samples and animal studies, to address these questions.

The first data chapter will address the hypothesis that FGF9 is related to affect and becomes dysregulated in MDD. Because the literature implicating FGF9 in affective dysregulation is still in its infancy, we will follow the path laid by researchers examining the role of FGF2 in MDD. Specifically, this chapter will re-examine previous findings that FGF9 expression is increased in prefrontal cortical regions of the depressed human brain, and it will describe MDD-related FGF9 expression changes in the hippocampus for the first time. Additionally, this chapter will also answer two questions that have not yet been addressed in relation to FGF9: 1) Is there evidence for individual variation in FGF9 expression that relates to affective dysregulation? (Alternatively stated: can we detect basal differences in FGF9 expression between the selectively-bred bHR and bLR rat

lines?) 2) Does knocking down FGF9 expression in the hippocampus alter affective behavior, and if so, which cells mediate this behavior?

The second data chapter will focus on the affective functions of FGF2 in the brain. While the literature describing its effects and dysregulation in MDD has been growing rapidly, much work remains to be done to reinforce FGF2's role in affective dysregulation. We extend previous findings by posing several outstanding questions using our selectively bred bHR and bLR rat lines: 1) Are the differences in basal FGF2 expression in the hippocampus replicable and stable across generations? 2) Are basal differences in hippocampal FGF2 expression necessary for the observed differences in spontaneous anxiety- and depression-like behavior between the lines? (Alternatively stated: does knocking down hippocampal FGF2 expression abolish basal differences in affective behavior?) Additionally, we revisit data derived from postmortem human samples to determine if the the previously-observed reductions in FGF2 expression across different brain regions in MDD can still be observed after controlling for a variety of confounding pre- and postmortem factors.

Because we have consistently observed opposing effects of FGF2 and FGF9 in MDD and animal models of affective disorders, we sought to determine whether FGFs become dysregulated in isolation and/or if expression of members of the FGF family become coordinately dysregulated in MDD. To this end, we examined three questions: 1) Can we find evidence of opposing effects in the relationships in expression between FGF2, FGF9, and the FGF receptors? 2) Do relationships in expression between FGF2, FGF9 and their receptors change with diagnosis? 3) What molecular networks (and their accompanying functions) are associated with dysregulated FGF2 and FGF9 in MDD?

References

Abe, K., Ishiyama, J., & Saito, H. (1992). Effects of epidermal growth factor and basic fibroblast growth factor on generation of long-term potentiation in the dentate gyrus of fimbria-fornix-lesioned rats. *Brain Research*, *593*(2), 335–338. http://doi.org/10.1016/0006-8993(92)91332-9

American Psychiatric Association. (2013). *Diagnostic and statistical manual of mental disorders: DSM-5*. Washington, D.C.

Bachis, A., Mallei, A., Cruz, M. I., Wellstein, A., & Mocchetti, I. (2008). Chronic antidepressant treatments increase basic fibroblast growth factor and fibroblast growth factor-binding protein in neurons. *Neuropharmacology*, *55*(7), 1114–1120. http://doi.org/10.1016/j.neuropharm.2008.07.014

Banasr, M., Dwyer, J. M., & Duman, R. S. (2011). Cell atrophy and loss in depression: reversal by antidepressant treatment. *Current Opinion in Cell Biology*, *23*(6), 730–737. http://doi.org/10.1016/j.ceb.2011.09.002

Bansal, R., Lakhina, V., Remedios, R., & Tole, S. (2003). Expression of FGF receptors 1, 2, 3 in the embryonic and postnatal mouse brain compared with Pdgfralpha, Olig2 and Plp/dm20: implications for oligodendrocyte development. *Developmental Neuroscience*, *25*(2-4), 83–95. http://doi.org/72258

Beenken, A., & Mohammadi, M. (2009). The FGF family: biology, pathophysiology and therapy. *Nature Reviews Drug Discovery*, *8*(3), 235–253. http://doi.org/10.1038/nrd2792

Belluardo, N., Wu, G., Mudo, G., Hansson, A. c., Pettersson, R., & Fuxe, K. (1997). Comparative localization of fibroblast growth factor receptor-1, -2, and -3 mRNAs in the rat brain: In situ hybridization analysis. *The Journal of Comparative Neurology*, *379*(2), 226–246. http://doi.org/10.1002/(SICI)1096-9861(19970310)379:2<226::AID-CNE5>3.0.CO;2-5

Berman, R. M., Cappiello, A., Anand, A., Oren, D. A., Heninger, G. R., Charney, D. S., & Krystal, J. H. (2000). Antidepressant effects of ketamine in depressed patients. *Biological Psychiatry*, *47*(4), 351–354. http://doi.org/10.1016/S0006-3223(99)00230-9

Bernard, R., Kerman, I. A., Thompson, R. C., Jones, E. G., Bunney, W. E., Barchas, J. D., … Watson, S. J. (2011). Altered expression of glutamate signaling, growth factor, and glia genes in the locus coeruleus of patients with major depression. *Molecular Psychiatry*, *16*(6), 634–646. http://doi.org/10.1038/mp.2010.44

Berton, O., & Nestler, E. J. (2006). New approaches to antidepressant drug discovery: beyond monoamines. *Nature Reviews Neuroscience*, *7*(2), 137–151. http://doi.org/10.1038/nrn1846

Bland, S. T., Tamlyn, J. P., Barrientos, R. M., Greenwood, B. N., Watkins, L. R., Campeau, S., … Maier, S. F. (2007). Expression of fibroblast growth factor-2 and brain-derived neurotrophic factor mRNA in the medial prefrontal cortex and hippocampus after uncontrollable or controllable stress. *Neuroscience*, *144*(4), 1219–1228. http://doi.org/10.1016/j.neuroscience.2006.11.026

Bora, E., Fornito, A., Pantelis, C., & Yücel, M. (2012). Gray matter abnormalities in Major Depressive Disorder: A meta-analysis of voxel based morphometry studies.

Journal of Affective Disorders, *138*(1–2), 9–18. http://doi.org/10.1016/j.jad.2011.03.049

Borroto-Escuela, D. O., Narvaez, M., Pérez-Alea, M., Tarakanov, A. O., Jiménez-Beristain, A., Mudó, G., … Fuxe, K. (2015). Evidence for the existence of FGFR1–5-HT1A heteroreceptor complexes in the midbrain raphe 5-HT system. *Biochemical and Biophysical Research Communications*, *456*(1), 489–493. http://doi.org/10.1016/j.bbrc.2014.11.112

Borroto-Escuela, D. O., Romero-Fernandez, W., Mudó, G., Pérez-Alea, M., Ciruela, F., Tarakanov, A. O., … Fuxe, K. (2012). Fibroblast Growth Factor Receptor 1– 5-Hydroxytryptamine 1A Heteroreceptor Complexes and Their Enhancement of Hippocampal Plasticity. *Biological Psychiatry*, *71*(1), 84–91. http://doi.org/10.1016/j.biopsych.2011.09.012

Bremner, J. D., Narayan, M., Anderson, E. R., Staib, L. H., Miller, H. L., Charney, D. S., & others. (2000). Hippocampal volume reduction in major depression. *American Journal of Psychiatry*, *157*(1), 115–117.

Bremner, J. D., Vythilingam, M., Vermetten, E., Nazeer, A., Adil, J., Khan, S., … Charney, D. S. (2002). Reduced volume of orbitofrontal cortex in major depression. *Biological Psychiatry*, *51*(4), 273–279. http://doi.org/10.1016/S0006-3223(01)01336-1

Brown, E. S., Rush, A. J., & McEwen, B. S. (1999). Hippocampal Remodeling and Damage by Corticosteroids: Implications for Mood Disorders. *Neuropsychopharmacology*, *21*(4), 474–484. http://doi.org/10.1016/S0893-133X(99)00054-8

Brown, T. A., Campbell, L. A., Lehman, C. L., Grisham, J. R., & Mancill, R. B. (2001). Current and lifetime comorbidity of the DSM-IV anxiety and mood disorders in a large clinical sample. *Journal of Abnormal Psychology*, *110*(4), 585–599. http://doi.org/10.1037/0021-843X.110.4.585

Chaudhury, S., Aurbach, E. L., Sharma, V., Blandino, P., Turner, C. A., Watson, S. J., & Akil, H. (2014). FGF2 is a target and a trigger of epigenetic mechanisms associated with differences in emotionality: Partnership with H3K9me3. *Proceedings of the National Academy of Sciences*, *111*(32), 11834–11839. http://doi.org/10.1073/pnas.1411618111

Chen, K., Ohkubo, Y., Shin, D., Doetschman, T., Sanford, P. L., Li, H., & Vaccarino, F. M. (2008). Decrease in excitatory neurons, astrocytes and proliferating progenitors in the cerebral cortex of mice lacking exon 3 from the Fgf2 gene. *BMC Neuroscience*, *9*, 94. http://doi.org/10.1186/1471-2202-9-94

Chuang, J.-I., Huang, J.-Y., Tsai, S.-J., Sun, H. S., Yang, S.-H., Chuang, P.-C., … Ching, C.-H. (2015). FGF9-induced changes in cellular redox status and HO-1 upregulation are FGFR-dependent and proceed through both ERK and AKT to induce CREB and Nrf2 activation. *Free Radical Biology and Medicine*, *89*, 274–286. http://doi.org/10.1016/j.freeradbiomed.2015.08.011

Cohen, R. I., & Chandross, K. J. (2000). Fibroblast growth factor-9 modulates the expression of myelin related proteins and multiple fibroblast growth factor receptors in developing oligodendrocytes. *Journal of Neuroscience Research*, *61*(3), 273–287. http://doi.org/10.1002/1097-4547(20000801)61:3<273::AID-JNR5>3.0.CO;2-I

Colvin, J. S., White, A. C., Pratt, S. J., & Ornitz, D. M. (2001). Lung hypoplasia and neonatal death in Fgf9-null mice identify this gene as an essential regulator of lung mesenchyme. *Development*, *128*(11), 2095 –2106.

Cuthbert, B. N., & Insel, T. (2010). The Data of Diagnosis: New Approaches to Psychiatric Classification. *Psychiatry: Interpersonal and Biological Processes*, *73*(4), 311–314. http://doi.org/10.1521/psyc.2010.73.4.311

Cuthbert, B. N., & Insel, T. R. (2013). Toward the future of psychiatric diagnosis: the seven pillars of RDoC. *BMC Medicine*, *11*, 126. http://doi.org/10.1186/1741-7015-11-126

Cuthbert, B. N., & Kozak, M. J. (2013). Constructing constructs for psychopathology: The NIMH research domain criteria. *Journal of Abnormal Psychology*, *122*(3), 928–937. http://doi.org/10.1037/a0034028

Dono, R. (1998). Impaired cerebral cortex development and blood pressure regulation in FGF-2-deficient mice. *The EMBO Journal*, *17*(15), 4213–4225. http://doi.org/10.1093/emboj/17.15.4213

Drevets, W. C., Price, J. L., & Furey, M. L. (2008). Brain structural and functional abnormalities in mood disorders: implications for neurocircuitry models of depression. *Brain Structure & Function*, *213*(1-2), 93–118. http://doi.org/10.1007/s00429-008-0189-x

Duman, R. S., Heninger, G. R., & Nestler, E. J. (1997). A molecular and cellular theory of depression. *Archives of General Psychiatry*, *54*(7), 597–606. http://doi.org/10.1001/archpsyc.1997.01830190015002

Duman, R. S., & Monteggia, L. M. (2006). A Neurotrophic Model for Stress-Related Mood Disorders. *Biological Psychiatry*, *59*(12), 1116–1127. http://doi.org/16/j.biopsych.2006.02.013

Duric, V., & Duman, R. S. (2012). Depression and treatment response: dynamic interplay of signaling pathways and altered neural processes. *Cellular and Molecular Life Sciences*, *70*(1), 39–53. http://doi.org/10.1007/s00018-012-1020-7

Elsayed, M., Banasr, M., Duric, V., Fournier, N. M., Licznerski, P., & Duman, R. S. (2012). Antidepressant Effects of Fibroblast Growth Factor-2 in Behavioral and Cellular Models of Depression. *Biological Psychiatry*, *72*(4), 258–265. http://doi.org/10.1016/j.biopsych.2012.03.003

Eren-Koçak, E., Turner, C. A., Watson, S. J., & Akil, H. (2011). Short-Hairpin RNA Silencing of Endogenous Fibroblast Growth Factor 2 in Rat Hippocampus Increases Anxiety Behavior. *Biological Psychiatry*, *69*(6), 534–540. http://doi.org/10.1016/j.biopsych.2010.11.020

Evans, S. J., Choudary, P. V., Neal, C. R., Li, J. Z., Vawter, M. P., Tomita, H., … Akil, H. (2004). Dysregulation of the fibroblast growth factor system in major depression. *Proceedings of the National Academy of Sciences of the United States of America*, *101*(43), 15506 –15511. http://doi.org/10.1073/pnas.0406788101

Falcone, C., Filippis, C., Granzotto, M., & Mallamaci, A. (2015). Emx2 expression levels in NSCs modulate astrogenesis rates by regulating EgfR and Fgf9. *Glia*, *63*(3), 412–422. http://doi.org/10.1002/glia.22761

Fava, M., & Davidson, K. G. (1996). DEFINITION AND EPIDEMIOLOGY OF TREATMENT-RESISTANT DEPRESSION. *Psychiatric Clinics of North America*, *19*(2), 179–200. http://doi.org/10.1016/S0193-953X(05)70283-5

Flagel, S. B., Waselus, M., Clinton, S. M., Watson, S. J., & Akil, H. (2014). Antecedents and consequences of drug abuse in rats selectively bred for high and low response to novelty. *Neuropharmacology*, *76, Part B*, 425–436. http://doi.org/10.1016/j.neuropharm.2013.04.033

Fumagalli, F., Bedogni, F., Slotkin, T. A., Racagni, G., & Riva, M. A. (2005). Prenatal stress elicits regionally selective changes in basal FGF-2 gene expression in adulthood and alters the adult response to acute or chronic stress. *Neurobiology of Disease*, *20*(3), 731–737. http://doi.org/10.1016/j.nbd.2005.05.005

Garcès, A., Nishimune, H., Philippe, J.-M., Pettmann, B., & deLapeyrière, O. (2000). FGF9: A motoneuron survival factor expressed by medial thoracic and sacral motoneurons. *Journal of Neuroscience Research*, *60*(1), 1–9. http://doi.org/10.1002/(SICI)1097-4547(20000401)60:1<1::AID-JNR1>3.0.CO;2-P

Gaughran, F., Payne, J., Sedgwick, P. M., Cotter, D., & Berry, M. (2006). Hippocampal FGF-2 and FGFR1 mRNA expression in major depression, schizophrenia and bipolar disorder. *Brain Research Bulletin*, *70*(3), 221–227. http://doi.org/10.1016/j.brainresbull.2006.04.008

Godsil, B. P., Kiss, J. P., Spedding, M., & Jay, T. M. (2013). The hippocampal–prefrontal pathway: The weak link in psychiatric disorders? *European Neuropsychopharmacology*, *23*(10), 1165–1181. http://doi.org/10.1016/j.euroneuro.2012.10.018

Gold, P. W., Machado-Vieira, R., Pavlatou, M. G., Gold, P. W., Machado-Vieira, R., & Pavlatou, M. G. (2015). Clinical and Biochemical Manifestations of Depression: Relation to the Neurobiology of Stress, Clinical and Biochemical Manifestations of Depression: Relation to the Neurobiology of Stress. *Neural Plasticity, Neural Plasticity*, *2015*, *2015*, e581976. http://doi.org/10.1155/2015/581976, 10.1155/2015/581976

Gómez-Pinilla, F., Dao, L., Choi, J., & Ryba, E. . (2000). Diazepam induces FGF-2 mRNA in the hippocampus and striatum. *Brain Research Bulletin*, *53*(3), 283–289. http://doi.org/10.1016/S0361-9230(00)00342-7

Gómez-Pinilla, F., Dao, L., & So, V. (1997). Physical exercise induces FGF-2 and its mRNA in the hippocampus. *Brain Research*, *764*(1–2), 1–8. http://doi.org/10.1016/S0006-8993(97)00375-2

Gonzalez, A. M., Berry, M., Maher, P. A., Logan, A., & Baird, A. (1995). A comprehensive analysis of the distribution of FGF-2 and FGFR1 in the rat brain. *Brain Research*, *701*(1–2), 201–226. http://doi.org/10.1016/0006-8993(95)01002-X

Goodkind, M., Eickhoff, S. B., Oathes, D. J., Jiang, Y., Chang, A., Jones-Hagata, L. B., … Etkin, A. (2015). Identification of a common neurobiological substrate for mental illness. *JAMA Psychiatry*, *72*(4), 305–315. http://doi.org/10.1001/jamapsychiatry.2014.2206

Grant, B. F., Hasin, D. S., Stinson, F. S., Dawson, D. A., Patricia Chou, S., June Ruan, W., & Huang, B. (2005). Co-occurrence of 12-month mood and anxiety disorders and personality disorders in the US: results from the national epidemiologic survey on alcohol and related conditions. *Journal of Psychiatric Research*, *39*(1), 1–9. http://doi.org/10.1016/j.jpsychires.2004.05.004

Guillemot, F., & Zimmer, C. (2011). From Cradle to Grave: The Multiple Roles of Fibroblast Growth Factors in Neural Development. *Neuron*, *71*(4), 574–588. http://doi.org/10.1016/j.neuron.2011.08.002

Gunaydin, L. A., & Kreitzer, A. C. (2016). Cortico–Basal Ganglia Circuit Function in Psychiatric Disease. *Annual Review of Physiology*, *78*(1), null. http://doi.org/10.1146/annurev-physiol-021115-105355

He, S., Zhang, T., Hong, B., Peng, D., Su, H., Lin, Z., … Li, H. (2014). Decreased serum fibroblast growth factor - 2 levels in pre- and post-treatment patients with major depressive disorder. *Neuroscience Letters*, *579*, 168–172. http://doi.org/10.1016/j.neulet.2014.07.035

Holtzheimer, P. E., & Mayberg, H. S. (2011). Deep Brain Stimulation for Psychiatric Disorders. *Annual Review of Neuroscience*, *34*(1), 289–307. http://doi.org/10.1146/annurev-neuro-061010-113638

Huang, J.-Y., Hong, Y.-T., & Chuang, J.-I. (2009). Fibroblast growth factor 9 prevents MPP+-induced death of dopaminergic neurons and is involved in melatonin neuroprotection in vivo and in vitro. *Journal of Neurochemistry*, *109*(5), 1400–1412. http://doi.org/10.1111/j.1471-4159.2009.06061.x

Hunt, C., Issakidis, C., & Andrews, G. (2002). DSM-IV generalized anxiety disorder in the Australian National Survey of Mental Health and Well-Being. *Psychological Medicine*, *32*(4), 649–659.

Ishiyama, J., Saito, H., & Abe, K. (1991). Epidermal growth factor and basic fibroblast growth factor promote the generation of long-term potentiation in the dentate gyrus of anaesthetized rats. *Neuroscience Research*, *12*(3), 403–411. http://doi.org/10.1016/0168-0102(91)90071-6

Itoh, N., & Ornitz, D. M. (2008). Functional evolutionary history of the mouse Fgf gene family. *Developmental Dynamics: An Official Publication of the American Association of Anatomists*, *237*(1), 18–27. http://doi.org/10.1002/dvdy.21388

Itoh, N., & Ornitz, D. M. (2011). Fibroblast growth factors: from molecular evolution to roles in development, metabolism and disease. *Journal of Biochemistry*, *149*(2), 121 –130. http://doi.org/10.1093/jb/mvq121

Jama, A., Cecchi, M., Calvo, N., Watson, S. J., & Akil, H. (2008). Inter-individual differences in novelty-seeking behavior in rats predict differential responses to desipramine in the forced swim test. *Psychopharmacology*, *198*(3), 333–340. http://doi.org/10.1007/s00213-008-1126-7

Kanda, T., Iwasaki, T., Nakamura, S., Kurokawa, T., Ikeda, K., & Mizusawa, H. (2000). Self-secretion of fibroblast growth factor-9 supports basal forebrain cholinergic neurons in an autocrine/paracrine manner. *Brain Research*, *876*(1–2), 22–30. http://doi.org/10.1016/S0006-8993(00)02563-4

Kanda, T., Iwasaki, T., Nakamura, S., Ueki, A., Kurokawa, T., Ikeda, K., & Mizusawa, H. (1999). FGF-9 is an autocrine/paracrine neurotrophic substance for spinal motoneurons. *International Journal of Developmental Neuroscience*, *17*(3), 191–200. http://doi.org/10.1016/S0736-5748(99)00026-X

Kato, M., Okugawa, G., Wakeno, M., Takekita, Y., Nonen, S., Tetsuo, S., … Serretti, A. (2009). Effect of basic fibroblast growth factor (FGF2) gene polymorphisms on SSRIs treatment response and side effects. *European Neuropsychopharmacology*, *19*(10), 718–725. http://doi.org/10.1016/j.euroneuro.2009.06.006

Kessler, R. C., Berglund, P., Demler, O., Jin, R., Koretz, D., Merikangas, K. R., … National Comorbidity Survey Replication. (2003). The epidemiology of major depressive disorder: results from the National Comorbidity Survey Replication (NCS-R). *JAMA*, *289*(23), 3095–3105. http://doi.org/10.1001/jama.289.23.3095

Kessler, R. C., Petukhova, M., Sampson, N. A., Zaslavsky, A. M., & Wittchen, H.-U. (2012). Twelve-month and lifetime prevalence and lifetime morbid risk of anxiety and mood disorders in the United States. *International Journal of Methods in Psychiatric Research*, *21*(3), 169–184. http://doi.org/10.1002/mpr.1359

Klint, P., & Claesson-Welsh, L. (1999). Signal transduction by fibroblast growth factor receptors. *Frontiers in Bioscience: A Journal and Virtual Library*, *4*, D165–177.

Korada, S., Zheng, W., Basilico, C., Schwartz, M. L., & Vaccarino, F. M. (2002). Fibroblast Growth Factor 2 Is Necessary for the Growth of Glutamate Projection Neurons in the Anterior Neocortex. *The Journal of Neuroscience*, *22*(3), 863–875.

Krejci, P., Prochazkova, J., Bryja, V., Kozubik, A., & Wilcox, W. R. (2009). Molecular pathology of the fibroblast growth factor family. *Human Mutation*, *30*(9), 1245–1255. http://doi.org/10.1002/humu.21067

Krishnan, V., & Nestler, E. J. (2008). The molecular neurobiology of depression. *Nature*, *455*(7215), 894–902. http://doi.org/10.1038/nature07455

Li, A., Suzuki, S., Suzuki, M., Mizukoshi, E., & Imamura, T. (2002). Fibroblast growth factor-2 increases functional excitatory synapses on hippocampal neurons. *European Journal of Neuroscience*, *16*(7), 1313–1324. http://doi.org/10.1046/j.1460-9568.2002.02193.x

Lindner, M., Thümmler, K., Arthur, A., Brunner, S., Elliott, C., McElroy, D., … Linington, C. (2015). Fibroblast growth factor signalling in multiple sclerosis: inhibition of myelination and induction of pro-inflammatory environment by FGF9. *Brain*, *138*(7), 1875–1893. http://doi.org/10.1093/brain/awv102

Lin, Y., Chen, L., Lin, C., Luo, Y., Tsai, R. Y. L., & Wang, F. (2009). Neuron-derived FGF9 is essential for scaffold formation of Bergmann radial fibers and migration of granule neurons in the cerebellum. *Developmental Biology*, *329*(1), 44–54. http://doi.org/10.1016/j.ydbio.2009.02.011

Lum, M., Turbic, A., Mitrovic, B., & Turnley, A. M. (2009). Fibroblast growth factor-9 inhibits astrocyte differentiation of adult mouse neural progenitor cells. *Journal of Neuroscience Research*, *87*(10), 2201–2210. http://doi.org/10.1002/jnr.22047

MacQueen, G., & Frodl, T. (2011). The hippocampus in major depression: evidence for the convergence of the bench and bedside in psychiatric research[quest]. *Mol Psychiatry*, *16*(3), 252–264.

Mallei, A., Shi, B., & Mocchetti, I. (2002). Antidepressant Treatments Induce the Expression of Basic Fibroblast Growth Factor in Cortical and Hippocampal Neurons. *Molecular Pharmacology*, *61*(5), 1017 –1024. http://doi.org/10.1124/mol.61.5.1017

Mason, I. (2007). Initiation to end point: the multiple roles of fibroblast growth factors in neural development. *Nature Reviews Neuroscience*, *8*(8), 583–596. http://doi.org/10.1038/nrn2189

Matsuo, A., Tooyama, I., Isobe, S., Oomura, Y., Akiguchi, I., Hanai, K., … Kimura, H. (1994). Immunohistochemical localization in the rat brain of an epitope

corresponding to the fibroblast growth factor receptor-1. *Neuroscience*, *60*(1), 49–66. http://doi.org/10.1016/0306-4522(94)90203-8

McClung, C. A., & Nestler, E. J. (2007). Neuroplasticity Mediated by Altered Gene Expression. *Neuropsychopharmacology*, *33*(1), 3–17. http://doi.org/10.1038/sj.npp.1301544

McEwen, B. S. (1999). Stress and hippocampal plasticity. *Annual Review of Neuroscience*, *22*, 105–122. http://doi.org/10.1146/annurev.neuro.22.1.105

Meier, F., Giesert, F., Delic, S., Faus-Kessler, T., Matheus, F., Simeone, A., … Prakash, N. (2014). FGF/FGFR2 Signaling Regulates the Generation and Correct Positioning of Bergmann Glia Cells in the Developing Mouse Cerebellum. *PLoS ONE*, *9*(7), e101124. http://doi.org/10.1371/journal.pone.0101124

Molteni, R., Fumagalli, F., Magnaghi, V., Roceri, M., Gennarelli, M., Racagni, G., … Riva, M. A. (2001). Modulation of fibroblast growth factor-2 by stress and corticosteroids: from developmental events to adult brain plasticity. *Brain Research Reviews*, *37*(1–3), 249–258. http://doi.org/10.1016/S0165-0173(01)00128-X

Morrow, T., Song, M.-R., & Ghosh, A. (2001). Sequential specification of neurons and glia by developmentally regulated extracellular factors. *Development*, *128*(18), 3585–3594.

Nakagami, Y., Saito, H., & Matsuki, N. (1997). Basic fibroblast growth factor and brain-derived neurotrophic factor promote survival and neuronal circuit formation in organotypic hippocampal culture. *Japanese Journal of Pharmacology*, *75*(4), 319–326.

Nakamura, S., Todo, T., Haga, S., Aizawa, T., Motoi, Y., Ueki, A., … Ikeda, K. (1997). Motor neurons in human and rat spinal cord synthesize fibroblast growth factor-9. *Neuroscience Letters*, *221*(2–3), 181–184. http://doi.org/10.1016/S0304-3940(96)13312-7

Nakamura, S., Todo, T., Motoi, Y., Haga, S., Aizawa, T., Ueki, A., & Ikeda, K. (1999). Glial expression of fibroblast growth factor-9 in rat central nervous system. *Glia*, *28*(1), 53–65. http://doi.org/10.1002/(SICI)1098-1136(199910)28:1<53::AID-GLIA7>3.0.CO;2-V

Nestler, E. J., Barrot, M., DiLeone, R. J., Eisch, A. J., Gold, S. J., & Monteggia, L. M. (2002). Neurobiology of depression. *Neuron*, *34*(1), 13–25.

Nestler, E. J., & Carlezon, W. A. (2006). The Mesolimbic Dopamine Reward Circuit in Depression. *Biological Psychiatry*, *59*(12), 1151–1159. http://doi.org/10.1016/j.biopsych.2005.09.018

Nestler, E. J., & Hyman, S. E. (2010). Animal models of neuropsychiatric disorders. *Nat Neurosci*, *13*(10), 1161–1169. http://doi.org/10.1038/nn.2647

Oh, L. Y. S., Denninger, A., Colvin, J. S., Vyas, A., Tole, S., Ornitz, D. M., & Bansal, R. (2003). Fibroblast Growth Factor Receptor 3 Signaling Regulates the Onset of Oligodendrocyte Terminal Differentiation. *The Journal of Neuroscience*, *23*(3), 883–894.

Ornitz, D. M., Xu, J., Colvin, J. S., McEwen, D. G., MacArthur, C. A., Coulier, F., … Goldfarb, M. (1996). Receptor Specificity of the Fibroblast Growth Factor Family. *Journal of Biological Chemistry*, *271*(25), 15292–15297. http://doi.org/10.1074/jbc.271.25.15292

Ortega, S., Ittmann, M., Tsang, S. H., Ehrlich, M., & Basilico, C. (1998). Neuronal defects and delayed wound healing in mice lacking fibroblast growth factor 2. *Proceedings of the National Academy of Sciences*, *95*(10), 5672 –5677.

Palmer, T. D., Markakis, E. A., Willhoite, A. R., Safar, F., & Gage, F. H. (1999). Fibroblast growth factor-2 activates a latent neurogenic program in neural stem cells from diverse regions of the adult CNS. *The Journal of Neuroscience: The Official Journal of the Society for Neuroscience*, *19*(19), 8487–8497.

Panksepp, J. (2003). Feeling the Pain of Social Loss. *Science*, *302*(5643), 237–239. http://doi.org/10.1126/science.1091062

Panksepp, J., Knutson, B., & Burgdorf, J. (2002). The role of brain emotional systems in addictions: a neuro-evolutionary perspective and new "self-report" animal model. *Addiction*, *97*(4), 459–469. http://doi.org/10.1046/j.1360-0443.2002.00025.x

Panksepp, J., Nelson, E., & Bekkedal, M. (1997). Brain Systems for the Mediation of Social Separation-Distress and Social-Reward Evolutionary Antecedents and Neuropeptide Intermediariesa. *Annals of the New York Academy of Sciences*, *807*(1), 78–100. http://doi.org/10.1111/j.1749-6632.1997.tb51914.x

Paradiso, B., Zucchini, S., & Simonato, M. (2013). Implication of fibroblast growth factors in epileptogenesis-associated circuit rearrangements. *Frontiers in Cellular Neuroscience*, *7*. http://doi.org/10.3389/fncel.2013.00152

Pataky, D. M., Borisoff, J. F., Fernandes, K. J. L., Tetzlaff, W., & Steeves, J. D. (2000). Fibroblast Growth Factor Treatment Produces Differential Effects on Survival and Neurite Outgrowth from Identified Bulbospinal Neurons in Vitro. *Experimental Neurology*, *163*(2), 357–372. http://doi.org/10.1006/exnr.2000.7365

Patel, M. N., & McNamara, J. O. (1995). Selective enhancement of axonal branching of cultured dentate gyrus neurons by neurotrophic factors. *Neuroscience*, *69*(3), 763–770. http://doi.org/10.1016/0306-4522(95)00281-M

Perez, J. A., Clinton, S. M., Turner, C. A., Watson, S. J., & Akil, H. (2009). A New Role for FGF2 as an Endogenous Inhibitor of Anxiety. *J. Neurosci.*, *29*(19), 6379–6387. http://doi.org/10.1523/JNEUROSCI.4829-08.2009

Peters, K., Ornitz, D., Werner, S., & Williams, L. (1993). Unique Expression Pattern of the FGF Receptor 3 Gene during Mouse Organogenesis. *Developmental Biology*, *155*(2), 423–430. http://doi.org/10.1006/dbio.1993.1040

Plotsky, P. M., Owens, M. J., & Nemeroff, C. B. (1998). PSYCHONEUROENDOCRINOLOGY OF DEPRESSION: Hypothalamic-Pituitary-Adrenal Axis. *Psychiatric Clinics of North America*, *21*(2), 293–307. http://doi.org/10.1016/S0193-953X(05)70006-X

Price, J. L., & Drevets, W. C. (2012). Neural circuits underlying the pathophysiology of mood disorders. *Trends in Cognitive Sciences*, *16*(1), 61–71. http://doi.org/10.1016/j.tics.2011.12.011

Pringle, N. P., Yu, W.-P., Howell, M., Colvin, J. S., Ornitz, D. M., & Richardson, W. D. (2003). Fgfr3 expression by astrocytes and their precursors: evidence that astrocytes and oligodendrocytes originate in distinct neuroepithelial domains. *Development*, *130*(1), 93–102. http://doi.org/10.1242/dev.00184

Qian, X., Davis, A. A., Goderie, S. K., & Temple, S. (1997). FGF2 Concentration Regulates the Generation of Neurons and Glia from Multipotent Cortical Stem Cells. *Neuron*, *18*(1), 81–93. http://doi.org/10.1016/S0896-6273(01)80048-9

Qian, X., Shen, Q., Goderie, S. K., He, W., Capela, A., Davis, A. A., & Temple, S. (2000). Timing of CNS Cell Generation. *Neuron*, *28*(1), 69–80. http://doi.org/10.1016/S0896-6273(00)00086-6

Rai, K. S., Hattiangady, B., & Shetty, A. K. (2007). Enhanced production and dendritic growth of new dentate granule cells in the middle-aged hippocampus following intracerebroventricular FGF-2 infusions. *European Journal of Neuroscience*, *26*(7), 1765–1779. http://doi.org/10.1111/j.1460-9568.2007.05820.x

Rash, B. G., Tomasi, S., Lim, H. D., Suh, C. Y., & Vaccarino, F. M. (2013). Cortical Gyrification Induced by Fibroblast Growth Factor 2 in the Mouse Brain. *The Journal of Neuroscience*, *33*(26), 10802–10814. http://doi.org/10.1523/JNEUROSCI.3621-12.2013

Ressler, K. J., & Mayberg, H. S. (2007). Targeting abnormal neural circuits in mood and anxiety disorders: from the laboratory to the clinic. *Nature Neuroscience*, *10*(9), 1116–1124. http://doi.org/10.1038/nn1944

Ressler, K. J., & Nemeroff, C. B. (2000). Role of serotonergic and noradrenergic systems in the pathophysiology of depression and anxiety disorders. *Depression and Anxiety*, *12*(S1), 2–19. http://doi.org/10.1002/1520-6394(2000)12:1+<2::AID-DA2>3.0.CO;2-4

Reuss, B., & von Bohlen und Halbach, O. (2003). Fibroblast growth factors and their receptors in the central nervous system. *Cell and Tissue Research*, *313*(2), 139–157. http://doi.org/10.1007/s00441-003-0756-7

Seuntjens, E., Nityanandam, A., Miquelajauregui, A., Debruyn, J., Stryjewska, A., Goebbels, S., … Tarabykin, V. (2009). Sip1 regulates sequential fate decisions by feedback signaling from postmitotic neurons to progenitors. *Nature Neuroscience*, *12*(11), 1373–1380. http://doi.org/10.1038/nn.2409

Sheline, Y. I., Gado, M. H., & Kraemer, H. C. (2003). Untreated depression and hippocampal volume loss. *American Journal of Psychiatry*, *160*(8), 1516–1518.

Shetty, A. K. (2004). Progenitor cells from the CA3 region of the embryonic day 19 rat hippocampus generate region-specific neuronal phenotypes in vitro. *Hippocampus*, *14*(5), 595–614. http://doi.org/10.1002/hipo.10206

Stead, J. D. H., Clinton, S., Neal, C., Schneider, J., Jama, A., Miller, S., … Akil, H. (2006). Selective Breeding for Divergence in Novelty-seeking Traits: Heritability and Enrichment in Spontaneous Anxiety-related Behaviors. *Behavior Genetics*, *36*(5), 697–712. http://doi.org/10.1007/s10519-006-9058-7

Stedenfeld, K. A., Clinton, S. M., Kerman, I. A., Akil, H., Watson, S. J., & Sved, A. F. (2011). Novelty-seeking behavior predicts vulnerability in a rodent model of depression. *Physiology & Behavior*, *103*(2), 210–216. http://doi.org/10.1016/j.physbeh.2011.02.001

Swaab, D. F., Bao, A.-M., & Lucassen, P. J. (2005). The stress system in the human brain in depression and neurodegeneration. *Ageing Research Reviews*, *4*(2), 141–194. http://doi.org/10.1016/j.arr.2005.03.003

Szebenyi, G., Dent, E. W., Callaway, J. L., Seys, C., Lueth, H., & Kalil, K. (2001). Fibroblast Growth Factor-2 Promotes Axon Branching of Cortical Neurons by Influencing Morphology and Behavior of the Primary Growth Cone. *The Journal of Neuroscience*, *21*(11), 3932–3941.

Tagashira, S., Ozaki, K., Ohta, M., & Itoh, N. (1995). Localization of fibroblast growth factor-9 mRNA in the rat brain. *Molecular Brain Research*, *30*(2), 233–241. http://doi.org/10.1016/0169-328X(95)00009-H

Takebayashi, M., Hashimoto, R., Hisaoka, K., Tsuchioka, M., & Kunugi, H. (2010). Plasma levels of vascular endothelial growth factor and fibroblast growth factor 2 in patients with major depressive disorders. *Journal of Neural Transmission*, *117*(9), 1119–1122. http://doi.org/10.1007/s00702-010-0452-1

Tao, Y., Black, I. B., & DiCicco-Bloom, E. (1997). In vivo neurogenesis is inhibited by neutralizing antibodies to basic fibroblast growth factor. *Journal of Neurobiology*, *33*(3), 289–296. http://doi.org/10.1002/(SICI)1097-4695(199709)33:3<289::AID-NEU7>3.0.CO;2-Y

Tekin, S., & Cummings, J. L. (2002). Frontal–subcortical neuronal circuits and clinical neuropsychiatry: An update. *Journal of Psychosomatic Research*, *53*(2), 647–654. http://doi.org/10.1016/S0022-3999(02)00428-2

Turner, C. A., Calvo, N., Frost, D. O., Akil, H., & Watson, S. J. (2008). The fibroblast growth factor system is downregulated following social defeat. *Neuroscience Letters*, *430*(2), 147–150.

Turner, C. A., Clinton, S. M., Thompson, R. C., Watson, S. J., & Akil, H. (2011). Fibroblast growth factor-2 (FGF2) augmentation early in life alters hippocampal development and rescues the anxiety phenotype in vulnerable animals. *Proceedings of the National Academy of Sciences*, *108*(19), 8021 –8025. http://doi.org/10.1073/pnas.1103732108

Turner, C. A., Eren-Koçak, E., Inui, E. G., Watson, S. J., & Akil, H. (2015). Dysregulated fibroblast growth factor (FGF) signaling in neurological and psychiatric disorders. *Seminars in Cell & Developmental Biology*. http://doi.org/10.1016/j.semcdb.2015.10.003

Turner, C. A., Gula, E. L., Taylor, L. P., Watson, S. J., & Akil, H. (2008). Antidepressant-like effects of intracerebroventricular FGF2 in rats. *Brain Research*, *1224*, 63–68. http://doi.org/16/j.brainres.2008.05.088

Turner, C. A., Watson, S. J., & Akil, H. (2012). The Fibroblast Growth Factor Family: Neuromodulation of Affective Behavior. *Neuron*, *76*(1), 160–174. http://doi.org/10.1016/j.neuron.2012.08.037

Vaccarino, F. M., Schwartz, M. L., Hartigan, D., & Leckman, J. F. (1995). Basic fibroblast growth factor increases the number of excitatory neurons containing glutamate in the cerebral cortex. *Cerebral Cortex (New York, N.Y.: 1991)*, *5*(1), 64–78.

Vaccarino, F. M., Schwartz, M. L., Raballo, R., Rhee, J., & Lyn-Cook, R. (1999). 6 Fibroblast Growth Factor Signaling Regulates Growth and Morphogenesis at Multiple Steps during Brain Development1. In R. A. P. and G. P. Schatten (Ed.), *Current Topics in Developmental Biology* (Vol. 46, pp. 179–200). Academic Press. Retrieved from http://www.sciencedirect.com/science/article/pii/S0070215308603294

Vescovi, A. L., Reynolds, B. A., Fraser, D. D., & Weiss, S. (1993). bFGF regulates the proliferative fate of unipotent (neuronal) and bipotent (neuronal/astroglial) EGF-generated CNS progenitor cells. *Neuron*, *11*(5), 951–966. http://doi.org/10.1016/0896-6273(93)90124-A

Vicario-Abejón, C., Johe, K. K., Hazel, T. G., Collazo, D., & McKay, R. D. G. (1995). Functions of basic fibroblast growth factor and neurotrophins in the differentiation of hippocampal neurons. *Neuron*, *15*(1), 105–114. http://doi.org/10.1016/0896-6273(95)90068-3

Wagner, J. P., Black, I. B., & DiCicco-Bloom, E. (1999). Stimulation of Neonatal and Adult Brain Neurogenesis by Subcutaneous Injection of Basic Fibroblast Growth Factor. *The Journal of Neuroscience*, *19*(14), 6006–6016.

Wing, L.-Y. C., Chen, H.-M., Chuang, P.-C., Wu, M.-H., & Tsai, S.-J. (2005). The Mammalian Target of Rapamycin-p70 Ribosomal S6 Kinase but Not Phosphatidylinositol 3-Kinase-Akt Signaling Is Responsible for Fibroblast Growth Factor-9-induced Cell Proliferation. *Journal of Biological Chemistry*, *280*(20), 19937–19947. http://doi.org/10.1074/jbc.M411865200

Woodbury, M. E., & Ikezu, T. (2013). Fibroblast Growth Factor-2 Signaling in Neurogenesis and Neurodegeneration. *Journal of Neuroimmune Pharmacology*, *9*(2), 92–101. http://doi.org/10.1007/s11481-013-9501-5

Yazaki, N., Hosoi, Y., Kawabata, K., Miyake, A., Minami, M., Satoh, M., … Itoh, N. (1994). Differential expression patterns of mRNAs for members of the fibroblast growth factor receptor family, FGFR-1–FGFR-4, in rat brain. *Journal of Neuroscience Research*, *37*(4), 445–452. http://doi.org/10.1002/jnr.490370403

Yoshimura, S., Takagi, Y., Harada, J., Teramoto, T., Thomas, S. S., Waeber, C., … Moskowitz, M. A. (2001). FGF-2 regulation of neurogenesis in adult hippocampus after brain injury. *Proceedings of the National Academy of Sciences*, *98*(10), 5874 –5879. http://doi.org/10.1073/pnas.101034998

Yu, H., & Chen, Z. (2011). The role of BDNF in depression on the basis of its location in the neural circuitry. *Acta Pharmacol Sin*, *32*(1), 3–11.

Zarate, C. A., Singh, J. B., Carlson, P. J., Brutsche, N. E., Ameli, R., Luckenbaugh, D. A., … Manji, H. K. (2006). A randomized trial of an N-methyl-D-aspartate antagonist in treatment-resistant major depression. *Archives of General Psychiatry*, *63*(8), 856–864. http://doi.org/10.1001/archpsyc.63.8.856

Zechel, S., Werner, S., Unsicker, K., & Von Bohlen Und Halbach, O. (2010). Expression and Functions of Fibroblast Growth Factor 2 (FGF-2) in Hippocampal Formation. *The Neuroscientist*, *16*(4), 357–373. http://doi.org/10.1177/1073858410371513

Zhang, X., Ibrahimi, O. A., Olsen, S. K., Umemori, H., Mohammadi, M., & Ornitz, D. M. (2006). Receptor Specificity of the Fibroblast Growth Factor Family THE COMPLETE MAMMALIAN FGF FAMILY. *Journal of Biological Chemistry*, *281*(23), 15694–15700. http://doi.org/10.1074/jbc.M601252200

Zheng, W., Nowakowski, R. S., & Vaccarino, F. M. (2004). Fibroblast Growth Factor 2 Is Required for Maintaining the Neural Stem Cell Pool in the Mouse Brain Subventricular Zone. *Developmental Neuroscience*, *26*(2-4), 181–196. http://doi.org/10.1159/000082136

Chapter 2.

Hippocampal Fibroblast Growth Factor 9 is Dysregulated in Depression and Modulates Negative Affect in Rodents[1]

Introduction

The neurotrophic hypothesis of major depressive disorder (MDD) posits that the neurobiological basis for mood disorders may be due to the dysregulation of growth factors and their effects on brain circuitry (Duman & Monteggia, 2006; Turner, Watson, & Akil, 2012). This hypothesis is supported by gene expression profiling experiments in postmortem human brains that implicate the fibroblast growth factor (FGF) family and other neurotrophins in MDD (Bernard et al., 2011; Evans et al., 2004; Gaughran, Payne, Sedgwick, Cotter, & Berry, 2006). Despite the evidence that multiple growth factors are involved in mood disorders, to date only a few, such as brain-derived neurotrophic factor (BDNF) and FGF2, have been studied in depth.

Our laboratory and others have demonstrated that FGF2 is dysregulated in several different regions of the brain (Bernard et al., 2011; Evans et al., 2004; Gaughran et al., 2006). Follow-up studies from our laboratory and others have focused on the FGF family

[1] *Note: Some data from this chapter has been published; specifically, Figures 2.4, 2.5, and 2.6, Tables 2.1 and 2.2, and Appendix 2.4 can be found in: Aurbach, E. L., Inui, E. G., Turner, C. A., Hagenauer, M. H., Prater, K. E., Li, J. Z., … Akil, H. (2015). Fibroblast growth factor 9 is a novel modulator of negative affect. Proceedings of the National Academy of Sciences, 112(38), 11953–11958. http://doi.org/10.1073/pnas.1510456112*

and have demonstrated a key role of FGF2 in the control of emotional behavior. Rats exposed to chronic social defeat stress, an animal model recapitulating some aspects of MDD, showed decreased hippocampal FGF2 expression relative to unstressed controls (Turner, Calvo, Frost, Akil, & Watson, 2008), whereas subchronic and chronic administration of FGF2 had antidepressant properties (Elsayed et al., 2012; Turner, Gula, Taylor, Watson, & Akil, 2008). Moreover, administration of antidepressants and anxiolytics induced FGF2 expression (Bachis, Mallei, Cruz, Wellstein, & Mocchetti, 2008; Gómez-Pinilla, Dao, Choi, & Ryba, 2000; Mallei, Shi, & Mocchetti, 2002), and recent studies suggested that this induction is necessary for the effectiveness of the antidepressants (Elsayed et al., 2012). In contrast, knocking down FGF2 in the dentate gyrus of the hippocampus increased anxiety-like behavior of outbred rats (Eren-Koçak, Turner, Watson, & Akil, 2011). Paralleling the human depression studies, hippocampal FGF2 was basally decreased in animals selectively bred for a low locomotor response to novelty (bred low responders, bLRs), who show high levels of spontaneous anxiety during adulthood. However, administering FGF2 either chronically during adulthood (Perez, Clinton, Turner, Watson, & Akil, 2009) or once during early development (Turner, Clinton, Thompson, Watson, & Akil, 2011) eliminated basal differences in behavior. Collectively, these studies suggest that FGF2 is an endogenous anxiolytic and anti-depressant molecule that plays both an organizational role during development and a role during adulthood to modulate emotional reactivity.

Other members of the FGF family showed different patterns of dysregulation in postmortem tissue from individuals with MDD. Most notably, FGF9 had a pattern of dysregulation opposite to FGF2; FGF9 expression was increased in the frontal cortices

(Evans et al., 2004) and locus coeruleus (Bernard et al., 2011) in patients with MDD. This relationship is intriguing because additional evidence suggests that FGF2 and FGF9 may be functionally opposed. For example, FGF2 expression was decreased and FGF9 expression was increased in an *in vitro* cellular model of chronic stress (Salaria et al., 2006).

Despite the preliminary evidence that FGF9 is altered in MDD, little is known about the function of this molecule in the brain. FGF9 is primarily expressed by neurons in the cortex, hippocampus, thalamus, cerebellum, and spinal cord (Garcès, Nishimune, Philippe, Pettmann, & deLapeyrière, 2000; Lin et al., 2009; Nakamura et al., 1997; Tagashira, Ozaki, Ohta, & Itoh, 1995; Todo et al., 1998), though it is also expressed by glia in the hindbrain and spinal cord (Nakamura et al., 1999). FGF9 interacts with several of the FGF receptors (FGFR) (Goetz & Mohammadi, 2013), binding preferentially to FGFR3 (Ornitz et al., 1996). One study implicated FGF9 in multiple sclerosis, suggesting that increased FGF9 signaling disrupted myelination (Lindner et al., 2015), and data from our laboratory indicates that FGF9 expression increases after brain injury (Turner, Eren-Koçak, Inui, Watson, & Akil, 2015). Many other reports suggest that FGF9 may promote cell survival. For example, FGF9 acts as a survival factor for nigrostriatal and mesencephalic dopamine neurons (Chuang et al., 2015; Huang, Hong, & Chuang, 2009), and FGF9 treatment can increase the survival and soma size of cultured basal forebrain cholinergic neurons (Kanda et al., 2000). FGF9 also weakly promoted proliferation and survival of cultured adult subventricular neural progenitor cells, inhibiting astrocyte differentiation (Lum, Turbic, Mitrovic, & Turnley, 2009). However, these effects may differ depending on the phase of development and brain region, because other reports

suggest that FGF9 may be particularly important for glia: FGF9 influences astrogenesis (Falcone, Filippis, Granzotto, & Mallamaci, 2015) and the generation, positioning, and patterning of cerebellar Bergmann glial cells (Lin et al., 2009; Meier et al., 2014). However, these are all studies of exogenous FGF9, which may be mimicking the actions of other FGFs. Moreover, none of the studies to date have examined the role of FGF9 in the hippocampus or its role in the regulation of emotionality.

In projects begun by previous members of our laboratory, we sought to elucidate the potential role of hippocampal FGF9 in the regulation of emotions and mood, given early observations that FGF9 expression was altered in the postmortem hippocampus of individuals with MDD. To this end, our laboratory showed that the effects of psychosocial stress, an animal model recapitulating some aspects of depression, significantly increased hippocampal FGF9 expression (Aurbach et al., 2015). Moreover, acute and chronic administration of FGF9 was sufficient to alter anxiety- and depression-like behavior: acute FGF9 was anxiolytic and pro-depressant, while chronic FGF9 was anxiogenic and pro-depressant (Aurbach et al., 2015).

Here, *we carried out several analyses in both human and rodent to further examine the role of FGF9 in affective dysregulation*. We examined FGF9 expression in postmortem human tissue across three brain regions to ascertain if FGF9 was dysregulated in major depression. Because the postmortem human data strongly implicated the hippocampus as a key site for FGF9 dysregulation, we studied the role of hippocampal FGF9 in both outbred and selectively bred rats. We examined FGF9 expression in animals selectively bred for differing locomotor responses to novelty, an animal model of individual differences in affective dysregulation, to determine if FGF9

expression consistently varied with concomitant differences in emotional behavior. To determine if endogenous FGF9 was necessary for expression of affective-like behavior, we characterized the effects of FGF9 knockdown on anxiety-like and depression-like behavior and hippocampal gene expression in outbred rats. Together with the previous data from our laboratory, the combination of these various strands of evidence provides strong support for FGF9 as an endogenous molecule that promotes negative affect and may play a role in vulnerability to major depression.

Materials and Methods

Human Studies

Subject Characteristics and Tissue Extraction. The human tissue samples used for all microarray experiments as well as the qRT-PCR validation were obtained from the Brain Donor Program at the University of California, Irvine with the consent of the next-of-kin of the deceased (Appendix 2.1, Appendix 2.2, & Appendix 2.3).

Dissection of Human DLPFC, ACC, and Hippocampus. For the first stage of dissection, a 1-cm coronal slab was placed on a block of dry ice. At the level of the genu of the corpus callosum, the middle frontal gyrus was identified by an expert in human neuroanatomy, and a fine jeweler's blade in a coping saw handle was used to excise the gyrus for dorsolateral prefrontal cortex samples. In some cases, more than one slab was used to cover the full extent of the structure. In the same plane, the cingulate gyrus was identified by an expert in human neuroanatomy, and a fine jeweler's blade in a coping saw handle was used to excise the gyrus for anterior cingulate cortex samples. In some cases, more than one slab was used to cover the full extent of the structure. In more caudal planes, the hippocampus was visually identified using the dentate gyrus by an

expert in human neuroanatomy, and a fine jeweler's blade in a coping saw handle was used to dissect the hippocampus and surrounding temporal cortex. Both sides of the slab were carefully inspected. In some cases, more than one slab was used to cover the full extent of the structure. For all structures, the block was wrapped in foil, placed on dry ice and then stored at -80°C until further processing (Jones et al., 1992). In the hippocampus, a second stage of finer dissection was then carried out. The temporal cortex was removed by visual inspection using the dentate gyrus and CA3 as landmarks. The dissected tissue was then processed for RNA extraction. Total RNA was extracted using procedures described previously (Evans et al., 2004; Li et al., 2004). Clinical information was obtained from medical examiners, coroners' medical records and a family member. Patients were diagnosed by consensus based on criteria from DSM-IV. For further information regarding data collection procedures, please see Li and colleagues (Li et al., 2013). Final Control and MDD sample sizes and demographics are in the appendices (DLPFC: Appendix 2.1, ACC: Appendix 2.2, HPC: Appendix 2.3).

Gene Expression Profiling. In general, the labeling and hybridization of sample mRNA with oligonucleotide probes followed standard manufacturer protocols. Analyses used the full microarray datasets (all probe data from the MDD and Control subjects, as well as from small samples of subjects with bipolar disorder and schizophrenia) to maximize our ability to identify technical artifacts and confounds, although the reported results focus on MDD vs. Control comparisons for a subset of growth factor probes.

Affymetrix Microarray. Microarray experiments were performed in eight separate experimental cohorts containing both patients and controls. The majority of RNA samples were analyzed in duplicate at two different laboratories using Affymetrix

Genechips (either U133A or U133Plus-v2). After extracting summarized probe signal data from scanned microarray image files, all downstream analyses were completed in the R statistical programming environment. We extracted the U133A subset of probes and applied RMA (Robust Multi-array Analysis) to summarize probe set expression levels using custom ENTREZ12.1 Chip Definition Files (CDF) which defined probe sets for 11,912 transcripts (as defined by ENTREZ in 03/2010) and 68 control probe sets. As is traditional, all probe signal values were log (base 2)-transformed to reduce heteroskedasticity and quantile-normalized to remove technical artifacts in the overall distribution of signal per sample. To ensure sample quality, we required an average sample to sample correlation coefficient (r) of 0.85-0.9, excluding 6 of 235 samples (2.6%) in the HPC, 30 of 367 samples (8.1%) in the DLPFC, and 6 of 283 samples (2.1%) in the ACC. Batch effects due to cohort, laboratory, and platform were removed by median-centering the data. Replicate samples were then averaged and any subjects that were missing information were removed from the dataset (n = 16 in DLPFC, n = 13 in ACC, n=8 in HPC), leaving a final sample size of 156 subjects in the DLPFC, 140 subjects in the ACC, and 129 subjects in the HPC. For further information regarding the Affymetrix data pre-processing procedures, please see Li and colleagues (Li et al., 2013). For all of the microarray experiments, as part of quality control, subjects were verified to have gene expression typical of their reported sex using data from genes XIST, EIF1AY, RPS4Y1, UTY, USP9Y, NLGN4Y, NCRNA00185, TTTY15, KDM5D, CYorf15B, and DDX3Y.

Microarray Analysis: Determining Diagnosis-Related Gene Expression while Correcting for Confounding Variables. Although agonal factor, brain pH, PMI, and

gender did not differ significantly by diagnosis, we found that it was necessary to control for these variables because they strongly correlated with the top principal components of variation in the data sets (PC1-4). We also controlled for the average age at the time of death, which sometimes varied significantly with diagnosis. The degree of severity and duration of physiological stress at the time of death was estimated by calculating an Agonal Factor Score (AFS) for each subject (Tomita et al., 2004). Additionally, we measured the pH of cerebellar tissue as an indicator of the extent of oxygen deprivation experienced around the time of death (Li et al., 2004). We also calculated the interval between the estimated time of death and the freezing of the brain tissue (the postmortem interval or PMI) using coroner records. We ensured high quality data by choosing samples with relatively high pH and low agonal factor (DLPFC: Appendix 2.1, ACC: Appendix 2.2, HPC: Appendix 2.3), but still decided that it was prudent to control for these variables as well as age and gender by including them as terms in our linear model:

Equation 1: $(probe\ signal) \approx \beta_0 + \beta_1(Brain\ pH) + \beta_2(Agonal\ Factor) + \beta_3(PMI) + \beta_4(Age) + \beta_5(Gender) + \beta_6(Diagnosis)$

We also ran our analyses on an uncleaned probe signal dataset and found similar, stronger relationships between FGF9 and other FGF family members. In the manuscript, we have only reported the more conservative results from the data cleaned of confounds.

qRT-PCR Validation. Microarray results represent relative levels of probe signal, so we validated hippocampal data using quantitative real-time PCR (qRT-PCR). After dissection, samples were stored at -80°C prior to RNA extraction. The starting blocks of hippocampal tissue weighed an average of 600 mg. Tissue was homogenized in TRIzol reagent and treated with chloroform. Samples were centrifuged to separate the suspended

RNA from other cellular materials, and the RNA was pelleted and washed with ethanol. All RNA pellets were resuspended in DEPC-treated water. The average concentration after extraction was of 0.7 µg/µl for an average of 420 µg of total RNA. 100 µg of the total RNA was purified using the RNeasy kit (Qiagen); RNA quality was assessed using the Agilent 2100 Bioanalyzer, and quantity was determined using a spectrophotometer (A260). All RNA concentrations were adjusted to 1 µg/µL with DEPC-treated water prior to first-strand DNA synthesis. Total RNA (1 µg) was reverse transcribed using the iScript cDNA synthesis kit (Bio-Rad Laboratories, Hercules, CA) in a total reaction volume of 20 µl. cDNA (1 µl) was used as the template for real-time PCR assays with a MyiQ real-time PCR system (Bio-Rad Laboratories). The quantitative PCR was conducted in duplicate using iQ SYBR Green Supermix, according to the manufacturer's instructions (Bio-Rad Laboratories). Sequences for the 5' forward primer (start) were as follows: *FGF9:* GGGGAGCTGTATGGATCAGA (1201); *FGFR3:* CTTGTGCCTGGGGTGTTAGT (3133), Sequences for the 3' reverse primer (start) were as follows: *FGF9:* GTGAATTTCTGGTGCCGTTT (1391); *FGFR3:* AAAGGCTCCCATCTTCAGGT (3240). Relative expression of the gene of interest was normalized to β-actin expression in each sample. The expression level of the gene of interest was evaluated using the $2^{-(\Delta\Delta Ct)}$ method (Livak & Schmittgen, 2001). The PCR product quality was monitored using post-PCR melt-curve analysis at the end of the amplification cycles.

Culture Studies

FGF9 Knockdown in vitro. We generated three 19-mer short interfering RNA (siRNA) sequences targeted against the coding region of the rat FGF9 mRNA sequence

(accession number: NM_012952.1) using an RNA interference design site (Dharmacon, Chicago, IL). The target sites on the FGF9 mRNA and corresponding siRNA sequences were: FGF9 siRNA1 (414-433; AGGAAAGACCACAGCCGAT), FGF9 siRNA2 (415-434; GGAAAGACCACAGCCGATT), and FGF9 siRNA3 (682-701; GGACCAGGACTAAACGGCA). Short-hairpin RNA (shRNA) sequences were generated by adding a nine-nucleotide loop sequence (UUCAAGAGA) and restriction enzyme sites; the shRNA constructs were then synthesized (Invitrogen, Carlsbad, CA) and subcloned into the pLentiLox3.7 lentiviral vector (https://www.addgene.org/11795/), which drives shRNA expression using a mouse U6 promoter and which contains an eGFP reporter tag under the control of a CMV promoter. All shRNA inserts were verified by sequencing. A scrambled, non-silencing shRNA (shNS) sequence was also used as a control construct (Eren-Koçak et al., 2011).

COS7 cells were grown in Hyclone Dulbecco's Modified Eagle Medium/High Glucose Media (Fisher Scientific) with 10% fetal bovine serum (Invitrogen) and gentamycin (10 µg/mL; Gibco). 24 hours after plating at 100,000 cells/well (6-well plates), cells were transfected with the psiCHECK2 vector (Promega) containing a clone of rat FGF9 (bp 37-869, of 1065 total length; encompasses entirety of the coding region; generated in-house) or FGF2 (Eren-Koçak et al., 2011) at 20 ng/µL and, in some conditions, either siRNA or shRNA constructs to knock down expression of FGF9, FGF2, or the control, non-silencing construct (shNS) at 7 ng/µL (Eren-Koçak et al., 2011). Dharmafect Duo transfection reagent (Dharmacon) was used to facilitate the transfection. Twenty-four hours after transfection, cells were lysed using passive lysis buffer, and lysates were collected for analysis of knockdown efficacy. The psiCHECK2

vector housing the FGF9 clone enabled us to use a Dual Luciferase Assay (Promega) to assess knockdown efficacy *in vitro*. The FGF9 clone was inserted downstream of the *Renilla* luciferase reporter, allowing the creation of a fusion *Renilla-FGF9* mRNA, while expression of the independent *Firefly* luciferase gene was used to normalize the chemoluminescence readout across wells. Lysates were exposed to chemolumiscent reagents in the Dual Luciferase Assay according to the manufacturer's instructions, and the ratio of *Renilla/Firefly* chemoluminescence was used to assess efficacy of FGF9 knockdown for each siRNA/shRNA candidate *in vitro*. Each experimental condition was normalized to the chemoluminescence of the baseline condition and expressed as a percentage (% knockdown = 100 * (chemoluminescence of FGF2/FGF9 clone + shRNA knockdown condition / chemoluminescence of FGF2/FGF9 clone-only baseline condition). Ultimately, shRNA3 was selected to be used *in vivo* because it was the most effective at reducing FGF9 expression *in vitro* (Figure 2.4) and showed the least toxic effects on cultured cells (<10% cell death, data not shown). The vector containing shRNA3 (hereafter referred to as shFGF9), as well as vector containing a scrambled, non-silencing sequence (shNS; Eren-Koçak et al., 2011) were submitted to the University of Michigan Viral Vector core for lentivirus synthesis. The returned lentiviruses (100X; LVshFGF9 and LVshNS) were used in FGF9 knockdown experiments *in vivo*.

Animal Studies

Animals. We used both outbred and selectively-bred male rats in these studies. In studies utilizing outbred animals, adult male Sprague-Dawley rats (Charles River Laboratories, Wilmington, MA), weighing between 220-350g were delivered from the supplier and housed in pairs. All outbred animals were allowed to acclimate to the

housing environment for at least seven days before any experiments began. In studies using selectively bred Sprague-Dawley rats, bred high responder (bHR) and low responder (bLR) rats were bred, weaned, and tested for a locomotor response to novelty between postnatal days 50-60, and housed in pairs in-house as previously described (Stead et al., 2006; Turner et al., 2011). All animals were maintained on a 12/12 light/dark schedule, with access to food and water *ad libitum*. Animals in the knockdown study were pair-housed until the surgery, singly housed for 3 days after surgery, then returned to pair-housing. All animals were treated in accordance with the National Institutes of Health *Guidelines on Laboratory Animal Use and Care* and in accordance with the guidelines set by the university committee on use and care of animals at the University of Michigan.

bHR and bLR studies. In the basal gene expression studies, bHR and bLR rats from generation F41 were born, weaned, and reared, and tested for locomotor response to novelty before being euthanized between postnatal day 60-70. Brains were extracted, snap-frozen in 2-methylbutane, and stored at -80C until processing. In the early-life FGF2 studies, bHR and bLR rats from generation F26 were subcutaneously (axillary space) injected with 20ng/g FGF2 or vehicle (0.1M phosphate buffered saline with 0.1% bovine serum albumin, Sigma) on the day after birth. Animals were reared and weaned as usual until between postnatal day 54-60, when animals were euthanized, the brains removed and snap-frozen in 2-methylbutane, and stored at -80C until processing.

FGF9 Knockdown in vivo. All animals underwent microinjection surgery (groups: LVshFGF9, LVshNS). Under isoflurane anesthesia and using standard stereotaxic protocols, a 33-gauge microinjector was lowered bilaterally just above the dentate gyrus

of the hippocampus (coordinates from bregma: A/P -5.0, M/L ±3.5, D/V -3.6-3.8). One µL of LVshNS or LVshFGF9 was infused over four minutes (Eren-Koçak et al., 2011), and two minutes were allowed for diffusion. We allowed 4 weeks for recovery, then animals were subjected to behavioral testing in the EPM and FST (Figure 2.6a). Twenty-four hours after the last behavioral test, animals were euthanized. Brains were rapidly extracted and frozen in 2-methylbutane at -30°C and stored at -80°C until processing.

Behavioral Testing.

Locomotion testing. Locomotion testing was used to assess the locomotor response to a novel environment, one measure of novelty-seeking and anxiety-like behavior. Rats were placed in a 43 x 21.5 x 25.5cm acrylic cage. Two panels of photocells recording beam breaks determined both horizontal and vertical locomotor activity, and activity was monitored in five minute bins for one hour. The horizontal and vertical components were added together for each animal and an average was found for each group (Stead et al., 2006). After each animal was tested, the apparatus was cleaned with 70% ethanol in water. All locomotor testing took place between 0800 and 1200 h. We interpreted low locomotor scores as indicating low levels of novelty-seeking and high spontaneous anxiety-like behavior.

Forced swim test (FST). We used the FST to determine effects on depression-like behavior (Lucki, 1997; Porsolt, Bertin, Blavet, Deniel, & Jalfre, 1979; Porsolt, Bertin, & Jalfre, 1977, 1978). The test occurs over two days. On day one, animals undergo a 10-minute pre-test swim. 24 hours later, animals undergo a five-minute test swim (Lucki, 1997; Porsolt et al., 1979, 1977, 1978). Animals were placed in cylinders filled with water at a depth at which the rats' tail could not touch the bottom and a temperature of

25-27°C. Water was changed between animals and all sessions were video recorded. All FST swim sessions took place between 0900 h and 1300 h. The videotaped behaviors were scored by an observer blind to the experimental conditions using The Observer software (Noldus Information Technology, The Netherlands) (Lucki, 1997). Swimming was scored when at least two paws were consistently moving in a horizontal direction. Climbing was defined by vertically-directed movement of at least two paws against the wall of the cylinder. Immobility was defined as floating or the minimal movement necessary to keep the head above water level. Percent total duration of swimming, climbing, and immobility episodes were determined and compared across groups. Animals who spent increased percent time immobile and decreased percent time swimming or climbing were interpreted to be exhibiting depression-like behavior.

Elevated plus-maze (EPM). We used the EPM to determine effects on anxiety-like behavior (Eren-Koçak et al., 2011; Pellow, Chopin, File, & Briley, 1985; Perez et al., 2009; Turner et al., 2011). The EPM apparatus consists of a plus-shaped platform elevated 70cm above the floor; two arms are enclosed in black plexiglass (the "anxiolytic" regions of the maze), while the other two arms are left open (the "anxiogenic" regions of the maze). At the intersection of the arms, there is a 12 x 12cm square platform allowing access to all four arms (Pellow et al., 1985; Pellow & File, 1986). During the five-minute test period, the room is dimly lit (~40 lux), and behavior is monitored using a computerized video tracking system (Noldus Ethovision). At the start of the five-minute test, the rat was placed in the center square platform. The tracking system recorded the latency to enter the open arm, the amount of time spent in arms, and the time spent in the center square. After every animal, the testing apparatus was wiped

down with 70% ethanol in water. We compared the amount of time spent in the open and closed arms of the maze across groups; animals that spent more time in the closed arms (and/or less time in the open arms) of the maze were considered to show elevated levels of spontaneous anxiety-like behavior.

Tissue Analysis.

mRNA in situ hybridization (ISH). All rats were euthanized by rapid decapitation; brains were removed, snap-frozen, and stored at -80°C. Ten-µm sections were taken every 100-200 µm (depending on the study) and mounted onto Superfrost Plus slides (Fisher, Waltham, MA). Slide-mounted tissue was fixed in 4% paraformadehyde solution for 60 minutes, washed three times with 2X SSC (1X SSC is 150 mM sodium chloride and 15 mM sodium citrate), and treated with 1M triethanolamine with 0.25% acetic anhydride. Slides were rinsed and dehydrated in graded ethanols before air-drying. All *in situ* probes were generated in-house; the rat mRNA sequences used for generating probes were complimentary to the following RefSeq database numbers: FGF2 (NM_019395, 716-994), FGF9 (NM_012952, 661-880), FGFR1 (NM_024146, 320-977), and eGFP was sub-cloned from the pLentiLox3.7 vector. All cDNA segments were extracted (Qiaquick Gel Extraction kit, Qiagen, Valencia, CA), subcloned in Bluescript SK (Stratagene, La Jolla, CA) and confirmed by nucleotide sequencing. The probes were labeled in a reaction mixture of 0.5-1µg of linearized plasmid specific to the probe of interest, 1X transcription buffer (Epicentre Technologies, Madison, WI), 125µCi of 35S-labeled UTP, 125µCi of 35-S labeled CTP, 150µM ATP and GTP, 12.5mM dithiothreitol, 1µl of RNAse inhibitor (4U/µl), and 1.5µl of T7 or T3 RNA polymerase (20U/µl). Labeled probes were purified on Micro Bio-Spin Chromatography Columns (BioRad, Berkeley,

CA) according to the manufacturer's instructions. After air-drying, slides were treated with hybridization buffer containing the labeled probe (1-2 x 10^6 counts/75 μL buffer) 50% formamide, 10% dextran sulfate, 3X SSC, 50 mM sodium phosphate buffer (pH = 7.4), 1X Denhardt's solution, 0.1 mg/ml yeast tRNA, and 10 mM dithiothreitol. All slides were cover-slipped and stored in humidified chambers at 55C during the 12-16 hour hybridization period. After hybridization, sections were washed three times in 2X SSC and incubated in an RNase solution (100 μg/mL RNase in Tris buffer with 0.5M NaCl, pH=8) at 37C. Sections were then sequentially washed in 2X, 1X, and 0.5X SSC before being incubated in 0.1X SSC at 65C for 1 hour. Sections were rinsed in distilled water and dehydrated through graded ethanols. Slides were exposed to Kodak BioMax MR Scientific Imaging Film (Sigma Aldrich), and exposure times were experimentally determined for optimal signal, as follows: FGF2 (7 days), FGF9 (14-21 days), FGFR1 (7 days), and eGFP (7 days).

Autoradiograph Quantification Procedures. mRNA expression signals from autoradiographic films were quantified using computer-assisted optical densitometry software ImageJ (National Institutes of Health, Bethesda, MD). For all experiments except FGF9 knockdown, mean signal was determined by outlining a hippocampal subfield (CA1, CA2, CA3, and dentate gyrus) on each hemisphere, thresholding for background plus 3.5 times its standard deviation. Background values were subtracted and data from >6 sections were averaged to create a mean signal measurement for each animal and group averages and standard error of the mean were calculated.

For quantification of sections from animals in the FGF9 knockdown study, we used a modified quantification approach to enable us to quantify expression of a gene

target (e.g., FGF9, FGF2, or FGFR1) only where we saw concurrent expression of eGFP. This approach also facilitated careful analysis of hippocampal anatomy impacted by viral infection on an animal-by-animal basis, which we then used to create inclusion/exclusion criteria for the behavioral analyses. We digitally overlaid a transparent copy of the eGFP autoradiogram (from an adjacent section) on the image of the probe of interest then outlined only the region with eGFP expression to take measurements for the probe of interest. Because there was variability in infection spread, we chose to disregard area in calculating the quantified measurements for these experiments; therefore, we present the data in terms of average optical density (mean signal-background).

eGFP autoradiograms were separately analyzed for the specific animal-by-animal infection pattern, and these data were used to define strict inclusion criteria for behavioral analysis. Animals in the knockdown study were only included in the final behavioral analysis if they showed robust expression of eGFP (the marker for successful viral infection) in the dentate gyrus of both hemispheres in at least four adjacent sections ($\geq$800 μm) without additional eGFP labeling of other hippocampal or extra-hippocampal structures. After exclusion, eight animals were included in the shNS control group, while ten animals were included in the shFGF9 group.

Immunohistochemistry. A separate cohort of animals (n = 4 animals/group; shNS, shFGF9) underwent the same surgical procedures as those described in the behavioral knockdown study. Three weeks post-surgery, animals were transcardially perfused using 4% PFA. Brains were removed and postfixed for 1 hour in fresh 4% PFA, then transferred to 30% sucrose solution for 24-48 hours. Brains were subsequently snap-frozen in 2-methylbutane and stored at -80°C until sectioned. Tissue was sectioned at -

15°C at 45 µm, and free-floating sections were stored in phosphate-buffered saline (PBS) + 0.1% sodium azide at 4°C until processing. Free-floating sections were rinsed 3 times in PBS then blocked for 1 hour at room temperature on an orbital shaker (blocking solution: 0.3% Triton X 100 (Sigma-Aldrich, St. Louis, Missouri), 1% normal goat serum (Invitrogen), 1% bovine serum albumin (Fisher Scientific) in 0.1 mol/L phosphate buffer). After blocking, sections were incubated overnight at room temperature on the orbital shaker in the blocking solution with the following primary antibodies: eGFP (chicken anti-GFP, 1:2000; Abcam), NeuN (rabbit anti-NeuN, 1:300, Abcam), and GFAP (mouse anti-GFAP, 1:500; Millipore). After primary antibody incubation, sections were washed three times in PBS then transferred to blocking solution with the following three secondary antibodies with conjugated fluorophores (AlexaFluor488 goat anti-chicken IgG, 1:200; AlexaFluor568 goat anti-rabbit IgG, 1:200; AlexaFluor 647 goat anti-mouse, 1:200; Life Technologies) to incubate for 2 hours at room temperature on the orbital shaker. After secondary incubation, sections were washed 4-5 times in PBS and mounted onto Superfrost Plus slides and coverslipped using ProLong Gold Antifade Reagent with DAPI (Fisher Scientific). Slides were imaged on an Olympus Fluoview FV1000 confocal microscope with a multi-line argon laser (457, 488, 515nm), 405nm diode laser, 543 and 633nm HeNe lasers. Microscope control and image acquisition was achieved using Olympus FV10 software.

Statistical Analyses. Analysis of human data is described above. All animal behavioral tests were analyzed by Student's t-test or 2-way ANOVA.

Results

Human Studies

FGF9 expression was increased in the hippocampus of depressed individuals. Based on published data indicating that specific growth factor transcripts are altered in depressed brains (Bernard et al., 2011; Evans et al., 2004; Gaughran et al., 2006), we selectively examined their differential expression in major depression across three brain regions: the hippocampus (HPC), anterior cingulate cortex (ACC), and dorsolateral prefrontal cortex (DLPFC) using human microarray datasets. We examined diagnosis-related gene expression using a linear model that controlled for a variety of confounding pre- and post-mortem variables. In the ACC and DLPFC, FGF9 expression in MDD was not significantly different from nonpsychiatric control subjects, though a visual trend for an increase in FGF9 expression was observed in both regions (Figure 2.1a, Figure 2.1b; ACC: $p = 0.163$; DLPFC: $p = 0.354$). In the hippocampus, we found that FGF9 expression was consistently increased in subjects with MDD relative to controls across three microarray datasets and platforms (Figure 2.1c, Table 2.1).

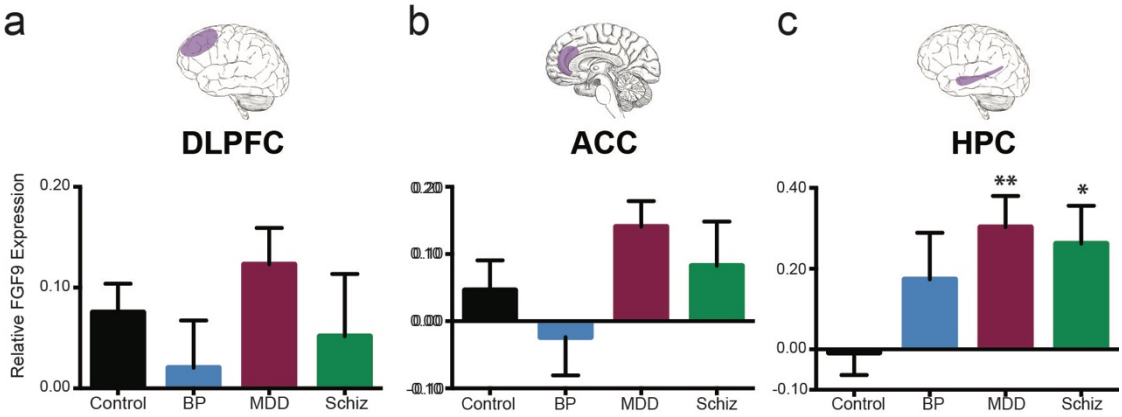


Figure 2.1. FGF9 expression is non-significantly elevated in the (a) dorsolateral prefrontal cortex (DLPFC), (b) anterior cingulate cortex (ACC), and hippocampus (HPC) of individuals with Major Depressive Disorder. Hippocampus data is replicated in Table 2.1. Note: All data is median-centered, which means that expression data is plotted relative to the median of the full sample (i.e., 0 on the y-axis is the median of the full sample). All statistics assess control vs. psychiatric diagnosis alterations in FGF9 expression while controlling for confounding pre- and postmortem variables. Sample sizes can be found in Appendix 2.1, Appendix 2.2, and Appendix 2.3.

Using quantitative RT-PCR we further found that hippocampal FGF9 expression was significantly increased by 32% in individuals with MDD relative to controls, with a Cohen's d effect size of 0.57, a medium-sized effect (Table 2.1). We further examined expression of other growth factors and receptors in the hippocampal microarray data: FGFR1 and FGFR2 appeared to be downregulated in individuals with MDD, but these effects were borderline significant and platform-specific (

Table 2.2). Expression of BDNF, glial-derived neurotrophic factor (GDNF), FGF2, and FGFR3 did not differ in MDD subjects relative to controls (

Table 2.2). Thus, these analyses pointed to FGF9 as one of the most clearly and consistently altered growth factors in MDD: we always observed it to be upregulated in the depressed hippocampus.

Table 2.1. FGF9 is increased in the hippocampus of individuals diagnosed with Major Depressive Disorder. Methods for Illumina data can be found in Aurbach et al. (2015) and overlaps in samples are described in Appendix 2.4. Affymetrix data is replicated

from Figure 2.1. Note that preprocessing (in particular, log transformation) of microarray data prevented us from calculating the degree of expression change, though it is likely to be similar to that observed in qPCR data. Significant effects are in bold.

Candidate	***Microarray: Affymetrix HG-U133A***	***Microarray: Illumina HT-12***	***Microarray: Illumina Ref-8_v2***	***qPCR***	***qPCR: Effect Size (Cohen's D)***
Control (n)	*56*	*24*	*45*	*22*	
MDD (n)	*36*	*21*	*33*	*23*	
FGF9 (raw p-value)	↑ **(0.0029)**	↑ **(0.0295)**	↑ **(0.0027)**	↑ **32% (0.0272)**	**0.57** ***(medium)***

Table 2.2. Other members of the FGF family, as well as BDNF and GDNF, are not significantly altered in the postmortem hippocampus from individuals diagnosed with Major Depressive Disorder. Note that FGF2 data is the same as the data presented in Chapter 3, Appendix 3.1. Methods for Illumina data can be found in Aurbach et al. (2015). Note that preprocessing (in particular, log transformation) of microarray data prevented us from calculating the degree of expression change, though it is likely to be similar to that observed in qPCR data. Significant effects are in bold.

Candidate	***Microarray: Affymetrix HG-U133A***	***Microarray: Illumina HT-12***	***Microarray: Illumina Ref-8_v2***	***qPCR***	***qRT-PCR Effect Size (Cohen's D)***
Control (n)	*56*	*24*	*45*	*22*	
MDD (n)	*36*	*21*	*33*	*23*	
FGF2 (raw p-value)	0.3427	0.5798	-	-	-
FGFR1 (raw p-value)	↓ 0.1199	**0.0248**	-	-	-
FGFR2 (raw p-value)	↓ 0.0647	0.4882	-	-	-
FGFR3 (raw p-value)	0.6228	0.6428	0.2473	↓ 13% (0.2400)	0.35 *(small)*
BDNF (raw p-value)	0.6515	0.8990	0.8317	-	-
GDNF (raw p-value)	0.3720	0.9227	-	-	-

Animal Studies

bHR animals had significantly more FGF9 expression in subfields of the ventral hippocampus. We examined endogenous FGF9 mRNA expression in two lines of rats

selectively bred for locomotor response to novelty. bHR animals displayed significantly higher locomotion scores than bLRs (Figure 2.2a; $t(10) = 42.43$, $p < 0.0001$). Contrary to our hypothesis, low-anxiety bHR animals had significantly more FGF9 mRNA expression in the ventral dentate gyrus and ventral CA1 subfields of the hippocampus during adulthood, relative to high-anxiety bLR animals (Figure 2.2c; vDG: $t(10) = 2.77$, $p = 0.02$; vCA1: $(10) = 2.32$, $p = 0.045$). Though there were visual trends in the ventral CA3 subfield and in all subfields of the dorsal hippocampus, these trends did not reach statistical significance Figure 2.2b; dDG: $t(10) = 1.81$, $p = 0.10$; dCA1: $t(10) = 1.72$, $p = 0.12$; dCA2: $t(10) = 1.252$, $p = 0.24$; dCA3: $t(10) = 1.42$, $p = 0.19$; Figure 2.2c; vCA3: $t(10) = 1.802$, $p = 0.11$).

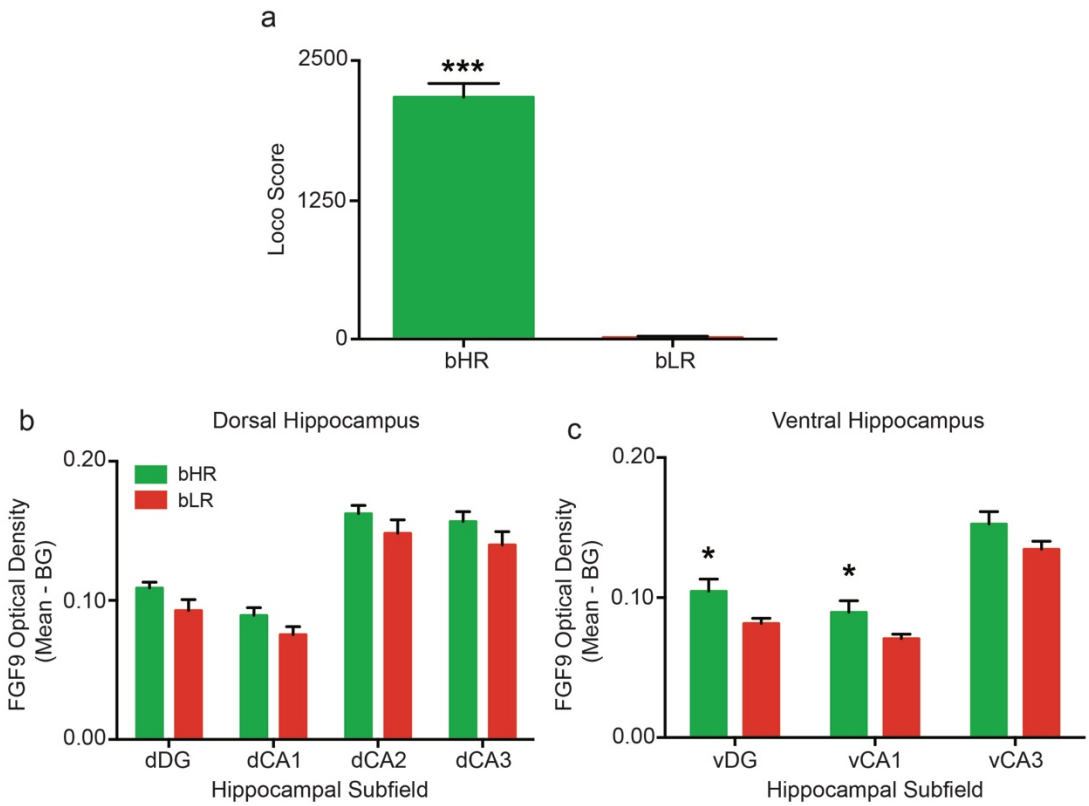


*Figure 2.2. (a) bHR animals had significantly higher locomotion scores than bLRs. (b) Basal FGF9 expression did not differ in the dorsal hippocampus, but (c) bHRs had significantly higher FGF9 expression in the dentate gyrus and the CA1 subfields of the ventral hippocampus. n = 8-10 animals/group; * indicates $p < 0.05$.*

Early-life injections eliminated basal differences in hippocampal FGF9 mRNA expression between bHR and bLR animals. We examined FGF9 expression in bHR and bLR animals that received a one-time injection of either FGF2 or vehicle on the day after birth; we have previously shown that this early-life intervention is sufficient to ameliorate the spontaneous anxiety-like phenotype of bLR animals during adulthood. We observed no differences in hippocampal FGF9 expression in any group (Figure 2.3a: dDG: Phenotype $F(1,28) = 0.04$, $p = 0.87$, Drug $F(1,28) = 0.45$, $p = 0.51$, Interaction $F(1,28) = 2.81$, $p = 0.10$; dCA1: Phenotype $F(1,28) = 1.14$, $p = 0.29$, Drug $F(1,28) = 0.93$, $p = 0.35$, Interaction $F(1,28) = 0.84$, $p = 0.37$; dCA2: Phenotype $F(1,28) = 0.05$, $p = 0.83$, Drug

F(1,28) = 1.30, p = 0.26, Interaction F(1,28) = 0.06, p = 0.81; dCA3: Phenotype F(1,28) =0.94 , p = 0.34, Drug F(1,28) = 1.88, p = 0.18, Interaction F(1,28) = 1.51, p = 0.23; Figure 2.3b: vDG: Phenotype F(1,28) = 0.02, p = 0.88, Drug F(1,28) = 0.04, p = 0.84, Interaction F(1,28) = 0.14, p = 0.71; vCA1: Phenotype F(1,28) = 0.42, p = 0.52, Drug F(1,28) = 0.02, p = 0.88, Interaction F(1,28) = 0.51, p = 0.48; vCA3: Phenotype F(1,28) = 0.02, p = 0.88, Drug F(1,28) = 0.04, p = 0.83, Interaction F(1,28) = 0.14, p = 0.71), suggesting that the previous results did not replicate or that the early-life stress of injection and brief maternal separation was sufficient to eliminate any basal differences in FGF9 expression between bHR and bLR animals.

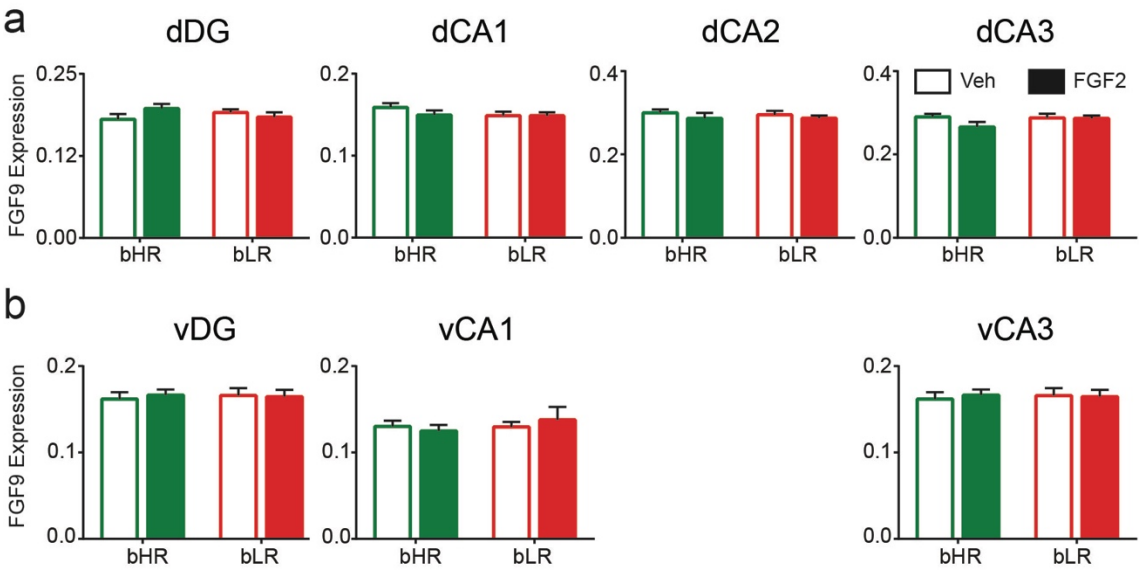


Figure 2.3. Early-life FGF2 treatment eliminates basal differences in FGF9 expression between bHRs and bLRs in all subfields of the (a) dorsal and (b) ventral hippocampus. n = 6-8 animals/group.

Both short interfering RNA (siRNA) and short hairpin RNA (shRNA) constructs targeted to the coding region of the primary FGF9 transcript reduce FGF9 expression in vitro. To determine the preliminary effectiveness of a lentiviral-mediated RNA

interference strategy to reduce endogenous FGF9 expression, we performed knockdown experiments using transfected COS7 cells and monitored expression levels of FGF9 basally and under different knockdown conditions using a dual luciferase assay. Three short interfering RNAs targeted to the coding region of the FGF9 transcript reduced FGF9 expression to one-third to one-half of basal levels (Figure 2.4a).

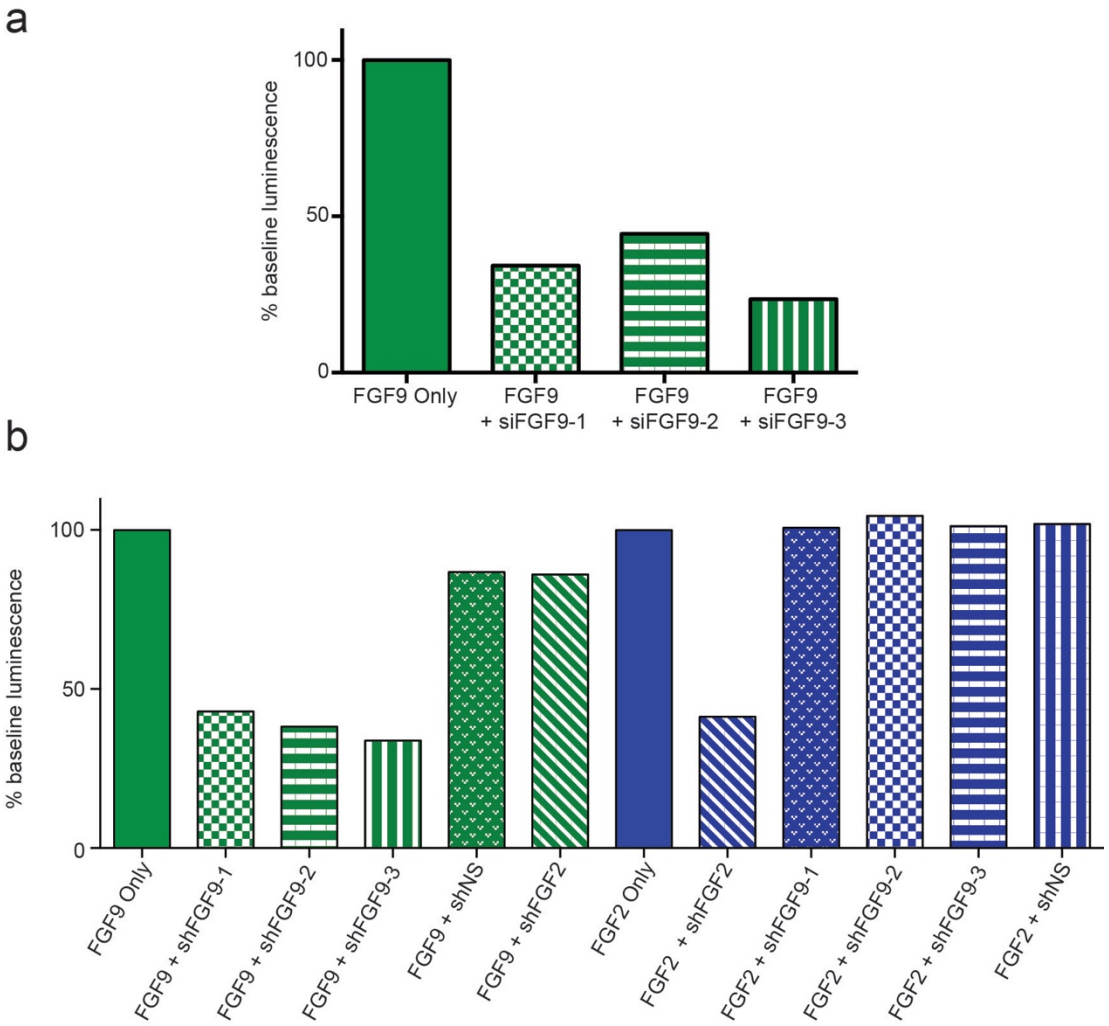


Figure 2.4. FGF9 (a) siRNA and (b) shRNA constructs effectively and selectively knock down expression of FGF9 in vitro. shRNAs targeted against FGF9 (3 candidates: shFGF9-1, shFGF9-2, shFGF9-3), FGF2 (shFGF2), and a control, non-silencing construct (shNS). COS7 cells were transfected with a construct containing either FGF9 or FGF2 and, in some cases, different si/shRNAs. 24 hours after transfection, cells were lysed; lysates were used in a dual luciferase assay to determine knockdown efficacy. All values are mean (2 replicate experiments).

These siRNA constructs were developed into shRNA constructs by adding a loop sequence and restriction enzyme sites; shRNAs were subcloned into a component vector of a 3rd generation lentiviral construct and tested in further transfection experiments. Similar to the siRNA constructs, shRNAs reduced FGF9 expression by approximately one-half of basal levels (Figure 2.4b). shRNA candidate 3 was chosen for use *in vivo* because it showed the greatest degree of knockdown (Figure 2.4b) and showed the least toxic effects on cultured cells (<10% cell death, data not shown).

Animals administered LVshFGF9 had significantly less FGF9 expression in the dentate gyrus relative to animals administered LVshNS. To determine whether endogenous FGF9 expression was necessary for the normal expression of affective behavior, we employed a lentiviral vector containing shRNA candidate 3 to knock down expression of FGF9. Four weeks after microinjecting the knockdown (LVshFGF9) or control (LVshNS) viruses bilaterally into the dentate gyrus, we assessed effects on behavior and euthanized all animals (Figure 2.6a). We confirmed the impact of the localized viral injection by examining eGFP expression with concurrent changes in FGF9 expression in the dentate gyrus. Animals who received the LVshFGF9 knockdown virus showed significantly less FGF9 expression relative to LVshNS control animals ($t(19) = 3.53$, $p < 0.01$; n = 8-10 animals per group), indicating that LVshFGF9 reduced FGF9 expression by approximately 30% (Figure 2.5a-b). In contrast, we found no effect of FGF9 knockdown on FGF2 or FGFR1 expression (Figure 2.6b-e; FGF2: $t(15) = 0.34$, $p = 0.74$; n = 8-10 animals per group; FGFR1: $t(16) = 1.36$, $p = 0.19$; n = 8-10 animals per

group). Therefore, the LVshFGF9 virus was both effective and selective in reducing FGF9 expression *in vivo*.

The LVshFGF9 and LVshNS viruses primarily infected dentate granule neurons. To examine the cell types impacted by lentiviral infection, we performed triple-label immunohistochemistry, using antibodies against eGFP to mark transduced cells, NeuN to label neurons, and GFAP to identify astrocytes. We observed that the vast majority of co-localization occurred between GFP and NeuN, with very few GFAP-labeled cells showing concurrent GFP expression (Figure 2.5c). The pattern of transduction and co-localization did not differ between LVshNS and LVshFGF9 animals. Therefore, the virus preferentially transduced dentate granule neurons, suggesting that the behavioral effects of FGF9 knockdown are likely mediated through these cells.

FGF9 knockdown decreased spontaneous anxiety-like behavior on the EPM. We analyzed GFP expression, a marker for successful viral transduction: only animals who expressed GFP in the dentate gyrus bilaterally in at least four serial sections were included, leaving 8 animals per group. LVshFGF9 animals spent significantly less time in the closed arms of the EPM than did LVshNS control animals (Figure 2.5d; $t(14) = 2.51$, $p = 0.025$), suggesting an anxiolytic effect. Similarly, LVshFGF9 animals spent more time in the open arms than did LVshNS animals, though this trend did not reach statistical significance (Figure 2.5d; $t(14) = 2.02$, $p = 0.063$). The impact of FGF9 knockdown was selective to anxiety-like behavior: we observed no differences between groups in the total distance travelled (Figure 2.6f; $t(14) = 1.78$, $p = 0.10$). There were no differences on the FST (Figure 2.5e; Climbing: $t(14) = 1.91$, $p = 0.07$; Swimming: $t(14) = 1.13$, $p = 0.28$; Immobility: $t(14) = 1.68$, $p = 0.011$; $n = 8$ animals/group).

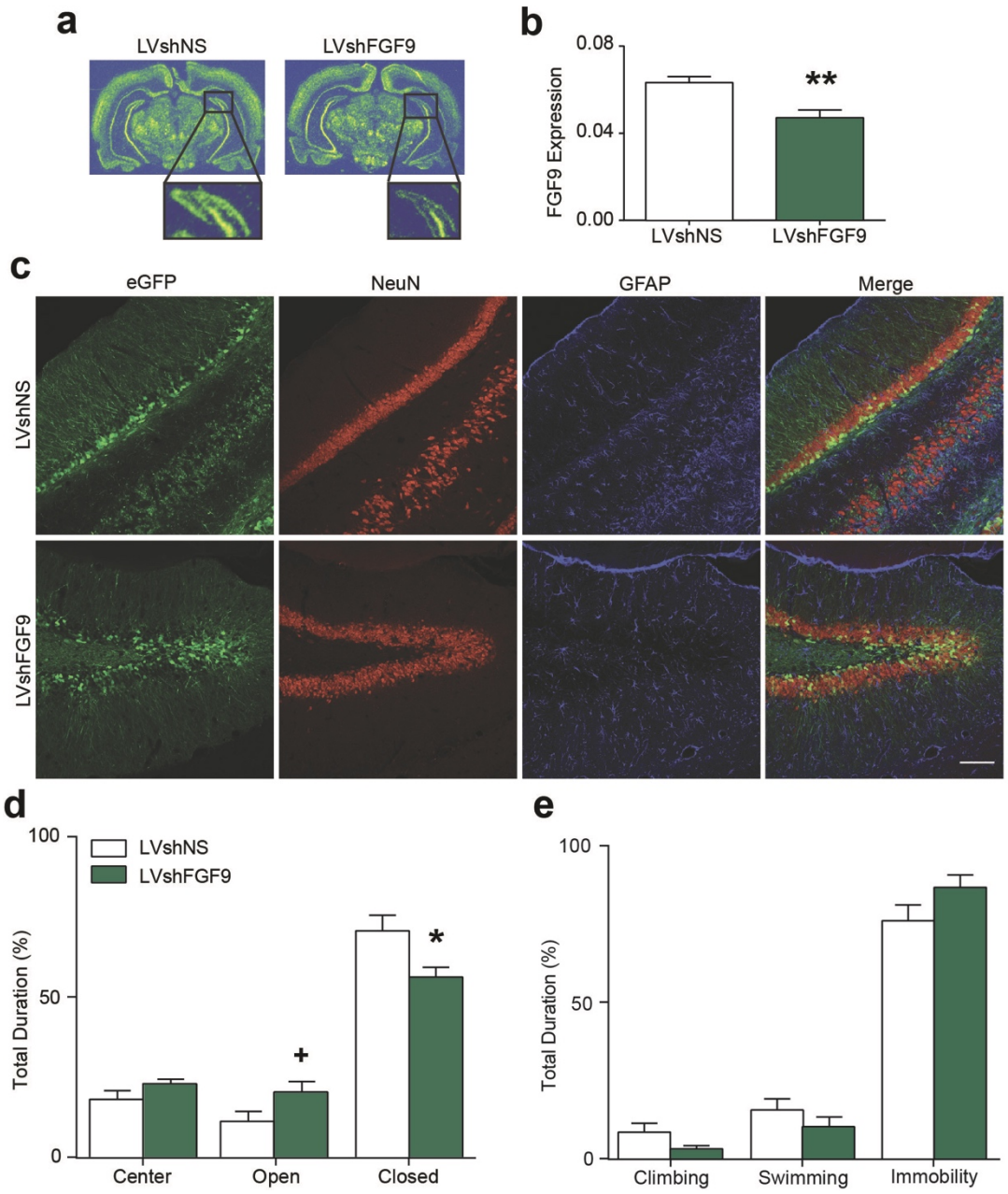


Figure 2.5. Lentiviral-mediated FGF9 knockdown reduced FGF9 mRNA expression in dentate granule neurons and decreased anxiety-like behavior. a) Representative pseudocolored autoradiograms from mRNA in situ hybridization against FGF9 for animals transduced with a control, non-silencing virus (LVshNS, left) and with the FGF9 knockdown virus (LVshFGF9, right). Dentate gryus is enlarged. b) FGF9 expression is significantly reduced in the dentate gyrus of rats transduced with the LVshFGF9 virus relative to LVshNS controls. c) Triple-label immunohistochemistry demonstrating cell-type specificity of viral transduction; eGFP = green, NeuN = red, GFAP = blue. The viruses seem to infect dentate granule neurons. Scale bar denotes 100 µm. d) Knocking down FGF9 expression in the dentate gyrus significantly decreased closed arm time, and there was a trend indicating increased open arm time in the EPM. e) FGF9 knockdown

*did not impact behavior in the FST. All values are mean ± SEM. n = 8 animals/group for behavioral studies and mRNA expression studies; n = 4 animals/group for IHC studies. + indicates 0.5 < p < 0.1; * indicates p < 0.05, ** indicates p < 0.01.*

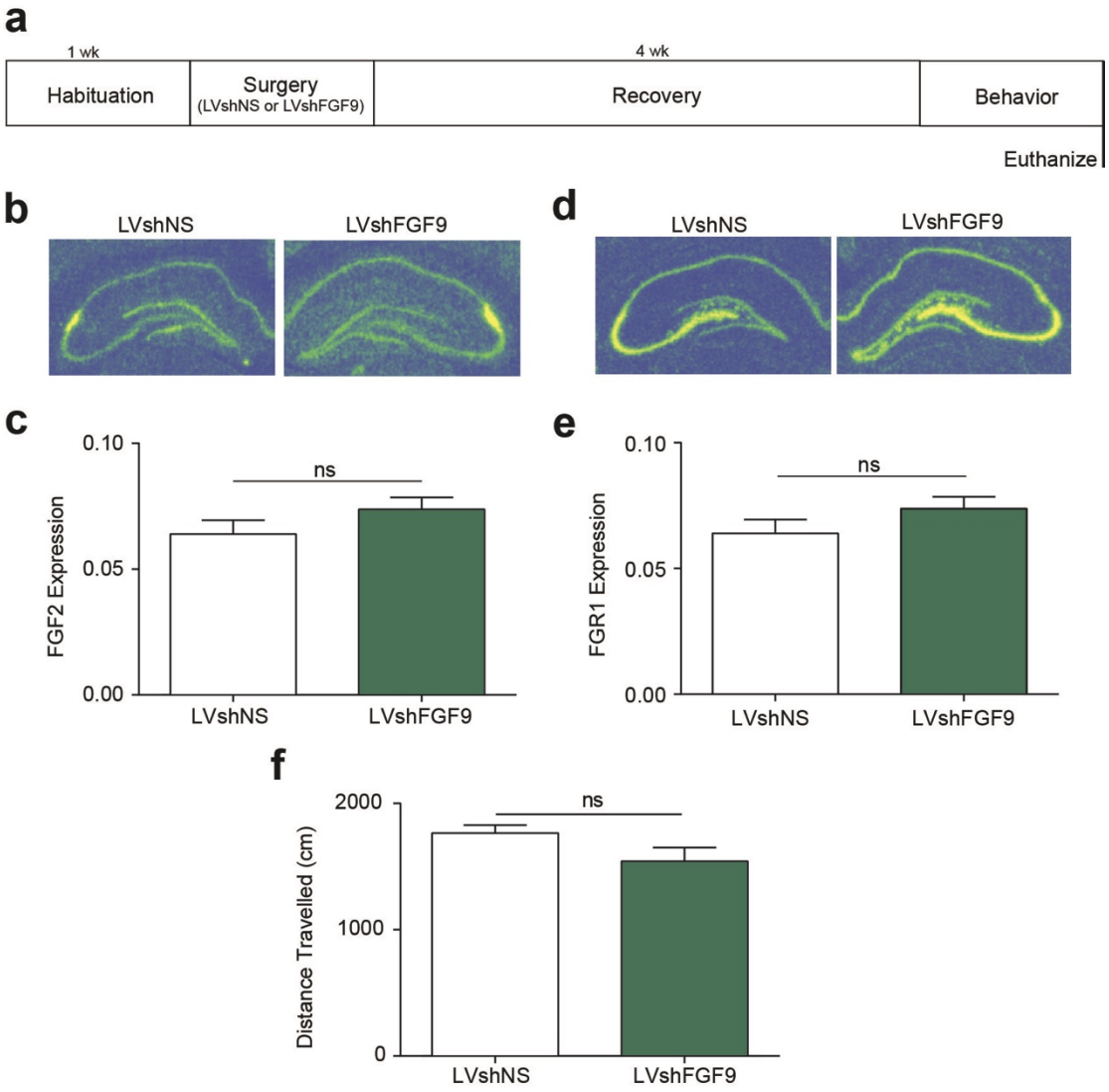


Figure 2.6. Knocking down FGF9 mRNA expression does not affect mRNA expression of FGF2 or FGFR1, and knocking down FGF9 selectively impacts anxiety-like behavior. a) Experimental schema for in vivo FGF9 knockdown experiments. b,c) Representative pseudocolored autoradiograms from mRNA in situ hybridization autoradiographs against FGF2 from shNS controls (left) and shFGF9 animals (right). Knocking down FGF9 expression had no effect on FGF2 gene expression in the hippocampal dentate gyrus compared to shNS controls. d,e) Representative pseudocolored autoradiograms from mRNA in situ hybridization autoradiographs against FGFR1 from shNS controls (left) and shFGF9 animals (right). Knocking down FGF9 expression had no effect on FGFR1 gene expression in the hippocampal dentate gyrus compared to shNS controls (n = 8 animals per group). f) FGF9 knockdown did not impact distance travelled on the

elevated plus maze, indicating that effects were selective for anxiety-like behavior (n = 8 animals per group). All values are mean ± SEM. n = 8 animals/group.

Discussion

This series of studies links for the first time the dysregulation of hippocampal FGF9 to affective disorders. Using microarray data from several analyses with qRT-PCR validation, we showed that FGF9 expression is significantly increased in the postmortem hippocampus of individuals diagnosed with MDD relative to non-psychiatric controls. We saw similar trends in the anterior cingulate and dorsolateral prefrontal cortices, though these effects did not reach statistical significance, suggesting that the hippocampus may be particularly important for the affective functions of FGF9. To address the functional significance of these observations in human brain, we performed a complementary series of experiments in a rodent model to better characterize the role of FGF9 in affective processes. We examined FGF9 expression in a rodent model of affective dysregulation, showing that there were basal differences specific to the ventral hippocampus between two lines of rats selectively bred for a locomotor response to novelty. Previous studies in our laboratory indicate that chronic social defeat stress decreased social interaction and body weight in our animals and was associated with increased hippocampal FGF9 expression. Similarly, prior experiments show that chronic FGF9 administration increased both anxiety- and depression-like behavior. Here, we demonstrate the opposite effect: knocking down FGF9 expression in the dentate gyrus decreased anxiety-like behavior. These animal studies provide converging, complementary evidence for the role of FGF9 as an anxiogenic and pro-depressant agent in the rodent. Together with the evidence from the human postmortem analyses, this body

of work suggests that FGF9 is an endogenous factor that enhances vulnerability to negative affect. Thus, an agent that blocks the effects of FGF9 may be useful in treating human affective disorders.

FGF9 expression is dysregulated in the post-mortem hippocampus from individuals diagnosed with MDD.

Using microarray and qRT-PCR, we observed a significant increase in hippocampal FGF9 expression in individuals with MDD. We did not observe a decrease in BDNF, GDNF, or FGF2 expression. However, other studies have reported MDD-associated decreases in FGF2 in the hippocampus (Gaughran et al., 2006) and locus coeruleus (Bernard et al., 2011), and this may indicate that these transcripts work in functional opposition.

We saw similar trends for increased FGF9 in MDD in both the anterior cingulate and dorsolateral prefrontal cortex, though these effects did not reach statistical significance. A previous study from our laboratory showed that FGF9 expression was significantly increased in these frontal cortex regions (Evans et al., 2004). Though we saw similar directionality in effects, the discrepancy in the strength of the effect may be explained by statistical power (we included many more samples in this series of studies than in the previous analysis) and/or analytical approach (we used a linear model that controlled for more confounding factors than the previous approach using a multiple comparisons-corrected mixed-model multivariate ANOVA).

Notably, these human studies are, by nature, correlational, and do not address whether these changes are a by-product of an underlying pathology associated with MDD or if they are part of the disease process. Moreover, many of the patients had a history of

antidepressant and other psychoactive drug treatment. Both clinical and toxicology data revealed a great deal of heterogeneity in drug exposure, making it difficult to analyze the impact of these drugs on FGF9 expression. Therefore, it will be important for future animal studies to ascertain whether psychoactive drugs can modify FGF9 expression. As noted above, such studies on FGF2 indicate that the dysregulation we observed in MDD is not secondary to these treatments but occurs in spite of them, as antidepressants induce FGF2 and mediate their actions in part through that induction (Elsayed et al., 2012). Whether antidepressants inhibit FGF9 expression remains to be determined.

Hippocampal FGF9 expression influences affect in an animal model.

We have previously demonstrated that hippocampal FGF2 expression is decreased in rats subjected to four days of social defeat stress relative to controls (Turner, Calvo, Frost, Akil, & Watson, 2008b). In contrast, our laboratory has previously demonstrated that ten days of social defeat stress upregulates hippocampal FGF9 expression while decreasing body weight and social interactions, correlates of increased depression-like behavior (Aurbach et al., 2015). These results are congruent with our observations from human studies and indicate that hippocampal FGF9 may play an important role in stress responsiveness. Furthermore, these results are another example that reinforce the idea that FGF2 and FGF9 act as physiological antagonists to mediate vulnerability to affective dysregulation.

We have also found that hippocampal FGF2 expression co-varies with anxiety- and depression-like behavior. For example, endogenous hippocampal FGF2 expression inversely correlated with spontaneous anxiety-like behavior on the EPM in outbred rats (Eren-Koçak et al., 2011). Moreover, we have previously observed differences in

hippocampal FGF2 expression in animals selectively bred for different locomotor responses to a novel environment. Bred low responders (bLRs) show a low locomotor response to a novel environment and exhibit high levels of spontaneous anxiety- and depression-like behavior, and bLRs have low levels of hippocampal FGF2. In contrast, bred high responders (bHRs) show a high locomotor response to a novel environment and exhibit low levels of spontaneous anxiety- and depression-like behavior, and bHRs have high levels of hippocampal FGF2 (Perez et al., 2009; Turner et al., 2011). We hypothesized that we would observe the opposite trend with FGF9 expression, predicting that bLRs would show higher hippocampal FGF9 expression than bHRs. Surprisingly, we observed that bHR animals had more FGF9 expression, but these effects were limited to the ventral hippocampus. This indicates that our interpretation of FGF9 as a purely anxiogenic and pro-depressant molecule may be too simplistic and/or that the behavioral effects of differential FGF9 expression could differ depending on region. Thus, these results indicate that FGF2 and FGF9 may have complicated region-specific effects, and further research is needed to examine this possibility.

Our laboratory has previously demonstrated that a one-time injection of FGF2 on the day after birth is sufficient to eliminate differences in affective-like behavior during adulthood (Turner et al., 2011). We examined if this manipulation was sufficient to alter the differences in FGF9 expression we previously observed in the ventral hippocampus. Surprisingly, we did not observe the differences in basal FGF9 expression between bHR and bLR rats in animals administered vehicle during early life, though this may have been due to experimental design, since we did not include no-injection control groups. Indeed, we have previously observed that the stress of brief maternal separation,

handling, and injection is sufficient to eliminate other basal differences in bHR and bLR animals (data not shown), so we hypothesize that this early stress is sufficient to alter FGF9 expression during adulthood. Unfortunately, because we did not observe differences in hippocampal FGF9 expression in the vehicle animals, we cannot determine if FGF2 treatment affected hippocampal FGF9 expression during adulthood.

Chronic administration of FGFs produced coordinate effects on anxiety- and depression-like behavior: FGF2 reduced anxiety- (Perez et al., 2009) and depression-like (Turner, Gula, et al., 2008) behavior. In contrast, our laboratory previously demonstrated that chronic FGF9 administration increases anxiety-like and depression-like behavior, demonstrating again that the two FGFs have opposing effects (Aurbach et al., 2015). Given that antidepressant and anxiolytic medications induce growth factor expression (Bachis et al., 2008; Gómez-Pinilla et al., 2000; Mallei et al., 2002), it may be that administering growth factors directly activates the same physiological mechanisms as classical anxiolytics and antidepressants. Indeed, work from others indicates FGF2 is necessary for the positive effect of antidepressants (Elsayed et al., 2012). However, one limitation of our FGF9 administration studies is the lack of anatomical specificity: that is, that administration into the lateral ventricle likely affects many brain regions in addition to the hippocampus. Other regions, including the prefrontal cortices, are also likely to be involved in the behavioral expression of these changes in negative affect, and future studies can better elucidate the function of FGF9 in these regions and determine if FGF2 and FGF9 have region-specific effects.

Altering endogenous gene expression using transgenic mice or viral-mediated knockdown demonstrates that hippocampal growth factor expression is necessary for

appropriate spontaneous regulation of affective behavior. We previously used a lentiviral vector to knock down FGF2 expression in the dentate gyrus of outbred rats, increasing anxiety-like behavior on the EPM (Eren-Koçak et al., 2011). In contrast, we demonstrate here that using a lentiviral vector to knock down FGF9 expression bilaterally in the dentate gyrus increases anxiety-like behavior. Interestingly, unlike the results of the administration experiments, our knockdown experiments have produced effects on anxiety-like, but not depression-like, behavior. These findings may result from several factors: a) magnitude: the extent of FGF9 knockdown (~30%) may not be sufficient to alter depression-like behavior; b) partial effect: FGF9 knockdown in the DG may increase vulnerability to depression-like behavior, but stress exposure may be required to uncover behavioral changes; and c) anatomical specificity: our injections were highly localized to the dentate gyrus and had limited spread. This region may be critical to the regulation of anxiety behavior, but may be less pivotal in the control of depression-like behavior. FGF9 may play somewhat different roles in different components of the negative affect circuitry, regulating anxiety in the DG but depression-like behavior in different brain regions, possibly including the prefrontal cortices and/or mesolimbic dopamine system. This is quite plausible as we have previously observed effects exclusive to anxiety-like behavior after knocking down FGF2 expression selectively in the dentate gyrus (Eren-Koçak et al., 2011). Future studies can examine these various possibilities.

It should be noted that MDD and anxiety disorders are often co-morbid. Typically 75% of MDD patients exhibit comorbid anxiety (Lamers et al., 2011). Our records do in fact document the existence of co-morbid clinical anxiety in 58% of the MDD subjects,

and this is likely an underestimate due to the methods for psychological autopsy in our brain bank. Additional studies are needed to examine whether other factors known to influence gene expression in MDD, including comorbid anxiety or a history of antidepressant treatment, impact hippocampal FGF9 expression. Further, it will be of interest to study the role of FGF9 in other brain regions implicated in MDD, including the prefrontal cortices and mesolimbic dopamine circuit. It may also be fruitful to explore the impact of antidepressants on hippocampal FGF9 levels in postmortem human tissue, and animal studies involving antidepressant administration and other resilience-inducing manipulations, including environmental enrichment, can be used to clarify effects.

Summary

We have demonstrated here that FGF9 is an anxiogenic growth factor in the hippocampus. FGF9 expression was upregulated in the post-mortem hippocampus of individuals with MDD, and we observed higher hippocampal FGF9 expression in bHR rats. Previous laboratory members reported that psychosocial stress, a model of depression in rodents, increased hippocampal FGF9 gene expression, and chronic FGF9 administration increased anxiety- and depression-like behavior. In contrast, we show here that knocking down FGF9 expression in the dentate gyrus reduced anxiety-like behavior. These results suggest that high levels of hippocampal FGF9 may increase vulnerability to affective dysregulation and mood disorder, and the data contrast to a body of work indicating that high levels of hippocampal FGF2 may promote resilience. Therefore, we hypothesize that FGF2 and FGF9 act as physiological antagonists to mediate emotionality and vulnerability to mood disorders. Together, this body of work suggests

that blocking the actions of hippocampal FGF9 offers a novel therapeutic approach to the treatment of anxiety and depression.

References

Aurbach, E. L., Inui, E. G., Turner, C. A., Hagenauer, M. H., Prater, K. E., Li, J. Z., … Akil, H. (2015). Fibroblast growth factor 9 is a novel modulator of negative affect. *Proceedings of the National Academy of Sciences*, *112*(38), 11953–11958. http://doi.org/10.1073/pnas.1510456112

Bachis, A., Mallei, A., Cruz, M. I., Wellstein, A., & Mocchetti, I. (2008). Chronic antidepressant treatments increase basic fibroblast growth factor and fibroblast growth factor-binding protein in neurons. *Neuropharmacology*, *55*(7), 1114–1120. http://doi.org/10.1016/j.neuropharm.2008.07.014

Bernard, R., Kerman, I. A., Thompson, R. C., Jones, E. G., Bunney, W. E., Barchas, J. D., … Watson, S. J. (2011). Altered expression of glutamate signaling, growth factor, and glia genes in the locus coeruleus of patients with major depression. *Molecular Psychiatry*, *16*(6), 634–646. http://doi.org/10.1038/mp.2010.44

Chaudhury, S., Aurbach, E. L., Sharma, V., Blandino, P., Turner, C. A., Watson, S. J., & Akil, H. (2014). FGF2 is a target and a trigger of epigenetic mechanisms associated with differences in emotionality: Partnership with H3K9me3. *Proceedings of the National Academy of Sciences*, *111*(32), 11834–11839. http://doi.org/10.1073/pnas.1411618111

Chuang, J.-I., Huang, J.-Y., Tsai, S.-J., Sun, H. S., Yang, S.-H., Chuang, P.-C., … Ching, C.-H. (2015). FGF9-induced changes in cellular redox status and HO-1 upregulation are FGFR-dependent and proceed through both ERK and AKT to induce CREB and Nrf2 activation. *Free Radical Biology and Medicine*, *89*, 274–286. http://doi.org/10.1016/j.freeradbiomed.2015.08.011

Duman, R. S., & Monteggia, L. M. (2006). A Neurotrophic Model for Stress-Related Mood Disorders. *Biological Psychiatry*, *59*(12), 1116–1127. http://doi.org/16/j.biopsych.2006.02.013

Elsayed, M., Banasr, M., Duric, V., Fournier, N. M., Licznerski, P., & Duman, R. S. (2012). Antidepressant Effects of Fibroblast Growth Factor-2 in Behavioral and Cellular Models of Depression. *Biological Psychiatry*, *72*(4), 258–265. http://doi.org/10.1016/j.biopsych.2012.03.003

Eren-Koçak, E., Turner, C. A., Watson, S. J., & Akil, H. (2011). Short-Hairpin RNA Silencing of Endogenous Fibroblast Growth Factor 2 in Rat Hippocampus Increases Anxiety Behavior. *Biological Psychiatry*, *69*(6), 534–540. http://doi.org/10.1016/j.biopsych.2010.11.020

Evans, S. J., Choudary, P. V., Neal, C. R., Li, J. Z., Vawter, M. P., Tomita, H., … Akil, H. (2004). Dysregulation of the fibroblast growth factor system in major depression. *Proceedings of the National Academy of Sciences of the United States of America*, *101*(43), 15506 –15511. http://doi.org/10.1073/pnas.0406788101

Falcone, C., Filippis, C., Granzotto, M., & Mallamaci, A. (2015). Emx2 expression levels in NSCs modulate astrogenesis rates by regulating EgfR and Fgf9. *Glia*, *63*(3), 412–422. http://doi.org/10.1002/glia.22761

Garcès, A., Nishimune, H., Philippe, J.-M., Pettmann, B., & deLapeyrière, O. (2000). FGF9: A motoneuron survival factor expressed by medial thoracic and sacral motoneurons. *Journal of Neuroscience Research*, *60*(1), 1–9.

http://doi.org/10.1002/(SICI)1097-4547(20000401)60:1<1::AID-JNR1>3.0.CO;2-P

Gaughran, F., Payne, J., Sedgwick, P. M., Cotter, D., & Berry, M. (2006). Hippocampal FGF-2 and FGFR1 mRNA expression in major depression, schizophrenia and bipolar disorder. *Brain Research Bulletin*, *70*(3), 221–227. http://doi.org/10.1016/j.brainresbull.2006.04.008

Goetz, R., & Mohammadi, M. (2013). Exploring mechanisms of FGF signalling through the lens of structural biology. *Nature Reviews. Molecular Cell Biology*, *14*(3), 166–180. http://doi.org/10.1038/nrm3528

Gómez-Pinilla, F., Dao, L., Choi, J., & Ryba, E. . (2000). Diazepam induces FGF-2 mRNA in the hippocampus and striatum. *Brain Research Bulletin*, *53*(3), 283–289. http://doi.org/10.1016/S0361-9230(00)00342-7

Huang, J.-Y., Hong, Y.-T., & Chuang, J.-I. (2009). Fibroblast growth factor 9 prevents MPP+-induced death of dopaminergic neurons and is involved in melatonin neuroprotection in vivo and in vitro. *Journal of Neurochemistry*, *109*(5), 1400–1412. http://doi.org/10.1111/j.1471-4159.2009.06061.x

Jones, E. G., Hendry, S. H. C., Liu, X.-B., Hodgins, S., Potkin, S. G., & Tourtellotte, W. W. (1992). A method for fixation of previously fresh-frozen human adult and fetal brains that preserves histological quality and immunoreactivity. *Journal of Neuroscience Methods*, *44*(2–3), 133–144. http://doi.org/10.1016/0165-0270(92)90006-Y

Kanda, T., Iwasaki, T., Nakamura, S., Kurokawa, T., Ikeda, K., & Mizusawa, H. (2000). Self-secretion of fibroblast growth factor-9 supports basal forebrain cholinergic neurons in an autocrine/paracrine manner. *Brain Research*, *876*(1-2), 22–30.

Lamers, F., van Oppen, P., Comijs, H. C., Smit, J. H., Spinhoven, P., van Balkom, A. J. L. M., … Penninx, B. W. J. H. (2011). Comorbidity patterns of anxiety and depressive disorders in a large cohort study: the Netherlands Study of Depression and Anxiety (NESDA). *The Journal of Clinical Psychiatry*, *72*(3), 341–348. http://doi.org/10.4088/JCP.10m06176blu

Li, J. Z., Bunney, B. G., Meng, F., Hagenauer, M. H., Walsh, D. M., Vawter, M. P., … Bunney, W. E. (2013). Circadian patterns of gene expression in the human brain and disruption in major depressive disorder. *Proceedings of the National Academy of Sciences*, *110*(24), 9950–9955. http://doi.org/10.1073/pnas.1305814110

Li, J. Z., Vawter, M. P., Walsh, D. M., Tomita, H., Evans, S. J., Choudary, P. V., … Myers, R. M. (2004). Systematic changes in gene expression in postmortem human brains associated with tissue pH and terminal medical conditions. *Human Molecular Genetics*, *13*(6), 609–616. http://doi.org/10.1093/hmg/ddh065

Lindner, M., Thümmler, K., Arthur, A., Brunner, S., Elliott, C., McElroy, D., … Linington, C. (2015). Fibroblast growth factor signalling in multiple sclerosis: inhibition of myelination and induction of pro-inflammatory environment by FGF9. *Brain*, *138*(7), 1875–1893. http://doi.org/10.1093/brain/awv102

Lin, Y., Chen, L., Lin, C., Luo, Y., Tsai, R. Y. L., & Wang, F. (2009). Neuron-derived FGF9 is essential for scaffold formation of Bergmann radial fibers and migration of granule neurons in the cerebellum. *Developmental Biology*, *329*(1), 44–54. http://doi.org/10.1016/j.ydbio.2009.02.011

Livak, K. J., & Schmittgen, T. D. (2001). Analysis of Relative Gene Expression Data Using Real-Time Quantitative PCR and the 2−ΔΔCT Method. *Methods*, *25*(4), 402–408. http://doi.org/10.1006/meth.2001.1262

Lucki, I. (1997). The forced swimming test as a model for core and component b... : Behavioural Pharmacology. Retrieved June 17, 2014, from http://journals.lww.com/behaviouralpharm/Fulltext/1997/11000/The_forced_swimming_test_as_a_model_for_core_and.10.aspx

Lum, M., Turbic, A., Mitrovic, B., & Turnley, A. M. (2009). Fibroblast growth factor-9 inhibits astrocyte differentiation of adult mouse neural progenitor cells. *Journal of Neuroscience Research*, *87*(10), 2201–2210. http://doi.org/10.1002/jnr.22047

Mallei, A., Shi, B., & Mocchetti, I. (2002). Antidepressant Treatments Induce the Expression of Basic Fibroblast Growth Factor in Cortical and Hippocampal Neurons. *Molecular Pharmacology*, *61*(5), 1017 –1024. http://doi.org/10.1124/mol.61.5.1017

Meier, F., Giesert, F., Delic, S., Faus-Kessler, T., Matheus, F., Simeone, A., … Prakash, N. (2014). FGF/FGFR2 Signaling Regulates the Generation and Correct Positioning of Bergmann Glia Cells in the Developing Mouse Cerebellum. *PLoS ONE*, *9*(7), e101124. http://doi.org/10.1371/journal.pone.0101124

Nakamura, S., Todo, T., Haga, S., Aizawa, T., Motoi, Y., Ueki, A., … Ikeda, K. (1997). Motor neurons in human and rat spinal cord synthesize fibroblast growth factor-9. *Neuroscience Letters*, *221*(2-3), 181–184. http://doi.org/16/S0304-3940(96)13312-7

Nakamura, S., Todo, T., Motoi, Y., Haga, S., Aizawa, T., Ueki, A., & Ikeda, K. (1999). Glial expression of fibroblast growth factor-9 in rat central nervous system. *Glia*, *28*(1), 53–65. http://doi.org/10.1002/(SICI)1098-1136(199910)28:1<53::AID-GLIA7>3.0.CO;2-V

Ornitz, D. M., Xu, J., Colvin, J. S., McEwen, D. G., MacArthur, C. A., Coulier, F., … Goldfarb, M. (1996). Receptor Specificity of the Fibroblast Growth Factor Family. *Journal of Biological Chemistry*, *271*(25), 15292–15297. http://doi.org/10.1074/jbc.271.25.15292

Pellow, S., Chopin, P., File, S. E., & Briley, M. (1985). Validation of open : closed arm entries in an elevated plus-maze as a measure of anxiety in the rat. *Journal of Neuroscience Methods*, *14*(3), 149–167. http://doi.org/10.1016/0165-0270(85)90031-7

Pellow, S., & File, S. E. (1986). Anxiolytic and anxiogenic drug effects on exploratory activity in an elevated plus-maze: A novel test of anxiety in the rat. *Pharmacology Biochemistry and Behavior*, *24*(3), 525–529. http://doi.org/10.1016/0091-3057(86)90552-6

Perez, J. A., Clinton, S. M., Turner, C. A., Watson, S. J., & Akil, H. (2009). A New Role for FGF2 as an Endogenous Inhibitor of Anxiety. *J. Neurosci.*, *29*(19), 6379–6387. http://doi.org/10.1523/JNEUROSCI.4829-08.2009

Porsolt, R. D., Bertin, A., Blavet, N., Deniel, M., & Jalfre, M. (1979). Immobility induced by forced swimming in rats: effects of agents which modify central catecholamine and serotonin activity. *European Journal of Pharmacology*, *57*(2), 201–210.

Porsolt, R. D., Bertin, A., & Jalfre, M. (1977). Behavioral despair in mice: a primary screening test for antidepressants. *Archives Internationales de Pharmacodynamie et de Thérapie*, *229*(2), 327–336.

Porsolt, R. D., Bertin, A., & Jalfre, M. (1978). "Behavioural despair" in rats and mice: strain differences and the effects of imipramine. *European Journal of Pharmacology*, *51*(3), 291–294.

Salaria, S., Chana, G., Caldara, F., Feltrin, E., Altieri, M., Faggioni, F., … Everall, I. P. (2006). Microarray analysis of cultured human brain aggregates following cortisol exposure: Implications for cellular functions relevant to mood disorders. *Neurobiology of Disease*, *23*(3), 630–636. http://doi.org/10.1016/j.nbd.2006.05.003

Stead, J. D. H., Clinton, S., Neal, C., Schneider, J., Jama, A., Miller, S., … Akil, H. (2006). Selective Breeding for Divergence in Novelty-seeking Traits: Heritability and Enrichment in Spontaneous Anxiety-related Behaviors. *Behavior Genetics*, *36*(5), 697–712. http://doi.org/10.1007/s10519-006-9058-7

Tagashira, S., Ozaki, K., Ohta, M., & Itoh, N. (1995). Localization of fibroblast growth factor-9 mRNA in the rat brain. *Molecular Brain Research*, *30*(2), 233–241. http://doi.org/10.1016/0169-328X(95)00009-H

Todo, T., Kondo, T., Nakamura, S., Kirino, T., Kurokawa, T., & Ikeda, K. (1998). Neuronal localization of fibroblast growth factor-9 immunoreactivity in human and rat brain. *Brain Research*, *783*(2), 179–187. http://doi.org/10.1016/S0006-8993(97)01340-1

Tomita, H., Vawter, M. P., Walsh, D. M., Evans, S. J., Choudary, P. V., Li, J., … Bunney Jr., W. E. (2004). Effect of agonal and postmortem factors on gene expression profile: quality control in microarray analyses of postmortem human brain. *Biological Psychiatry*, *55*(4), 346–352. http://doi.org/10.1016/j.biopsych.2003.10.013

Turner, C. A., Calvo, N., Frost, D. O., Akil, H., & Watson, S. J. (2008). The fibroblast growth factor system is downregulated following social defeat. *Neuroscience Letters*, *430*(2), 147–150.

Turner, C. A., Clinton, S. M., Thompson, R. C., Watson, S. J., & Akil, H. (2011). Fibroblast growth factor-2 (FGF2) augmentation early in life alters hippocampal development and rescues the anxiety phenotype in vulnerable animals. *Proceedings of the National Academy of Sciences*, *108*(19), 8021 –8025. http://doi.org/10.1073/pnas.1103732108

Turner, C. A., Eren-Koçak, E., Inui, E. G., Watson, S. J., & Akil, H. (2015). Dysregulated fibroblast growth factor (FGF) signaling in neurological and psychiatric disorders. *Seminars in Cell & Developmental Biology*. http://doi.org/10.1016/j.semcdb.2015.10.003

Turner, C. A., Gula, E. L., Taylor, L. P., Watson, S. J., & Akil, H. (2008). Antidepressant-like effects of intracerebroventricular FGF2 in rats. *Brain Research*, *1224*, 63–68.

Turner, C. A., Watson, S. J., & Akil, H. (2012). The Fibroblast Growth Factor Family: Neuromodulation of Affective Behavior. *Neuron*, *76*(1), 160–174. http://doi.org/10.1016/j.neuron.2012.08.037

Appendix 2.1. DLPFC Demographics.

Diagnosis	Age (median)	Gender (% Female)	Suicide (% Suicide)	Agonal Factor (% 0)	Brain pH (median)	Post-Mortem Interval (median)
Control (n=70)	58 (SD: 13.9; range: 18-78)	23%	0%	73%	6.82 (SD: 0.29; range: 6.06-7.51)	21.9 (SD: 21.9; range: 6.5-45.5)
MD (n=40)	48.5 (SD: 16.3; range: 19-84)	20%	55%	85%	6.91(SD: 0.28; range: 6.3-7.21)	27.8 (SD: 27.75; range: 9.5-37)
BP (n=24)	54 (SD: 15.6; range: 23-73)	45.83%	38%	75%	6.82 (SD: 0.21; range: 6.41-7.2)	26.8 (SD: 26.8; range: 9-54.8)
Schiz (n=22)	47.5 (SD: 10.2; range: 31-64)	32%	23%	77%	6.78 (SD: 0.29; range: 6.24-7.21)	23 (SD: 23; range: 6.25-46)

Appendix 2.2. ACG Demographics.

Diagnosis	Age (median)	Gender (% Female)	Suicide (% Suicide)	Agonal Factor (% 0)	Brain pH (median)	Post-Mortem Interval (median)
Control (n=62)	57.5 (SD: 13.1; range: 18-78)	23%	0%	86%	6.88 (SD: 0.25; range: 6.15-7.25)	22.4 (SD: 7.9; range: 7.3-45.5)
MD (n=37)	48 (SD: 15.0; range: 19-80)	22%	57%	92%	6.95 (SD: 0.27; range: 6.3-7.39)	27.5 (SD: 7.4; range: 9.5-37)
BP (n=20)	57.5 (SD: 15.9; range: 23-73)	45%	40%	80%	6.87 (SD: 0.18; range: 6.55-7.2)	27.3 (SD: 12.3; range: 9-54.8)
Schiz (n=21)	47 (SD: 10.5; range: 31-64)	33%	24%	81%	6.78 (SD: 0.28; range: 6.24-7.21)	24 (SD: 11.8; range: 6.3-46)

Appendix 2.3. HPC Demographics.

Diagnosis	Age (median)	Gender (% Female)	Suicide (% Suicide)	Agonal Factor (% 0)	Brain pH (median)	Post-Mortem Interval (median)
Control (n=56)	58 (SD: 12; range: 30-77)	23%	0%	86%	6.9 (SD: -0.26; range: 6.3-7.5)	21 (SD: 7; range: 7-40)
MDD (n=36)	48 (SD: 16; range: 19-84)	25%	58%	92%	7 (SD: 0.28; range: 6.3-7.4)	28 (SD: 8; range: 10-37)
BP (n=18)	51.5 (SD: 16.1; range: 23-73)	39%	44%	83%	6.87 (SD: 0.18; range: 6.44-7.12)	26.25 (SD: 10.8; range: 9-54.8)
Schiz (n=20)	47.5 (SD: 9.7; range: 31-64)	35%	25%	75%	6.775 (SD: 0.29; range: 6.24-7.21)	21 (SD: 10.6; range: 6.3-44.3)

Appendix 2.4. Overlap between the final MDD and control samples used in the four human postmortem experiments examining the hippocampus, following outlier removal and the removal of subjects with missing or incorrect information. Note that methods describing Illumina microarray data can be found in Aurbach et al. (2015).

Subject Overlap in Replicate Human Studies

	Affymetrix HG-U133A Microarray:	***Illumina HT-12 Microarray:***	***Illumina Ref-8_v2 Microarray:***	***qRT-PCR:***
Affymetrix HG-U133A Microarray:	***n=92***	42% *(39/92)*	80% *(74/92)*	49% *(45/92)*
Illumina HT-12 Microarray:	87% *(39/45)*	***n=45***	87% *(39/45)*	87% *(39/45)*
Illumina Ref-8_v2 Microarray:	95% *(74/78)*	50% *(39/78)*	***n=78***	58% *(45/78)*
qRT-PCR:	100% *(45/45)*	87% *(39/45)*	100% *(45/45)*	***n=45***

Chapter 3.

Hippocampal Fibroblast Growth Factor 2 Expression Predisposes Individuals to Differences in Affect Regulation.[2]

Introduction

Increasingly, many researchers accept that the pathophysiology of major depressive disorder (MDD) arises from malfunctioning brain circuits, with a number of brain regions implicated in this dysregulation. These regions are thought to interact in complex ways to underlie cognitive, emotional, behavioral, and physiological states, and disruption of these functions can be linked to the symptoms of mood disorders (Drevets, Price, & Furey, 2008; Price & Drevets, 2012). Some components of this circuitry, including limbic areas such as the hippocampus, medial prefrontal cortex, and amygdala (Godsil, Kiss, Spedding, & Jay, 2013; Price & Drevets, 2012), thalamic regions including the dorsomedial nucleus (Tekin & Cummings, 2002), mesolimbic components including the ventral tegmental area and basal ganglia, especially the nucleus accumbens (Nestler

[2] *Note that some of the work discussed in this chapter has been published: Figures 3.2b and 3.2c were published in Chaudhury, S., Aurbach, E. L., Sharma, V., Blandino, P., Turner, C. A., Watson, S. J., & Akil, H. (2014). FGF2 is a target and a trigger of epigenetic mechanisms associated with differences in emotionality: Partnership with H3K9me3. Proceedings of the National Academy of Sciences, 111(32), 11834–11839. http://doi.org/10.1073/pnas.1411618111; data from Appendix 3.1 was published in Aurbach, E. L., Inui, E. G., Turner, C. A., Hagenauer, M. H., Prater, K. E., Li, J. Z., … Akil, H. (2015). Fibroblast growth factor 9 is a novel modulator of negative affect. Proceedings of the National Academy of Sciences, 112(38), 11953–11958. http://doi.org/10.1073/pnas.1510456112.*

& Carlezon, 2006; Drevets et al., 2008; Gunaydin & Kreitzer, 2016), and areas in the hypothalamus (Plotsky, Owens, & Nemeroff, 1998; Swaab, Bao, & Lucassen, 2005; Drevets et al., 2008) and brainstem (Drevets et al., 2008; Ressler & Nemeroff, 2000; Bernard et al., 2011; Gold et al., 2015) have been studied extensively in mood disorders. These regions underlie many functions, and many have been studied both in the context of their individual properties as microcircuits (e.g., the hippocampus) and as collective assemblies (e.g., the separation distress circuit; Panksepp, Nelson, & Bekkedal, 1997; Panksepp, Knutson, & Burgdorf, 2002; Panksepp, 2003) to better understand their dysregulation in mood disorders. Notably, there are competing – though not mutually exclusive – theories about the relative importance and involvement of different brain regions and circuits in MDD pathophysiology. Regions including the hippocampus and prefrontal cortices are thought to be primarily related to the cognitive components of MDD symptomology, while more primitive regions in the hypothalamus and brainstem are understood to underlie some physiological symptoms of MDD, including autonomic aberrations (Drevets et al., 2008).

The neurotrophic hypothesis of major depression has emerged to provide a unifying framework to understand this circuit dysregulation across regions, and it posits that changes in growth factors may be related to the circuit abnormalities that arise in major depression (Duman & Monteggia, 2006; Turner, Watson, & Akil, 2012). Among the best-characterized is fibroblast growth factor 2 (FGF2): our laboratory and others have consistently observed FGF2 to be downregulated in brain tissue derived from individuals diagnosed with MDD (Evans et al., 2004; Gaughran, Payne, Sedgwick, Cotter, & Berry, 2006; Bernard et al., 2011) and animal models of mood disorders

(Turner, Calvo, Frost, Akil, & Watson, 2008; Perez, Clinton, Turner, Watson, & Akil, 2009). While much progress has been made in the last 10 years, gaps remain in our understanding of FGF2's function to mediate affective dysregulation. These questions should be addressed before investing resources into developing novel therapeutic agents that could target FGF2 to treat MDD.

Previous studies have shown that FGF2 expression is reduced in the postmortem brain tissue of individuals diagnosed with MDD during life; these changes have been observed in the dorsolateral prefrontal cortex (DLPFC), anterior cingulate cortex (ACC) (Evans et al., 2004), hippocampus (Gaughran et al., 2006), and locus coeruleus (Bernard et al., 2011). However, these studies do not provide insight into whether reduced FGF2 levels predispose individuals to developing MDD or if they arise from the disease process.

Studies of FGF2 function in rodents have helped to clarify the role of FGF2 in affective regulation. For example, FGF2 has been repeatedly emphasized as a player in the rodent brain's response to stress. After an acute stressor or exposure to gluccocorticoids, FGF2 levels in adult animals are elevated, perhaps in a neuroprotective function to buffer the brain against exposure to deleterious effects of stress (Molteni et al., 2001). However, social defeat stress decreased hippocampal expression of FGF2 (Turner, Calvo, et al., 2008) indicating that prolonged social stress might downregulate FGF2, similar to the results seen in human postmortem brains. Manipulations known to combat the detrimental effects of stress, such as environmental enrichment (Perez et al., 2009) or treatment with antidepressant or anxiolytic drugs (Gómez-Pinilla, Dao, Choi, &

Ryba, 2000; Mallei, Shi, & Mocchetti, 2002; Bachis, Mallei, Cruz, Wellstein, & Mocchetti, 2008), increased FGF2 expression across several brain regions.

Directly manipulating FGF2, either by injecting it or by knocking down its expression using RNA interference, had effects on depression-like and anxiety-like behavior. Acute microinjections of FGF2 into the lateral ventricles decreased immobility time in the Porsolt forced swim test (FST), and chronic microinjections over seven days decreased latency to feed in the novelty suppressed feeding task (NSF), both indicating a decrease in depressive-like behavior (Turner, Gula, Taylor, Watson, & Akil, 2008). Similarly, using a lentiviral-mediated short-hairpin RNA targeted against FGF2, Eren-Koçak and colleagues showed that knocking down FGF2 expression in the hippocampus increased time that rats spent in the closed arms of an elevated plus maze (EPM), an anxiogenic effect (Eren-Koçak, Turner, Watson, & Akil, 2011). These results further confirm the role of FGF2 in mediating anxiety- and depression-like behavior in rodents.

While FGF2 is down-regulated in human brains, hippocampal FGF2 expression and regulation varies in animal models of individual vulnerability to affective dysregulation. Eren-Kocak et al. (2011) demonstrated that hippocampal FGF2 expression levels correlated with anxiety-like behavior on the elevated plus maze, indicating that the degree of endogenous FGF2 expression is associated with spontaneous affective-like behavior. Moreover, Perez and colleagues examined endogenous FGF2 expression in animals selectively bred for a low locomotor response to novelty (bred low responders, bLRs), that also have increased anxiety-like behavior. Anxiety- and depression-vulnerable bLRs showed lower FGF2 expression in the hippocampus compared to animals selectively bred for a high locomotor response to novelty (bred high responders,

bHRs; Perez et al., 2009), who have been previously characterized as showing less anxiety-like behavior than bLRs (Stead et al., 2006), though it is unknown if these differences in basal FGF2 expression in the hippocampus are stable across generations.

Supporting the idea that FGF2 expression is critical during development, Turner and colleagues demonstrated that administering a single dose of FGF2 on the day after birth decreased bLR's anxiety-like behavior during adulthood. bLRs treated with early-life FGF2 showed increases in the time spent in the open arms of the EPM, increases in time spent in the light side of the light-dark box, and increased locomotor scores in a novel environment, all indicators of reduced anxiety-like behavior (Turner, Clinton, Thompson, Watson, & Akil, 2011). In a related phenomenon, both Perez et al. (2009) and Turner et al. (2011) showed that FGF2 administration increased the proliferation of early postnatal cells in the subgranule zone and survival of both the early postnatal cells and adult-born dentate granule cells in the dentate gyrus of the hippocampus, which is also associated with successful antidepressant and anxiolytic treatments in animal models of mood and anxiety disorders. Furthermore, these alterations in phenotype and FGF2 expression have been linked to the epigenetic marker H3K9me3, reinforcing the findings that both genetic background and environment can directly impact FGF2 expression (Chaudhury et al., 2014). Collectively, these results indicate that FGF2 acts as an anxiolytic and an antidepressant for animals that are genetically vulnerable to anxiety-like and depression-like behavior, though it is unknown if altering endogenous expression of hippocampal FGF2 would impact affective vulnerability in high-anxiety animals.

This section of the chapter examines the hypothesis that *differential FGF2 expression is necessary for selective vulnerability to affective dysregulation, continuing*

to build the case that FGF2 is an important molecular target that becomes dysregulated in MDD. Therefore, we determined if variation in FGF2 levels predisposes individuals to affective dysregulation. We examined this question by asking two sub-questions, including: 1) Are the basal differences in hippocampal FGF2 expression between bHR and bLR rats stable across generations? And 2) is this difference in hippocampal FGF2 expression necessary for the differences in spontaneous affective-like behavior between bHR and bLR rats?

Materials and Methods

Animals. We used selectively-bred male rats in these studies. Bred high responder and low responder rats were bred, weaned, and tested for their locomotor response to novelty between postnatal days 50-60, and housed in pairs in-house as previously described (Perez et al., 2009; Stead et al., 2006; Turner et al., 2011). All animals were maintained on a 12/12 light/dark schedule, with access to food and water *ad libitum*. All animals were treated in accordance with the National Institutes of Health *Guidelines on Laboratory Animal Use and Care* and in accordance with the guidelines set by the university committee on use and care of animals at the University of Michigan.

bHR and bLR studies. In the basal gene expression studies, bHR and bLR rats from generations F25 and F35 were tested for locomotor response to novelty before being euthanized between postnatal day 50-60. Brains were extracted, snap-frozen in 2-methylbutane, and stored at -80C until processing. In the knockdown studies, bHR and bLR rats from generation F34 underwent microinjection surgery at postnatal day 60 (groups: LVshFGF2, LVshNS; Eren-Koçak et al., 2011). Under isoflurane anesthesia and using standard stereotaxic protocols, a 33-gauge microinjector was lowered

bilaterally just above the hippocampus (coordinates from bregma: A/P -5.0, M/L ±3.5, D/V -2.6). One µL of LVshNS or LVshFGF2 was infused over four minutes, and two minutes were allowed for diffusion. We allowed 4 weeks for recovery, then animals were subjected to behavioral testing in the elevated plus maze and forced swim test. Twenty-four hours after the last behavioral test, animals were euthanized. Brains were rapidly extracted and frozen in 2-methylbutane at -30°C and stored at -80°C until processing.

Behavioral Testing.

Locomotion testing. Locomotion testing was used to assess the locomotor response to a novel environment, one measure of novelty-seeking and anxiety-like behavior. Rats were placed in a 43 x 21.5 x 25.5cm acrylic cage. Two panels of photocells recording beam breaks determined both horizontal and vertical locomotor activity, and activity was monitored in five minute bins for one hour. The horizontal and vertical components were added together for each animal and an average was found for each group (Stead et al., 2006). After each animal was tested, the apparatus was cleaned with 70% ethanol in water. All locomotor testing took place between 0800 and 1200 h. We interpreted low locomotor scores as indicating low levels of novelty-seeking and high spontaneous anxiety-like behavior.

Forced swim test (FST). We used the FST to determine effects on depression-like behavior (Lucki, 1997; Porsolt, Bertin, & Jalfre, 1977; Porsolt, Bertin, & Jalfre, 1978; Porsolt, Bertin, Blavet, Deniel, & Jalfre, 1979). The test occurs over two days. On day one, animals undergo a 15 minute pre-test swim. 24 hours later, animals undergo a five minute test swim (Lucki, 1997; Porsolt et al., 1977; Porsolt et al., 1979, 1978). Animals were placed in cylinders filled with water at a temperature of 25-27°C. Water was

changed between animals and all sessions were video recorded. All FST swim sessions took place between 0900 h and 1300 h. The videotaped behaviors were scored by an observer blind to the experimental conditions using The Observer software (Noldus Information Technology, The Netherlands). Swimming was scored when at least two paws were consistently moving in a horizontal direction. Climbing was defined by vertically-directed movement of at least two paws against the wall of the cylinder. Immobility was defined as floating or the minimal movement necessary to keep the head above water level. Percent total duration of swimming, climbing, and immobility episodes were determined and compared across groups. Animals who spent increased percent time immobile and decreased percent time swimming or climbing were interpreted to be exhibiting enhanced depression-like behavior.

Elevated plus-maze (EPM). We used the EPM to determine effects on anxiety-like behavior (Pellow, Chopin, File, & Briley, 1985; Pellow & File, 1986; Turner et al., 2011; Eren-Koçak et al., 2011). The EPM apparatus consists of a plus-shaped platform elevated 70cm above the floor; two arms are enclosed in black plexiglass (the "anxiolytic" regions of the maze), while the other two arms are left open (the "anxiogenic" regions of the maze). At the intersection of the arms, there is a 12 x 12cm square platform allowing access to all four arms (Pellow et al., 1985; Pellow & File, 1986). During the five minute test period, the room is dimly lit (~40 lux), and behavior is monitored using a computerized video tracking system (Noldus Ethovision). At the start of the five minute test, the rat was placed in the center square platform. The tracking system recorded the latency to enter the open arm, the amount of time spent in arms, and the time spent in the center square. After every animal, the testing apparatus was wiped

down with 70% ethanol in water. We compared the amount of time spent in the open and closed arms of the maze across groups; animals that spent more time in the closed arms (and/or less time in the open arms) of the maze were considered to show elevated levels of spontaneous anxiety-like behavior.

Tissue Analysis.

mRNA in situ hybridization (ISH). All rats were euthanized by rapid decapitation; brains were removed, snap-frozen, and stored at -80°C. Ten-µm sections were taken every 100-200 µm (depending on the study) and mounted onto Superfrost Plus slides at -20°C (Fisher, Waltham, MA). Slide-mounted tissue was fixed in 4% paraformadehyde solution for 60 minutes, washed three times with 2X SSC (1X SSC is 150 mM sodium chloride and 15 mM sodium citrate), and treated with 1M triethanolamine with 0.25% acetic anhydride. Slides were rinsed and dehydraded in graded ethanols before air-drying. All *in situ* probes were synthesized in-house; the rat FGF2 mRNA sequences used for generating probes were complimentary to NM_019395 (RefSeq database number), and eGFP was sub-cloned from the pLentiLox3.7 vector. All cDNA segments were extracted (Qiaquick Gel Extraction kit, Qiagen, Valencia, CA), subcloned in Bluescript SK (Stratagene, La Jolla, CA) and confirmed by nucleotide sequencing. The probes were labeled in a reaction mixture of 0.5-1µg of linearized plasmid specific to the probe of interest, 1X transcription buffer (Epicentre Technologies, Madison, WI), 125µCi of ^{35}S-labeled UTP, 125µCi of ^{35}S labeled CTP, 150µM ATP and GTP, 12.5mM dithiothreitol, 1µl of RNAse inhibitor (4U/µl), and 1.5µl of T7 or T3 RNA polymerase (20U/µl). Labeled probes were purified on Micro Bio-Spin Chromatography Columns (BioRad, Berkeley, CA) according to the manufacturer's instructions. After air-drying, slides were

treated with hybridization buffer containing the labeled probe (1-2 x 10^6 counts/75 μL buffer) 50% formamide, 10% dextran sulfate, 3X SSC, 50 mM sodium phosphate buffer (pH = 7.4), 1X Denhardt's solution, 0.1 mg/ml yeast tRNA, and 10 mM dithiothreitol. All slides were cover-slipped and stored in humidified chambers at 55°C during the 12-16 hour hybridization period. After hybridization, sections were washed three times in 2X SSC and incubated in an RNase solution (100 μg/mL RNase in Tris buffer with 0.5M NaCl, pH=8) at 37C. Sections were then sequentially washed in 2X, 1X, and 0.5X SSC before being incubated in 0.1X SSC at 65C for 1 hour. Sections were rinsed in distilled water and dehydrated through graded ethanols. Slides were exposed to Kodak BioMax MR Scientific Imaging Film (Sigma Aldrich), and exposure times were experimentally determined for optimal signal, as follows: FGF2 (21 days), eGFP (7 days).

Autoradiograph Quantification Procedures. mRNA expression signals from autoradiographic films were quantified using the computer-assisted optical densitometry software ImageJ (National Institutes of Health, Bethesda, MDD).

For the basal bHR/bLR experiments, integrated optical densities were determined by outlining a hippocampal subfield (CA1, CA2, CA3, and dentate gyrus) on each hemisphere, correcting for background plus 3.5 times its standard deviation. Data from >6 sections were averaged to create a mean signal measurement for each animal and group averages and standard error of the mean were calculated.

For quantification of sections from animals in the FGF2 knockdown study, we used a modified quantification approach to enable us to quantify expression of FGF2 only where we saw concurrent expression of eGFP. We digitally overlaid a transparent copy of the eGFP autoradiogram (from an adjacent section) on the image of the probe of interest

then outlined only the region with eGFP expression to take measurements for the probe of interest. Because there was variability in infection spread, we chose to disregard area in calculating the quantified measurements for these experiments; therefore, we present the data in terms of average optical density (mean signal-background).

Statistical Analyses. All animal behavioral tests were analyzed by Student's t-test or 2-way ANOVA followed by Fisher's LSD posthoc tests. All gene expression data was analyzed on an individual region-to-region basis.

Results

bHR animals have higher FGF2 expression in the hippocampus relative to bLR animals across generations. Previous studies have shown that bred high responder rats have increased levels of FGF2 expression in various subfields of the hippocampus; here, we examined if this result was stable across generations. In generation F25 (Figure 3.1a), bHRs had more FGF2 expression in the CA1 ($t(17) = 2.57$, $p = 0.019$) and CA3 ($t(17) = 2.17$, $p = 0.044$) subfields of the hippocampus, but not in the dentate gyrus ($t(17) = 1.58$, $p = 0.131$) or in CA2 ($t(17) = 0.29$, $p = 0.774$). Similarly, in generation F35 (Figure 3.1b), we observed higher FGF2 expression in CA1 ($t(11) = 3.36$, $p = 0.006$) and CA3 ($t(11) = 3.44$, $p = 0.005$), with no differences in expression levels in the dentate gyrus ($t(11) = 0.17$, 0.866) or in CA2 ($t(11) = 0.58$, $p = 0.570$). Collectively, these results reinforce the findings that bHR and bLR rats have differing basal levels of hippocampal FGF2, though we observed differential expression in subfields other than those previously reported (Perez et al. (2009) observed differences in the dentate gyrus and CA2 subfields).

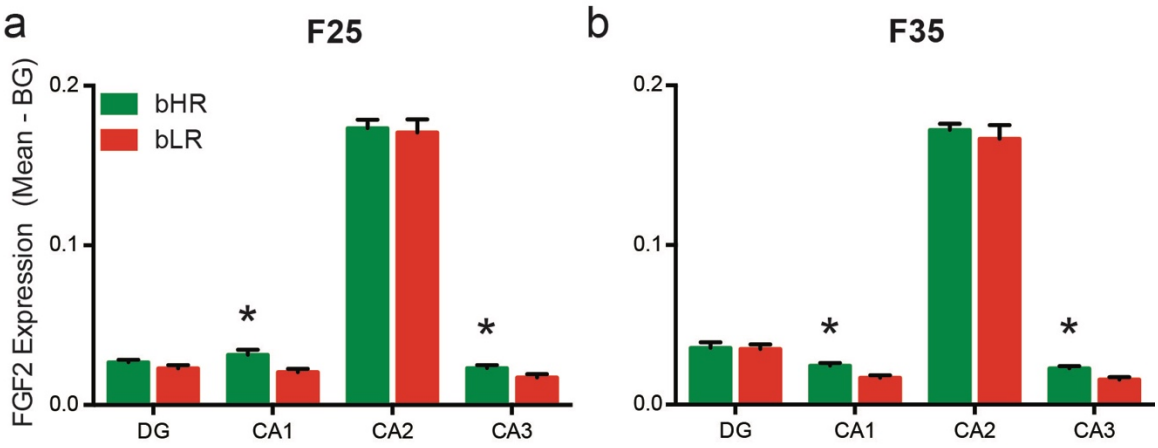


Figure 3.1. FGF2 mRNA expression is consistently higher in the CA1 and CA3 subfields of the hippocampus of bHRs relative to bLRs across generations. There were no statistically significant differences between FGF2 expression in the dentate gyrus or CA2 subfields. n = 8-10 animals/group.

Knocking down hippocampal FGF2 reduces expression levels and eliminates differences in spontaneous anxiety- and depression-like behavior in bHR and bLR rats. Based on previous work demonstrating that knocking down FGF2 expression in the dentate gyrus of outbred rats increased their spontaneous anxiety-like behavior on the elevated plus maze, we examined if knocking down FGF2 expression in the hippocampus of bHR and bLR rats altered FGF2 expression levels and affective behavior. Analysis of autoradiograms against GFP, a marker for successful viral transduction, revealed that the viruses primarily affected the CA1 subfield of the hippocampus for both bHR and bLR rats. In that region, we observed a modest but significant reduction in FGF2 expression in all animals who received the LVshFGF2 virus compared to animals who received the nonsilencing control LVshNS virus (Figure 3.2a; $t(51) = 2.13$, $p = 0.038$), and this reduction did not differ between bHR and bLR animals ($F(1,49) = 0.71$, $p = 0.79$), suggesting that knockdown efficacy was similar for both strains of rats. This modest knockdown produced a significant effect on behavior: bLR animals who were

administered the nonsilencing LVshNS virus spent more time in the closed arms ($t(10) = 7.07$, $p < 0.001$) and less time of the open arms ($t(10) = 4.38$, $p = 0.001$) of the elevated plus maze than bHR animals (Figure 3.2b), but these differences were eliminated for animals who received the LVshFGF2 virus (Figure 3.2c; closed arms: $t(11) = 0.022$, $p = 0.983$; open arms: $t(11) = 0.861$, $p = 0.408$). We saw similar effects of viral knockdown on behavior in the forced swim test: after treatment with the nonsilencing LVshNS virus, and bHR and bLR animals displayed characteristic spontaneous depression-like behavior (Figure 3.2d) with bLRs spending more time immobile ($t(10) = 3.209$, $p = 0.009$) and less time swimming ($t(10) = 3.556$, $p = 0.012$). In contrast, after treatment with the LVshFGF2 virus, these basal differences were eliminated (Figure 3.2e; immobility: $t(11) = 1.11$, $p = 0.29$; swimming: $t(11) = 0.658$, $p = 0.523$). Collectively, these results demonstrate that even modest reductions in FGF2 expression in the CA1 subfield of the hippocampus are sufficient to eliminate basal differences in anxiety- and depression-like behavior between bHR and bLR rats.

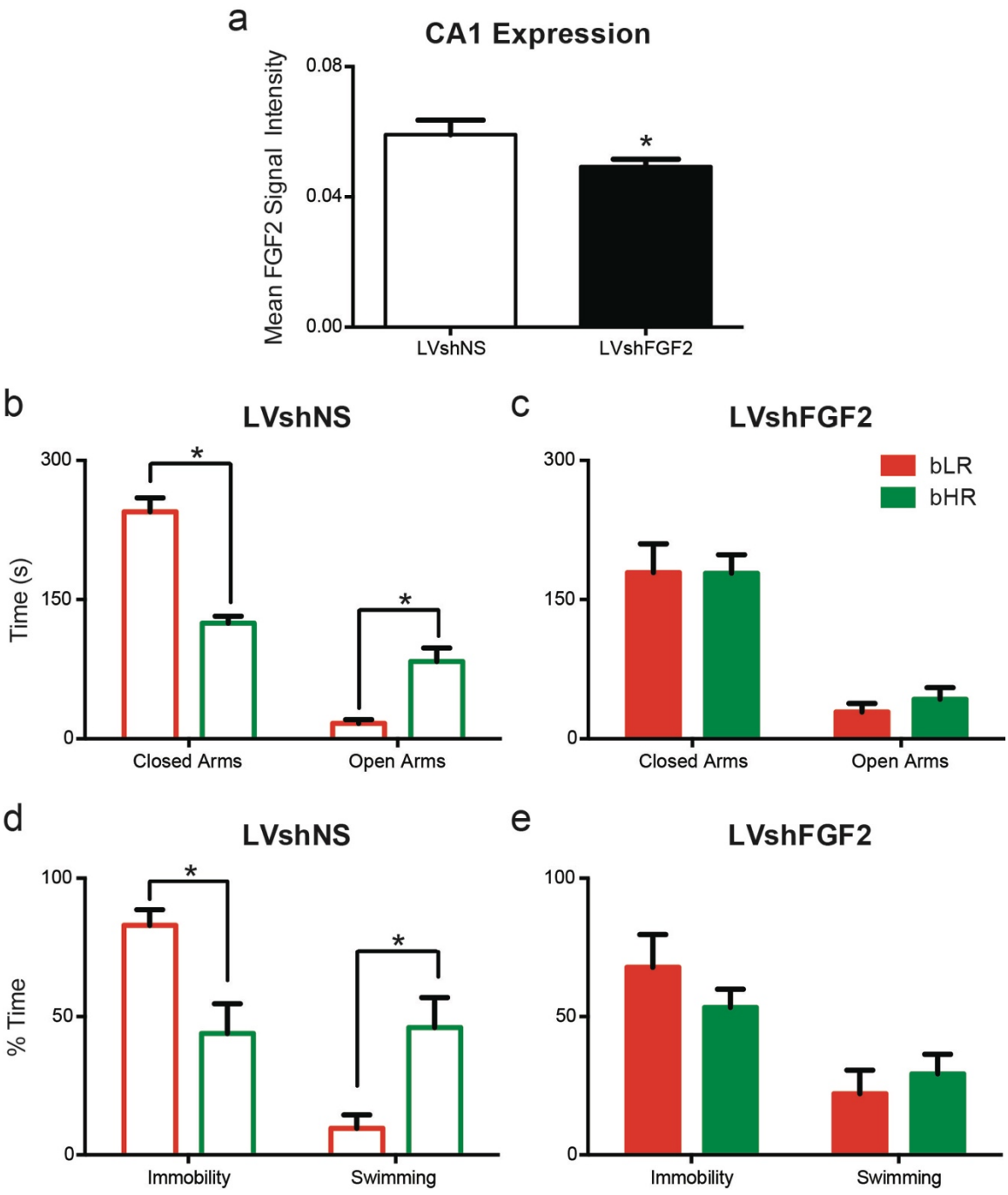


*Figure 3.2. Lentiviral-mediated knockdown of FGF2 expression in CA1 a) reduces FGF2 expression and eliminates basal differences in spontaneous anxiety- (b, c) and depression-like (d,e) behavior between bHR and bLR rats. n = 6 animals/group; * indicates $p < 0.05$.*

Discussion

Collectively, these data reinforce FGF2 as an anxiolytic and antidepressant molecule in hippocampal microcircuitry. In an animal model of individual differences and vulnerability to affective dysregulation, we observed an increase in hippocampal FGF2 expression that was stable across generations in animals selectively bred for a high locomotor response to novelty and who spontaneously exhibit low levels of anxiety- and depression-like behavior. Furthermore, we demonstrated that knocking down expression of FGF2 in the CA1 subfield of the hippocampus was sufficient to eliminate differences in spontaneous anxiety- and depression-like behavior between these selectively-bred lines of rats. Together, these studies indicate that FGF2 levels vary across individuals and are important to predispose these individuals to different patterns of affective behavior. These data support the hypothesis that FGF2 is an important player in the pathophysiology underlying MDD.

<u>Bred high responders have higher hippocampal FGF2 expression than bred low responder rats across generations, and knocking down FGF2 expression in CA1 eliminates behavioral differences between bHR and bLR animals.</u>

Previous studies have shown that hippocampal FGF2 expression correlates with anxiety-like behavior on the elevated plus maze: in particular, CA2 expression positively correlated with open-arm time in outbred rats (Eren-Koçak et al., 2011). Furthermore, previous studies have also reported that bHR animals have higher levels of FGF2 expression in the dentate gyrus and CA2 subfields of the hippocampus than do bLR animals (Perez et al., 2009; Turner et al., 2011). Because we were interested in examining if this variation in FGF2 expression was crucial to the individual differences in

behavioral phenotype, we sought to determine if this differential expression across bHR and bLR animals was stable across generations. We examined hippocampal FGF2 expression in two generations of bHR and bLR rats, 10 generations apart, and found consistent increases in expression in the CA1 and CA3 subfields of the hippocampus of bHRs relative to bLRs. It is interesting to note that while the overall pattern was the same across studies, we observed differential FGF2 expression in subfields other than those previously described (Perez et al., 2009). There could be several reasons for these differences, including that there may be subtle alterations in the methods with which tissue was collected, processed, and quantified. However, given that we did observe consistent and stable differential FGF2 expression in the hippocampus across generations, we sought to determine if this distinctive pattern was necessary for the differences in spontaneous affective-like behavior that we have previously observed between bHRs and bLRs.

Reducing endogenous gene expression using viral-mediated knockdown allows researchers to probe the region-specific necessity of particular transcripts for physiological and behavioral outputs. Our laboratory has previously used a lentiviral vector to knock down FGF2 expression in the dentate gyrus of outbred rats, demonstrating that this reduction of endogenous expression increased anxiety-like behavior on the EPM (Eren-Koçak et al., 2011). Here, we used viral vectors to knock down FGF2 expression in the CA1 subfield of the hippocampus of selectively-bred bHR and bLR animals. Control animals who received the non-silencing LVshNS virus exhibited behavior prototypical to the selectively-bred lines, with bHRs showing less anxiety- and depressive-like behavior than bLR rats. In contrast, animals who received

the LVshFGF2 knockdown virus did not display these phenotype-specific differences in either anxiety- or depression-like behavior, suggesting that even modest reductions in FGF2 expression are sufficient to eliminate behavioral differences between these selectively-bred rats by altering anxiety and depressive-like behavior. These results are paralleled with basal differences in the epigenetic marker H3K9me3 in animals treated with LVshNS that are eliminated in animals who received LVshFGF2 (Chaudhury et al., 2014), a possible route for environmental manipulations to mediate similar effects.

Interestingly, bHR rats who received the LVshFGF2 virus showed greater differences in affective-like behavior than did bLR rats, which could indicate that bHR animals, like the outbred rats previously reported (Eren-Koçak et al., 2011), could sustain decreases in hippocampal FGF2 levels, while bLR animals may already express hippocampal FGF2 at minimal levels necessary for basic regional function. These data are also interesting because the majority of findings that our laboratory has previously reported have indicated that bLR rats are more responsive to positive manipulations like early-life (Turner et al., 2011) or chronic FGF2 administration and/or environmental enrichment (Perez et al., 2009) than bHR rats. Therefore, this indicates that differential FGF2 expression in the hippocampus is important to the behavioral phenotypes for both bHR and bLR animals, indicating that the expression of this molecule is strongly related to individual variation in affective dysregulation. It is notable, however, that other regions and molecular systems are also likely to be critical for their behavioral differences.

Summary

We have shown that FGF2 may play an organizational capacity in the human and rodent brains to mediate affective regulation. We observed consistent basal differences in hippocampal FGF2 expression between rats selectively bred for a locomotor response to novelty, one measure of anxiety-like behavior. We demonstrated that knocking down expression of FGF2 in the CA1 subfield of the hippocampus was sufficient to eliminate prototypical differences in spontaneous anxiety- and depression-like behavior between these phenotypes, suggesting that this difference in FGF2 expression is important and necessary for the expression of these genetically-based individual differences in behavioral phenotypes. Collectively, these studies highlight FGF2 as a critical mediator of genetic predisposition to affective dysregulation and support the hypothesis that it is dysregulated in MDD.

References

Bachis, A., Mallei, A., Cruz, M. I., Wellstein, A., & Mocchetti, I. (2008). Chronic antidepressant treatments increase basic fibroblast growth factor and fibroblast growth factor-binding protein in neurons. *Neuropharmacology*, *55*(7), 1114–1120. http://doi.org/10.1016/j.neuropharm.2008.07.014

Bernard, R., Kerman, I. A., Thompson, R. C., Jones, E. G., Bunney, W. E., Barchas, J. D., … Watson, S. J. (2011). Altered expression of glutamate signaling, growth factor, and glia genes in the locus coeruleus of patients with major depression. *Molecular Psychiatry*, *16*(6), 634–646. http://doi.org/10.1038/mp.2010.44

Borroto-Escuela, D. O., Narvaez, M., Pérez-Alea, M., Tarakanov, A. O., Jiménez-Beristain, A., Mudó, G., … Fuxe, K. (2015). Evidence for the existence of FGFR1–5-HT1A heteroreceptor complexes in the midbrain raphe 5-HT system. *Biochemical and Biophysical Research Communications*, *456*(1), 489–493. http://doi.org/10.1016/j.bbrc.2014.11.112

Borroto-Escuela, D. O., Romero-Fernandez, W., Mudó, G., Pérez-Alea, M., Ciruela, F., Tarakanov, A. O., … Fuxe, K. (2012). Fibroblast Growth Factor Receptor 1– 5-Hydroxytryptamine 1A Heteroreceptor Complexes and Their Enhancement of Hippocampal Plasticity. *Biological Psychiatry*, *71*(1), 84–91. http://doi.org/10.1016/j.biopsych.2011.09.012

Chaudhury, S., Aurbach, E. L., Sharma, V., Blandino, P., Turner, C. A., Watson, S. J., & Akil, H. (2014). FGF2 is a target and a trigger of epigenetic mechanisms associated with differences in emotionality: Partnership with H3K9me3. *Proceedings of the National Academy of Sciences*, *111*(32), 11834–11839. http://doi.org/10.1073/pnas.1411618111

Drevets, W. C., Price, J. L., & Furey, M. L. (2008). Brain structural and functional abnormalities in mood disorders: implications for neurocircuitry models of depression. *Brain Structure & Function*, *213*(1-2), 93–118. http://doi.org/10.1007/s00429-008-0189-x

Duman, R. S., & Monteggia, L. M. (2006). A Neurotrophic Model for Stress-Related Mood Disorders. *Biological Psychiatry*, *59*(12), 1116–1127. http://doi.org/16/j.biopsych.2006.02.013

Elsayed, M., Banasr, M., Duric, V., Fournier, N. M., Licznerski, P., & Duman, R. S. (2012). Antidepressant Effects of Fibroblast Growth Factor-2 in Behavioral and Cellular Models of Depression. *Biological Psychiatry*, *72*(4), 258–265. http://doi.org/10.1016/j.biopsych.2012.03.003

Eren-Koçak, E., Turner, C. A., Watson, S. J., & Akil, H. (2011). Short-Hairpin RNA Silencing of Endogenous Fibroblast Growth Factor 2 in Rat Hippocampus Increases Anxiety Behavior. *Biological Psychiatry*, *69*(6), 534–540. http://doi.org/10.1016/j.biopsych.2010.11.020

Evans, S. J., Choudary, P. V., Neal, C. R., Li, J. Z., Vawter, M. P., Tomita, H., … Akil, H. (2004). Dysregulation of the fibroblast growth factor system in major depression. *Proceedings of the National Academy of Sciences of the United States of America*, *101*(43), 15506 –15511. http://doi.org/10.1073/pnas.0406788101

Gaughran, F., Payne, J., Sedgwick, P. M., Cotter, D., & Berry, M. (2006). Hippocampal FGF-2 and FGFR1 mRNA expression in major depression, schizophrenia and

bipolar disorder. *Brain Research Bulletin*, *70*(3), 221–227. http://doi.org/10.1016/j.brainresbull.2006.04.008

Godsil, B. P., Kiss, J. P., Spedding, M., & Jay, T. M. (2013). The hippocampal–prefrontal pathway: The weak link in psychiatric disorders? *European Neuropsychopharmacology*, *23*(10), 1165–1181. http://doi.org/10.1016/j.euroneuro.2012.10.018

Gold, P. W., Machado-Vieira, R., Pavlatou, M. G., Gold, P. W., Machado-Vieira, R., & Pavlatou, M. G. (2015). Clinical and Biochemical Manifestations of Depression: Relation to the Neurobiology of Stress, Clinical and Biochemical Manifestations of Depression: Relation to the Neurobiology of Stress. *Neural Plasticity*, *Neural Plasticity*, *2015*, *2015*, e581976. http://doi.org/10.1155/2015/581976, 10.1155/2015/581976

Gómez-Pinilla, F., Dao, L., Choi, J., & Ryba, E. A. (2000). Diazepam induces FGF-2 mRNA in the hippocampus and striatum. *Brain Research Bulletin*, *53*(3), 283–289. http://doi.org/10.1016/S0361-9230(00)00342-7

Gunaydin, L. A., & Kreitzer, A. C. (2016). Cortico–Basal Ganglia Circuit Function in Psychiatric Disease. *Annual Review of Physiology*, *78*(1), null. http://doi.org/10.1146/annurev-physiol-021115-105355

Jones, E. G., Hendry, S. H. C., Liu, X.-B., Hodgins, S., Potkin, S. G., & Tourtellotte, W. W. (1992). A method for fixation of previously fresh-frozen human adult and fetal brains that preserves histological quality and immunoreactivity. *Journal of Neuroscience Methods*, *44*(2–3), 133–144. http://doi.org/10.1016/0165-0270(92)90006-Y

Li, J. Z., Bunney, B. G., Meng, F., Hagenauer, M. H., Walsh, D. M., Vawter, M. P., … Bunney, W. E. (2013). Circadian patterns of gene expression in the human brain and disruption in major depressive disorder. *Proceedings of the National Academy of Sciences*, *110*(24), 9950–9955. http://doi.org/10.1073/pnas.1305814110

Li, J. Z., Vawter, M. P., Walsh, D. M., Tomita, H., Evans, S. J., Choudary, P. V., … Myers, R. M. (2004). Systematic changes in gene expression in postmortem human brains associated with tissue pH and terminal medical conditions. *Human Molecular Genetics*, *13*(6), 609–616. http://doi.org/10.1093/hmg/ddh065

Lucki, I. (1997). The forced swimming test as a model for core and component b... : Behavioural Pharmacology. Retrieved June 17, 2014, from http://journals.lww.com/behaviouralpharm/Fulltext/1997/11000/The_forced_swimming_test_as_a_model_for_core_and.10.aspx

Mallei, A., Shi, B., & Mocchetti, I. (2002). Antidepressant Treatments Induce the Expression of Basic Fibroblast Growth Factor in Cortical and Hippocampal Neurons. *Molecular Pharmacology*, *61*(5), 1017 –1024. http://doi.org/10.1124/mol.61.5.1017

Maragnoli, M. E., Fumagalli, F., Gennarelli, M., Racagni, G., & Riva, M. A. (2004). Fluoxetine and olanzapine have synergistic effects in the modulation of fibroblast growth factor 2 expression within the rat brain. *Biological Psychiatry*, *55*(11), 1095–1102. http://doi.org/10.1016/j.biopsych.2004.02.003

Molteni, R., Fumagalli, F., Magnaghi, V., Roceri, M., Gennarelli, M., Racagni, G., … Riva, M. A. (2001). Modulation of fibroblast growth factor-2 by stress and

corticosteroids: from developmental events to adult brain plasticity. *Brain Research Reviews*, *37*(1–3), 249–258. http://doi.org/10.1016/S0165-0173(01)00128-X

Nestler, E. J., & Carlezon, W. A. (2006). The Mesolimbic Dopamine Reward Circuit in Depression. *Biological Psychiatry*, *59*(12), 1151–1159. http://doi.org/10.1016/j.biopsych.2005.09.018

Panksepp, J. (2003). Feeling the Pain of Social Loss. *Science*, *302*(5643), 237–239. http://doi.org/10.1126/science.1091062

Panksepp, J., Knutson, B., & Burgdorf, J. (2002). The role of brain emotional systems in addictions: a neuro-evolutionary perspective and new "self-report" animal model. *Addiction*, *97*(4), 459–469. http://doi.org/10.1046/j.1360-0443.2002.00025.x

Panksepp, J., Nelson, E., & Bekkedal, M. (1997). Brain Systems for the Mediation of Social Separation-Distress and Social-Reward Evolutionary Antecedents and Neuropeptide Intermediariesa. *Annals of the New York Academy of Sciences*, *807*(1), 78–100. http://doi.org/10.1111/j.1749-6632.1997.tb51914.x

Paxinos, G., & Watson, C. (2007). *The Rat Brain in Stereotaxic Coordinates: Hard Cover Edition*. Academic Press.

Pellow, S., Chopin, P., File, S. E., & Briley, M. (1985). Validation of open : closed arm entries in an elevated plus-maze as a measure of anxiety in the rat. *Journal of Neuroscience Methods*, *14*(3), 149–167. http://doi.org/10.1016/0165-0270(85)90031-7

Pellow, S., & File, S. E. (1986). Anxiolytic and anxiogenic drug effects on exploratory activity in an elevated plus-maze: A novel test of anxiety in the rat. *Pharmacology Biochemistry and Behavior*, *24*(3), 525–529. http://doi.org/10.1016/0091-3057(86)90552-6

Perez, J. A., Clinton, S. M., Turner, C. A., Watson, S. J., & Akil, H. (2009). A New Role for FGF2 as an Endogenous Inhibitor of Anxiety. *The Journal of Neuroscience*, *29*(19), 6379 –6387. http://doi.org/10.1523/JNEUROSCI.4829-08.2009

Plotsky, P. M., Owens, M. J., & Nemeroff, C. B. (1998). PSYCHONEUROENDOCRINOLOGY OF DEPRESSION: Hypothalamic-Pituitary-Adrenal Axis. *Psychiatric Clinics of North America*, *21*(2), 293–307. http://doi.org/10.1016/S0193-953X(05)70006-X

Porsolt, R. D., Bertin, A., Blavet, N., Deniel, M., & Jalfre, M. (1979). Immobility induced by forced swimming in rats: effects of agents which modify central catecholamine and serotonin activity. *European Journal of Pharmacology*, *57*(2), 201–210.

Porsolt, R. D., Bertin, A., & Jalfre, M. (1977). Behavioral despair in mice: a primary screening test for antidepressants. *Archives Internationales de Pharmacodynamie et de Thérapie*, *229*(2), 327–336.

Porsolt, R. D., Bertin, A., & Jalfre, M. (1978). "Behavioural despair" in rats and mice: strain differences and the effects of imipramine. *European Journal of Pharmacology*, *51*(3), 291–294.

Price, J. L., & Drevets, W. C. (2012). Neural circuits underlying the pathophysiology of mood disorders. *Trends in Cognitive Sciences*, *16*(1), 61–71. http://doi.org/10.1016/j.tics.2011.12.011

Ressler, K. J., & Nemeroff, C. B. (2000). Role of serotonergic and noradrenergic systems in the pathophysiology of depression and anxiety disorders. *Depression and Anxiety, 12*(S1), 2–19. http://doi.org/10.1002/1520-6394(2000)12:1+<2::AID-DA2>3.0.CO;2-4

Stead, J. D. H., Clinton, S., Neal, C., Schneider, J., Jama, A., Miller, S., … Akil, H. (2006). Selective Breeding for Divergence in Novelty-seeking Traits: Heritability and Enrichment in Spontaneous Anxiety-related Behaviors. *Behavior Genetics, 36*(5), 697–712. http://doi.org/10.1007/s10519-006-9058-7

Swaab, D. F., Bao, A.-M., & Lucassen, P. J. (2005). The stress system in the human brain in depression and neurodegeneration. *Ageing Research Reviews*, *4*(2), 141–194. http://doi.org/10.1016/j.arr.2005.03.003

Tekin, S., & Cummings, J. L. (2002). Frontal–subcortical neuronal circuits and clinical neuropsychiatry: An update. *Journal of Psychosomatic Research*, *53*(2), 647–654. http://doi.org/10.1016/S0022-3999(02)00428-2

Tomita, H., Vawter, M. P., Walsh, D. M., Evans, S. J., Choudary, P. V., Li, J., … Bunney Jr., W. E. (2004). Effect of agonal and postmortem factors on gene expression profile: quality control in microarray analyses of postmortem human brain. *Biological Psychiatry*, *55*(4), 346–352. http://doi.org/10.1016/j.biopsych.2003.10.013

Turner, C. A., Calvo, N., Frost, D. O., Akil, H., & Watson, S. J. (2008). The fibroblast growth factor system is downregulated following social defeat. *Neuroscience Letters*, *430*(2), 147–150. http://doi.org/10.1016/j.neulet.2007.10.041

Turner, C. A., Clinton, S. M., Thompson, R. C., Watson, S. J., & Akil, H. (2011). Fibroblast growth factor-2 (FGF2) augmentation early in life alters hippocampal development and rescues the anxiety phenotype in vulnerable animals. *Proceedings of the National Academy of Sciences*, *108*(19), 8021 –8025. http://doi.org/10.1073/pnas.1103732108

Turner, C. A., Gula, E. L., Taylor, L. P., Watson, S. J., & Akil, H. (2008). Antidepressant-like effects of intracerebroventricular FGF2 in rats. *Brain Research, 1224*, 63–68. http://doi.org/16/j.brainres.2008.05.088

Turner, C. A., Watson, S. J., & Akil, H. (2012). The Fibroblast Growth Factor Family: Neuromodulation of Affective Behavior. *Neuron*, *76*(1), 160–174. http://doi.org/10.1016/j.neuron.2012.08.037

Appendix 3.1: Reevaluating MDD-specific alterations in FGF2 expression in postmortem human tissue using a statistical approach that controls for confounding variables

Introduction

Previous studies have shown that FGF2 expression is reduced in the postmortem brain tissue of individuals diagnosed with MDD during life; these changes have been observed in the dorsolateral prefrontal cortex (DLPFC), anterior cingulate cortex (ACC) (Evans et al., 2004), hippocampus (Gaughran et al., 2006), and locus coeruleus (Bernard et al., 2011). While this consistency is striking, the majority of these findings are derived from microarray analyses that do not fully control for some confounding variables, including age, and gender, and factors impacting gene expression including pH and agonal factor (Li et al., 2004; Tomita et al., 2004). Here, we asked a single question: Are the effects of FGF2 dysregulation in postmortem human brain tissue still observable using a statistical approach that controls for a variety of confounding pre- and postmortem variables?

Materials and Methods

Subject Characteristics and Tissue Extraction. The human tissue samples used for all microarray experiments as well as the qRT-PCR validation were obtained from the

Brain Donor Program at the University of California, Irvine with the consent of the next-of-kin of the deceased (Appendix 2.1, Appendix 2.2, & Appendix 2.3).

Dissection of Human DLPFC, ACC, and Hippocampus. For the first stage of dissection, a 1-cm coronal slab was placed on a block of dry ice. At the level of the genu of the corpus callosum, the middle frontal gyrus was identified by an expert in human neuroanatomy, and a fine jeweler's blade in a coping saw handle was used to excise the gyrus for dorsolateral prefrontal cortex samples. In some cases, more than one slab was used to cover the full extent of the structure. In the same plane, the cingulate gyrus was identified by an expert in human neuroanatomy, and a fine jeweler's blade in a coping saw handle was used to excise the gyrus for anterior cingulate cortex samples. In some cases, more than one slab was used to cover the full extent of the structure. In more caudal planes, the hippocampus was visually identified using the dentate gyrus by an expert in human neuroanatomy, and a fine jeweler's blade in a coping saw handle was used to dissect the hippocampus and surrounding temporal cortex. Both sides of the slab were carefully inspected. In some cases, more than one slab was used to cover the full extent of the structure. For all structures, the block was wrapped in foil, placed on dry ice and then stored at -80°C until further processing (Jones et al., 1992). In the hippocampus, a second stage of finer dissection was then carried out. The temporal cortex was removed by visual inspection using the dentate gyrus and CA3 as landmarks. The dissected tissue was then processed for RNA extraction. Total RNA was extracted using procedures described previously (Evans et al., 2004; Li et al., 2004). Clinical information was obtained from medical examiners, coroners' medical records and a family member. Patients were diagnosed by consensus based on criteria from DSM-IV. For further

information regarding data collection procedures, please see Li and colleagues (Li et al., 2013). Final Control and MDD sample sizes and demographics are in the appendices (DLPFC: Appendix 2.1, ACG: Appendix 2.2, HPC: Appendix 2.3).

Gene Expression Profiling. In general, the labeling and hybridization of sample mRNA with oligonucleotide probes followed standard manufacturer protocols. Analyses used the full microarray datasets (all probe data from the MDD and Control subjects, as well as from small samples of subjects with bipolar disorder and schizophrenia) to maximize our ability to identify technical artifacts and confounds, although the reported results focus on MDD vs. Control comparisons for a subset of growth factor probes.

Affymetrix Microarray. Microarray experiments were performed in eight separate experimental cohorts containing both patients and controls. The majority of RNA samples were analyzed in duplicate at two different laboratories using Affymetrix Genechips (either U133A or U133Plus-v2). After extracting summarized probe signal data from scanned microarray image files, all downstream analyses were completed in the R statistical programming environment. We extracted the U133A subset of probes and applied RMA (Robust Multi-array Analysis) to summarize probe set expression levels using custom ENTREZ12.1 Chip Definition Files (CDF) which defined probe sets for 11,912 transcripts (as defined by ENTREZ in 03/2010) and 68 control probe sets. As is traditional, all probe signal values were log (base 2)-transformed to reduce heteroskedasticity and quantile-normalized to remove technical artifacts in the overall distribution of signal per sample. To ensure sample quality, we required an average sample to sample correlation coefficient (r) of 0.85-0.9, excluding 6 of 235 samples (2.6%) in the HPC, 30 of 367 samples (8.1%) in the DLPFC, and 6 of 283 samples

(2.1%) in the ACC. Batch effects due to cohort, laboratory, and platform were removed by median-centering the data. Replicate samples were then averaged and any subjects that were missing information were removed from the dataset (n = 16 in DLPFC, n = 13 in ACC, n=8 in HPC), leaving a final sample size of 156 subjects in the DLPFC, 140 subjects in the ACC, and 129 subjects in the HPC. For further information regarding the Affymetrix data pre-processing procedures, please see Li and colleagues (Li et al., 2013). For all of the microarray experiments, as part of quality control, subjects were verified to have gene expression typical of their reported sex using data from genes XIST, EIF1AY, RPS4Y1, UTY, USP9Y, NLGN4Y, NCRNA00185, TTTY15, KDM5D, CYorf15B, and DDX3Y.

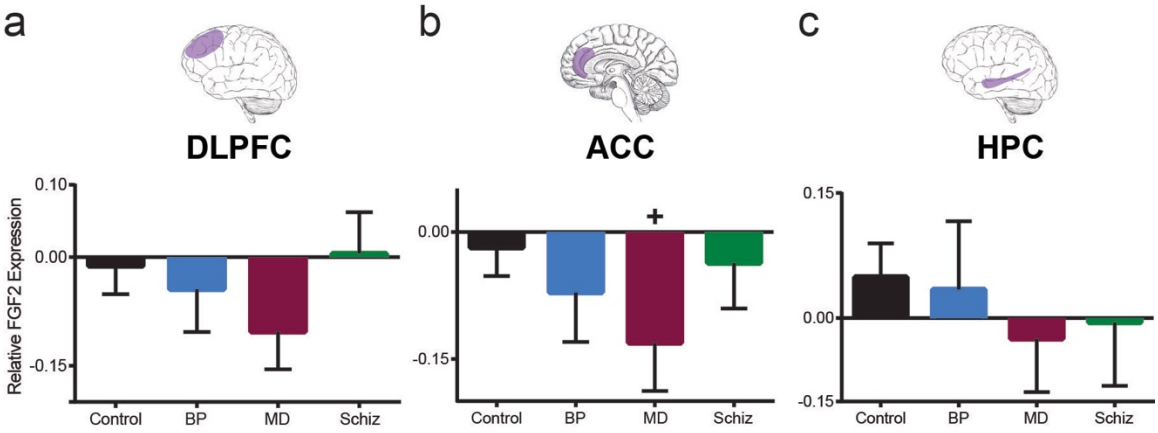
Microarray Analysis: Determining Diagnosis-Related Gene Expression while Correcting for Confounding Variables. Although agonal factor, brain pH, PMI, and gender did not differ significantly by diagnosis, we found that it was necessary to control for these variables because they strongly correlated with the top principal components of variation in the data sets (PC1-4). We also controlled for the average age at the time of death, which sometimes varied significantly with diagnosis. The degree of severity and duration of physiological stress at the time of death was estimated by calculating an Agonal Factor Score (AFS) for each subject (Tomita et al., 2004). Additionally, we measured the pH of cerebellar tissue as an indicator of the extent of oxygen deprivation experienced around the time of death (Li et al., 2004). We also calculated the interval between the estimated time of death and the freezing of the brain tissue (the postmortem interval or PMI) using coroner records. We ensured high quality data by choosing samples with relatively high pH and low agonal factor (DLPFC: Appendix 2.1, ACG:

Appendix 2.2, HPC: Appendix 2.3), but still decided that it was prudent to control for these variables as well as age and gender by including them as terms in our linear model:

Equation 1: $(probe\ signal) \approx \beta_0 + \beta_1(Brain\ pH) + \beta_2(Agonal\ Factor) + \beta_3(PMI) + \beta_4(Age) + \beta_5(Gender) + \beta_6(Diagnosis)$

Results

FGF2 expression trends towards a decrease in the anterior cingulate cortex of depressed individuals. Based on published data indicating that specific growth factor transcripts are altered in depressed brains (Bernard et al., 2011; Evans et al., 2004; Gaughran et al., 2006), we examined the relationship between FGF2 and major depression across three brain regions: the hippocampus, anterior cingulate cortex, and dorsolateral prefrontal cortex using human microarray datasets. We used linear regression to determine diagnosis-related gene expression while controlling for a variety of confounding pre- and post-mortem variables, including age, pH, gender, etc. In the hippocampus and DLPFC, FGF2 expression was not significantly different from nonpsychiatric control subjects, though we observed a visual trend for a decrease in FGF2 expression in the DLPFC (Appendix 3.1a; Appendix 3.1c; DLPFC: $p = 0.172$; HPC: $p = 0.344$). In the anterior cingulate cortex, there was a statistical trend for a decrease in FGF2 expression (Appendix 3.1b; $p=0.077$). Although we observed consistent trends for a reduction in FGF2 expression related to depression, we cannot conclude that we observed depression-related dysregulation of FGF2 in these brain regions because these trends did not reach statistical significance.



Appendix 3.1. FGF2 expression is consistently decreased in MDD relative to control across brain regions, though these trends do not reach statistical significance. a) dorsolateral prefrontal cortex, b) anterior cingulate cortex, c) hippocampus. All data is median-centered, which means that expression data is plotted relative to the median of the full sample (i.e., 0 on the y-axis is the median of the full sample). All statistics assess control vs. psychiatric diagnosis alterations in FGF2 expression while controlling for confounding pre- and postmortem variables. Sample sizes can be found in Appendix 2.1, Appendix 2.2, and Appendix 2.3.

Discussion

FGF2 expression is non-significantly decreased in the frontal cortices and hippocampus in MDD.

Contrary to previous reports, we did not observe statistically significant differences in FGF2 expression in the dorsolateral prefrontal cortex or anterior cingulate cortex, though we did observe consistent visual and statistical trends for decreased FGF2 expression (especially in the ACC), consistent with previous studies that used overlapping data and reported decreased FGF2 expression in postmortem brains of patients with MDD (Evans et al., 2004). Though we saw similar directionality, we hypothesize that there are several reasons that may underlie the lack of statistical significance: 1) statistical power (the sample sizes in our study were larger than in the previous report); 2) statistical approach (we used a linear model which controls for a

variety of confounding pre- and postmortem variables including age, gender, postmortem interval, agonal factor, and pH instead of mixed-model multivariate ANOVA with correction for multiple comparisons that conflates these important factors); and/or 3) treatment history (Evans et al. (2004) was able to much more accurately examine effects of a treatment history including antidepressant drugs than we were able to do with our larger dataset, which included missing and/or contradictory information related to antidepressant use).

In previous studies, other groups have observed a decrease in hippocampal FGF2 expression in depressed patients using mRNA *in situ* hybridization (Gaughran et al., 2006). The reason behind the discrepancy in our results is not clear, but may be due to differences in technique (microarray versus mRNA *in situ* hybridization) or because tissue pH was higher in our study, and low pH can impact gene expression (Li et al., 2004), reducing the expression of certain genes. Moreover, factors such as comorbid anxiety or a treatment history that includes antidepressant drugs would likely influence FGF2 expression and we could not fully account for these factors.

Indeed, administration of compounds with antidepressant and anxiolytic properties is known to increase FGF2 expression in human (Evans et al., 2004) and rodent brains. For example, chronic, but not acute, administration of several antidepressant and anxiolytic compounds increases FGF2 expression in the rodent cortex and hippocampus (Gómez-Pinilla et al., 2000; Mallei et al., 2002; Bachis et al., 2008). Interestingly, this effect seems to be additive: co-administering two antidepressants increased FGF2 expression in the hippocampus, and the increase was higher than either treatment alone (Maragnoli, Fumagalli, Gennarelli, Racagni, & Riva, 2004). A recent

study indicates that this induction of FGF2 expression is necessary for the positive effects of antidepressant drugs (Elsayed et al., 2012), which strongly suggests that one reason we were not able to observe strong decreases in FGF2 expression across brain regions in our postmortem human data may be because we could not fully account for a history of antidepressant drug usage in our MDD sample. Moreover, heterogeneity in drug type, administration chronicity, whether the individual was taking antidepressants at the time of death, and if there were interactions with other compounds in an individual's system, are all likely to have an impact on FGF2 expression. Due to the difficulties of full psychological autopsy, we could not adequately account for all these factors.

Summary

In postmortem human samples, we did not find strong evidence for FGF2 reductions in MDD, though we observed consistent visual trends for reduced expression across regions. The strongest effect was in the anterior cingulate cortex, where there was a statistical trend for a decrease in FGF2 expression in depressed samples.

Chapter 4.

Fibroblast Growth Factors 2 and 9 May Act as Molecular Organizers in Anterior Cingulate Cortex and Hippocampus to Mediate Circuit Function in MDD[3]

Introduction

A growing body of evidence strongly suggests that neurotrophic factors like FGF2 and FGF9 are dysregulated in Major Depressive Disorder (MDD; Aurbach et al., 2015; Evans et al., 2004; Perez, Clinton, Turner, Watson, & Akil, 2009; Turner, Calvo, Frost, Akil, & Watson, 2008; Turner, Clinton, Thompson, Watson, & Akil, 2011). However, the precise roles that these molecules play, including the myriad ways that they may impact neurotransmission, brain circuit structure and function, and large molecular networks remains to be determined. Furthermore, given that a number of growth factors, including FGF2, FGF9, brain-derived neurotrophic factor, glial cell line derived neurotrophic factor, and vascular endothelial growth factor, and others have been implicated in MDD (Björkholm & Monteggia, 2016; Carvalho et al., 2015; Duclot & Kabbaj, 2015; Duman & Monteggia, 2006; Miller, 2011; Turner, Watson, & Akil, 2012; Warner-Schmidt & Duman, 2007), greater effort should be made to understand if and

[3] *Data from this chapter will be prepared for publication after completing additional validation studies. Appendix 4.2 was published in Aurbach, E. L., Inui, E. G., Turner, C. A., Hagenauer, M. H., Prater, K. E., Li, J. Z., … Akil, H. (2015). Fibroblast growth factor 9 is a novel modulator of negative affect. Proceedings of the National Academy of Sciences, 112(38), 11953–11958. http://doi.org/10.1073/pnas.1510456112*

how these molecules interact with each other in the brain to mediate effects on emotionality. Indeed, building this deep understanding of the organizational and functional capacity of growth factors both inside and outside the adult central nervous system will be critical to avoid harmful side effects of pharmacological interventions and harness the potential to target growth factors with new antidepressant medications.

Previous literature and preceding chapters of this dissertation have highlighted FGF2 and FGF9 as particular targets of interest in the disrupted neural circuitry thought to underlie MDD. Indeed, previous studies demonstrated that FGF2 is disrupted in MDD in humans, and it acts as an endogenous anxiolytic and antidepressant substance in rodents (Bernard et al., 2011; Chaudhury et al., 2014; Evans et al., 2004; Gaughran, Payne, Sedgwick, Cotter, & Berry, 2006; Perez et al., 2009; Turner, Calvo, et al., 2008; Turner, Gula, Taylor, Watson, & Akil, 2008; Turner et al., 2012). While many fewer studies have examined FGF9's role in affect, the available evidence is striking and suggests that FGF9 may work in functional opposition to FGF2 (Aurbach et al., 2015; Salaria et al., 2006).

A variety of different types of data support the hypothesis that FGF2 and FGF9 act as physiological antagonists in the brain to mediate affect. Though some analyses, including the approach described in this thesis, have not all supported differential expression of FGF2 and FGF9 in MDD across all brain regions, we found some significant differences and report that FGF2 is decreased and FGF9 is increased in different regions of the depressed human brain (Aurbach et al., 2015; Bernard et al., 2011; Evans et al., 2004; Gaughran et al., 2006). This opposite pattern of dysregulation can also be observed in rodent models of MDD. In rodents, chronic stress decreases

hippocampal FGF2 expression, while the same manipulation increases FGF9 expression (Aurbach et al., 2015; Turner, Calvo, et al., 2008). Chronic administration of FGF2 has anxiolytic and antidepressant properties, while chronic administration of FGF9 is anxiogenic and pro-depressant (Aurbach et al., 2015; Perez et al., 2009; Turner et al., 2011; Turner, Gula, et al., 2008). In contrast, knocking down endogenous expression of FGF2 in the dentate gyrus of the hippocampus is anxiogenic, while knocking down FGF9 in the same region is anxiolytic. These studies and others support the idea that FGF2 and FGF9 act in opposite ways to mediate effects on emotionality in certain brain regions.

Although few studies have examined the effects of manipulating both FGF2 and FGF9 in the same samples, those that have support the hypothesis that FGF2 and FGF9 have opposite effects in MDD. In addition to the microarray studies described above, Salaria and colleagues (2006) examined the effects of three weeks of cortisol exposure on fetal brain aggregates, an *in vitro* model of chronic stress. Using microarray and immunohistochemistry, they found that FGF2 expression decreased and FGF9 expression increased after three weeks of cortisol (Salaria et al., 2006), supporting the idea that these FGFs are dysregulated in opposite directions in this model of chronic stress.

If FGF2 and FGF9 are coordinately and/or oppositely dysregulated to mediate affect and vulnerability in MDD, there are several possibilities for how these effects may be borne out biologically. In the simplest case, FGF2 and FGF9 may directly converge on the same receptor system to exert opposite effects on subsequent signaling pathways, which could mediate different molecular and cellular responses to each FGF. We attempted to examine this possibility experimentally after acute administration of both FGF2 and FGF9 *in vivo* (data not shown), but limitations to our approach prevented us

from drawing conclusions. Alternatively, *FGF2 and FGF9 may play important, but non-overlapping, organizational roles in molecular networks and/or brain circuitry, and the appearance of opposite effects may arise because we have previously chosen to examine affective behavior, which is a final common pathway influenced by a number of brain circuits and molecular systems*. Evidence supporting this interpretation might show different networks of molecules which are co-regulated with FGF2 and FGF9. Other approaches will not be examined in this chapter, but they could include probing other systems including brain circuit activation and/or molecular network co-regulation.

Because we have previously observed important – but sometimes inconsistent – alterations in FGF2 and FGF9 expression in MDD, we chose to determine if FGF family members might be acting as an ensemble of genes to search for more consistent effects. *Specifically, to examine the hypothesis that FGF2 and FGF9 played coordinate roles in the brain to mediate affective dysregulation, we explored if and how there were relationships in the patterns of expression between members of the FGF family (particularly FGF2, FGF9, and the FGF receptors), and we further examined whether these possible patterns might change as a function of MDD diagnosis.* Therefore, we returned to the postmortem human datasets described in Chapters 2 and 3, which include data on the expression of thousands of different transcripts in controls and individuals diagnosed with psychiatric illnesses.

To probe different molecular networks that may be co-regulated with FGF2 and FGF9, we examined patterns of expression between different FGF genes (data will be presented in later sections). We first sought to determine if we could observe relationships in expression between FGF2, FGF9, and the FGF receptors across all

samples, hypothesizing that if we did observe relationships, they might emerge in opposite directions. Indeed, we demonstrated that FGF2 showed positive relationships with the FGF receptors, while FGF9 showed negative relationships with FGF2 and with the FGF receptors. This opposite pattern in directionality in the relationship between expression of genes related to FGF2 and FGF9 was striking, but it did not help us to determine if and how these relationships might be altered with MDD. To that end, we examined how the relationships between FGF2, FGF9, and the FGF receptors altered with MDD diagnosis. We found that the relationship between a number of FGF gene pairs in the anterior cingulate cortex and the hippocampus was significantly altered with MDD diagnosis, so we decided to examine if and how these altered relationships impacted other genes and biological functions that they underlie.

To determine how these altered relationships co-varied with the expression of other genes, we chose two candidate gene pairs, FGF2-FGF9 in the anterior cingulate cortex and FGF9-FGFR1 in the hippocampus, whose relationships were altered as a function of MDD diagnosis, as a proof of concept. To our surprise, we observed a very interesting pattern: when there was a diagnosis-specific relationship between the gene pair, the expression of hundreds of other genes also correlated with both FGFs in the pair (the "overlapping" pool). In contrast, when there was no diagnosis-specific relationship between the gene pair, very few genes correlated with both FGFs in the pair. To understand if this pattern was biologically meaningful, we performed several ontology analyses to parse gene function. Specifically, we took two approaches: we used Ingenuity Pathway Analysis® to determine the broad categories to which the genes in the "overlapping" pools aligned, and we subsequently used a more fine-grained approach,

Gene Set Enrichment Analysis, to identify individual pathways or gene sets that were enriched in samples displaying a diagnosis-specific relationship with both FGF genes.

Because our analyses were intended to generate hypotheses that we could test in other samples and/or model systems, we partially validated the altered relationships discovered in human analyses between several FGF genes in the hippocampi of selectively bred high responder (bHR) and low responder (bLR) animals previously exposed to a chronic variable stress paradigm. However, we believe that this preliminary effort at cross-validation should be expanded to other datasets.

Materials and Methods

Human Studies

Subject Characteristics and Tissue Extraction. The human tissue samples used for all microarray experiments as well as the qRT-PCR validation were obtained from the Brain Donor Program at the University of California, Irvine with the consent of the next-of-kin of the deceased (Appendix 2.1, Appendix 2.2, & Appendix 2.3).

Dissection of Human Dorsolateral Prefrontal Cortex (DLPFC), Anterior Cingulate Cortex (ACC), and Hippocampus (HPC). For the first stage of dissection, a 1-cm coronal slab was placed on a block of dry ice. At the level of the genu of the corpus callosum, the middle frontal gyrus was identified by an expert in human neuroanatomy, and a fine jeweler's blade in a coping saw handle was used to excise the gyrus for dorsolateral prefrontal cortex samples. In some cases, more than one slab was used to cover the full extent of the structure. In the same plane, the cingulate gyrus was identified by an expert in human neuroanatomy, and a fine jeweler's blade in a coping saw handle was used to excise the gyrus for anterior cingulate cortex samples. In some cases, more

than one slab was used to cover the full extent of the structure. In more caudal planes, the hippocampus was visually identified using the dentate gyrus by an expert in human neuroanatomy, and a fine jeweler's blade in a coping saw handle was used to dissect the hippocampus and surrounding temporal cortex. Both sides of the slab were carefully inspected. In some cases, more than one slab was used to cover the full extent of the structure. For all structures, the block was wrapped in foil, placed on dry ice and then stored at -80°C until further processing (Jones et al., 1992). In the hippocampus, a second stage of finer dissection was then carried out. The temporal cortex was removed by visual inspection using the dentate gyrus and CA3 as landmarks. The dissected tissue was then processed for RNA extraction. Total RNA was extracted using procedures described previously (Evans et al., 2004; Li et al., 2004). Clinical information was obtained from medical examiners, coroners' medical records and a family member. Patients were diagnosed by consensus based on criteria from DSM-IV. For further information regarding data collection procedures, please see Li and colleagues (Li et al., 2013). Final Control and MDD sample sizes and demographics are in the appendices (DLPFC: Appendix 2.1, ACG: Appendix 2.2, HPC: Appendix 2.3).

Gene Expression Profiling. In general, the labeling and hybridization of sample mRNA with oligonucleotide probes followed standard manufacturer protocols. Analyses used the full microarray datasets (all probe data from the MDD and Control subjects, as well as from small samples of subjects with bipolar disorder and schizophrenia) to maximize our ability to identify technical artifacts and confounds, although the reported results focus on MDD vs. Control comparisons for a subset of growth factor probes.

Affymetrix Microarray. Microarray experiments were performed in eight separate experimental cohorts containing both patients and controls. The majority of RNA samples were analyzed in duplicate at two different laboratories using Affymetrix Genechips (either U133A or U133Plus-v2). After extracting summarized probe signal data from scanned microarray image files, all downstream analyses were completed in the R statistical programming environment. We extracted the U133A subset of probes and applied RMA (Robust Multi-array Analysis) to summarize probe set expression levels using custom ENTREZ12.1 Chip Definition Files (CDF) which defined probe sets for 11,912 transcripts (as defined by ENTREZ in 03/2010) and 68 control probe sets. As is traditional, all probe signal values were log (base 2)-transformed to reduce heteroskedasticity and quantile-normalized to remove technical artifacts in the overall distribution of signal per sample. To ensure sample quality, we required an average sample to sample correlation coefficient (r) of 0.85-0.9, excluding 6 of 235 samples (2.6%) in the HPC, 30 of 367 samples (8.1%) in the DLPFC, and 6 of 283 samples (2.1%) in the ACC. Batch effects due to cohort, laboratory, and platform were removed by median-centering the data. Replicate samples were then averaged and any subjects that were missing information were removed from the dataset (n = 16 in DLPFC, n = 13 in ACC, n=8 in HPC), leaving a final sample size of 156 subjects in the DLPFC, 140 subjects in the ACC, and 129 subjects in the HPC. For further information regarding the Affymetrix data pre-processing procedures, please see Li and colleagues (Li et al., 2013). For all of the microarray experiments, as part of quality control, subjects were verified to have gene expression typical of their reported sex using data from genes XIST, EIF1AY,

RPS4Y1, UTY, USP9Y, NLGN4Y, NCRNA00185, TTTY15, KDM5D, CYorf15B, and DDX3Y.

Microarray Analysis: Determining Diagnosis-Related Gene Expression while Correcting for Confounding Variables. Although agonal factor, brain pH, PMI, and gender did not differ significantly by diagnosis, we found that it was necessary to control for these variables because they strongly correlated with the top principal components of variation in the data sets (PC1-4). We also controlled for the average age at the time of death, which sometimes varied significantly with diagnosis. The degree of severity and duration of physiological stress at the time of death was estimated by calculating an Agonal Factor Score (AFS) for each subject (Tomita et al., 2004). Additionally, we measured the pH of cerebellar tissue as an indicator of the extent of oxygen deprivation experienced around the time of death (Li et al., 2004). We also calculated the interval between the estimated time of death and the freezing of the brain tissue (the postmortem interval or PMI) using coroner records. We ensured high quality data by choosing samples with relatively high pH and low agonal factor (DLPFC: Appendix 2.1, ACG: Appendix 2.2, HPC: Appendix 2.3), but still decided that it was prudent to control for these variables as well as age and gender by including them as terms in our linear model:

Equation 1: $(probe\ signal) \approx \beta_0 + \beta_1(Brain\ pH) + \beta_2(Agonal\ Factor) + \beta_3(PMI) + \beta_4(Age) + \beta_5(Gender) + \beta_6(Diagnosis)$

We further explored the relationships between FGF2, FGF9, and each of the FGF receptors. To ensure that the relationships that we observed were not due to the major confounds present in the dataset, we reduced their influence using estimates of their effects provided by the model in Equation 1:

Equation 2: $(\text{cleaned probe signal}) \approx (\text{probe signal}) - \beta_1(\text{Brain pH} - 6.88) + \beta_2(\text{Agonal Factor}) + \beta_3(\text{PMI} - 23.5) + \beta_4(\text{Age} - 52) + \beta_5(\text{Gender})$

This means that the data were projected to the median brain pH of 6.88, PMI of 23.5 hours, agonal factor of 0, age 52 years, and male gender. This allowed us to examine the relationship between the FGF-related genes in data resembling that from subjects with consistent pre- and postmortem factors. We examined the relationship between pairs of FGF genes across all samples using the linear model:

Equation 3: $(\text{cleaned probe signal for FGF2/9/R1/R2/R3}) \approx \beta_0 + \beta_1 (\text{cleaned probe signal for FGF2/9/R1/R2/R3})$

Example: $(\text{cleaned probe signal for FGF2 in the ACG}) \approx \beta_0 + \beta_1 (\text{cleaned probe signal for FGF9 in ACG})$

We were also interested in determining whether mood disorder diagnoses altered the relationships between FGF family members in different brain regions, and we examined this possibility using the linear model:

Equation 4: $(\text{cleaned probe signal for FGF2/9/R1/R2/R3}) \approx \beta_0 + \beta_1 (\text{cleaned probe signal for FGF2/9/R1/R2/R3}) + \beta_2(\text{Diagnosis}) + \beta_3((\text{Diagnosis}) * (\text{cleaned probe signal for FGF2/9/R1/R2/R3}))$

Example: $(\text{cleaned probe signal for FGF2 in the ACG}) \approx \beta_0 + \beta_1 (\text{cleaned probe signal for FGF9 in ACG}) + \beta_2(\text{Diagnosis}) + \beta_3((\text{Diagnosis}) * (\text{cleaned probe signal for FGF9 in ACG}))$

Microarray analysis: Determining other diagnosis-specific correlates of FGF family members. Because we observed diagnosis-specific relationships between individual FGF family members that differed across regions, we chose two pairs of genes whose interaction varied with diagnosis in different ways for further examination, as a

proof-of-concept. We hypothesized if these diagnosis-specific alterations in the relationships between FGF family members were meaningful, we might see concurrent changes in the expression of other genes as they related to FGF expression. To this end, we examined the relationships between (1) FGF2 and FGF9 in the ACG, whose relationship is lost in MDD, and (2) FGF9 and FGFR1 in the HPC, which show no relationship in expression in controls but a relationship emerges in MDD. We sought to understand the relationships between these FGFs with all the other genes in the dataset to determine if there were gene subsets whose region- and diagnosis-specific expression varied with these FGFs of interest. Furthermore, we examined if there were diagnosis-specific patterns in the numbers and sets genes that correlated with both FGFs in each pair. Therefore, within a diagnostic category (i.e., Control or MDD), we ran Pearson's correlations between each FGF (i.e., FGF2 or FGF9 in ACG and FGF9 or FGFR1 in the HPC) and all the other genes in the dataset for the region of interest. Because we examined nearly 12,000 pairs for each FGF, we corrected for multiple comparisons using the Benjamini-Hochberg method with a false detection rate of 0.05 (i.e., accepting 5% of results as false positives). We examined the proportion of genes that uniquely correlated with either FGF in the pair (the "non-overlapping" pool), as well as the proportion of genes that correlated with both FGFs (the "overlapping" pool). We assessed the statistical significance of these effects using Fisher's exact test and (the more conservative) McNemar's test.

Analysis of microarray data: Analyzing the biological functions of all genes correlating with FGFs of interest. We sought to understand if there were biological functions lost or gained with observed diagnosis-specific relationships between FGFs

(i.e., FGF2 or FGF9 in ACG and FGF9 or FGFR1 in the HPC). We used QIAGEN's Ingenuity® Pathway Analysis (IPA®, QIAGEN Redwood City, www.qiagen.com/ingenuity) to assess functional ontology, with a particular interest in the biological and physiological functions of the genes whose expression correlated with both FGFs (the "overlapping" pools of correlated genes, as opposed to those whose expression uniquely correlated with a single FGF; the "non-overlapping" pools of correlated genes). To that end, we calculated the mean expression level for each gene in the overlapping pool in both Control and MDD samples (after mathematically correcting for confounding factors, as described in Equation 2), and we used the results from the linear model described in Equation 1 to indicate diagnosis-specific effects. The proprietary IPA® software generated lists of top biological and physiological functions associated with the overlapping pool of each gene pair of interest.

To better parse specific networks of genes in the overlapping pools, we used gene set enrichment analysis (GSEA; GSEA software and Molecular Signature Database, MSigDB: Subramanian et al., 2005; http://www.broad.mit.edu/gsea/). The GSEA approach is more fine-grained than some ontology algorithms and examines if gene sets (i.e., curated lists of genes corresponding to known pathways or biological functions) are selectively enriched in different experimental samples – in this case, diagnosis (groups: Control, MDD). This differs from previous analyses because GSEA assesses how gene sets are altered with diagnosis (as opposed to individual genes). As before, we selectively examined the overlapping pools for each gene pair of interest to determine which gene sets were enriched in the Controls in the ACG (whose functions may be lost or altered in MDD) and which gene sets were enriched in the MDD subjects in the HPC (whose

functions emerge in MDD). We extracted the signal information for each gene target (corrected for confounding factors, as described in Equation 2, and annotated using the custom approach described above) for each subject in the Control and MDD samples, and we set the algorithm to run 1000 permutations. We restricted the analyses to the curated lists of reactome pathways (within MSigDB, the C2 REACTOME sets), to reduce the number of overall comparisons (from ~10,000 to 674) and focus directly on curated gene sets with known, associated biological functions. We focused our analyses on the leading edge subset of genes for each set, corresponding to the genes most enriched within each set in the diagnosis group of interest, as is common using the approach (Subramanian et al., 2005).

Animal Studies

Animals. Bred high responder (bHR) and low responder (bLR) rats were bred, weaned, and tested for a locomotor response to novelty between postnatal days 50-60, and housed in pairs in-house as previously described (Stead et al., 2006; Turner et al., 2011). All animals were maintained on a 12/12 light/dark schedule, with access to food and water *ad libitum*. All animals were treated in accordance with the National Institutes of Health *Guidelines on Laboratory Animal Use and Care* and in accordance with the guidelines set by the university committee on use and care of animals at the University of Michigan.

bHR and bLR chronic variable stress studies. Other members of our laboratory performed a chronic variable stress experiment and analyzed tissue for gene expression changes. bHR and bLR animals were reared without disruption until postnatal day 80-83. Animals were divided into chronic variable stress and no-stress control groups (n = 10

per group). Animals undergoing chronic variable stress experienced one of several stressors (cage tilt, isolation, novel environment, or restraint) per day for 14 days. Affective behavior was evaluated (forced swim test, social interaction test, sucrose preference test, and elevated plus maze) over four days following cessation of the chronic variable stress paradigm. Thirty minutes after elevated plus maze testing on the last day, animals were euthanized. Brains were rapidly extracted and hemisected. One hemisphere was snap-frozen in 2-methylbutane at -30°C and stored at -80°C until processing. The other hemisphere was dissected: dorsal hippocampal tissues were collected, stored in 1.5 mL Eppendorf tubes, frozen on dry ice, and stored at -80°C until processing.

Tissue Analysis.

Analysis of hippocampal gene expression using qRT-PCR. After dissection, samples were stored at -80°C prior to RNA extraction. Dorsal hippocampal RNA was extracted and purified using the Direct-Zol RNA Mini-Prep Kit according to manufacturer's instructions (Zymo Research, Irvine, CA), and concentrations were determined with a NanoDrop Spectophotometer (Thermo Scientific). Total RNA (200 ng) was reverse transcribed using the iScript cDNA synthesis kit (Bio-Rad Laboratories, Hercules, CA). cDNA (1 µl, diluted 1:10) was used as the template for real-time PCR assays with a MyiQ real-time PCR system (Bio-Rad Laboratories). The quantitative PCR was conducted in duplicate using iQ SYBR Green Supermix, according to the manufacturer's instructions (Bio-Rad Laboratories). Sequences for the 3' primer (start) were as follows: *GAPDH: GTTTGTGATGGGTGTGAACC; FGF2: TATGAAGGAAGATGGACGGC; FGF9: TTTCACTTAGAAATCTTCCCCAAC; FGFR1: CAGAGACCAACTTTCAAGCAGTT;* and *FGFR3: GAACAGCGCCTATAGCATCC.*

Sequences for the 5’ primer (start) were as follows: *GAPDH: TCTTCTGAGTGGCAGTGATG; FGF2: TCGTTTCAGTGCCACATACC; FGF9: TCCTGTGTTAGTTTTTCTGATCCA; FGFR1: ATGGCTCATGAGAGAAGACAGAG;* and *FGFR3: TCCGTTCCACAAACTCACAC*. (Note that these primers for FGFR1 and FGFR3 do not reference regions of the gene which determine splice variants, so these data do not address if there are differences in FGFR isoforms.) Relative expression of the gene of interest was normalized to GAPDH expression in each sample. The expression level of the gene of interest was evaluated using the $2^{-(\Delta\Delta Ct)}$ method (Livak & Schmittgen, 2001). The PCR product quality was monitored using post-PCR melt-curve analysis at the end of the amplification cycles. Because there was insufficient statistical power to include both bHRs and bLRs in a single linear model, we used the following model to examine the relationships between expression of FGF family members, stress, and their interaction within-phenotype. (Note that this approach is almost identical to that used in the human analyses above, but the stress manipulation replaces the diagnosis terms in the model.)

Equation 5: *[(Expression of FGF2/9/R1/ R3)* $\approx \beta_1$ *(Expression of FGF2/9/R1 /R3)+* β_2*(Stress) +* β_3*((Stress)* (Expression of FGF2/9/R1/ R3))] in either bHRs or bLRs*

Example: *[(Expression of FGF2)* $\approx \beta_1$ *(expression of FGF9)+* β_2*(Stress)+* β_3*((Stress)* (Expression of FGF9))] in bLRs*

Results

FGF2 expression positively correlates with expression of the FGF receptors in all samples across regions in the postmortem human brain. We explored possible

relationships between the expression of FGF2 and the FGF receptors (FGFRs) across all three brain regions (Figure 4.1). We found that FGF2 expression strongly correlated positively with FGFR2 and FGFR3 across diagnoses, but the relationship between FGF2 and FGFR1 was less strong or nonexistent across brain regions: FGFR1 (DLPFC: R^2 = 0.007, p = 0.313; ACC: R^2 = 0.064, p = 0.0026; HPC: R^2 = 0.036, p = 0.030), FGFR2 (DLPFC: R^2 = 0.257, p = 1 x 10^{-10}; ACC: R^2 = 0.177, p = 1 x 10^{-10}; HPC: R^2 = 0.163, p = 2 x 10^{-6}), and FGFR3 (DLPFC: R^2 = 0.273, p = 1 x 10^{-10}; ACC: R^2 = 0.264, p = 1 x 10^{-10}; HPC: R^2 = 0.14, p = 1.1 x 10^{-5}). This indicates that FGF2 may be acting in a molecule- and region-specific manner, though notably, the relationships between FGF2 and the FGF receptors are all positive in direction. These data support the idea that the frontal cortices and hippocampus may be sensitive to FGF2 dysregulation, even if it is subtle.

Opposite to FGF2, FGF9 expression negatively correlates with expression of FGF2 and the FGF receptors in all samples across regions in the postmortem human brain. We further explored whether the relationship between the expression of FGF9 and other candidate members of the FGF family across brain regions (Figure 4.2). We found that the expression of FGF9 was significantly negatively related to FGF2 (DLPFC: R^2 = 0.056, p = 0.0024; ACC: R^2 = 0.126, p = 1.7 x 10^{-5}; HPC: R^2 = 0.15, p = 5 x 10^{-6}) and to three FGF receptors: FGFR1 (DLPFC: R^2 = 0.036, p = 0.017; ACC: R^2 = 0.082, p = 0.0009; HPC: R^2 = 0.082, p = 0.0009), FGFR2 (DLPFC: R^2 = 0.201, p = 1 x 10^{-10}; ACC: R^2 = 0.201, p = 1 x 10^{-10}; HPC: R^2 = 0.172, p = 5 x 10^{-6}), and FGFR3 (DLPFC: R^2 = 0.141, p = 5 x 10^{-6}; ACC: R^2 = 0.193, p = 1 x 10^{-10}; HPC: R^2 = 0.193, p = 1 x 10^{-10}) across all three brain regions. This suggests a general dysregulation in the FGF system in MDD, with FGF9 appearing to be one of the key players. The strength of the relationship

varied by region, with the hippocampus and anterior cingulate showing the strongest relationships, and by FGF family member, with FGF2, FGFR2, and FGFR3 consistently showing strong relationships with FGF9. This suggests that there may be region- and molecule-specific effects that are worthy of further investigation. Similar to FGF2, these data support the idea that the frontal cortices and hippocampus are sensitive to FGF9 dysregulation, and that other members of the FGF family may be particularly sensitive to subtle dysregulation. Notably, the negative relationships between FGF9 and the FGF receptors stand in contrast to the positive relationships we observed with FGF2.

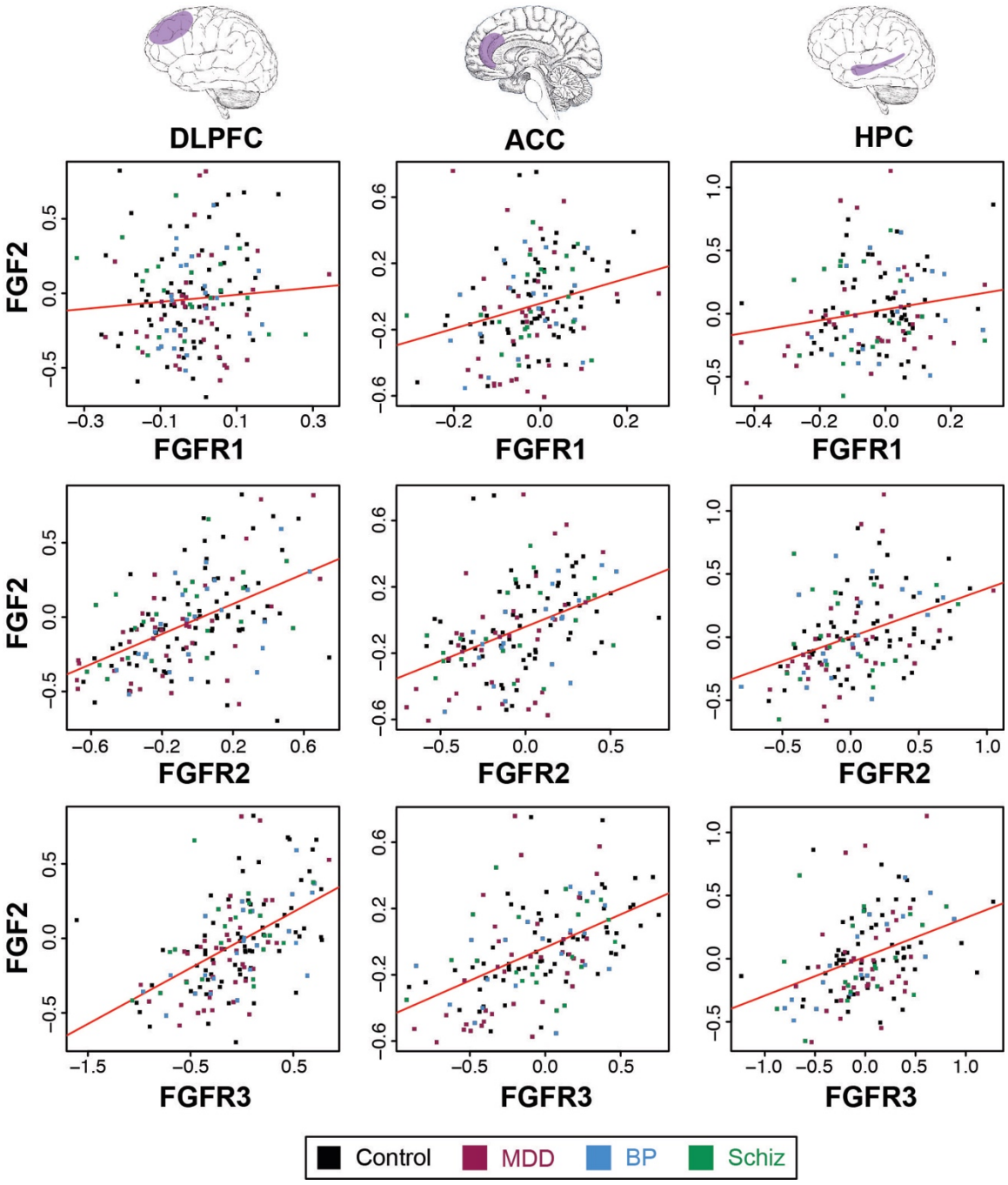


Figure 4.1. FGF2 gene expression correlates with gene expression of FGFR1, FGFR2, and FGFR3 across brain regions in the full sample, with one exception: the relationship between FGF2 and FGFR1 is not significant in the dorsolateral prefrontal cortex. MDD: major depressive disorder; BP: bipolar disorder; Schiz: Schizophrenia. Sample sizes can be found in Appendix 2.1, Appendix 2.2, and Appendix 2.3.

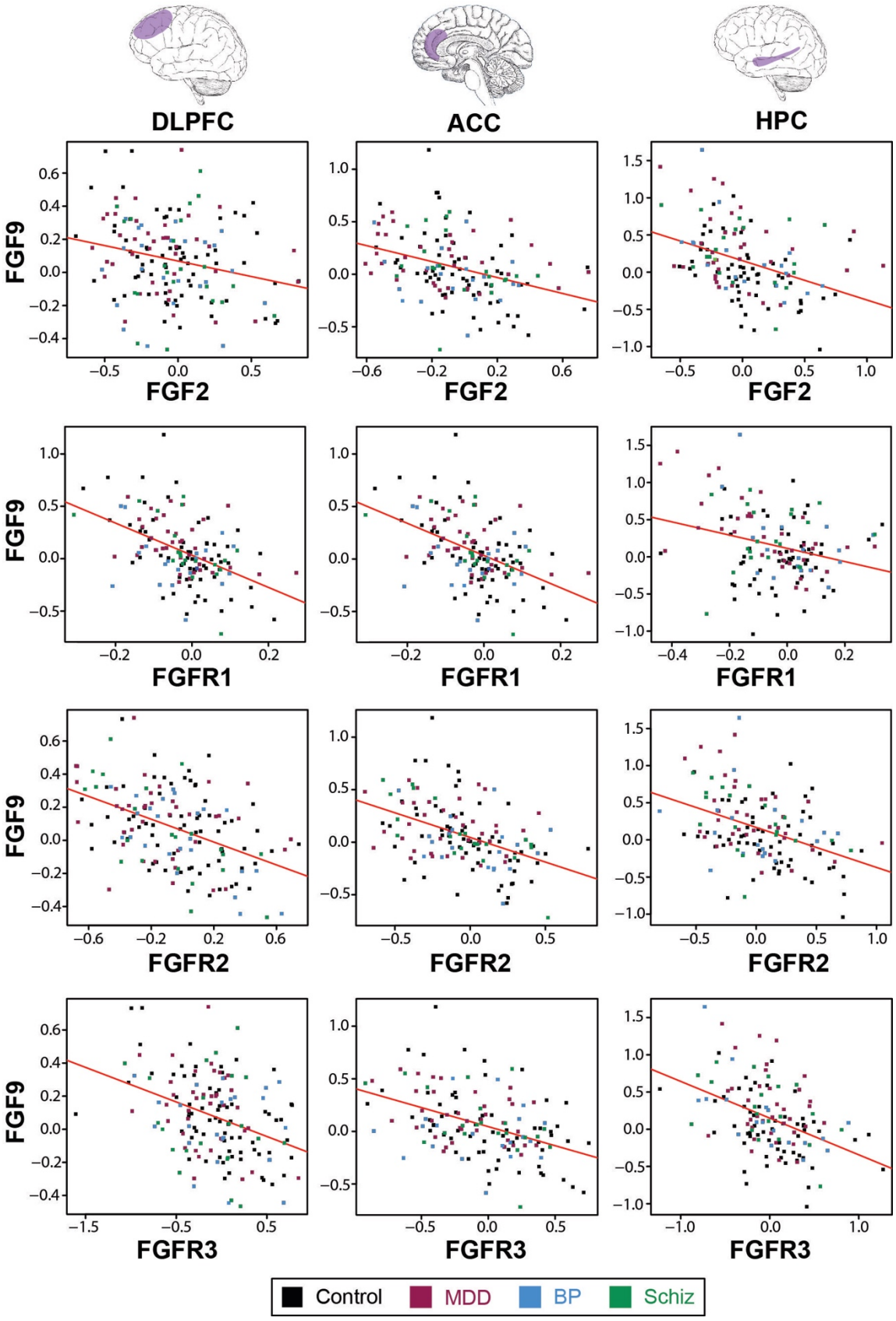

DLPFC
ACC
HPC
FGF9
FGF2
FGFR1
FGFR2
FGFR3
Control
MDD
BP
Schiz

Figure 4.2. FGF9 gene expression negatively correlates with FGF2, FGFR1, FGFR2, and FGFR3 gene expression in the dorsolateral prefrontal cortex, anterior cingulate cortex, and hippocampus across the full sample. MDD: major depressive disorder; BP: bipolar disorder; Schiz: Schizophrenia.Sample sizes can be found in Appendix 2.1, Appendix 2.2, and Appendix 2.3.

FGF2 and FGF9 show diagnosis-specific correlations with each other and with specific FGF receptors, and these correlations vary by region. Given that we previously observed robust correlations between FGF2 (Figure 4.1), FGF9 (Figure 4.2), and the FGF receptors across diagnosis categories in DLPFC, ACC, and HPC, we sought to determine if some of these relationships were diagnosis-specific. (That is, we re-analyzed the data to determine if there were particular relationships between pairs of FGF genes that emerged, were lost, or changed direction in MDD tissue relative to nonpsychiatric control tissue). We examined diagnosis-specific relationships between pairs of FGF genes within each region (e.g., FGF2-FGF9 in the ACC) using a linear model. We found that several FGF gene pairs showed diagnosis-specific relationships (results are summarized in Table 4.1), and these relationships differed by region. The DLPFC showed no changes in any gene-gene relationships as a result of diagnosis. In the ACC, relationships between FGF family member genes were generally lost in MDD and BP tissue relative to controls. In the HPC, relationships emerged in MDD and BP tissue relative to controls. We selected two such relationships, FGF2-FGF9 in the ACC (relationship lost in MDD, Figure 4.3) and FGF9-FGFR1 in the HPC (relationship emerges in MDD, Figure 4.4) for further analysis as a proof-of-concept, to determine if these altered relationships were biologically meaningful.

*Table 4.1. Relationships between FGF ligands and receptors are lost in the ACC and gained in the HPC in mood disorders. + indicates 0.1<p<0.05; * indicates p<0.05; ** indicates p<0.01. Gene pairs selected for further analysis are in bold.*

Region	*Gene Pair*	*Diagnosis-Specific Interaction (p-value)*	*Significance*	*Nature of Relationship in MDD relative to Control tissue*
	FGF2-FGF9	0.6523		no change
	FGF2-FGFR1	0.723		no change
	FGF2-FGFR2	0.505		no change
DLPFC	FGF2-FGFR3	0.198		no change
	FGF9-FGFR1	0.2141		no change
	FGF9-FGFR2	0.59726		no change
	FGF9-FGFR3	0.7359		no change
	FGF2-FGF9	**0.0397**	*****	**lost**
	FGF2-FGFR1	0.3335		no change
	FGF2-FGFR2	0.0738	+	lost
ACC	FGF2-FGFR3	0.172		no change
	FGF9-FGFR1	0.0945	+	lost
	FGF9-FGFR2	0.0447	*	lost
	FGF9-FGFR3[†]	0.3719		no change
	FGF2-FGF9	0.23998		no change
	FGF2-FGFR1	0.458		no change
	FGF2-FGFR2	0.27552		no change
HPC	FGF2-FGFR3[††]	0.1558		no change
	FGF9-FGFR1[††]	**0.00123**	******	**emerges**
	FGF9-FGFR2	0.43099		no change
	FGF9-FGFR3	0.849458		no change

[†] A significant relationship is lost in bipolar disorder (ACC, FGF9-FGFR3: p = 0.0224).
[††] A significant relationship emerges in bipolar disorder (HPC, FGF2-FGFR3: p = 0.0379; HPC, FGF9-FGFR1: p = 0.0384).

Diagnosis-specific relationships between FGF family members are associated with larger pools of genes correlating with both members of the pair. Because we are interested in understanding the molecular networks impacted by FGF dysregulation, we examined the within-region relationships between specific FGFs (i.e., FGF2-FGF9 in the ACC and FGF9-FGFR1 in the HPC) and all other genes in the Affymetrix dataset. For the candidate gene pairs whose relationship changed with diagnosis (i.e., FGF2-FGF9 in the ACC and FGF9-FGFR1 in the HPC), we examined the proportion of genes

correlating with both members of the target pair (the "overlapping pool") as well as the proportion of genes which uniquely correlated with one member of the target pair (the "non-overlapping pool") for both control and MDD tissue. In both regions, we found that the overlapping pools were large when there was a correlation between the gene pair, but the overlapping pools were small when there was no correlation between the gene pair, suggesting that the pattern held for both of our proof-of-concept cases. Case 1: In the ACC, there is a significant correlation between FGF2 and FGF9 in control tissue, and 624 genes correlate with both FGF2 and FGF9. In MDD tissue, the correlation between FGF2 and FGF9 is lost, and only 20 genes correlate with both FGF2 and FGF9. The relationship between diagnosis and overlapping pool size (i.e., change in the number of transcripts correlating with both FGF2 and FGF9 in Control vs. MDD tissue) is highly significant (Figure 4.3; Fisher's exact test: $p < 0.0001$; [more conservative] McNemar's $\chi^2 = 2991.5$, $p < 0.0001$). Case 2: In contrast, there is no relationship between FGF9 and FGFR1 in control tissue in the HPC, and only 73 genes correlate with both FGF9 and FGFR1. In MDD, a relationship between FGF9 and FGFR1 emerges, and 678 genes correlate with both FGF9 and FGFR1. This relationship between diagnosis and overlapping pool size with this pair is also highly significant (Figure 4.4; Fisher's exact test: $p < 0.0001$; [more conservative] McNemar's $\chi^2 = 1229.3$, $p < 0.0001$).

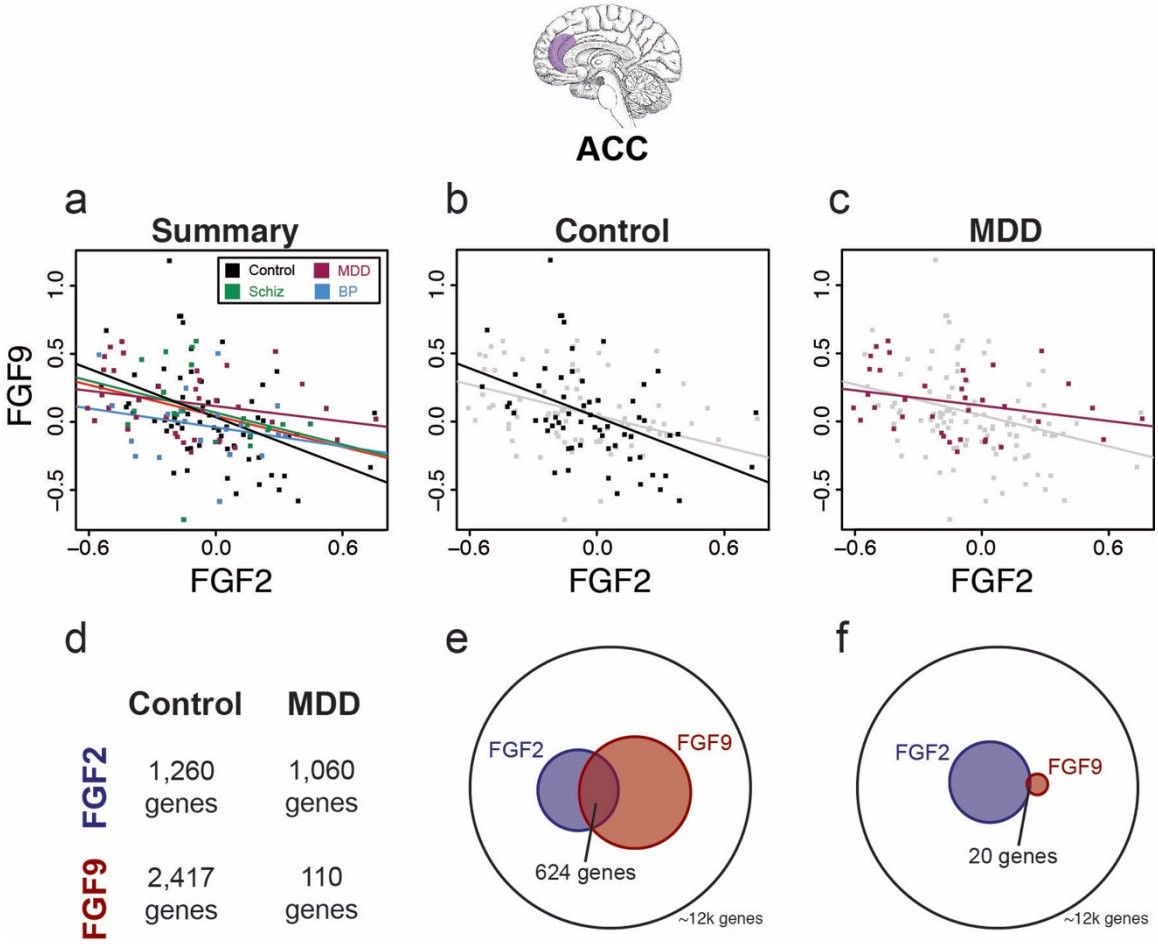


Figure 4.3. Anterior cingulate cortex. In control tissue, there is a correlation between gene expression of FGF2 and FGF9, and many genes correlate with both FGF2 and FGF9. In MDD tissue, this relationship is lost, and very few genes correlate with both FGF2 and FGF9. Note: genes were required to survive correction for multiple comparisons in order to be included in this analysis. (a) Summary of all diagnostic categories with associated regression lines (bright red indicates cumulative regression line). (b) Plot from a) with only control data highlighted; grey line indicates cumulative regression line (red in a). (c) Plot from a) with only MDD data highlighted; grey line indicates cumulative regression line (red in a). (d) Total number of genes that correlate (and survive multiple comparisons correction) with either FGF2 or FGF9 in control or MDD tissue. (e) Overlapping pool size in control tissue. (f) Overlapping pool size in MDD tissue. Sample sizes can be found in Appendix 2.1, Appendix 2.2, and Appendix 2.3.

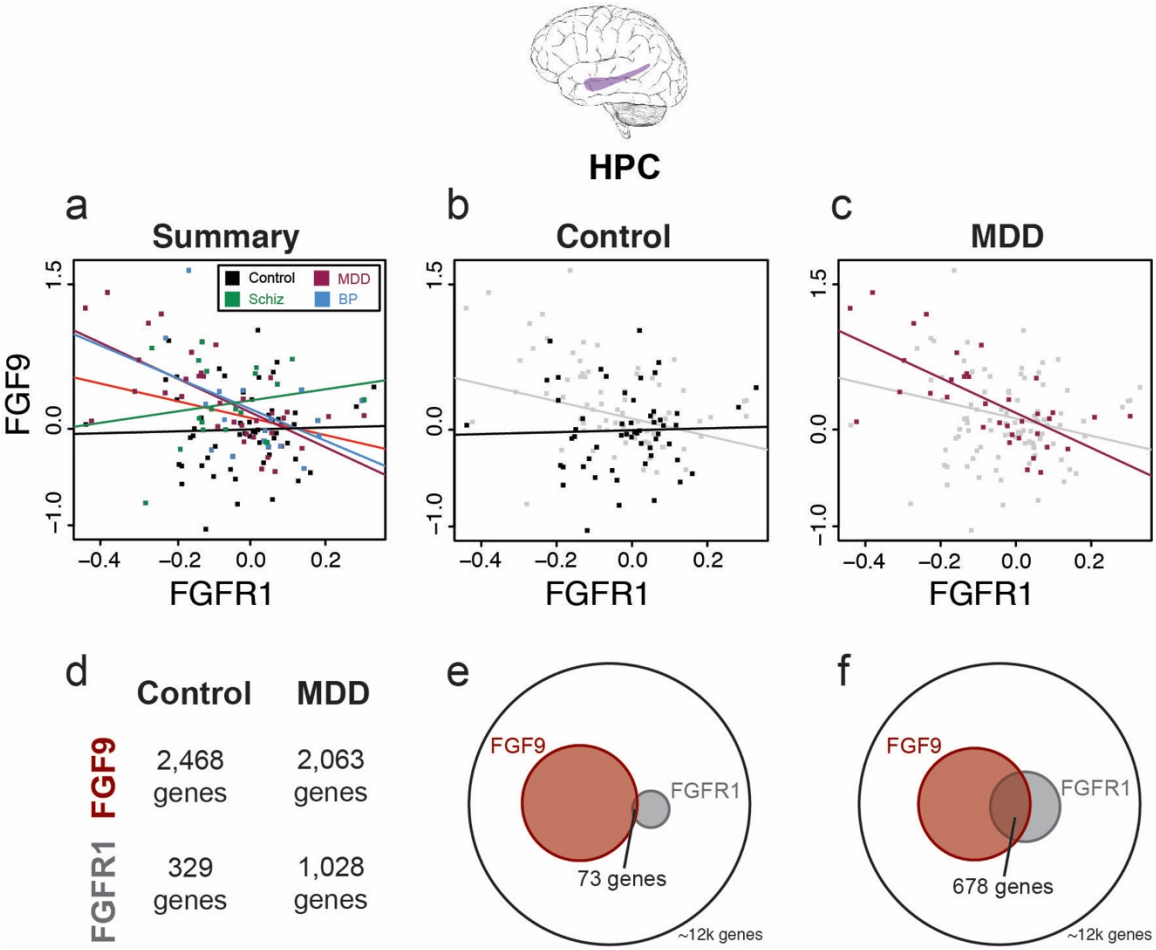


Figure 4.4. Hippocampus. In control tissue, there is no correlation between expression of FGF9 and FGFR1, and very few genes correlate with both FGF9 and FGFR1. In MDD tissue, a relationship between FGF9 and FGFR1 emerges, and many genes correlate with both FGF9 and FGFR1. Note: genes were required to survive correction for multiple comparisons in order to be included in this analysis. (a) Summary of all diagnostic categories with associated regression lines (bright red indicates cumulative regression line). (b) Plot from a) with only control data highlighted; grey line indicates cumulative regression line (red in a). (c) Plot from a) with only MDD data highlighted; grey line indicates cumulative regression line (red in a). (d) Total number of genes that correlate (and survive multiple comparisons correction) with either FGF9 or FGFR1 in control or MDD tissue. (e) Overlapping pool size in control tissue. (f) Overlapping pool size in MDD tissue. Sample sizes can be found in Appendix 2.1, Appendix 2.2, and Appendix 2.3.

Gene ontology analysis suggests that genes related to circuit formation and maintenance are particularly associated with FGF dysregulation. In order to better understand the biological significance of the genes in the overlapping pools, we

examined genetic ontology of the overlapping pools for both regions and gene-pairs of interest using Ingenuity® Pathway Analysis (QIAGEN) to determine the top biological and physiological functions lost and gained with FGF dysregulation in MDD. In the ACC, the 624 genes that correlate with both FGF2 and FGF9 in control tissue (and are lost in MDD tissue) primarily corresponded to biological functions that were involved in cellular microcircuit formation and maintenance, including Cell Morphology and Cell Death and Survival, suggesting that these are among the functions likely altered in the ACC in MDD (Table 4.2). Similarly, in the HPC, few genes correlate with both FGF9 and FGFR1 in control tissue, but the 678 genes that emerge in the overlapping pool for MDD tissue also have biological functions related to circuit formation and maintenance, including Cell Morphology, Cell-to-Cell Signaling and Interaction, and Cellular Function and Maintenance (Table 4.3). In both examples, the top physiological function relates to Nervous System Development and Function (Table 4.2 & Table 4.3).

Table 4.2. Top associated functions with the 624 genes that correlate with both FGF2 and FGF9 (the overlapping pool) in ACC control tissue.

Biological Functions

Function	***p-value range***	***# molecules***
Cell Morphology	2.64×10^{-2}- 6.08 x $^{10-6}$	78
Molecular Transport	2.41×10^{-2} - 2.79×10^{-5}	130
Carbohydrate metabolism	2.86×10^{-2} - 1.23×10^{-4}	63
Post-Translational Modification	2.53×10^{-2} - 1.48×10^{-4}	33
Cell Death and Survival	2.72×10^{-2} - 2.06×10^{-4}	226

Physiological System & Development Functions

Function	***p-value range***	***# molecules***
Nervous System Development and Function	2.72×10^{-2} - 7.39×10^{-5}	133
Tissue Morphology	2.96×10^{-2} - 7.39×10^{-5}	80
Behavior	7.00×10^{-3} - 1.143×10^{-3}	9
Embryonic Development	2.92×10^{-2} - 1.95×10^{-3}	91
Organ Development	2.41×10^{-2} - 1.95×10^{-3}	31

Table 4.3. Top associated functions with the 678 genes that correlate with both FGF9 and FGFR1 (the overlapping pool) in HPC MDD tissue.

Biological Functions

Function	***p-value range***	***# genes***
Cellular Assembly and Organization	4.90×10^{-2} - 1.56×10^{-5}	194
Cellular Function and Maintenance	4.99×10^{-2} - 1.56×10^{-5}	185
Cell-to-Cell Signaling and Interaction	4.99×10^{-2} - 1.90×10^{-5}	101
Cell Morphology	4.99×10^{-2} - 1.97×10^{-5}	138
Cellular Development	4.99×10^{-2} - 8.35×10^{-5}	120

Physiological System & Development Functions

Function	***p-value range***	***# genes***
Nervous System Development and Function	4.99×10^{-2} - 1.90×10^{-5}	148
Tissue Development	4.86×10^{-2} - 8.35×10^{-5}	142
Embryonic Development	4.29×10^{-2} - 5.21×10^{-4}	77
Organismal Development	4.99×10^{-2} - 5.81×10^{-4}	68
Cardiovascular System Development and Function	4.99×10^{-2} - 1.30×10^{-3}	60

Gene Set Enrichment Analysis identifies NCAM1 Interactions among gene sets "lost" in the ACC and FGFR Activation and Transcription/mRNA Processing among gene sets "emerging" in the HPC in MDD. To better understand the broad results from IPA® with more detail, we used a GSEA algorithm (Subramanian et al., 2005) to examine the diagnosis-specific biological functions within each pool of overlapping genes. For both gene pairs whose relationship varies with diagnosis, several gene sets were nominally significant (Table 4.4 & Table 4.5), though none survived correction for multiple comparisons using a false detection rate of 0.25. In the ACC, none of the gene sets passed a 0.01 significance threshold, though 12 passed a 0.05 threshold; several of these are related to FGF signaling (e.g., NCAM1 Interaction) or have been implicated in psychiatric dysregulation (e.g., GABA-B Receptor Activation). Other gene sets enriched in this pool relate to fundamental biology, including Pyrimidine Metabolism and Catabolism and Chondroitin Sulfate Synthesis and Metabolism (Table 4.4).

Table 4.4. Significant gene sets enriched in the pool of genes correlating with both FGF2 and FGF9 (the overlapping pool) in the ACC in Control samples. Lost functions can be grouped into categories, including GABA-B Receptor Activation, NCAM1 Interactions, Inhibition of Voltage-Gated Calcium Channels, Lipid and Pyrimidine Metabolism/Catabolism, and Chondroitin Sulfate Synthesis and Metabolism.

MSigDB Reactome Gene Set	***|Normalized Enrichment Score|***	***Nominal p-value***
GAB1 Signalosome	1.511	0.011
GABA-B Receptor Activation	1.559	0.017
TRAF6-Mediated Induction of TAK1 Complex	1.381	0.026
Metabolism of Lipids and Lipoproteins	1.567	0.027
Pyrimidine Catabolism	1.305	0.027
Inhibition of Voltage-Gated CA_2 Channels via GBeta Gamma Subunits	1.440	0.027
Pyrimidine Metabolism	1.483	0.037
NCAM1 Interactions	1.432	0.043
Inactivation of IRF3 IRF7 by TBK1 IKK Epsilon	1.293	0.045
IKK Complex Recruitment Mediated by RIP1	1.293	0.045
Chondroitin Sulfate Biosynthesis	1.391	0.049
Chondroitin Sulfate Dermatan Sulfate Metabolism	1.391	0.049

In the HPC, 5 gene sets passed a 0.01 significance threshold, and an additional 11 gene sets passed a 0.05 significance threshold. Among the gene sets enriched in MDD, we can extract several notable commonalities, including FGF receptor ligand binding and activation (including mutants), DNA replication, and transcription/mRNA processing, providing face validity to the approach. Additionally, fundamental biological functions including vitamin metabolism was significantly enriched in MDD among the genes whose expression correlates with both FGF9 and FGFR1 in the HPC (Table 4.5). Notably, Cell to Cell Communication was the top gene set enriched in MDD samples in this dataset that just missed the 0.05 cutoff ($p = 0.0517$).

Table 4.5. Significant gene sets enriched in the pool of genes correlating with both FGF9 and FGFR1 (the overlapping pool) in the HPC in MDD samples. Emergent functions can be grouped into categories including FGF receptor activation (including activated mutants), transcription and mRNA processing, and vitamin metabolism.

MSigDB Reactome Gene Set	***|Normalized Enrichment Score|***	***Nominal p-value***
Activation of Rac	1.394	<0.001
Metabolism of Vitamins and Cofactors	1.468	0.002
mRNA Splicing - Minor Pathway	1.450	0.004
Striated Muscle Contraction	1.453	0.008
Muscle Contraction	1.453	0.008
Vitamin B5 Pantothenate Metabolism	1.346	0.024
SLBP-dependent Processing of Replication-Dependent Histone Pre-mRNAs	1.325	0.032
Processing of Capped Intronless Pre-mRNA	1.325	0.032
Activation of the Pre-Replicative Complex	1.409	0.041
Activation of ATR in Response to Replication Stress	1.409	0.041
G2 M Checkpoints	1.409	0.041
Activated Point Mutants of FGFR2	1.310	0.043
Signaling by FGFR3 Mutants	1.310	0.043
FGFR2c Ligand Binding and Activation	1.310	0.043
FGFR4 Ligand Binding and Activation	1.310	0.043
DSCAM Interactions	1.393	0.044

Hippocampal gene expression of FGF2 correlates with FGF9 and FGF receptors in bLRs but not bHRs, and in some cases, stress alters these relationships. We sought additional evidence to reinforce the microarray results demonstrating that the relationship between FGF family members was altered with diagnosis. To that end, we examined expression of FGF2, FGF9, and two FGF receptors, FGFR1 and FGFR3, in the hippocampus of bHR and bLR rats after undergoing chronic variable stress during adulthood. We observed that FGF gene expression was unrelated in bHR animals (Gene-Gene Relationship (p-value) column of Table 4.6). In contrast, we observed trends or significant relationships between several FGFs pairs in bLRs, including FGF2-FGF9, FGF2-FGFR1, FGF2-FGFR3, and FGF9-FGFR3 (Gene-Gene Relationship (p-value)

column of Table 4.6). In two cases, these expression relationships in bLRs were altered by stress: a relationship between FGF2 and FGFR3 changed direction after chronic variable stress (Figure 4.5; $p = 0.043$), while a relationship between FGF9 and FGFR3 was lost after stress (Table 4.6; $p = 0.028$). (Note that the primers for FGFR1 and FGFR3 did not encompass the coding region of the gene that determines ligand binding specificity through splice variants, so these data do not address if there are differences in expression for the IIIb and IIIc isoforms.) These results partially reinforce the findings in the postmortem human microarray studies.

*Table 4.6. In bLRs but not bHRs, hippocampal expression of different FGF family members correlates and, in some cases, is modulated by stress. qPCR data courtesy of Cigdem Aydin and Pamela Maras; analyses by Elyse L. Aurbach. Data in bold is plotted in Figure 4.5. + indicates 0.1>p>0.05; * indicates p < 0.05; ** indicates p < 0.01.*

Phenotype	*Gene Pair*	*Gene-Gene Relationship (p-value)*	*Stress Interaction (p-value)*	*Nature of Relationship in Stress Relative to Control*
	FGF2-FGF9	0.544	0.578	no change
	FGF2-FGFR1	0.181	0.496	no change
bHR	FGF2-FGFR3	0.343	0.483	no change
	FGF9-FGFR1	0.848	0.517	no change
	FGF9-FGFR3	0.740	0.390	no change
	FGF2-FGF9	0.009**	0.577	no change
	FGF2-FGFR1	0.013*	0.335	no change
bLR	**FGF2-FGFR3**	**0.032***	**0.043***	**changes direction**
	FGF9-FGFR1	0.316	0.618	no change
	FGF9-FGFR3	0.092^{+}	0.028*	lost

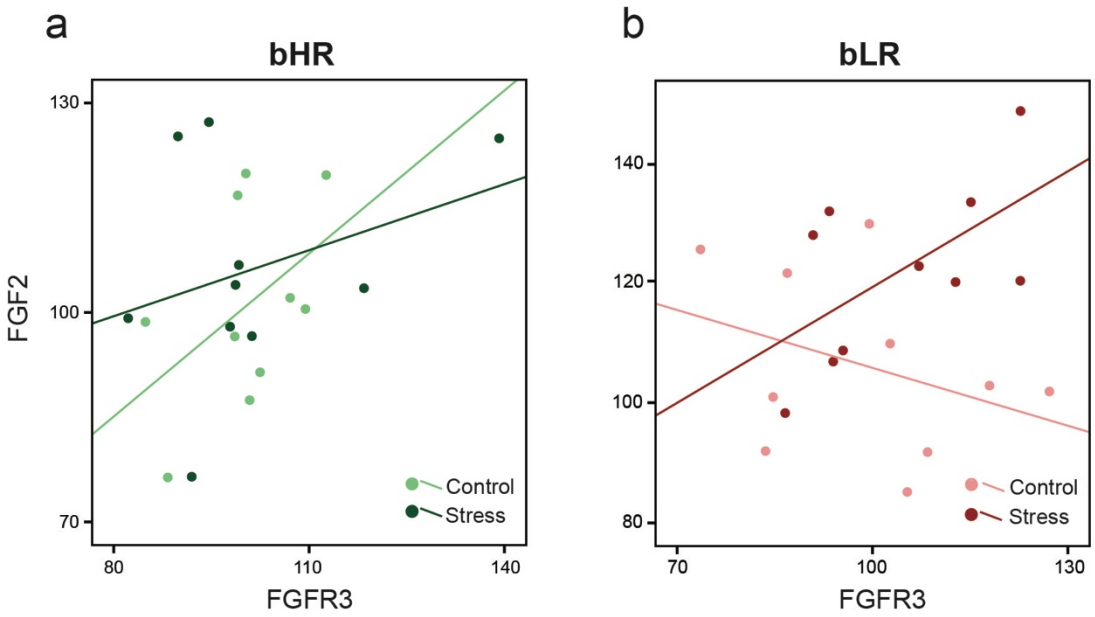


Figure 4.5. Stress changes the direction of the relationship between FGF2 and FGFR3 in (b) bLR animals (from a weak negative relationship to a strong positive one), while there is no relationship between expression of FGF2 and FGFR3 in (a) bHR animals. n = 8-10 animals/group.

Discussion

In this chapter, we sought to examine if and how FGF2 and FGF9 were coordinately dysregulated both with each other and with FGF receptors in MDD. In postmortem human samples, expression of FGF2 positively correlated with expression of all three FGF receptors across regions (except FGFR1 in the DLPFC), and FGF9 expression negatively correlated with FGF2 expression as well as with all three FGF receptors across regions. This pattern supports the idea that these transcripts are oppositely dysregulated and further indicates that absolute levels of FGF2 and FGF9 may matter less than their interactions with family members.

To understand if these relationships were altered in mood disorders, we examined if the relationships between FGF2, FGF9, and several FGF receptors changed with

diagnosis. We found that there were region- and diagnosis-specific alterations in the relationships between various FGF family members: we generally observed a loss of relationships between FGF family members in the anterior cingulate cortex in MDD, while new relationships emerged in the hippocampus in MDD or bipolar disorder. We selected one such pair of FGF genes from each region for further study as a proof-of-concept, and we examined the genes whose expression correlated with both members of that gene pair using functional ontology and gene set enrichment analysis. In the ACC, a relationship between FGF2 and FGF9 is present in Control tissue but lost in MDD samples. This diagnosis-specific relationship co-occurs with a significant reduction in the number of genes correlating with both FGF2 and FGF9, and further analyses indicate that the functions of these genes relate to circuit maintenance. In the HPC, there is no relationship between FGF9 and FGFR1 in control tissue, but a significant relationship emerges in both MDD and bipolar samples. This change co-occurs with a significant increase in the number of genes correlating with both FGF9 and FGFR1 in MDD. Biological functions related to circuit formation and maintenance are again implicated: particular targets for further examination are related to FGFR activation, DNA replication, and transcription and mRNA processing.

To reinforce the diagnosis-specific relationships between FGF family members, we examined relationships between hippocampal expression of FGF2, FGF9, FGFR1, and FGFR3 in an animal model which combines differential vulnerability to affective dysregulation with stress exposure. We observed no relationships between FGF family members and no effect of stress in resilient bHR animals. In contrast, vulnerable bLR animals showed significant relationships in expression for several pairs of FGFs, and in

some cases, these relationships were modulated by stress. These studies partially reinforce the findings in the postmortem human data, suggesting that the relationships between FGF family members may be particularly sensitive to disruption in an animal model of vulnerability to MDD.

Collectively, these data highlight the idea that individual FGFs may not be working independently to mediate affective dysregulation. Indeed, this work indicates that the patterns in relationships between FGF ligands and receptors may be particularly vulnerable to disruption in MDD, and these disruptions may influence a number of other biological functions necessary for appropriate neural circuit maintenance and function. Therefore, these data collectively support the hypothesis that FGFs may act as molecular "organizers" to mediate affective dysregulation. (Here, we operationally define this organizational capacity as mediating broad influence over different functions, including gene expression, to impact molecular networks and cellular microcircuits.)

FGF2 and FGF9 negatively correlate with each other and demonstrate opposite patterns of correlations with FGF receptors across samples and brain regions.

We uncovered robust positive relationships between FGF2 and the three FGF receptors expressed in the brain, and the consistency of these relationships suggests that even subtle alterations in FGF2 expression may have dramatic effects on other members of its molecular family or vice versa. Surprisingly, the strongest relationships with FGF2 were between FGFR2 and FGFR3, not FGFR1, which *in vitro* studies suggest is FGF2's putative primary binding partner (Ornitz et al., 1996; Zhang et al., 2006; Goetz & Mohammadi, 2013). Ligand-receptor affinity relationships for the FGF family are not known *in vivo*, and similarly, it is not known whether receptor affinity or density is more

important for the observed effects on affect previously reported for individual FGFs. Because the alterations in FGF2 expression are concurrent with changes in expression of other FGF family members, we suggest that the relationship with expression of FGFR2 and FGFR3 may be particularly important for FGF2 function across brain regions.

In contrast, we uncovered a significant negative correlation between FGF9 expression and FGF2 expression in postmortem tissue across all three brain regions, and this relationship supports the idea that these transcripts work in functional opposition. Furthermore, we observed that FGF9 negatively correlated with all three FGF receptors, with the strongest relationships with FGF9 again appearing between FGFR2 and FGFR3, which are FGF9's putative preferential binding partners according to *in vitro* data (Ornitz et al., 1996; Zhang et al., 2006; Goetz & Mohammadi, 2013). These data again stand in contrast to the strong positive relationships that FGF2 shows with FGFR2 and FGFR3, and it provides additional evidence that these transcripts may work in functional opposition in the brain to mediate affect.

The relationships in expression between FGF2, FGF9, and FGFRs underscore the biological significance of FGF family dysregulation in affective disorders: these relationships indicate that both FGF2 and FGF9 may be acting in a complex network of molecules, and these complex network interactions may have more relevance to MDD pathophysiology than examining absolute levels of either transcript in isolation. Furthermore, these relationships indicate that even subtle changes in FGF2 or FGF9 expression levels may unbalance the expression of other FGF family members or vice versa. To better understand this possibility as it relates to MDD, we examined how the

expression relationships between individual FGF family members changed with MDD diagnosis.

Relationships between some FGF family members change with diagnosis, and these changes co-occur with alterations in gene expression underlying circuit function.

We sought to understand if there were diagnosis-specific relationships between FGF family members, which would indicate that the relationships between FGFs were altered as a function of disease. We found that the general trends in relationship differed by region: in the ACC, relationships between FGF genes that were typically present in control tissue were lost, while in the HPC, new relationships between FGF family members emerged in MDD (and in some cases, bipolar disorder) tissue. To determine the biological impact of these alterations in FGF gene-gene relationships, we have initially focused on two gene pairs of interest as a proof-of-concept, FGF2 and FGF9 in the ACC (whose relationship is lost in MDD tissue) and FGF9 and FGFR1 in the HPC (whose relationship emerges in MDD tissue), and examined if other genes in the Affymetrix dataset correlated within-region with one or both members of the target gene pair. We found that between 600-700 genes correlated with both FGF genes when there was a diagnosis-specific relationship (i.e., 624 genes correlated with both FGF2 and FGF9 in control tissue, Figure 4.3e; 678 genes correlated with both FGF9 and FGFR1 in MDD tissue, Figure 4.4f), but very few genes correlated with both FGF genes when there was no relationship (e.g, only 20 genes correlated with both FGF2 and FGF9 in MDD tissue, Figure 4.3f; only 73 genes correlated with both FGF9 and FGFR1 in control tissue, Figure 4.4e).

Although the size of the overlapping pool (the number of genes whose expression correlates with both FGFs in the pair) and the relationship with diagnosis was highly significant, we noticed that the pools of genes uniquely correlating with FGF9 in the ACC and FGFR1 in the HPC were quite small (110 genes and 329 genes, respectively). We reasoned that a reduction in variability for each sample could influence the size of the correlating pool, so we calculated the range and standard deviation for each FGF gene (or GAPDH as a comparison, "control" gene, which did not vary with MDD diagnosis, $p = 0.504$) within diagnosis groups. Additionally, we examined if the variance in FGF (or GAPDH) gene expression was statistically different between Control and MDD tissue (Appendix 4.1). We found that hippocampal FGFR1 displayed significantly different variances between Control and MDD samples, with the distribution of data clustered more tightly in the middle of the distribution in the MDD group. Similarly, variance of FGF9 expression between Control and MDD tissue in the anterior cingulate was trending towards significance, with a less expansive range of data in the MDD group relative to the Control samples. In contrast, FGF2 in the ACC, FGF9 in the HPC, and GAPDH in both regions showed equivalent variability for both Control and MDD samples (Appendix 4.1). It may be that this alteration in variability represents a meaningful biological change in the interplay of genes associated with FGF9 expression in the ACC and FGFR1 expression in the HPC. Alternatively, this result might reflect a mathematical artifact that could be driving the diagnosis-specific effects in the linear model, although further analysis of the variability of other genes in this dataset argues against this possibility. Furthermore, it should be noted that these microarray data have been normalized during preprocessing and further altered by removing effects of confounding

factors including age, gender, tissue pH, agonal factor, and postmortem interval before tissue harvest. Given that all these mathematical transformations affect data variability, future analyses should assess the effects of these manipulations on the variability of these genes of interest by examining the gene-gene relationships in data at different stages of processing.

Because we concluded that changes in variability did not fully account for the changes in pool size, we sought to understand the biological significance of the large pools of overlapping genes in both regions by examining gene ontology as an initial pass and gene set enrichment analysis for finer-grained analysis. *While these analyses are interesting and quite promising, we believe that the best approach with these analyses is to use them to generate hypotheses for further testing, especially since none of the ontology results survive correction for multiple comparisons. Moreover, both approaches will benefit strongly from cross-validation using similar datasets across platforms (including microarray, RNA-seq, etc.), quantitative PCR for specific gene targets, and additional experiments (including similar analyses using FGFs as "seeds" to examine patterns of gene expression) in animal models.*

In the anterior cingulate cortex, there was a diagnosis-specific relationship between FGF2 and FGF9, and 624 genes correlated with both FGF2 and FGF9 in control tissue. Among these genes, Ingenuity Pathway Analysis® (IPA) of the top biological and physiological functions suggested that cellular functions important to neural circuit maintenance, including Cell Morphology and Cell Death and Survival were impacted (Table 4.2). To better parse the biological significance of these effects and generate hypotheses for future study, we used gene set enrichment analysis to identify the top

groups of genes enriched in these samples. Among the significant gene sets in the large overlapping pool in control tissue were those genes whose functions were associated with NCAM1 Interactions, which provides face validity to the analysis because NCAMs are known to interact with FGF receptors (Jacobsen, Kiselyov, Bock, & Berezin, 2008; Kiselyov, Soroka, Berezin, & Bock, 2005; Sanchez-Heras, Howell, Williams, & Doherty, 2006). Furthermore, genes enriched in NCAM1 set in control samples include GFRA2, or the glia cell line-derived growth factor family receptor alpha 2, and neurocan, or NCAN, which has been implicated as a risk factor in bipolar disorder and schizophrenia (Cichon et al., 2011; Mühleisen et al., 2012; Oruč, Kapur-Pojskić, Ramić, Pojskić, & Bajrović, 2012). Other gene sets included those associated with neurotransmission, like GABA-B Receptor Activation and/or cellular responses to stimuli, including Inhibition of Voltage-Gated Calcium Channels (Table 4.4). Collectively, these top gene sets support the idea that expression of many genes—including those associated with growth factors—underlying fundamental aspects of neural circuit maintenance are associated with diagnosis-specific expression of FGFs. Furthermore, the loss of the relationship between FGFs and these gene sets in MDD may indicate that circuits in the anterior cingulate cortex are particularly impacted. Indeed, evidence from MDD patients supports this interpretation, as targeting the subgenual cingulate white matter with deep brain stimulation has been effective in alleviating symptoms among treatment-resistant patients (Holtzheimer & Mayberg, 2011; Ressler & Mayberg, 2007), though the FGF system has not been directly implicated in those studies. Furthermore, these analyses highlight additional relationships between the FGF family and other molecular systems, reinforcing the hypothesis that FGFs act as molecular organizers in the ACC to mediate affect.

In the hippocampus, there is no relationship between the expression of FGF9 and FGFR1 in control tissue, but a strong negative relationship emerges in MDD samples and is accompanied by a large pool of genes whose expression correlates with both FGF9 and FGFR1. As in the ACC, our ontology analyses using IPA® implicated the functions of these genes as strongly related to circuit development and maintenance – indeed, all five of the top biological functions, including Cellular Assembly and Organization, Cellular Function and Maintenance, Cell-to-Cell Signaling and Interaction, Cell Morphology, and Cellular Development, relate to neural circuits (Table 4.3). This suggests that there may be circuit-specific function loss, or alternatively, aberrant neural circuits may arise in the hippocampus as a function of MDD. Indeed, the gene set enrichment analyses support this interpretation and reinforce the importance of FGF signaling in this region. Genes associated with FGF receptor activation sets (including activation related to mutant versions of FGFRs) were among the most consistent significantly enriched gene sets in the overlapping pool of MDD samples, providing face validity to the results of the analysis. Other gene sets significantly enriched in the overlapping pool included those related to DNA replication and mRNA transcription and processing. These data are consistent with several different literatures implicating the hippocampus as a site of circuit alterations in MDD: MDD patients have decreased hippocampal volume (Bremner et al., 2000; MacQueen & Frodl, 2011; Sheline, Gado, & Kraemer, 2003), and indeed, the FGF family has been itself strongly implicated in hippocampal cell genesis both during development and adulthood (Perez et al., 2009; Turner et al., 2011; Zechel, Werner, Unsicker, & Von Bohlen Und Halbach, 2010). Of note, Cell to Cell Communication was trending towards enrichment in MDD samples, though the statistical significance of this

gene set barely missed the threshold (though notably, as a category, it had the largest proportion of enriched genes within the gene set compared to all the other gene sets in the overlapping pool). Cadherins (CDH7, CDH12, and CDH13), genes known to be critical for cell adhesion, circuit formation and synaptogenesis (Seong, Yuan, & Arikkath, 2015), were among the core genes enriched in this set, and the emergence of these molecules reinforces the interpretation that circuit function may be disrupted in MDD. As in the ACC, these data support the hypothesis that disruptions in FGF levels have widespread effects throughout the hippocampus, reinforcing the idea that FGFs act as molecular "organizers" in the brain to influence neural circuitry and mediate affect.

While the implications of these results are quite interesting, these data are best interpreted as preliminary, and further work is needed to validate these findings. *Indeed, both approaches should be replicated with additional large-scale expression datasets (e.g., microarray, RNA-seq) before pursuing additional research to understand the relationships between FGFs and individual genes or gene sets implicated by these analyses*. In particular, the findings that individual pairs of FGFs alter their relationships in expression by diagnosis is provocative, but may be due in part to mathematical artifact, and should thus be replicated in other datasets before resources are invested in follow-up studies. Similarly, gene ontology analyses have been criticized for their lack of replicability, though some have suggested that gene set enrichment analyses provide better study-to-study replicability than either single-gene or ontology approaches (Subramanian et al., 2005). Indeed, the GSEA approach may also be applied more flexibly than a single-gene approach and may be of particular relevance to the current studies: one algorithm allows analysts to examine the gene sets correlated with a specific

gene of interest (Broad Institute), similar to the gene-by-gene correlative approach used here. Indeed, this approach may increase our statistical power, because it will utilize comparisons with the entire dataset instead of a limited pool, and this may address the concern that none of the GSEA results in our current studies survive correction for multiple comparisons.

Expression of FGFs are related in bLR, but not bHR animals, and in some cases, stress modulates these relationships.

Because we are interested in replicating the diagnosis-specific results demonstrating that region-specific expression relationships between FGFs are gained and lost in MDD, we examined the relationships between FGF2, FGF9, FGFR1, and FGFR3 derived from hippocampal samples of bHR and bLR animals exposed to stress. As discussed in other chapters, bHR animals show a high locomotor response to a novel environment and low levels of spontaneous anxiety- and depression-like behavior, while bLR animals exhibit a low locomotor response to a novel environment and high levels of spontaneous anxiety- and depression-like behavior (Stead et al., 2006; Stedenfeld et al., 2011). Previous data indicates that hippocampal FGF2 is important to this phenotype and manipulations involving FGF2 alter affective behavior (Chaudhury et al., 2014; Perez et al., 2009; Turner et al., 2011), and stress is known to modulate both FGF2 and FGF9 expression (Aurbach et al., 2015; Bland et al., 2007; Molteni et al., 2001; Turner, Calvo, et al., 2008). We hypothesized that we might see phenotype-specific relationships between expression of FGF ligands and receptors in the hippocampus, and that these relationships would be altered by stress. In accordance with our hypothesis, we observed that bLR animals, but not bHRs, showed significant relationships between expression of

different pairs of FGFs, including FGF2-FGF9, FGF2-FGFR1, FGF2-FGFR3, and FGF9-FGFR3. Furthermore, the within-bLR relationships between both FGF2 and FGF9 with FGFR3 were altered by stress exposure. We observed similar diagnosis-specific interactions in the hippocampus between FGF2 and FGFR3 in particular, indicating that our animal data partially replicated the human findings. Similarly, a previous student in our laboratory examined the effects of chronic FGF9 administration on FGFR1 expression in the hippocampus; she found that chronic FGF9 decreased FGFR1 expression in the dentate gyrus (Appendix 4.2), further reinforcing our human results. While there are many limitations to animal models of psychiatric disorders (Nestler & Hyman, 2010), we are encouraged by these results and view the general pattern as reinforcing of our postmortem human data. We plan to pursue further avenues for replication in future analyses.

Summary

In this chapter, we examined the hypothesis that FGF2 and FGF9 act in concert to modulate molecular networks in different brain regions. We chose to return to postmortem human datasets to examine if there were relationships in expression between FGF2, FGF9, and FGF receptors, and further determine if these relationships altered with MDD diagnosis. We showed that FGF2 positively correlated with FGF receptors, while FGF9 negatively correlated with FGF2 and FGF receptors across regions, supporting the idea that FGF2 and FGF9 may play coordinate and opposite roles in MDD. We further determined that some relationships between FGF2, FGF9, and FGF receptor gene expression changed as a function of diagnosis, and these alterations were region-specific. Specifically, we generally observed that relationships in expression between FGF family

members were lost in the ACC in MDD, but in contrast, new relationships between FGFs emerged in the HPC in MDD or bipolar disorder. We further found that region- and diagnosis-specific effects co-occurred with alterations in other genes correlating with FGF expression. Upon further analysis of these correlated genes, we consistently found that molecules whose expression was associated with diagnosis-specific alterations of FGF relationships were related to cell morphology, survival, and communication, reinforcing the hypothesis that FGFs act upon cellular microcircuitry as well as a wide range of other systems to mediate the circuit dysregulation believed to underlie the symptoms of MDD. We partially validated these findings using an animal model of genetically-linked affective dysregulation and stress exposure, though we believe that further replication in other large datasets should be a major goal for follow-up analyses.

Future studies should continue to examine convergence points both between members of the FGF family as well as relationships between FGFs with other molecular systems known to impact the biology associated with MDD. In particular, we believe that it will be valuable to determine if there is evidence for region- and diagnosis-specific alterations in the relationships in expression between different FGF family members, and if so, how this relates to other genes of interest. Moreover, it may be useful to apply machine learning algorithms to these data to determine if there are particular patterns of expression changes in ensembles of genes that can differentiate tissue derived from control and MDD subjects. This could provide a unique biological signature (that may differ by brain region) for MDD, and this could inform future studies.

References

Aurbach, E. L., Inui, E. G., Turner, C. A., Hagenauer, M. H., Prater, K. E., Li, J. Z., … Akil, H. (2015). Fibroblast growth factor 9 is a novel modulator of negative affect. *Proceedings of the National Academy of Sciences*, *112*(38), 11953–11958. http://doi.org/10.1073/pnas.1510456112

Bernard, R., Kerman, I. A., Thompson, R. C., Jones, E. G., Bunney, W. E., Barchas, J. D., … Watson, S. J. (2011). Altered expression of glutamate signaling, growth factor, and glia genes in the locus coeruleus of patients with major depression. *Molecular Psychiatry*, *16*(6), 634–646. http://doi.org/10.1038/mp.2010.44

Björkholm, C., & Monteggia, L. M. (2016). BDNF – a key transducer of antidepressant effects. *Neuropharmacology*, *102*, 72–79. http://doi.org/10.1016/j.neuropharm.2015.10.034

Bland, S. T., Tamlyn, J. P., Barrientos, R. M., Greenwood, B. N., Watkins, L. R., Campeau, S., … Maier, S. F. (2007). Expression of fibroblast growth factor-2 and brain-derived neurotrophic factor mRNA in the medial prefrontal cortex and hippocampus after uncontrollable or controllable stress. *Neuroscience*, *144*(4), 1219–1228. http://doi.org/10.1016/j.neuroscience.2006.11.026

Bremner, J. D., Narayan, M., Anderson, E. R., Staib, L. H., Miller, H. L., Charney, D. S., & others. (2000). Hippocampal volume reduction in major depression. *American Journal of Psychiatry*, *157*(1), 115–117.

Carvalho, A. F., Köhler, C. A., McIntyre, R. S., Knöchel, C., Brunoni, A. R., Thase, M. E., … Berk, M. (2015). Peripheral vascular endothelial growth factor as a novel depression biomarker: A meta-analysis. *Psychoneuroendocrinology*, *62*, 18–26. http://doi.org/10.1016/j.psyneuen.2015.07.002

Chaudhury, S., Aurbach, E. L., Sharma, V., Blandino, P., Turner, C. A., Watson, S. J., & Akil, H. (2014). FGF2 is a target and a trigger of epigenetic mechanisms associated with differences in emotionality: Partnership with H3K9me3. *Proceedings of the National Academy of Sciences*, *111*(32), 11834–11839. http://doi.org/10.1073/pnas.1411618111

Cichon, S., Mühleisen, T. W., Degenhardt, F. A., Mattheisen, M., Miró, X., Strohmaier, J., … Nöthen, M. M. (2011). Genome-wide Association Study Identifies Genetic Variation in Neurocan as a Susceptibility Factor for Bipolar Disorder. *American Journal of Human Genetics*, *88*(3), 372–381. http://doi.org/10.1016/j.ajhg.2011.01.017

Duclot, F., & Kabbaj, M. (2015). Epigenetic mechanisms underlying the role of brain-derived neurotrophic factor in depression and response to antidepressants. *Journal of Experimental Biology*, *218*(1), 21–31. http://doi.org/10.1242/jeb.107086

Duman, R. S., & Monteggia, L. M. (2006). A Neurotrophic Model for Stress-Related Mood Disorders. *Biological Psychiatry*, *59*(12), 1116–1127. http://doi.org/16/j.biopsych.2006.02.013

Eren-Koçak, E., Turner, C. A., Watson, S. J., & Akil, H. (2011). Short-Hairpin RNA Silencing of Endogenous Fibroblast Growth Factor 2 in Rat Hippocampus Increases Anxiety Behavior. *Biological Psychiatry*, *69*(6), 534–540. http://doi.org/10.1016/j.biopsych.2010.11.020

Evans, S. J., Choudary, P. V., Neal, C. R., Li, J. Z., Vawter, M. P., Tomita, H., … Akil, H. (2004). Dysregulation of the fibroblast growth factor system in major depression. *Proceedings of the National Academy of Sciences of the United States of America*, *101*(43), 15506 –15511. http://doi.org/10.1073/pnas.0406788101

Gaughran, F., Payne, J., Sedgwick, P. M., Cotter, D., & Berry, M. (2006). Hippocampal FGF-2 and FGFR1 mRNA expression in major depression, schizophrenia and bipolar disorder. *Brain Research Bulletin*, *70*(3), 221–227. http://doi.org/10.1016/j.brainresbull.2006.04.008

Goetz, R., & Mohammadi, M. (2013). Exploring mechanisms of FGF signalling through the lens of structural biology. *Nature Reviews. Molecular Cell Biology*, *14*(3), 166–180. http://doi.org/10.1038/nrm3528

Holtzheimer, P. E., & Mayberg, H. S. (2011). Deep Brain Stimulation for Psychiatric Disorders. *Annual Review of Neuroscience*, *34*(1), 289–307. http://doi.org/10.1146/annurev-neuro-061010-113638

Jacobsen, J., Kiselyov, V., Bock, E., & Berezin, V. (2008). A Peptide Motif from the Second Fibronectin Module of the Neural Cell Adhesion Molecule, NCAM, NLIKQDDGGSPIRHY, is a Binding Site for the FGF Receptor. *Neurochemical Research*, *33*(12), 2532–2539. http://doi.org/10.1007/s11064-008-9680-2

Jones, E. G., Hendry, S. H. C., Liu, X.-B., Hodgins, S., Potkin, S. G., & Tourtellotte, W. W. (1992). A method for fixation of previously fresh-frozen human adult and fetal brains that preserves histological quality and immunoreactivity. *Journal of Neuroscience Methods*, *44*(2–3), 133–144. http://doi.org/10.1016/0165-0270(92)90006-Y

Kiselyov, V. V., Soroka, V., Berezin, V., & Bock, E. (2005). Structural biology of NCAM homophilic binding and activation of FGFR. *Journal of Neurochemistry*, *94*(5), 1169–1179. http://doi.org/10.1111/j.1471-4159.2005.03284.x

Li, J. Z., Bunney, B. G., Meng, F., Hagenauer, M. H., Walsh, D. M., Vawter, M. P., … Bunney, W. E. (2013). Circadian patterns of gene expression in the human brain and disruption in major depressive disorder. *Proceedings of the National Academy of Sciences*, *110*(24), 9950–9955. http://doi.org/10.1073/pnas.1305814110

Li, J. Z., Vawter, M. P., Walsh, D. M., Tomita, H., Evans, S. J., Choudary, P. V., … Myers, R. M. (2004). Systematic changes in gene expression in postmortem human brains associated with tissue pH and terminal medical conditions. *Human Molecular Genetics*, *13*(6), 609–616. http://doi.org/10.1093/hmg/ddh065

Livak, K. J., & Schmittgen, T. D. (2001). Analysis of Relative Gene Expression Data Using Real-Time Quantitative PCR and the 2−ΔΔCT Method. *Methods*, *25*(4), 402–408. http://doi.org/10.1006/meth.2001.1262

MacQueen, G., & Frodl, T. (2011). The hippocampus in major depression: evidence for the convergence of the bench and bedside in psychiatric research[quest]. *Mol Psychiatry*, *16*(3), 252–264.

Miller, C. A. (2011). Stressed and Depressed? Check Your GDNF for Epigenetic Repression. *Neuron*, *69*(2), 188–190. http://doi.org/10.1016/j.neuron.2011.01.006

Molteni, R., Fumagalli, F., Magnaghi, V., Roceri, M., Gennarelli, M., Racagni, G., … Riva, M. A. (2001). Modulation of fibroblast growth factor-2 by stress and corticosteroids: from developmental events to adult brain plasticity. *Brain*

Research Reviews, *37*(1–3), 249–258. http://doi.org/10.1016/S0165-0173(01)00128-X

Mühleisen, T. W., Mattheisen, M., Strohmaier, J., Degenhardt, F., Priebe, L., Schultz, C. C., … Cichon, S. (2012). Association between schizophrenia and common variation in neurocan (NCAN), a genetic risk factor for bipolar disorder. *Schizophrenia Research*, *138*(1), 69–73. http://doi.org/10.1016/j.schres.2012.03.007

Nestler, E. J., & Hyman, S. E. (2010). Animal models of neuropsychiatric disorders. *Nat Neurosci*, *13*(10), 1161–1169. http://doi.org/10.1038/nn.2647

Ornitz, D. M., Xu, J., Colvin, J. S., McEwen, D. G., MacArthur, C. A., Coulier, F., … Goldfarb, M. (1996). Receptor Specificity of the Fibroblast Growth Factor Family. *Journal of Biological Chemistry*, *271*(25), 15292–15297. http://doi.org/10.1074/jbc.271.25.15292

Oruč, L., Kapur-Pojskić, L., Ramić, J., Pojskić, N., & Bajrović, K. (2012). Assessment of relatedness between neurocan gene as bipolar disorder susceptibility locus and schizophrenia. *Bosnian Journal of Basic Medical Sciences*, *12*(4), 245–248.

Paxinos, G., & Watson, C. (2006). The Rat Brain in Stereotaxic Coordinates, 6th Edition. Retrieved December 23, 2015, from http://store.elsevier.com/The-Rat-Brain-in-Stereotaxic-Coordinates/George-Paxinos/isbn-9780080475158/

Perez, J. A., Clinton, S. M., Turner, C. A., Watson, S. J., & Akil, H. (2009). A New Role for FGF2 as an Endogenous Inhibitor of Anxiety. *J. Neurosci.*, *29*(19), 6379–6387. http://doi.org/10.1523/JNEUROSCI.4829-08.2009

Ressler, K. J., & Mayberg, H. S. (2007). Targeting abnormal neural circuits in mood and anxiety disorders: from the laboratory to the clinic. *Nature Neuroscience*, *10*(9), 1116–1124. http://doi.org/10.1038/nn1944

Salaria, S., Chana, G., Caldara, F., Feltrin, E., Altieri, M., Faggioni, F., … Everall, I. P. (2006). Microarray analysis of cultured human brain aggregates following cortisol exposure: Implications for cellular functions relevant to mood disorders. *Neurobiology of Disease*, *23*(3), 630–636. http://doi.org/10.1016/j.nbd.2006.05.003

Sanchez-Heras, E., Howell, F. V., Williams, G., & Doherty, P. (2006). The Fibroblast Growth Factor Receptor Acid Box Is Essential for Interactions with N-Cadherin and All of the Major Isoforms of Neural Cell Adhesion Molecule. *Journal of Biological Chemistry*, *281*(46), 35208–35216. http://doi.org/10.1074/jbc.M608655200

Seong, E., Yuan, L., & Arikkath, J. (2015). Cadherins and catenins in dendrite and synapse morphogenesis. *Cell Adhesion & Migration*, *9*(3), 202–213. http://doi.org/10.4161/19336918.2014.994919

Sheline, Y. I., Gado, M. H., & Kraemer, H. C. (2003). Untreated depression and hippocampal volume loss. *American Journal of Psychiatry*, *160*(8), 1516–1518.

Stead, J. D. H., Clinton, S., Neal, C., Schneider, J., Jama, A., Miller, S., … Akil, H. (2006). Selective Breeding for Divergence in Novelty-seeking Traits: Heritability and Enrichment in Spontaneous Anxiety-related Behaviors. *Behavior Genetics*, *36*(5), 697–712. http://doi.org/10.1007/s10519-006-9058-7

Stedenfeld, K. A., Clinton, S. M., Kerman, I. A., Akil, H., Watson, S. J., & Sved, A. F. (2011). Novelty-seeking behavior predicts vulnerability in a rodent model of

depression. *Physiology & Behavior*, *103*(2), 210–216. http://doi.org/10.1016/j.physbeh.2011.02.001

Subramanian, A., Tamayo, P., Mootha, V. K., Mukherjee, S., Ebert, B. L., Gillette, M. A., … Mesirov, J. P. (2005). Gene set enrichment analysis: a knowledge-based approach for interpreting genome-wide expression profiles. *Proceedings of the National Academy of Sciences of the United States of America*, *102*(43), 15545–15550. http://doi.org/10.1073/pnas.0506580102

Tomita, H., Vawter, M. P., Walsh, D. M., Evans, S. J., Choudary, P. V., Li, J., … Bunney Jr., W. E. (2004). Effect of agonal and postmortem factors on gene expression profile: quality control in microarray analyses of postmortem human brain. *Biological Psychiatry*, *55*(4), 346–352. http://doi.org/10.1016/j.biopsych.2003.10.013

Turner, C. A., Calvo, N., Frost, D. O., Akil, H., & Watson, S. J. (2008). The fibroblast growth factor system is downregulated following social defeat. *Neuroscience Letters*, *430*(2), 147–150.

Turner, C. A., Clinton, S. M., Thompson, R. C., Watson, S. J., & Akil, H. (2011). Fibroblast growth factor-2 (FGF2) augmentation early in life alters hippocampal development and rescues the anxiety phenotype in vulnerable animals. *Proceedings of the National Academy of Sciences*, *108*(19), 8021 –8025. http://doi.org/10.1073/pnas.1103732108

Turner, C. A., Gula, E. L., Taylor, L. P., Watson, S. J., & Akil, H. (2008). Antidepressant-like effects of intracerebroventricular FGF2 in rats. *Brain Research*, *1224*, 63–68. http://doi.org/16/j.brainres.2008.05.088

Turner, C. A., Watson, S. J., & Akil, H. (2012). The Fibroblast Growth Factor Family: Neuromodulation of Affective Behavior. *Neuron*, *76*(1), 160–174. http://doi.org/10.1016/j.neuron.2012.08.037

Warner-Schmidt, J. L., & Duman, R. S. (2007). VEGF is an essential mediator of the neurogenic and behavioral actions of antidepressants. *Proceedings of the National Academy of Sciences*, *104*(11), 4647–4652. http://doi.org/10.1073/pnas.0610282104

Zechel, S., Werner, S., Unsicker, K., & Von Bohlen Und Halbach, O. (2010). Expression and Functions of Fibroblast Growth Factor 2 (FGF-2) in Hippocampal Formation. *The Neuroscientist*, *16*(4), 357–373. http://doi.org/10.1177/1073858410371513

Zhang, X., Ibrahimi, O. A., Olsen, S. K., Umemori, H., Mohammadi, M., & Ornitz, D. M. (2006). Receptor Specificity of the Fibroblast Growth Factor Family THE COMPLETE MAMMALIAN FGF FAMILY. *Journal of Biological Chemistry*, *281*(23), 15694–15700. http://doi.org/10.1074/jbc.M601252200

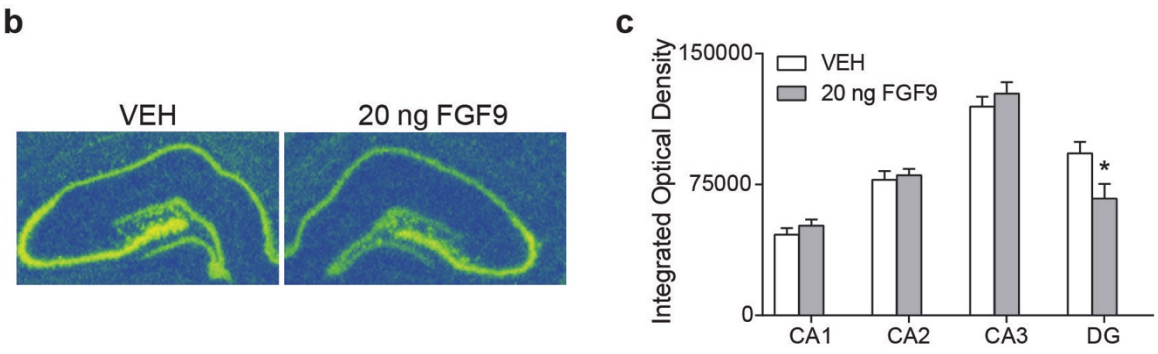
*Appendix 4.1. Statistics describing the region-, diagnosis-, and gene-specific variation in the corrected Affymetrix expression data for FGF2, FGF9, FGFR1, and a control comparison, GAPDH (which does not show MDD-related changes in expression). + indicates 0.1>p>0.05; * indicates p < 0.05.*

ACC

	FGF2		***FGF9***		***GAPDH***	
	Control	*MDD*	*Control*	*MDD*	*Control*	*MDD*
Range (Corrected Expression)	-0.54 - 0.75	-0.61 - 0.76	-0.58 - 1.18	-0.22 - 0.59	-0.35 - 0.21	-0.24 - 0.10
Standard Deviation	0.263	0.345	0.351	0.233	0.084	0.082
Levene's Test for Equal Variances (p-value)	0.101		0.097^{+}		0.536	

HPC

	FGF9		***FGFR1***		***GAPDH***	
	Control	*MDD*	*Control*	*MDD*	*Control*	*MDD*
Range (Corrected Expression)	-1.04 - 1.02	-0.44 - 1.42	-0.439 - 0.33	-0.44 - 0.30	-0.33 - 0.59	-0.34 - 0.59
Standard Deviation	0.419	0.464	0.132	0.174	0.212	0.184
Levene's Test for Equal Variances (p-value)	0.387		**0.048***		0.193	



*Appendix 4.2. Chronic FGF9 administration (20 ng, i.c.v., for 14 days) reduces expression of FGFR1 in the dentate gyrus, reinforcing the hypothesis that FGFs act as molecular organizers to mediate affective dysregulation. * indicates p< 0.05. Data courtesy of Edny Gula Inui.*

Chapter 5.

Discussion

The research in this dissertation addresses two broad hypotheses: *1) FGF2 and FGF9 play coordinate roles in the brain to mediate affect; 2) dysregulation of FGF2 and FGF9, either in isolation or relative to each other, may be important to the neural circuitry and pathophysiology underlying major depressive disorder (MDD).* We sought many different types of evidence to test these hypotheses, including examining postmortem human brain tissue, an animal model of differential vulnerability to affective dysregulation, and various in-vivo manipulations of FGF2 and FGF9, to determine their impact on affect and molecular networks across brain regions.

Both human and animal data support the hypotheses that FGF2 and FGF9 are critical to affect and are disrupted in MDD.

We set out to address a number of questions to test the hypotheses that FGF2 and FGF9 were implicated in affect and were dysregulated in MDD. In the introduction, we proposed that several lines of evidence would support FGF2 and/or FGF9 in these roles, including (but not limited to): 1) FGF2 and/or FGF9 would show selective or region-specific alterations in postmortem brain tissue derived from individuals diagnosed with MDD; 2) FGF2 and/or FGF9's expression would be sensitive to stress in animal models (though we did not perform additional experiments to add to this literature in this dissertation); 3) altering endogenous FGF2 and/or FGF9 levels would be sufficient to

alter affective-like behavior in animal models; and 4) endogenous FGF2 and/or FGF9 expression would vary across individuals and be associated with differential vulnerability or resilience to developing affective pathology. The research presented in this thesis joins the work of other scholars to address these questions.

Hippocampal FGF9 mediates affect and is dysregulated in MDD. When we began this research, the literature supporting the role of FGF2 in affect regulation was already quite large. By comparison, very little was known about FGF9 and its role in the brain to mediate affective behavior. In this dissertation, we significantly added to preliminary data to implicate FGF9 as an endogenous mediator of negative affect for the first time. We demonstrated that FGF9 shows a notably opposite pattern to those effects that we have previously observed with FGF2. We presented three key pieces of data supporting FGF9 in this role: 1) we showed that FGF9 expression was significantly upregulated in the postmortem hippocampus of individuals diagnosed with MDD during life; 2) we demonstrated that FGF9 levels in the ventral hippocampus were associated with differential vulnerability to affective dysregulation, and 3) we found that knocking down endogenous expression of FGF9 in hippocampal dentate granule neurons was sufficient to reduce anxiety-like behavior. These data joined studies previously performed in our laboratory showing that chronic stress exposure increased hippocampal FGF9 expression and that chronic administration of FGF9 increased anxiety- and depression-like behavior (Aurbach et al., 2015). Collectively, these data supported the hypothesis that FGF9 acts in the brain as a key mediator of negative affect, and furthermore, that its expression is dysregulated in MDD. Because our data are among the first to implicate FGF9 in affective dysregulation, a number of outstanding questions remain, including whether

antidepressant and anxiolytic medications alter FGF9 expression, similar to the manner in which they increase FGF2 expression (Bachis, Mallei, Cruz, Wellstein, & Mocchetti, 2008; Gómez-Pinilla, Dao, Choi, & Ryba, 2000; Mallei, Shi, & Mocchetti, 2002).

Hippocampal FGF2 expression varies in an animal model of differential vulnerability to affective dysregulation and is required for differences in spontaneous anxiety- and depression-like behavior. Because the literature supporting FGF2's role in affective dysregulation was much more advanced relative to the literature on FGF9, our research focused on several important and outstanding questions. Previous studies suggested that FGF2 levels vary across individuals and relate to differential vulnerability to affective dysregulation. FGF2 expression levels in the hippocampus correlated with affective-like behavior across outbred rats (Eren-Koçak, Turner, Watson, & Akil, 2011), and rats selectively bred for differing locomotor responses to a novel environment show accompanying differences in hippocampal FGF2 levels, with bred high responders (bHRs) showing higher FGF2 expression than bred low responders (bLRS) (Perez, Clinton, Turner, Watson, & Akil, 2009; Turner, Clinton, Thompson, Watson, & Akil, 2011). Here, we demonstrated that the differences in FGF2 expression between the lines of selectively-bred rats are consistent and stable across generations. Furthermore, we demonstrated that knocking down hippocampal FGF2 was sufficient to eliminate basal behavioral differences between the selectively bred lines, suggesting that this differential FGF2 expression is necessary for previously observed differences in spontaneous anxiety- and depression-like behavior in these rats. Collectively, these studies strongly implicate hippocampal FGF2 as a key mediator of temperament and affective behavior.

The relationships between FGF2, FGF9, and the FGF receptors may be comparably important to the individual roles of FGF2 and FGF9 in MDD. Because we consistently observed opposite effects of FGF2 and FGF9 in animal models, we hypothesized that their relative expression and activity would be important to their individual roles in mediating affect. We found evidence that FGF2 and FGF9 expression correlated strongly with each other and with FGF receptors across the DLPFC, ACC, and HPC (though microarray data do not address splice variant specificity): interestingly, the relationships between the FGF ligands and FGFR1 was consistently the weakest, while the relationships between the ligands and FGFR2 and FGFR3 were quite robust, and this trend was consistent across regions. The relationships between FGF2 and the FGF receptors were particularly surprising in light of FGF binding affinities determined *in vitro*. Specifically, FGF2 binds most strongly to FGFR1 and least strongly to FGFR3, while FGF9 binds most strongly to FGFR3 and least strongly to FGFR1 (Ornitz et al., 1996; Zhang et al., 2006), though it is unknown if these patterns change *in vivo* or are modulated by factors including the heparin sulfate proteoglycans, which can impact FGF binding in other systems (Guillemot & Zimmer, 2011; Mason, 2007). Notably, the relationships between FGF2, FGF9, and the FGF receptors were opposite in directionality. This finding reinforced the opposing effects that we previously observed in animal models and support the hypothesis that FGF2 and FGF9 may play coordinate roles across brain regions.

To understand how these relationships were altered in mood disorders, we further examined these data and found a striking pattern of results that varied across brain regions. In the anterior cingulate cortex, correlations between FGF family members were

consistently lost in MDD samples, while in the hippocampus, new relationships that were not present in Control tissue emerged in mood disorders. We partially validated these findings in an animal model, reinforcing the hypothesis that the coordinate relationships of FGFs are important for affective dysregulation. As a proof-of-concept, we selected one pair of genes whose relationship was altered in MDD in both the anterior cingulate cortex and the hippocampus for further analysis. We found that many other genes co-correlated with FGFs as a function of diagnosis, and we determined that these genes were broadly related to fundamental biology and cellular microcircuitry. These data support the hypothesis that the FGF family may act as an ensemble to mediate gene expression, and expression of different FGFs relative to each other may be important for MDD pathophysiology. Furthermore, these data provide preliminary evidence that the FGFs may have broad organizing effects on other genes.

Collectively, these data support the hypothesis that FGF2 and FGF9 play opposite or coordinate roles in the brain to mediate affect. These data further support the hypothesis that FGF2 and FGF9 become dysregulated in MDD (
Table 5.1***).***

This series of observations is striking in light of the history implicating the FGF family in MDD. The role of the FGFs was first discovered in the human brain over ten years ago, and subsequent studies in animal models have strongly reinforced the roles of FGF2 and FGF9 in affective dysregulation. *The studies in this dissertation add to this literature by further examining the individual and coordinate roles of FGF2 and FGF9 in mediating affect and becoming dysregulated in MDD. We performed key studies to implicate hippocampal FGF9 in negative affect and MDD for the first time, and we*

extended our understanding of FGF2's role in the brain by showing that hippocampal FGF2 levels were necessary for behavioral differences in selectively bred lines of rats. Because we were interested in determining how members of the FGF family impacted each other, we returned to the postmortem human data to further explore relationships with FGFs and generate novel hypotheses. We showed that expression correlated between different FGF family members, and the patterns of these relationships were altered by MDD.

Table 5.1. FGF2 and FGF9 show opposing or coordinating effects in postmortem human tissue and animal models. Citations which include data in this dissertation are in bold.

Manipulation	***Effect of/on FGF2***	***Effect of/on FGF9***	***Citation***
Human brain expression MDD relative to control	↓	↑	Evans et al. (2004); Gaughran et al. (2006); Bernard et al. (2011); **Aurbach et al. (2015)**
Individual variation in vulnerability to affective dysregulation	Dorsal HPC (↑ in bHR)	Ventral HPC (↑ in bHR)	Perez et al. (2009); **Chapter 2; Chapter 3**
Rat brain expression social defeat relative to control	↓	↑	Turner et al. (2008); **Aurbach et al. (2015)**
Chronic Administration anxiety-like behavior depression-like behavior	↓ ↓	↑ ↑	Turner et al. (2008); Perez et al. (2009); **Aurbach et al. (2015)**
Effect of knockdown on anxiety-like behavior in bHR/bLR animals	↑ **=**	↓ **?**	Eren-Kocak et al. (2011); **Aurbach et al. (2015); Chaudhury et al. (2014)**
Effect of antidepressant drug administration	↑	**?**	Mallei et al. (2002); Bachis et al. (2008)

While these broad observations are quite valuable, we can gain a richer understanding of FGF2 and FGF9's functions by reframing the data in a different light.

FGF2 and FGF9 dysregulation may be region-specific but still influence similar genes and biological functions across regions.

There are a number of published reports indicating that FGF2 expression is downregulated and FGF9 expression is upregulated across regions in brain samples deriving from individuals diagnosed with MDD during life (Bernard et al., 2011; Evans et al., 2004; Gaughran, Payne, Sedgwick, Cotter, & Berry, 2006). While we did not fully replicate those results in this dissertation, we consistently observed strong relationships between both FGF2 and FGF9 with each other and with FGF receptors across diagnostic categories in all three regions that we examined. This suggests that the relationships between FGF family members may play an important role in the brain, and these relationships may indicate that FGF2 and FGF9 can impact expression of other FGF family members. These data also provide preliminary evidence that FGF2 and FGF9 may be regulated in opposite directions consistently across regions, though these data do not speak to how these relationships might change with diagnosis.

However, research in this dissertation also suggests that there may be region-specific effects related to FGF2 and FGF9 dysregulation in MDD. As previously discussed, we found region-specific effects of diagnosis where FGF2 or FGF9 were significantly dysregulated in different brain regions; these results partially reinforce or extend some previous findings, but they do not fully replicate other studies (Bernard et al., 2011; Evans et al., 2004; Gaughran et al., 2006). Because we have discussed possible reasons underlying these discrepancies in replication in previous chapters, we will now examine the idea that this regional specificity is meaningful and may reflect biological differences in the effects of growth factor dysregulation across brain regions and circuits.

After controlling for a variety of confounding pre- and postmortem factors, we observed regional differences in diagnosis-specific FGF expression. Specifically, FGF2 expression was weakly decreased in the ACC. In contrast, we observed strong, consistent evidence that FGF9 expression was selectively increased in the hippocampus. In parallel, we observed striking region-specific trends when the relationships between pairs of FGF genes with mood disorder diagnoses. In the ACC, we generally observed a loss of relationship between pairs of FGF genes in MDD, while in the HPC, relationships between FGF genes emerged in MDD and bipolar disorder. These region-specific effects support the idea that FGFs may play slightly different roles in the ACC and HPC.

Other growth factors have shown region-specific differences in effects on behavior. Most notably, manipulations involving brain-derived neurotrophic factor (BDNF) have shown striking differences in directionality depending on region. In the hippocampus, BDNF has antidepressant properties and is important for the positive effects of antidepressants (Adachi, Barrot, Autry, Theobald, & Monteggia, 2008; Govindarajan et al., 2006; Monteggia et al., 2004; Shirayama, Chen, Nakagawa, Russell, & Duman, 2002). However, high levels of BDNF in the amygdala or ventral striatal regions are anxiogenic or pro-depressant and are associated with behavioral susceptibility to chronic social defeat stress (Berton et al., 2006; Eisch et al., 2003; Govindarajan et al., 2006; Krishnan et al., 2007). Collectively, these data indicate that BDNF, one of the best studied growth factors in affective dysregulation, shows substantial variation in effects depending on region: high levels of BDNF are protective in the hippocampus but have negative effects in the amygdala or ventral striatum, though the cellular mechanisms by which these effects arise have not been well described. By extension, the preliminary

evidence suggesting that different FGFs may show region-specific effects could be meaningful and should be further examined.

Unlike the BDNF literature, few experiments utilizing animal models have attempted to dissect the regional effects of FGF2 or FGF9. Many studies implicating FGFs in affective dysregulation in animal models have employed methods that do not give insight into region-specific anatomy, but instead administer FGF2 and FGF9 peripherally or intracerebroventricularly, which allows the molecules to spread throughout the brain (Aurbach et al., 2015; Perez et al., 2009; Turner et al., 2011; Turner, Gula, Taylor, Watson, & Akil, 2008). There are several notable exceptions. Elsayed and colleagues (2012) demonstrated that chronic site-specific infusions of FGF2 into prelimbic cortex (but not the dorsal striatum) reduced depression-like behavior, and knockdown studies demonstrated that reduction of FGF2 and FGF9 expression in the dentate gyrus had opposite effects on anxiety-like behavior (Aurbach et al., 2015; Eren-Koçak et al., 2011). While the knockdown studies are one example where both FGFs have opposing effects in the same region, the lack of studies differentiating regional effects remains a limitation to fully understanding the roles that FGF2 and FGF9 play in affective dysregulation, particularly in the prefrontal cortices.

The ACC and HPC have different roles in the pathophysiology underlying MDD, and both regions show volumetric reductions in individuals diagnosed with MDD. FGFs mediate biological functions, including cell survival, that may influence this volumetric loss (reviewed in Chapter 1). Since it is unlikely that FGF2 and FGF9's molecular functions change across regions, the most likely explanation for the region-specific effects we observe is that there are other biological differences between the regions that

mediate their unique functions. Some of these differences include (but are not limited to): connected regional circuitry (including different regional inputs and outgoing projections), within-region cell-type balance, predominant neurotransmitter systems, and differentially balanced molecular networks. Data in this dissertation suggests that FGF2 and FGF9 dysregulation might reflect or mediate within-region cell-type balance and/or alterations in molecular networks (which will be discussed in a later section).

While there are regional exceptions, many FGFs are expressed in either neurons or in glia. In particular, FGF2, FGFR2, and FGFR3 are primarily glial, while FGF9 and FGFR1 are primarily neuronal (Bansal, Lakhina, Remedios, & Tole, 2003; Belluardo et al., 1997; Garcès, Nishimune, Philippe, Pettmann, & deLapeyrière, 2000; Gonzalez, Berry, Maher, Logan, & Baird, 1995; Tagashira, Ozaki, Ohta, & Itoh, 1995). Given that both FGF2 and FGF9 have been implicated in proliferation and survival of both neurons and glia (Cheng, Black, & DiCicco-Bloom, 2002; Chuang et al., 2015; Falcone, Filippis, Granzotto, & Mallamaci, 2015; Huang, Hong, & Chuang, 2009; Kanda et al., 2000; Korada, Zheng, Basilico, Schwartz, & Vaccarino, 2002; Lum, Turbic, Mitrovic, & Turnley, 2009; Vicario-Abejón, Johe, Hazel, Collazo, & McKay, 1995; Wagner, Black, & DiCicco-Bloom, 1999), alterations in FGF2 or FGF9 levels could indicate shifts in the numbers and types of cells within each region. In both the ACC and the HPC, these shifts might alter the glial-neuronal balance of the region to influence regional microcircuitry and potentially contribute to MDD symptoms. Our data supports this interpretation and contributes to the hypothesis that cell type balance is altered in MDD, though additional studies are needed to prove this conclusively. Moreover, our research does not address if FGF changes reflect or are driving these possible differences in cell type balance.

In the ACC, we observed a decrease in FGF2 expression and a loss of correlation in expression between FGF2 and FGF9 in MDD. In parallel, a number of studies indicate that there is a dramatic reduction in the number of glia in the ACC in MDD (Chana, Landau, Beasley, Everall, & Cotter, 2003; Cotter et al., 2002; Cotter, Pariante, & Everall, 2001; Cotter, Landau, & Everall 2001; Öngür, Drevets, & Price, 1998). Because FGF2 is primarily expressed in glia, this reduction in glial cells might reflect the expression change in FGF2, though the causality of the effect is impossible to determine with these data (that is, we cannot determine if FGF2 reduction drives the loss of glial cells or vice versa). If this hypothesis is correct, these reductions in FGF2 expression and glial cell number might alter the glial-neuronal balance of the region, and potentially contribute to MDD symptoms. Indeed, work in animals supports this hypothesis: reducing the astrocytic population in prefrontal cortical regions induced depression-like behavior in an animal model (Banasr & Duman, 2008). However, this hypothesis is highly speculative, and studies examining the relationship between FGF2 expression, glial cell number, and alterations in MDD is necessary to lend clarity to these observations. In addition to these proposed histology studies, we may be able to examine this hypothesis by applying algorithms designed to estimate the proportion of different tissue types based on cell type-specific markers within a homogenous tissue set (Hagenauer et al., *unpublished*). Applying these approaches to our microarray data may also clarify our results.

In contrast, we observed an increase in FGF9 expression in the HPC. Because FGF9 is primarily expressed in neurons, this increase might arise if there were more neurons in the sample (alternatively, the relative number of glial cells could be reduced, which would proportionally increase the number of neurons). Supporting this hypothesis,

one previous study showed that subfields of the hippocampus show increases in the number of neurons (the somas of these cells are more densely packed in MDD than in control tissue), and the previously observed volumetric decreases in the hippocampus of depressed patients was likely due to a reduction in volume of the neuropil (Stockmeier et al., 2004). Other studies indicate that adult hippocampal neurogenesis is reduced in MDD (for reviews, see: Malberg, Eisch, Nestler, & Duman, 2000; Schmidt & Duman, 2007; Warner-Schmidt & Duman, 2006). In our data, we observed that a relationship between FGF9 and FGFR1 emerges in the depressed HPC. Given that FGFR1 has been linked to hippocampal neurogenesis (Zhao et al., 2007), our results might also reflect alterations in hippocampal neurogenesis, and the emergent inverse correlation between FGF9 and FGFR1 in MDD samples could reflect a relationship with altered cell type balance. Specifically, the hippocampus shows overall increases in neurons (which might increase FGF9) but decreases in adult neurogenesis (which might decrease FGFR1) in MDD. However, this interpretation is also highly speculative and studies examining the relationship between cell type balance and FGF expression are needed.

Collectively, these data may indicate that FGFs can play both universal and region-specific roles simultaneously: molecular function may not differ across regions, but cell type balance and/or other mechanisms may be region-specific. These interactions could dramatically influence basic biology and circuit function in the brain, and these functions are impacted with FGF disruption in MDD. Therefore, our data support the interpretation that *FGF2 and FGF9 can both widely influence gene expression within and across regions and may play individual, region-specific roles*.

FGF2 and FGF9 could both act as predisposing factors for affective dysregulation and become increasingly dysregulated over time with repeated stress exposure.

Because postmortem human data does not allow us to determine if FGF2 and FGF9 levels are predisposing to MDD or if they arise from the disease process, we both examined these questions in animal models of selective vulnerability to affective dysregulation and drew on the work of others. As reviewed in Chapter 1, FGF2 varies across individuals and is associated with MDD diagnosis and treatment response in humans.

In the brain, hippocampal FGF2 levels similarly differ in an animal model of affective dysregulation. Bred high responders (bHRs) show high levels of locomotion in a novel environment and low levels of spontaneous anxiety- and depression-like behavior, while bred low responders (bLRs) show low levels of locomotion in a novel environment and high spontaneous anxiety- and depression-like behavior (Flagel, Waselus, Clinton, Watson, & Akil, 2014; Stead et al., 2006; Stedenfeld et al., 2011). Previous studies in our laboratory demonstrated that bHR rats in earlier generations showed higher hippocampal expression of FGF2 (Perez et al., 2009), and data in this dissertation reinforced those findings, showing that these differences in hippocampal FGF2 expression were stable across generations. Similarly, we observed that FGF9 levels are also selectively elevated in bHR animals relative to bLRs, indicating that the interpretation of FGF9 as purely anxiogenic and pro-depressant may be too simplistic. However, these findings were restricted to the ventral hippocampus (and specifically ventral dentate gyrus and CA1), which may indicate that regional specificity is important for the impact of FGFs on affective dysregulation. Collectively, these studies indicate that

hippocampal FGF2 and FGF9 levels are associated with genetic vulnerability to affective dysregulation, though additional studies were necessary to address whether these differences in expression were crucial for the baseline behavioral differences displayed between the selectively-bred lines.

To address this limitation, we performed experiments to knock down endogenous expression of FGF2 and FGF9 in the hippocampus. In outbred rats, knocking down FGF2 is anxiogenic, while knocking down FGF9 is anxiolytic (Aurbach et al., 2015; Eren-Koçak et al., 2011), strongly suggesting that levels of both FGF2 and FGF9 in the hippocampus are critical for affective behavior. We performed a similar knockdown experiment in bHR and bLR animals, and showed that decreased hippocampal FGF2 expression eliminated differences in spontaneous anxiety- and depression-like behavior between the lines (Chaudhury et al., 2014). This result strongly indicates that hippocampal expression of FGF2 is necessary to differentiate vulnerability to affective dysregulation, and a future study should examine if FGF9 is similarly critical for affective differences between bHR and bLR animals.

Reactivity to stress exposure is known to be a vulnerability factor for MDD, and stress is frequently associated with the initiation of a depressive episode in humans (American Psychiatric Association, 2013). As previously noted, hippocampal FGF2 and FGF9 levels are dramatically and oppositely impacted by stress. Hippocampal FGF2 expression decreases after chronic stress exposure during development (Fumagalli, Bedogni, Slotkin, Racagni, & Riva, 2005; Molteni et al., 2001) and in adulthood (Turner, Calvo, Frost, Akil, & Watson, 2008), while hippocampal FGF9 expression increases

(Aurbach et al., 2015). These data support the idea that FGF2 and FGF9 dysregulation after stress could impact the progression of the disease.

Together with data indicating that hippocampal FGF2 and FGF9 expression predisposes individuals to affective dysregulation differently, the interaction between stress and FGF expression raises the interesting possibility that FGF2 and FGF9 may both predispose individuals for affective dysregulation *and* become altered themselves before or during depressive episodes. Indeed, if *FGF2 and FGF9 both act as predisposing factors and become dysregulated as a function of the disease process*, their disrupted expression could contribute to the development of a negative feedback loop, at least in the hippocampus (Figure 5.1), and this loop could possibly become associated with cyclic or repeated depressive episodes in MDD.

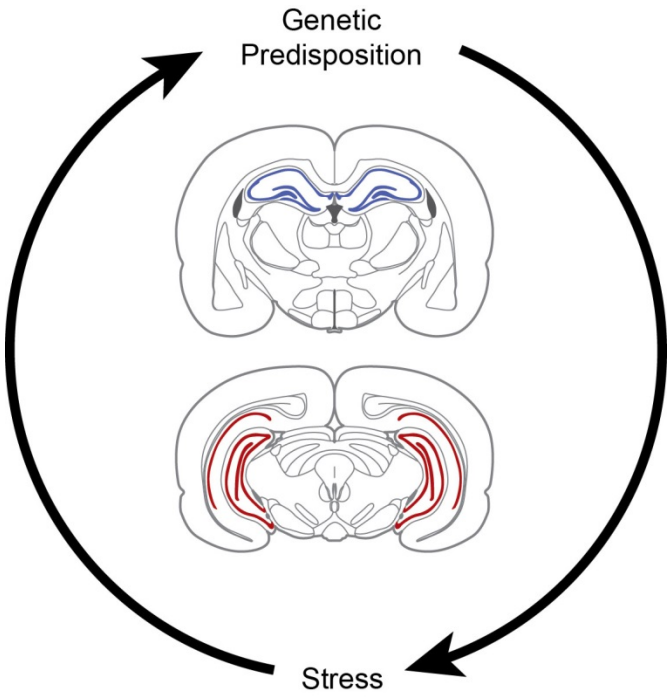


Figure 5.1. Proposed model: hippocampal FGF expression both predisposes individuals to be vulnerable to affective dysregulation and is responsive to stress. With repeated stressful encounters, FGF expression may become part of a negative feedback loop that

contributes to affective dysregulation. Future studies should examine if a similar loop exists in humans, and if and how it might be related to MDD. Brain templates adapted from Paxinos & Watson, 2006.

Indeed, evidence from our laboratory implicates epigenetic regulation as a key mediator that links environmental experience with FGF2 gene expression, which could provide an avenue to explore how stressful experiences might mediate changes in FGF expression. Chaudhury and colleagues showed that the transcription-repressive epigenetic marker H3K9me3 is elevated in bLR animals, and that treatment with FGF2 during early life or selective FGF2 knockdown during adulthood eliminates these differences (Chaudhury et al., 2014). While this study did not examine the effects of stress exposure on H3K9me3 levels, these data provide a possible mechanistic avenue whereby FGF2 levels may be altered by environment or prior experience.

Since it is known that a history of repeated depressive episodes in MDD predicts the likelihood of relapse (Keller, Lavori, Lewis, & Klerman, 1983) and since repeated episodes also relate to decreased hippocampal volume (Sheline, Gado, & Kraemer, 2003; Sheline, Sanghavi, Mintun, & Gado, 1999; Sheline, Wang, Gado, Csernansky, & Vannier, 1996), future studies should examine whether FGF levels become progressively dysregulated in this kind of negative feedback loop, and the evidence from animal models should be related back to human patients. Several studies can address this hypothesis. For example, it should be determined whether H3K9me3 (or another epigenetic marker) levels relate to FGF expression, are altered after stress, and if these alterations persist or resolve over time. In conjunction, a crucial experiment would examine how FGF2 and FGF9 levels change over a prolonged time period after stress exposure. Possible data supporting the negative feedback loop hypothesis would indicate that FGF2 and/or FGF9

levels would never fully return to pre-stress levels or become progressively dysregulated over time, tracking with stress-related changes in epigenetic markers. These studies will begin to address whether FGFs participate in a negative feedback loop to impact affective dysregulation, though additional evidence would be needed to implicate a similar loop in human patients.

FGF2 and FGF9 may act as molecular organizers to influence neural circuitry and mediate affective dysregulation

One emergent theme is that FGF2 and FGF9 could act as molecular organizers to mediate affective dysregulation. Here, we operationally define this organizational capacity as mediating broad influence over different functions, including gene expression, neurotransmission, cellular proliferation and survival, and cell morphology, to impact molecular networks and cellular microcircuits. Specifically, FGF2 and FGF9 have been shown to act in this capacity and influence the circuits underlying MDD by 1) altering the physical circuitry by influencing cells or synapses, 2) modulating neurotransmission, and 3) impacting the expression of molecular networks that underlie critical biological functions, including those regulating circuit tuning and maintenance. FGFs have previously been implicated in the first two roles (reviewed in Chapter 1), though notably, studies examining these functions have primarily probed the biological capacities of these molecules, and relatively few have assessed the relationship between cellular circuitry and affect or if and/or how these functions come to be altered in MDD. Briefly, FGF2 has been strongly implicated in a number of functions underlying neural circuit development, tuning, and maintenance, including cellular proliferation, survival, and fate determination (Perez et al., 2009; Rai, Hattiangady, & Shetty, 2007; Turner et

al., 2011; Wagner et al., 1999), as well as synaptogenesis (Li, Suzuki, Suzuki, Mizukoshi, & Imamura, 2002). While many fewer studies have examined the roles of FGF9 in these capacities, those that have strongly implicate it as a survival factor (Chuang et al., 2015; Garcès et al., 2000; Huang et al., 2009; Kanda et al., 1999, 2000; Lum et al., 2009; Pataky, Borisoff, Fernandes, Tetzlaff, & Steeves, 2000), and several studies directly implicate it in circuit formation in the cerebellum (Lin et al., 2009; Meier et al., 2014). Similarly, several studies indicate that FGF receptors heterodimerize with serotonin receptors, providing a mechanistic hypothesis whereby FGF signaling can influence neurotransmission in a system that is a known target of antidepressant drugs (Borroto-Escuela et al., 2012, 2015). However, the function of FGFs as organizers of molecular networks has been much less studied, and that is one area that this dissertation has contributed significantly to the field.

We showed that relationships between FGF2 and FGF9 and other FGF family members become altered in MDD, and these alterations are region-specific. In the ACC, we observed a general loss of interactions between FGF family members in postmortem human tissue, while in the HPC, we saw new relationships between FGF ligands and receptors emerge with mood disorder diagnoses. Reinforcing these data, we observed selective alterations between pairs of FGF genes in the hippocampus specifically in bLR animals, and moreover, some of these relationships were sensitive to stress. Upon further examining the postmortem human data using gene ontology approaches, we found preliminary evidence that there were wider gene networks associated with these diagnosis-specific interactions between FGFs. We may be able to apply additional analyses to determine if these patterns of alterations could serve as molecular

"signatures" to differentiate control and MDD tissue, which could generate novel hypotheses for future testing. Moreover, this could indicate that the interplay between FGF genes may have an organizational effect to impact the interplay between many other genes across the genome. Furthermore, we determined that there were region-specific differences in the families of genes which were altered with diagnosis. Core biological functions were altered in association with the lost relationship between FGF2 and FGF9 in MDD. In contrast, genes related to DNA replication, transcription and mRNA processing, and FGF receptor activation emerged in relation to FGF9 and FGFR1 in the HPC, similarly indicating that these core genes were selectively altered in MDD. The emergent data in the hippocampus are particularly interesting, and there are several possibilities regarding what these changes could indicate. These changes could signal that there is a selective alteration of circuits and/or circuit function in MDD, or these emergent relationships indicate that aberrant circuits could be forming in hippocampal microcircuitry. However, as discussed in Chapter 4, these conclusions are quite preliminary and would benefit greatly from additional studies that replicate or extend these results. Furthermore, while these relationships are very interesting, these data are by nature correlational, and additional studies are needed to provide causal evidence that FGF2 and FGF9 can act as molecular organizers.

Indeed, other data not shown in this dissertation provides preliminary evidence that FGF2 plays an organizational role with another molecular system across a brain circuit implicated in affective regulation, though additional work is needed to similarly implicate a causal role for FGF9 as a molecular organizer. We have unpublished data indicating that FGF2 influenced expression of genes in the endogenous opioid system in

the separation distress circuit, providing evidence that FGF2 influences patterning of both ligands and receptors in a separate molecular family to influence affective-like behavior in an animal model of affective dysregulation. These data join other work demonstrating that early life FGF2 also impacts the adult expression of other genes to influence cellular microcircuitry and affect: ntrk3 and bcl212 showed similar patterns in separate studies (Turner et al., 2011). While preliminary, these data lend credence to the correlational results in the human data and reinforce the need for future studies to further examine FGF2 and FGF9 as molecular organizers.

However, we do not currently have a holistic understanding about how alterations at the level of gene expression translate into the physiological changes that we have observed with changes in cell proliferation, survival, and circuit formation; this is one notable gap in understanding how FGFs influence brain circuitry. Some studies have implicated particular molecular pathways downstream of FGF receptors in this capacity, but few have connected the function of particular genes to changes in circuit dysregulation. Furthermore, while FGF2 has been the focus of a body of research, very few studies have examined the effects of FGF9 on other molecular systems related to affective dysregulation. Addressing this deficiency will help to differentiate the roles that different FGFs play in their molecular organizational capacities. Regardless of these gaps, the hypothesis that *FGF2 and FGF9 may act as molecular organizers in a number of different capacities, including on the molecular, cellular, and microcircuit levels*, generates a number of interesting hypotheses and should be studied further.

Future work should emphasize the potential roles of FGF2 and FGF9 in circuit-mediating functions, including synaptogenesis and dendritic arborization.

Taking a wide view, we have discussed how FGF2 and possibly FGF9 could influence the underlying biology and maintenance functions of brain circuits associated with MDD. In particular, they influence circuits at three or more different physiological levels, including: 1) organization of molecular networks that may relate to affective dysregulation, 2) neurotransmission, and 3) cellular proliferation and survival (including possible effects on within-region cell type balance) to directly influence regional microcircuitry (Figure 5.2).

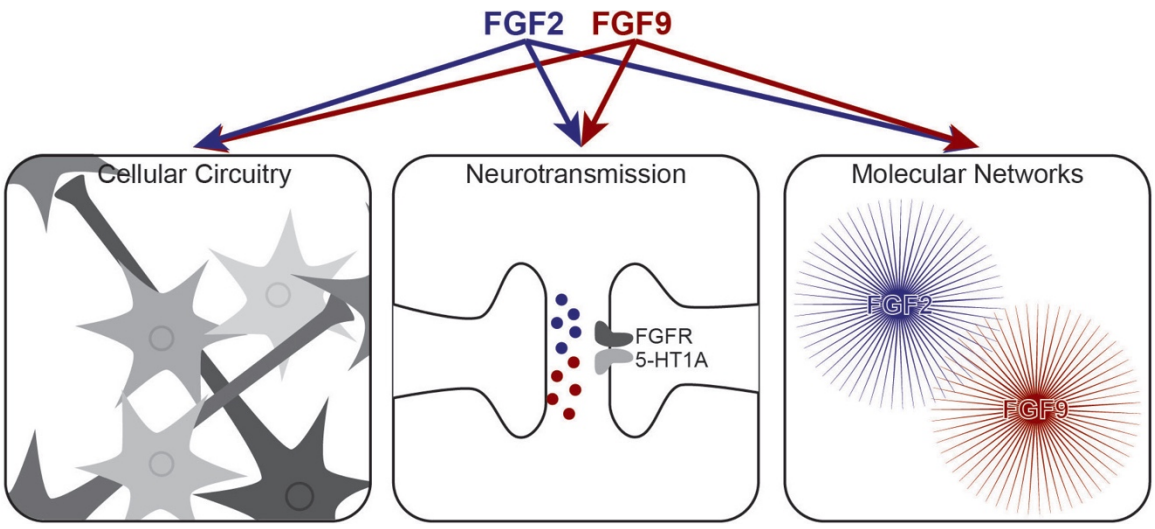


Figure 5.2. FGFs may influence neural circuits underlying affective dysregulation by impacting cells, modulating neurotransmission, and impacting the expression of molecular networks.

In addition, there may be other areas related to circuit maintenance and tuning in which FGF2 and FGF9 could play additional roles. Notably, the roles of FGF2 and FGF9 in synaptogenesis are not well understood: only two studies have examined FGF2 in this capacity, and to our knowledge, none have examined FGF9. While there is much room

for expansion, the literature provides preliminary evidence that FGF2 is important for synaptogenesis and spine morphology: in culture, FGF2 treatment increases functional excitatory synapses on hippocampal neurons (Li et al., 2002), while FGF2 knockout mice displayed longer, thinner spines (but no differences in spine number) on CA3 pyramidal neurons than wildtype littermates (Zechel, Unsicker, & von Bohlen und Halbach, 2009). Similarly, other literature has highlighted BDNF as a modulator of dendritic arborization (Magariños et al., 2011), though neither FGF2 nor FGF9 have not been studied in this capacity. Future studies should examine the roles of both FGF2 and FGF9 in dendritic arborization and synapses, with a particular emphasis on how synaptogenesis, spine maintenance, and pruning may be related to FGF dysregulation in affective disorders.

Collectively, these data situate FGF2, and possibly FGF9, as organizing factors with the potential capability to impact molecular interactions that mediate cell-type balance and microcircuitry. ***Specifically, this dissertation argues that FGF2 and FGF9 have region-specific patterns of disruption in MDD, that their expression and function in particular regions of the brain might be both predisposing to and progressively dysregulated during affective dysfunction, and that the molecular organizing functions of these molecules is altered in MDD. Given their regional disruption in expression and their altered molecular relationships in MDD, future work should further examine these roles, as well as other major ways that FGFs can influence neural circuitry (including possible roles in synaptogenesis, spine maintenance, and dendritic arborization), to holistically describe how FGF dysregulation mediates effects on the brain in MDD.*** Indeed, this gap in knowledge – how FGFs and other growth factors enact changes on the cellular and microcircuit level – is one major limitation of the

neurotrophic hypothesis of depression. Furthermore, a better understanding of these functions may clarify how region-level disruptions, including volumetric decreases in the ACC and HPC, arise in MDD (Bora, Fornito, Pantelis, & Yücel, 2012; Bremner et al., 2000, 2002; Goodkind et al., 2015; Sheline et al., 2003).

Moving forward with the neurotrophic hypothesis of MDD

As previously discussed, the neurotrophic hypothesis of MDD posits that changes in the expression and function of growth factors may be related to the pathophysiology underlying MDD. Over the past several decades, a number of growth factors have been implicated in MDD. (Because it its beyond the scope of this chapter to fully review all the evidence for every growth factor implicated in MDD, the reader is directed to papers and reviews that summarize findings for these various factors, including BDNF (Björkholm & Monteggia, 2016; Duclot & Kabbaj, 2015; Duman & Duman, 2015, Duman & Monteggia, 2006), glial cell line derived neurotrophic factor, GDNF (Miller, 2011; Uchida et al., 2011), nerve growth factor, NGF (Angelucci, A. Math, & Aloe, 2004), vascular endothelial growth factor, VEGF (Carvalho et al., 2015; Clark-Raymond & Halaris, 2013; Warner-Schmidt & Duman, 2007), insulin-like growth factors, IGF (Anderson, Åberg, Nilsson, & Eriksson, 2002; Duman et al., 2009; Szczęsny et al., 2013), other FGFs (Evans et al., 2004; Turner, Watson, & Akil, 2012; Williams & Umemori, 2014), and others) Like the FGF system, other growth factors can influence and organize functions at the level of molecular dynamics, neurotransmission, cellular function and subtype balance, or microcircuits. Because expression of many of these factors is selectively impacted in MDD, additional work needs to be done to differentiate the roles of different growth factors in various functions implicated in affective dysregulation.

As has been noted in the stress literature, similar molecular or cellular changes may have very different consequences depending on the brain region and associated regional circuitry and function. For example, in the amygdala, stress increases dendritic arbor complexity (Johnson et al., 2009), while the same manipulation decreases dendritic arbor complexity in the hippocampus (Magariños, Deslandes, & McEwen, 1999). By extension, we hypothesize that altered growth factor expression may have differential impacts depending on brain region, and regional dissociation of growth factor functions on cellular effects may help to develop a better understanding between the molecular and cellular functions of these molecules. Because BDNF is known to have region-specific effects (Adachi et al., 2008; Berton et al., 2006; Eisch et al., 2003; Govindarajan et al., 2006; Krishnan et al., 2007; Monteggia et al., 2004), it may be that other growth factors also play different roles depending on the region, and indeed, evidence from this thesis suggests that FGF2 and FGF9 may have region-specific effects. This region specificity may be one good avenue to dissociate the specific roles of different growth factors in molecular, cellular, and behavioral functions.

Some growth factors have shown similar effects on affective dysregulation within-region, raising the possibility that disruptions in one growth factor system may spur or induce adaptive or compensatory functions in another. This idea is supported by evidence suggesting that different growth factors can play very similar roles in the same brain region. For example, experiments examining the affective functions of hippocampal FGF2 and BDNF have yielded remarkably similar results. Early-life or chronic stress decreased hippocampal BDNF expression (Hill et al., 2014; Naert, Ixart, Maurice, Tapia-Arancibia, & Givalois, 2011; Roceri, Hendriks, Racagni, Ellenbroek, & Riva, 2002), and

our laboratory has shown similar effects of chronic social defeat on hippocampal FGF2 expression (Turner, Calvo, et al., 2008). Lentiviral-mediated knockdown of both FGF2 (Chaudhury et al., 2014; Eren-Koçak et al., 2011) and BDNF (Taliaz, Stall, Dar, & Zangen, 2010) in the dentate gyrus had negative effects on affective behaviors in rats. Similarly, antidepressant treatment strongly induce both BDNF (Adachi et al., 2008; Garcia et al., 2008) and FGF2 (Bachis et al., 2008; Elsayed et al., 2012; Mallei et al., 2002) expression and this induction is necessary for treatment efficacy. Because of the dramatic parallels between hippocampal BDNF and FGF2, we hypothesize that BDNF and FGF2 impact the same hippocampal microcircuits, and we hope that future research will examine how the manipulations involving one growth factor impact the other.

Similarly, because many growth factors have effects on the same cellular processes, future research will need to dissociate the effects of individual growth factors on neural circuit functions. Some scholars have already begun to assemble these comparisons in specific fields: for example, one review compares effects of different growth factors on cellular proliferation, differentiation, and survival in the adult hippocampus, finding that growth factors mediate different aspects of neurogenesis. Among other effects, BDNF generally influences cell survival, while IGF has more consistent effects on proliferation (Fournier & Duman, 2012). However, many fewer studies have begun to differentiate the roles of different growth factors in other areas, and effects of growth factor manipulations on synaptogenesis and dendritic arborization are areas for substantial expansion. Furthermore, to fully address the neurotrophic hypothesis of MDD, these various effects will need to be analyzed in the context of affective

dysregulation to determine how different growth factors contribute to the aberrant neural circuitry that may underlie MDD symptoms.

While it is theoretically possible that different growth factor systems work independently and, in many regions, in parallel, there are a number of reasons to believe that signaling or functions associated with many growth factors could converge at different points (Figure 5.3). At the molecular level, there is evidence that single molecules can bind multiple growth factors simultaneously (e.g., betaglycan can simultaneously bind FGF2 and transforming growth factor-beta; Andres, DeFalcis, Noda, & Massagué, 1992), while other molecules can singly bind to many classes of growth factors (e.g., fibronectin can bind >25 growth factors with relatively high affinity; Martino & Hubbell, 2010). Similarly, both growth factor ligands and receptors can heterodimerize to potentially modulate signaling or neurotransmission (e.g., BDNF, NT-3, and NGF can form heterodimers, Arakawa et al., 1994; both BDNF and FGF receptors can interact with the serotonin system, Borroto-Escuela et al., 2012, 2015; Martinowich & Lu, 2007). Even if individual growth factors do not converge on the same receptors, there is evidence that intracellular signaling pathways might provide an additional avenue for convergence (e.g., FGF2, IGF and BDNF all converge on the Akt pathway, while only FGF2 and BDNF significantly activate Erk, Johnson-Farley, Patel, Kim, & Cowen, 2007; both FGF2 and FGF9 activate mTOR to mediate autophagy, Chen, Xiong, Tong, & Mao, 2013; Wang et al., 2015). Interestingly, activation of growth factor ligands and receptors can even induce expression of other growth factors from different molecular families (e.g., serotonin activates FGFR2 to induce expression of GDNF, Tsuchioka, Takebayashi, Hisaoka, Maeda, & Nakata, 2008; and FGF2 induces GDNF expression,

Shin et al., 2009). In addition, many growth factors and their receptors have differential patterns of expression that are regionally restricted by cell type, and this cellular heterogeneity adds another layer of complexity to understanding how different growth factor systems interact.

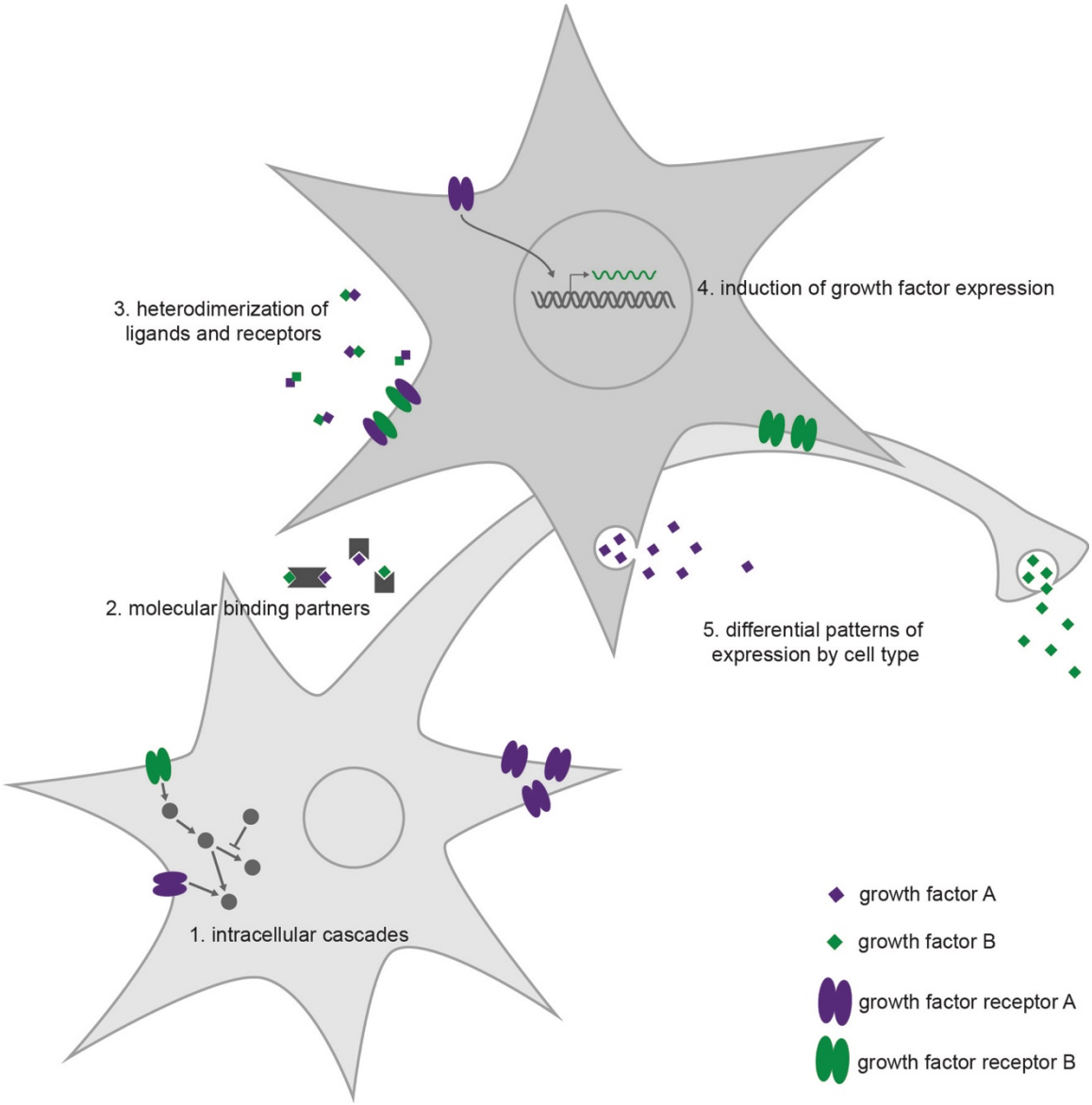


Figure 5.3. Schematic of previously demonstrated points of possible convergence for different growth factor systems.

To create a clear accounting of the unique, parallel, and compensatory roles of different growth factors in MDD, these distinctions will need to be separated and better described, requiring many studies to compare different growth factors head-to-head.

Future studies can begin to systematically parse different functions and interactions for different growth factors implicated in MDD by taking a number of approaches. As a field, it will be helpful to revisit basic experiments, like stress exposure, antidepressant administration, and others to systematically compare the molecular, cellular, and behavioral effects associated with different growth factors. To their credit, some researchers have already begun this endeavor (Angelucci, Aloe, Jiménez-Vasquez, & Mathé, 2003; Bland et al., 2007; First et al., 2011; Fournier & Duman, 2012; Fumagalli et al., 2004; Johnson-Farley et al., 2007), but it should be expanded dramatically to include many more growth factors and manipulations. Similarly, a greater emphasis should be placed on parsing the region-specific roles, including examining the interactions between neurons and glia and effects on cell-type balance, that different growth factors may play in brain regions and circuits underlying affective dysregulation. Furthermore, greater emphasis should be placed on understanding how alterations to one growth factor system impact others, and a better grasp of the points of downstream molecular and cellular convergence will facilitate this insight. Together, these additional studies will bring additional clarity to the neurotrophic hypothesis of MDD, allowing a better understanding of the development and maintenance of the disorder and creating the potential to develop better future antidepressants.

Limitations and strengths of the dissertation

While this dissertation focuses on growth factors and how alterations in growth factor expression alters vulnerability to affective dysregulation, it does not provide data on a cellular or circuit level that address how these alterations may be borne out in physical changes in brain circuitry. Indeed, as discussed above, this is one major challenge to the neurotrophic hypothesis of MDD as a whole, and a greater emphasis should be placed on addressing this gap. While there is abundant literature linking different growth factors to MDD (or different growth factors to processes which affect neural circuitry, including cellular proliferation, synaptogenesis, etc.), only a subset of these studies have attempted to link these types of observations in the same model or in the same tissue. The studies which have made these links provide clues suggesting that this avenue may be one way to untangle overlapping effects of different growth factor systems in circuit function (Elsayed et al., 2012; Perez et al., 2009; Taliaz et al., 2010; Turner et al., 2011), though for practical reasons, many focus on one cellular process and therefore do not differentiate between different circuit-altering mechanisms. Therefore, future studies involving FGF2 and FGF9 should better integrate multiple analyses related to circuitry, with a particular focus on analyzing changes in synaptogenesis and/or dendritic arborization, which have been studied with much less frequency than cellular processes like proliferation and/or survival.

Despite this limitation, this dissertation has a number of strengths. Chief among them, we have consistently worked to integrate MDD data from postmortem human brain tissue with animal models of affective dysregulation to parse the roles of FGF2 and FGF9 in different regions of the brain. Similarly, we have used a wide variety of techniques and

approaches, including “big data” approaches to analyze data derived from postmortem human tissue, animal behavior, administration paradigms, viral-mediated expression knockdown, and gene expression and protein activity assays, to draw our conclusions. This convergence on a set of conclusions using a wide variety of techniques increases the likelihood that our data are not due to random chance.

Furthermore, these different approaches have also enabled us to generate some striking conclusions as well as promising avenues for future study. We showed for the first time that FGF9 is disrupted in the hippocampus of depressed subjects, that an animal model of differing affective vulnerability showed divergent levels of hippocampal FGF9, and that altering endogenous expression of FGF9 in the hippocampus was sufficient to alter affective behavior. We contributed to the literature by reinforcing the hypothesis that hippocampal FGF2 has remarkable abilities to confer vulnerability or resilience in affective-like behavior. Moreover, we explored the impact of FGF2 and FGF9 dysregulation in MDD by probing how their expression was associated with different gene families and how these relationships changed with diagnosis, implicating circuit-related functions and generating hypotheses that can be addressed in future studies.

Future Directions

While the research in this dissertation generated some striking conclusions, we also formulated interesting hypotheses. We believe that there are many avenues for future studies to augment the data that we’ve showcased here to better describe the roles of FGF2 and FGF9 in MDD. Specifically, we see three main categories for future experiments that build on the findings of this body of work, including 1) further studying the role of FGF9 in affective dysregulation, 2) systematically differentiating FGF2,

FGF9, and other growth factors in various molecular, cellular, circuit, and behavioral processes (including additional studies to determine if FGF2 and FGF9 act as physiological antagonists to mediate opposite effects on affective behavior), and 3) further examining the molecular networks that FGF2, FGF9, and other growth factor systems impact to affect the interplay between genes.

Given that the data described in Chapter 2 are among the first to implicate FGF9 in MDD, it is evident that the literature examining the role of FGF9 in affective dysregulation is in its infancy. There are many studies yet to be completed, including a number of follow-up experiments to clarify our results. It will be interesting to determine if FGF9 knockdown eliminates behavioral differences between selectively bred bHR and bLR rats, and we are interested to know whether FGF9 knockdown in the dentate gyrus is protective against the deleterious effects of stress. Furthermore, we did not assess the effects of antidepressant drug administration on FGF9 expression, and we will be interested to determine whether there are differential region-specific effects of FGF9 dysregulation. Given the striking parallels in our preliminary data to manipulations involving FGF2, it is reasonable to ask whether FGF2 and FGF9 act as physiological antagonists to mediate affective dysregulation, and additional studies on possible points of convergence (including intracellular signaling) will clarify this.

While previous studies have shown that FGF2 and FGF9 impact cellular and circuit-level processes, there is much work to be done to elucidate the intersection between physical changes that the brain undergoes after chronic stress and MDD and the role of FGFs in these alterations. In particular, while FGF2 has been implicated in cell genesis and survival, no studies of FGF9 have been performed to evaluate its effects on

neurogenesis or gliogenesis. Similarly, very little is known about the roles of FGF2 or FGF9 in other cellular processes that impact circuit function, including synaptogenesis and dendritic arborization, especially with regards to possible effects of dysregulation in MDD. Therefore, we believe that an area of future emphasis should be determining how molecular alterations in FGF2 or FGF9 expression (or in the interactions between FGF2 and FGF9 with other molecular networks) are borne out on a cellular and circuit level.

Similarly, given the preliminary evidence that FGF2 and FGF9 may play organizational roles in the brain to mediate circuit function, we believe that our results implicating molecular network dysfunction should be further studied in other datasets. It will be interesting to see if the relationships that we observed in our postmortem human data replicate with other samples and other platforms, and any promising hypotheses should be systematically examined in animal models or other systems.

Final Conclusions

We have provided evidence that FGF2 and FGF9 may work coordinately in the brain to mediate affective dysregulation in MDD. We showed that FGF2 expression is reduced in the anterior cingulate cortex and FGF9 expression is elevated in the hippocampus of individuals diagnosed with MDD during life. Additionally, we showed that the expression of FGF2 and FGF9 correlates across regions, and we similarly showed that expression of both ligands correlates with FGF receptors. We found evidence that there are region-specific disruptions in the relationships between FGF family members in MDD, and these disruptions in relationships co-occurred with expression changes of genes related to basic biological and circuit function. Collectively, these data indicate that FGF2 and FGF9 are important organizational mediators in the depressed brain, and that

their effects may be coordinate and/or oppositional. However, these studies of postmortem human tissue are correlational and do not address if these altered patterns of expression are predisposing factors or if they arise from the disease process.

We used several animal models to reinforce these findings and address this limitation. In outbred animals, we demonstrated that knocking down endogenous expression of FGF9 in the dentate gyrus altered affective behavior. We also showed that both FGF2 and FGF9 are expressed at different levels in selectively-bred animals who differ in locomotor responses to a novel environment, one model of affective dysregulation, and we further showed that this difference in FGF2 expression was critical to the behavioral phenotype. Together with previous data demonstrating that FGF2 and FGF9 levels in the hippocampus are sensitive to stress, we argue that FGF2 and FGF9 could both act as predisposing factors for affective dysregulation and become increasingly dysregulated over time, contributing to a possible negative feedback cycle to influence the progression of the disease. We also partially validated preliminary findings from postmortem human data in an animal model of affective dysregulation, supporting the idea that FGF2 and FGF9 act as molecular organizers to mediate affect: specifically, we demonstrated that the relationships between FGF family members are phenotype-specific and, in some cases, modulated by stress. Collectively, these data reinforce the postmortem human data and strongly implicate FGF2 and FGF9 as molecular organizers in the brain whose functions can mediate affective dysregulation.

Furthermore, the data in this dissertation both proposes and addresses novel hypotheses. Together with previous data from our laboratory, the results in Chapter 2 suggesting that FGF9 is a novel modulator of negative affect are among the first data to

implicate this molecule in affective dysregulation and MDD. Moreover, to our knowledge, the data in Chapter 4 are among the first to examine diagnosis-specific changes in molecular networks associated with FGF2 and FGF9 expression, which have generated interesting preliminary data further implicating circuit dysregulation as a major underlying factor in MDD. We believe that these data are worthy of future study.

References

Adachi, M., Barrot, M., Autry, A. E., Theobald, D., & Monteggia, L. M. (2008). Selective Loss of Brain-Derived Neurotrophic Factor in the Dentate Gyrus Attenuates Antidepressant Efficacy. *Biological Psychiatry*, *63*(7), 642–649. http://doi.org/10.1016/j.biopsych.2007.09.019

American Psychiatric Association. (2013). *Diagnostic and statistical manual of mental disorders: DSM-5*. Washington, D.C.

Anderson, M. F., Åberg, M. A. I., Nilsson, M., & Eriksson, P. S. (2002). Insulin-like growth factor-I and neurogenesis in the adult mammalian brain. *Developmental Brain Research*, *134*(1–2), 115–122. http://doi.org/10.1016/S0165-3806(02)00277-8

Andres, J. L., DeFalcis, D., Noda, M., & Massagué, J. (1992). Binding of two growth factor families to separate domains of the proteoglycan betaglycan. *Journal of Biological Chemistry*, *267*(9), 5927–5930.

Angelucci, F., Aloe, L., Jiménez-Vasquez, P., & Mathé, A. A. (2003). Lithium treatment alters brain concentrations of nerve growth factor, brain-derived neurotrophic factor and glial cell line-derived neurotrophic factor in a rat model of depression. *The International Journal of Neuropsychopharmacology*, *6*(03), 225–231. http://doi.org/10.1017/S1461145703003468

Angelucci, F., A. Math, A., & Aloe, L. (2004). Neurotrophic factors and CNS disorders: findings in rodent models of depression and schizophrenia. In B.-P. in B. Research (Ed.), (Vol. 146, pp. 151–165). Elsevier. Retrieved from http://www.sciencedirect.com/science/article/pii/S0079612303460111

Arakawa, T., Haniu, M., Narhi, L. O., Miller, J. A., Talvenheimo, J., Philo, J. S., … Louis, J. C. (1994). Formation of heterodimers from three neurotrophins, nerve growth factor, neurotrophin-3, and brain-derived neurotrophic factor. *Journal of Biological Chemistry*, *269*(45), 27833–27839.

Aurbach, E. L., Inui, E. G., Turner, C. A., Hagenauer, M. H., Prater, K. E., Li, J. Z., … Akil, H. (2015). Fibroblast growth factor 9 is a novel modulator of negative affect. *Proceedings of the National Academy of Sciences*, *112*(38), 11953–11958. http://doi.org/10.1073/pnas.1510456112

Bachis, A., Mallei, A., Cruz, M. I., Wellstein, A., & Mocchetti, I. (2008). Chronic antidepressant treatments increase basic fibroblast growth factor and fibroblast growth factor-binding protein in neurons. *Neuropharmacology*, *55*(7), 1114–1120. http://doi.org/10.1016/j.neuropharm.2008.07.014

Banasr, M., & Duman, R. S. (2008). Glial Loss in the Prefrontal Cortex Is Sufficient to Induce Depressive-like Behaviors. *Biological Psychiatry*, *64*(10), 863–870. http://doi.org/10.1016/j.biopsych.2008.06.008

Bansal, R., Lakhina, V., Remedios, R., & Tole, S. (2003). Expression of FGF receptors 1, 2, 3 in the embryonic and postnatal mouse brain compared with Pdgfralpha, Olig2 and Plp/dm20: implications for oligodendrocyte development. *Developmental Neuroscience*, *25*(2-4), 83–95. http://doi.org/72258

Belluardo, N., Wu, G., Mudo, G., Hansson, A. c., Pettersson, R., & Fuxe, K. (1997). Comparative localization of fibroblast growth factor receptor-1, -2, and -3 mRNAs in the rat brain: In situ hybridization analysis. *The Journal of*

Comparative Neurology, *379*(2), 226–246. http://doi.org/10.1002/(SICI)1096-9861(19970310)379:2<226::AID-CNE5>3.0.CO;2-5

Bernard, R., Kerman, I. A., Thompson, R. C., Jones, E. G., Bunney, W. E., Barchas, J. D., … Watson, S. J. (2011). Altered expression of glutamate signaling, growth factor, and glia genes in the locus coeruleus of patients with major depression. *Molecular Psychiatry*, *16*(6), 634–646. http://doi.org/10.1038/mp.2010.44

Berton, O., McClung, C. A., DiLeone, R. J., Krishnan, V., Renthal, W., Russo, S. J., … others. (2006). Essential role of BDNF in the mesolimbic dopamine pathway in social defeat stress. *Science*, *311*(5762), 864.

Björkholm, C., & Monteggia, L. M. (2016). BDNF – a key transducer of antidepressant effects. *Neuropharmacology*, *102*, 72–79. http://doi.org/10.1016/j.neuropharm.2015.10.034

Bland, S. T., Tamlyn, J. P., Barrientos, R. M., Greenwood, B. N., Watkins, L. R., Campeau, S., … Maier, S. F. (2007). Expression of fibroblast growth factor-2 and brain-derived neurotrophic factor mRNA in the medial prefrontal cortex and hippocampus after uncontrollable or controllable stress. *Neuroscience*, *144*(4), 1219–1228. http://doi.org/10.1016/j.neuroscience.2006.11.026

Bora, E., Fornito, A., Pantelis, C., & Yücel, M. (2012). Gray matter abnormalities in Major Depressive Disorder: A meta-analysis of voxel based morphometry studies. *Journal of Affective Disorders*, *138*(1–2), 9–18. http://doi.org/10.1016/j.jad.2011.03.049

Borroto-Escuela, D. O., Narvaez, M., Pérez-Alea, M., Tarakanov, A. O., Jiménez-Beristain, A., Mudó, G., … Fuxe, K. (2015). Evidence for the existence of FGFR1–5-HT1A heteroreceptor complexes in the midbrain raphe 5-HT system. *Biochemical and Biophysical Research Communications*, *456*(1), 489–493. http://doi.org/10.1016/j.bbrc.2014.11.112

Borroto-Escuela, D. O., Romero-Fernandez, W., Mudó, G., Pérez-Alea, M., Ciruela, F., Tarakanov, A. O., … Fuxe, K. (2012). Fibroblast Growth Factor Receptor 1– 5-Hydroxytryptamine 1A Heteroreceptor Complexes and Their Enhancement of Hippocampal Plasticity. *Biological Psychiatry*, *71*(1), 84–91. http://doi.org/10.1016/j.biopsych.2011.09.012

Bremner, J. D., Narayan, M., Anderson, E. R., Staib, L. H., Miller, H. L., Charney, D. S., & others. (2000). Hippocampal volume reduction in major depression. *American Journal of Psychiatry*, *157*(1), 115–117.

Bremner, J. D., Vythilingam, M., Vermetten, E., Nazeer, A., Adil, J., Khan, S., … Charney, D. S. (2002). Reduced volume of orbitofrontal cortex in major depression. *Biological Psychiatry*, *51*(4), 273–279. http://doi.org/10.1016/S0006-3223(01)01336-1

Carvalho, A. F., Köhler, C. A., McIntyre, R. S., Knöchel, C., Brunoni, A. R., Thase, M. E., … Berk, M. (2015). Peripheral vascular endothelial growth factor as a novel depression biomarker: A meta-analysis. *Psychoneuroendocrinology*, *62*, 18–26. http://doi.org/10.1016/j.psyneuen.2015.07.002

Chana, G., Landau, S., Beasley, C., Everall, I. P., & Cotter, D. R. (2003). Two-dimensional assessment of cytoarchitecture in the anterior cingulate cortex in major depressive disorder, bipolar disorder, and schizophrenia: evidence for

decreased neuronal somal size and increased neuronal density. *Biological Psychiatry*, *53*(12), 1086–1098. http://doi.org/10.1016/S0006-3223(03)00114-8

Chaudhury, S., Aurbach, E. L., Sharma, V., Blandino, P., Turner, C. A., Watson, S. J., & Akil, H. (2014). FGF2 is a target and a trigger of epigenetic mechanisms associated with differences in emotionality: Partnership with H3K9me3. *Proceedings of the National Academy of Sciences*, *111*(32), 11834–11839. http://doi.org/10.1073/pnas.1411618111

Chen, A., Xiong, L.-J., Tong, Y., & Mao, M. (2013). Neuroprotective effect of brain-derived neurotrophic factor mediated by autophagy through the PI3K/Akt/mTOR pathway. *Molecular Medicine Reports*, *8*(4), 1011–1016. http://doi.org/10.3892/mmr.2013.1628

Cheng, Y., Black, I. B., & DiCicco-Bloom, E. (2002). Hippocampal granule neuron production and population size are regulated by levels of bFGF. *European Journal of Neuroscience*, *15*(1), 3–12. http://doi.org/10.1046/j.0953-816x.2001.01832.x

Chuang, J.-I., Huang, J.-Y., Tsai, S.-J., Sun, H. S., Yang, S.-H., Chuang, P.-C., … Ching, C.-H. (2015). FGF9-induced changes in cellular redox status and HO-1 upregulation are FGFR-dependent and proceed through both ERK and AKT to induce CREB and Nrf2 activation. *Free Radical Biology and Medicine*, *89*, 274–286. http://doi.org/10.1016/j.freeradbiomed.2015.08.011

Clark-Raymond, A., & Halaris, A. (2013). VEGF and depression: A comprehensive assessment of clinical data. *Journal of Psychiatric Research*, *47*(8), 1080–1087. http://doi.org/10.1016/j.jpsychires.2013.04.008

Cotter, D. R., Mackay, D., Chana, G., Beasley, C., Landau, S., & Everall, I. P. (2002). Reduced Neuronal Size and Glial Cell Density in Area 9 of the Dorsolateral Prefrontal Cortex in Subjects with Major Depressive Disorder. *Cerebral Cortex*, *12*(4), 386–394. http://doi.org/10.1093/cercor/12.4.386

Cotter, D. R., Pariante, C. M., & Everall, I. P. (2001). Glial cell abnormalities in major psychiatric disorders: the evidence and implications. *Brain Research Bulletin*, *55*(5), 585–595. http://doi.org/10.1016/S0361-9230(01)00527-5

Cotter, D. R., S, L., & I, E. (2001). Reduced glial cell density and neuronal size in the anterior cingulate cortex in major depressive disorder. *Archives of General Psychiatry*, *58*(6), 545–553. http://doi.org/10.1001/archpsyc.58.6.545

Duclot, F., & Kabbaj, M. (2015). Epigenetic mechanisms underlying the role of brain-derived neurotrophic factor in depression and response to antidepressants. *Journal of Experimental Biology*, *218*(1), 21–31. http://doi.org/10.1242/jeb.107086

Duman, C. H., & Duman, R. S. (2015). Spine synapse remodeling in the pathophysiology and treatment of depression. *Neuroscience Letters*, *601*, 20–29. http://doi.org/10.1016/j.neulet.2015.01.022

Duman, R. S., & Monteggia, L. M. (2006). A Neurotrophic Model for Stress-Related Mood Disorders. *Biological Psychiatry*, *59*(12), 1116–1127. http://doi.org/16/j.biopsych.2006.02.013

Duman, C. H., Schlesinger, L., Terwilliger, R., Russell, D. S., Newton, S. S., & Duman, R. S. (2009). Peripheral insulin-like growth factor-I produces antidepressant-like behavior and contributes to the effect of exercise. *Behavioural Brain Research*, *198*(2), 366–371. http://doi.org/10.1016/j.bbr.2008.11.016

Eisch, A. J., Bolaños, C. A., de Wit, J., Simonak, R. D., Pudiak, C. M., Barrot, M., … Nestler, E. J. (2003). Brain-derived neurotrophic factor in the ventral midbrain–nucleus accumbens pathway: a role in depression. *Biological Psychiatry*, *54*(10), 994–1005. http://doi.org/10.1016/j.biopsych.2003.08.003

Elsayed, M., Banasr, M., Duric, V., Fournier, N. M., Licznerski, P., & Duman, R. S. (2012). Antidepressant Effects of Fibroblast Growth Factor-2 in Behavioral and Cellular Models of Depression. *Biological Psychiatry*, *72*(4), 258–265. http://doi.org/10.1016/j.biopsych.2012.03.003

Eren-Koçak, E., Turner, C. A., Watson, S. J., & Akil, H. (2011). Short-Hairpin RNA Silencing of Endogenous Fibroblast Growth Factor 2 in Rat Hippocampus Increases Anxiety Behavior. *Biological Psychiatry*, *69*(6), 534–540. http://doi.org/10.1016/j.biopsych.2010.11.020

Evans, S. J., Choudary, P. V., Neal, C. R., Li, J. Z., Vawter, M. P., Tomita, H., … Akil, H. (2004). Dysregulation of the fibroblast growth factor system in major depression. *Proceedings of the National Academy of Sciences of the United States of America*, *101*(43), 15506 –15511. http://doi.org/10.1073/pnas.0406788101

Falcone, C., Filippis, C., Granzotto, M., & Mallamaci, A. (2015). Emx2 expression levels in NSCs modulate astrogenesis rates by regulating EgfR and Fgf9. *Glia*, *63*(3), 412–422. http://doi.org/10.1002/glia.22761

First, M., Gil-Ad, I., Taler, M., Tarasenko, I., Novak, N., & Weizman, A. (2011). The Effects of Fluoxetine Treatment in a Chronic Mild Stress Rat Model on Depression-Related Behavior, Brain Neurotrophins and ERK Expression. *Journal of Molecular Neuroscience*, *45*(2), 246–255. http://doi.org/10.1007/s12031-011-9515-5

Flagel, S. B., Waselus, M., Clinton, S. M., Watson, S. J., & Akil, H. (2014). Antecedents and consequences of drug abuse in rats selectively bred for high and low response to novelty. *Neuropharmacology*, *76, Part B*, 425–436. http://doi.org/10.1016/j.neuropharm.2013.04.033

Fournier, N. M., & Duman, R. S. (2012). Role of vascular endothelial growth factor in adult hippocampal neurogenesis: Implications for the pathophysiology and treatment of depression. *Behavioural Brain Research*, *227*(2), 440–449. http://doi.org/10.1016/j.bbr.2011.04.022

Fumagalli, F., Bedogni, F., Slotkin, T. A., Racagni, G., & Riva, M. A. (2005). Prenatal stress elicits regionally selective changes in basal FGF-2 gene expression in adulthood and alters the adult response to acute or chronic stress. *Neurobiology of Disease*, *20*(3), 731–737. http://doi.org/10.1016/j.nbd.2005.05.005

Fumagalli, F., Molteni, R., Bedogni, F., Gennarelli, M., Perez, J., Racagni, G., & Riva, M. A. (2004). Quetiapine regulates FGF-2 and BDNF expression in the hippocampus of animals treated with MK-801. *Neuroreport*, *15*(13), 2109–2112.

Garcès, A., Nishimune, H., Philippe, J.-M., Pettmann, B., & deLapeyrière, O. (2000). FGF9: A motoneuron survival factor expressed by medial thoracic and sacral motoneurons. *Journal of Neuroscience Research*, *60*(1), 1–9. http://doi.org/10.1002/(SICI)1097-4547(20000401)60:1<1::AID-JNR1>3.0.CO;2-P

Garcia, L. S. B., Comim, C. M., Valvassori, S. S., Réus, G. Z., Barbosa, L. M., Andreazza, A. C., … Quevedo, J. (2008). Acute administration of ketamine

induces antidepressant-like effects in the forced swimming test and increases BDNF levels in the rat hippocampus. *Progress in Neuro-Psychopharmacology and Biological Psychiatry*, *32*(1), 140–144. http://doi.org/10.1016/j.pnpbp.2007.07.027

Gaughran, F., Payne, J., Sedgwick, P. M., Cotter, D., & Berry, M. (2006). Hippocampal FGF-2 and FGFR1 mRNA expression in major depression, schizophrenia and bipolar disorder. *Brain Research Bulletin*, *70*(3), 221–227. http://doi.org/10.1016/j.brainresbull.2006.04.008

Gómez-Pinilla, F., Dao, L., Choi, J., & Ryba, E. . (2000). Diazepam induces FGF-2 mRNA in the hippocampus and striatum. *Brain Research Bulletin*, *53*(3), 283–289. http://doi.org/10.1016/S0361-9230(00)00342-7

Gonzalez, A. M., Berry, M., Maher, P. A., Logan, A., & Baird, A. (1995). A comprehensive analysis of the distribution of FGF-2 and FGFR1 in the rat brain. *Brain Research*, *701*(1–2), 201–226. http://doi.org/10.1016/0006-8993(95)01002-X

Goodkind, M., Eickhoff, S. B., Oathes, D. J., Jiang, Y., Chang, A., Jones-Hagata, L. B., … Etkin, A. (2015). Identification of a common neurobiological substrate for mental illness. *JAMA Psychiatry*, *72*(4), 305–315. http://doi.org/10.1001/jamapsychiatry.2014.2206

Govindarajan, A., Rao, B. S. S., Nair, D., Trinh, M., Mawjee, N., Tonegawa, S., & Chattarji, S. (2006). Transgenic brain-derived neurotrophic factor expression causes both anxiogenic and antidepressant effects. *Proceedings of the National Academy of Sciences*, *103*(35), 13208–13213. http://doi.org/10.1073/pnas.0605180103

Guillemot, F., & Zimmer, C. (2011). From Cradle to Grave: The Multiple Roles of Fibroblast Growth Factors in Neural Development. *Neuron*, *71*(4), 574–588. http://doi.org/10.1016/j.neuron.2011.08.002

Hill, R. A., Klug, M., Kiss Von Soly, S., Binder, M. D., Hannan, A. J., & van den Buuse, M. (2014). Sex-specific disruptions in spatial memory and anhedonia in a “two hit” rat model correspond with alterations in hippocampal brain-derived neurotrophic factor expression and signaling. *Hippocampus*, *24*(10), 1197–1211. http://doi.org/10.1002/hipo.22302

Huang, J.-Y., Hong, Y.-T., & Chuang, J.-I. (2009). Fibroblast growth factor 9 prevents MPP+-induced death of dopaminergic neurons and is involved in melatonin neuroprotection in vivo and in vitro. *Journal of Neurochemistry*, *109*(5), 1400–1412. http://doi.org/10.1111/j.1471-4159.2009.06061.x

Johnson-Farley, N. N., Patel, K., Kim, D., & Cowen, D. S. (2007). Interaction of FGF-2 with IGF-1 and BDNF in stimulating Akt, ERK, and neuronal survival in hippocampal cultures. *Brain Research*, *1154*, 40–49. http://doi.org/10.1016/j.brainres.2007.04.026

Johnson, S. A., Wang, J.-F., Sun, X., McEwen, B. S., Chattarji, S., & Young, L. T. (2009). Lithium treatment prevents stress-induced dendritic remodeling in the rodent amygdala. *Neuroscience*, *163*(1), 34–39. http://doi.org/10.1016/j.neuroscience.2009.06.005

Kanda, T., Iwasaki, T., Nakamura, S., Kurokawa, T., Ikeda, K., & Mizusawa, H. (2000). Self-secretion of fibroblast growth factor-9 supports basal forebrain cholinergic

neurons in an autocrine/paracrine manner. *Brain Research*, *876*(1–2), 22–30. http://doi.org/10.1016/S0006-8993(00)02563-4

Kanda, T., Iwasaki, T., Nakamura, S., Ueki, A., Kurokawa, T., Ikeda, K., & Mizusawa, H. (1999). FGF-9 is an autocrine/paracrine neurotrophic substance for spinal motoneurons. *International Journal of Developmental Neuroscience*, *17*(3), 191–200. http://doi.org/10.1016/S0736-5748(99)00026-X

Keller, M. B., Lavori, P. W., Lewis, C. E., & Klerman, G. L. (1983). Predictors of relapse in major depressive disorder. *JAMA*, *250*(24), 3299–3304.

Korada, S., Zheng, W., Basilico, C., Schwartz, M. L., & Vaccarino, F. M. (2002). Fibroblast Growth Factor 2 Is Necessary for the Growth of Glutamate Projection Neurons in the Anterior Neocortex. *The Journal of Neuroscience*, *22*(3), 863–875.

Krishnan, V., Han, M.-H., Graham, D. L., Berton, O., Renthal, W., Russo, S. J., … Nestler, E. J. (2007). Molecular Adaptations Underlying Susceptibility and Resistance to Social Defeat in Brain Reward Regions. *Cell*, *131*(2), 391–404. http://doi.org/10.1016/j.cell.2007.09.018

Li, A., Suzuki, S., Suzuki, M., Mizukoshi, E., & Imamura, T. (2002). Fibroblast growth factor-2 increases functional excitatory synapses on hippocampal neurons. *European Journal of Neuroscience*, *16*(7), 1313–1324. http://doi.org/10.1046/j.1460-9568.2002.02193.x

Lin, Y., Chen, L., Lin, C., Luo, Y., Tsai, R. Y. L., & Wang, F. (2009). Neuron-derived FGF9 is essential for scaffold formation of Bergmann radial fibers and migration of granule neurons in the cerebellum. *Developmental Biology*, *329*(1), 44–54. http://doi.org/10.1016/j.ydbio.2009.02.011

Lum, M., Turbic, A., Mitrovic, B., & Turnley, A. M. (2009). Fibroblast growth factor-9 inhibits astrocyte differentiation of adult mouse neural progenitor cells. *Journal of Neuroscience Research*, *87*(10), 2201–2210. http://doi.org/10.1002/jnr.22047

Magariños, A. M., Deslandes, A., & McEwen, B. S. (1999). Effects of antidepressants and benzodiazepine treatments on the dendritic structure of CA3 pyramidal neurons after chronic stress. *European Journal of Pharmacology*, *371*(2-3), 113–122.

Magariños, A. M., Li, C. J., Gal Toth, J., Bath, K. G., Jing, D., Lee, F. S., & McEwen, B. S. (2011). Effect of brain-derived neurotrophic factor haploinsufficiency on stress-induced remodeling of hippocampal neurons. *Hippocampus*, *21*(3), 253–264. http://doi.org/10.1002/hipo.20744

Malberg, J. E., Eisch, A. J., Nestler, E. J., & Duman, R. S. (2000). Chronic Antidepressant Treatment Increases Neurogenesis in Adult Rat Hippocampus. *The Journal of Neuroscience*, *20*(24), 9104 –9110.

Mallei, A., Shi, B., & Mocchetti, I. (2002). Antidepressant Treatments Induce the Expression of Basic Fibroblast Growth Factor in Cortical and Hippocampal Neurons. *Molecular Pharmacology*, *61*(5), 1017 –1024. http://doi.org/10.1124/mol.61.5.1017

Martino, M. M., & Hubbell, J. A. (2010). The 12th–14th type III repeats of fibronectin function as a highly promiscuous growth factor-binding domain. *The FASEB Journal*, *24*(12), 4711–4721. http://doi.org/10.1096/fj.09-151282

Martinowich, K., & Lu, B. (2007). Interaction between BDNF and Serotonin: Role in Mood Disorders. *Neuropsychopharmacology*, *33*(1), 73–83. http://doi.org/10.1038/sj.npp.1301571

Mason, I. (2007). Initiation to end point: the multiple roles of fibroblast growth factors in neural development. *Nature Reviews Neuroscience*, *8*(8), 583–596. http://doi.org/10.1038/nrn2189

Meier, F., Giesert, F., Delic, S., Faus-Kessler, T., Matheus, F., Simeone, A., … Prakash, N. (2014). FGF/FGFR2 Signaling Regulates the Generation and Correct Positioning of Bergmann Glia Cells in the Developing Mouse Cerebellum. *PLoS ONE*, *9*(7), e101124. http://doi.org/10.1371/journal.pone.0101124

Miller, C. A. (2011). Stressed and Depressed? Check Your GDNF for Epigenetic Repression. *Neuron*, *69*(2), 188–190. http://doi.org/10.1016/j.neuron.2011.01.006

Molteni, R., Fumagalli, F., Magnaghi, V., Roceri, M., Gennarelli, M., Racagni, G., … Riva, M. A. (2001). Modulation of fibroblast growth factor-2 by stress and corticosteroids: from developmental events to adult brain plasticity. *Brain Research Reviews*, *37*(1–3), 249–258. http://doi.org/10.1016/S0165-0173(01)00128-X

Monteggia, L. M., Barrot, M., Powell, C. M., Berton, O., Galanis, V., Gemelli, T., … Nestler, E. J. (2004). Essential role of brain-derived neurotrophic factor in adult hippocampal function. *Proceedings of the National Academy of Sciences of the United States of America*, *101*(29), 10827–10832. http://doi.org/10.1073/pnas.0402141101

Naert, G., Ixart, G., Maurice, T., Tapia-Arancibia, L., & Givalois, L. (2011). Brain-derived neurotrophic factor and hypothalamic-pituitary-adrenal axis adaptation processes in a depressive-like state induced by chronic restraint stress. *Molecular and Cellular Neuroscience*, *46*(1), 55–66. http://doi.org/10.1016/j.mcn.2010.08.006

Öngür, D., Drevets, W. C., & Price, J. L. (1998). Glial reduction in the subgenual prefrontal cortex in mood disorders. *Proceedings of the National Academy of Sciences*, *95*(22), 13290–13295. http://doi.org/10.1073/pnas.95.22.13290

Ornitz, D. M., Xu, J., Colvin, J. S., McEwen, D. G., MacArthur, C. A., Coulier, F., … Goldfarb, M. (1996). Receptor Specificity of the Fibroblast Growth Factor Family. *Journal of Biological Chemistry*, *271*(25), 15292–15297. http://doi.org/10.1074/jbc.271.25.15292

Panksepp, J. (2003). Feeling the Pain of Social Loss. *Science*, *302*(5643), 237 –239. http://doi.org/10.1126/science.1091062

Panksepp, J., Herman, B., Conner, R., Bishop, P., & Scott, J. P. (1978). The biology of social attachments: opiates alleviate separation distress. *Biological Psychiatry*, *13*(5), 607–618.

Panksepp, J., Knutson, B., & Burgdorf, J. (2002). The role of brain emotional systems in addictions: a neuro-evolutionary perspective and new “self-report” animal model. *Addiction*, *97*(4), 459–469. http://doi.org/10.1046/j.1360-0443.2002.00025.x

Panksepp, J., Nelson, E., & Bekkedal, M. (1997). Brain systems for the mediation of social separation-distress and social-reward. Evolutionary antecedents and neuropeptide intermediaries. *Annals of the New York Academy of Sciences*, *807*, 78–100.

Pataky, D. M., Borisoff, J. F., Fernandes, K. J. L., Tetzlaff, W., & Steeves, J. D. (2000). Fibroblast Growth Factor Treatment Produces Differential Effects on Survival and Neurite Outgrowth from Identified Bulbospinal Neurons in Vitro. *Experimental Neurology*, *163*(2), 357–372. http://doi.org/10.1006/exnr.2000.7365

Paxinos, G., & Watson, C. (2006). The Rat Brain in Stereotaxic Coordinates, 6th Edition. Retrieved December 23, 2015, from http://store.elsevier.com/The-Rat-Brain-in-Stereotaxic-Coordinates/George-Paxinos/isbn-9780080475158/

Perez, J. A., Clinton, S. M., Turner, C. A., Watson, S. J., & Akil, H. (2009). A New Role for FGF2 as an Endogenous Inhibitor of Anxiety. *The Journal of Neuroscience*, *29*(19), 6379 –6387. http://doi.org/10.1523/JNEUROSCI.4829-08.2009

Rai, K. S., Hattiangady, B., & Shetty, A. K. (2007). Enhanced production and dendritic growth of new dentate granule cells in the middle-aged hippocampus following intracerebroventricular FGF-2 infusions. *European Journal of Neuroscience*, *26*(7), 1765–1779. http://doi.org/10.1111/j.1460-9568.2007.05820.x

Roceri, M., Hendriks, W., Racagni, G., Ellenbroek, B. A., & Riva, M. A. (2002). Early maternal deprivation reduces the expression of BDNF and NMDA receptor subunits in rat hippocampus. *Molecular Psychiatry*, *7*(6), 609–616. http://doi.org/10.1038/sj.mp.4001036

Schmidt, H. D., & Duman, R. S. (2007). The role of neurotrophic factors in adult hippocampal neurogenesis, antidepressant treatments and animal models of depressive-like behavior. *Behavioural Pharmacology*, *18*(5-6), 391–418. http://doi.org/10.1097/FBP.0b013e3282ee2aa8

Sheline, Y. I., Gado, M. H., & Kraemer, H. C. (2003). Untreated depression and hippocampal volume loss. *American Journal of Psychiatry*, *160*(8), 1516–1518.

Sheline, Y. I., Sanghavi, M., Mintun, M. A., & Gado, M. H. (1999). Depression Duration But Not Age Predicts Hippocampal Volume Loss in Medically Healthy Women with Recurrent Major Depression. *The Journal of Neuroscience*, *19*(12), 5034–5043.

Sheline, Y. I., Wang, P. W., Gado, M. H., Csernansky, J. G., & Vannier, M. W. (1996). Hippocampal atrophy in recurrent major depression. *Proceedings of the National Academy of Sciences*, *93*(9), 3908–3913.

Shin, S. Y., Song, H., Kim, C. G., Choi, Y.-K., Lee, K. S., Lee, S.-J., … Lee, Y. H. (2009). Egr-1 Is Necessary for Fibroblast Growth Factor-2-induced Transcriptional Activation of the Glial Cell Line-derived Neurotrophic Factor in Murine Astrocytes. *The Journal of Biological Chemistry*, *284*(44), 30583–30593. http://doi.org/10.1074/jbc.M109.010678

Shirayama, Y., Chen, A. C.-H., Nakagawa, S., Russell, D. S., & Duman, R. S. (2002). Brain-Derived Neurotrophic Factor Produces Antidepressant Effects in Behavioral Models of Depression. *The Journal of Neuroscience*, *22*(8), 3251–3261.

Stead, J. D. H., Clinton, S., Neal, C., Schneider, J., Jama, A., Miller, S., … Akil, H. (2006). Selective Breeding for Divergence in Novelty-seeking Traits: Heritability and Enrichment in Spontaneous Anxiety-related Behaviors. *Behavior Genetics*, *36*(5), 697–712. http://doi.org/10.1007/s10519-006-9058-7

Stedenfeld, K. A., Clinton, S. M., Kerman, I. A., Akil, H., Watson, S. J., & Sved, A. F. (2011). Novelty-seeking behavior predicts vulnerability in a rodent model of

depression. *Physiology & Behavior*, *103*(2), 210–216. http://doi.org/10.1016/j.physbeh.2011.02.001

Stockmeier, C. A., Mahajan, G. J., Konick, L. C., Overholser, J. C., Jurjus, G. J., Meltzer, H. Y., … Rajkowska, G. (2004). Cellular Changes in the Postmortem Hippocampus in Major Depression. *Biological Psychiatry*, *56*(9), 640–650. http://doi.org/10.1016/j.biopsych.2004.08.022

Szczęsny, E., Slusarczyk, J., Głombik, K., Budziszewska, B., Kubera, M., Lasoń, W., & Basta-Kaim, A. (2013). Possible contribution of IGF-1 to depressive disorder. *Pharmacological Reports: PR*, *65*(6), 1622–1631.

Tagashira, S., Ozaki, K., Ohta, M., & Itoh, N. (1995). Localization of fibroblast growth factor-9 mRNA in the rat brain. *Molecular Brain Research*, *30*(2), 233–241. http://doi.org/10.1016/0169-328X(95)00009-H

Taliaz, D., Stall, N., Dar, D. E., & Zangen, A. (2010). Knockdown of brain-derived neurotrophic factor in specific brain sites precipitates behaviors associated with depression and reduces neurogenesis. *Molecular Psychiatry*, *15*(1), 80–92. http://doi.org/10.1038/mp.2009.67

Tsuchioka, M., Takebayashi, M., Hisaoka, K., Maeda, N., & Nakata, Y. (2008). Serotonin (5-HT) induces glial cell line-derived neurotrophic factor (GDNF) mRNA expression via the transactivation of fibroblast growth factor receptor 2 (FGFR2) in rat C6 glioma cells. *Journal of Neurochemistry*, *106*(1), 244–257. http://doi.org/10.1111/j.1471-4159.2008.05357.x

Turner, C. A., Calvo, N., Frost, D. O., Akil, H., & Watson, S. J. (2008). The fibroblast growth factor system is downregulated following social defeat. *Neuroscience Letters*, *430*(2), 147–150.

Turner, C. A., Clinton, S. M., Thompson, R. C., Watson, S. J., & Akil, H. (2011). Fibroblast growth factor-2 (FGF2) augmentation early in life alters hippocampal development and rescues the anxiety phenotype in vulnerable animals. *Proceedings of the National Academy of Sciences*, *108*(19), 8021 –8025. http://doi.org/10.1073/pnas.1103732108

Turner, C. A., Gula, E. L., Taylor, L. P., Watson, S. J., & Akil, H. (2008). Antidepressant-like effects of intracerebroventricular FGF2 in rats. *Brain Research*, *1224*, 63–68. http://doi.org/16/j.brainres.2008.05.088

Turner, C. A., Watson, S. J., & Akil, H. (2012). The Fibroblast Growth Factor Family: Neuromodulation of Affective Behavior. *Neuron*, *76*(1), 160–174. http://doi.org/10.1016/j.neuron.2012.08.037

Uchida, S., Hara, K., Kobayashi, A., Otsuki, K., Yamagata, H., Hobara, T., … Watanabe, Y. (2011). Epigenetic Status of Gdnf in the Ventral Striatum Determines Susceptibility and Adaptation to Daily Stressful Events. *Neuron*, *69*(2), 359–372. http://doi.org/10.1016/j.neuron.2010.12.023

Vicario-Abejón, C., Johe, K. K., Hazel, T. G., Collazo, D., & McKay, R. D. G. (1995). Functions of basic fibroblast growth factor and neurotrophins in the differentiation of hippocampal neurons. *Neuron*, *15*(1), 105–114. http://doi.org/10.1016/0896-6273(95)90068-3

Wagner, J. P., Black, I. B., & DiCicco-Bloom, E. (1999). Stimulation of Neonatal and Adult Brain Neurogenesis by Subcutaneous Injection of Basic Fibroblast Growth Factor. *The Journal of Neuroscience*, *19*(14), 6006–6016.

Wang, Z.-G., Wang, Y., Huang, Y., Lu, Q., Zheng, L., Hu, D., … Xiao, J. (2015). bFGF regulates autophagy and ubiquitinated protein accumulation induced by myocardial ischemia/reperfusion via the activation of the PI3K/Akt/mTOR pathway. *Scientific Reports*, *5*. http://doi.org/10.1038/srep09287

Warner-Schmidt, J. L., & Duman, R. S. (2006). Hippocampal neurogenesis: Opposing effects of stress and antidepressant treatment. *Hippocampus*, *16*(3), 239–249. http://doi.org/10.1002/hipo.20156

Warner-Schmidt, J. L., & Duman, R. S. (2007). VEGF is an essential mediator of the neurogenic and behavioral actions of antidepressants. *Proceedings of the National Academy of Sciences*, *104*(11), 4647–4652. http://doi.org/10.1073/pnas.0610282104

Williams, A. J., & Umemori, H. (2014). The best-laid plans go oft awry: synaptogenic growth factor signaling in neuropsychiatric disease. *Frontiers in Synaptic Neuroscience*, *6*, 4. http://doi.org/10.3389/fnsyn.2014.00004

Zechel, S., Unsicker, K., & von Bohlen und Halbach, O. (2009). Fibroblast growth factor-2 deficiency affects hippocampal spine morphology, but not hippocampal catecholaminergic or cholinergic innervation. *Developmental Dynamics*, *238*(2), 343–350. http://doi.org/10.1002/dvdy.21839

Zhang, X., Ibrahimi, O. A., Olsen, S. K., Umemori, H., Mohammadi, M., & Ornitz, D. M. (2006). Receptor Specificity of the Fibroblast Growth Factor Family THE COMPLETE MAMMALIAN FGF FAMILY. *Journal of Biological Chemistry*, *281*(23), 15694–15700. http://doi.org/10.1074/jbc.M601252200

Zhao, M., Li, D., Shimazu, K., Zhou, Y.-X., Lu, B., & Deng, C.-X. (2007). Fibroblast Growth Factor Receptor-1 is Required for Long-Term Potentiation, Memory Consolidation, and Neurogenesis. *Biological Psychiatry*, *62*(5), 381–390. http://doi.org/10.1016/j.biopsych.2006.10.019