A Role for the Fibroblast Growth Factor Family in Depression and Circadian Rhythms

by

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Dedication

To my persistent husband who continued to make the drive to Ann Arbor, so that we could take the rest of this journey together: “Tazo, your support and encouragement means the world to me, and I look forward to all that God has in store for two stubborn souls. I love you and would go to the end of this Earth for you; yes, even Cleveland.”

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Abstract

An unbiased microarray analysis of post-mortem tissue of individuals diagnosed with major depressive disorder (MDD) reported on the dysregulation of the fibroblast growth factor (FGF) family in cortical and limbic regions. This was the first study to implicate this system of growth factors in mood disorders. While FGF2 gene expression was consistently downregulated in a number of brain regions in MDD, FGF9 gene expression was consistently upregulated in MDD. While we have recently characterized the behavioral and molecular effects of the well-known FGF2 ligand in emotionality, the role of FGF9 in affective behavior had yet to be explored. Initial studies that paralleled FGF2 administration provided important information about method of administration, dosing, and time-sensitivity of testing. While chronic FGF2 had antidepressant-like and anxiolytic effects in animal models of depression, chronic FGF9 increased depression-like behavior and was anxiogenic. Chronic injections of FGF9 decreased expression of FGFR1 in the dentate gyrus in the hippocampus suggesting that FGF9 may alter the level of FGF2/FGFR1 signaling. In addition to the role of exogenous FGF9 in altering affective behaviors, endogenous FGF9 expression was increased by chronic social stress. These findings support the hypothesis that FGF9 mediates the long-term negative aspects of neurotrophic factor dysregulation in the hippocampus. As circadian disruptions are often implicated

in mechanisms underlying MDD, we also investigated the basal expression of FGF family members in the hippocampus over time. FGF2 and FGFR1 both displayed endogenous circadian gene expression in the hippocampus. Exogenous administration of FGF2 also altered circadian locomotor activity. These data support the hypothesis that dysregulation of fibroblast growth factor system gene expression may cause a disruption in circadian rhythms that underlie sleep/wake cycles. This series of studies increased our understanding of the role of the FGF family in affective behavior and circadian activity. Additionally, these studies suggest that dysregulation of FGF system gene expression in MDD may be a potential mechanism for alteration of circadian rhythms in individuals with this disorder. Taken together, these findings implicate FGF9 as a novel mediator of anxiety-like and depression-like behavior, endogenous antagonist of FGF2, and potential modulator of circadian rhythms.

Chapter 1

Introduction

There is increasing evidence that both genetic and environmental components contribute to psychiatric illness. Data also suggests that circadian activity may be affected by both genetic and environmental factors. As circadian dysregulation is often observed in patients diagnosed with mood disorders, it is possible that a common mediator affects mood, environmental interaction, and circadian activity. However, further study of the *mechanisms* underlying the development of mood disorders and disruption of circadian activity is necessary.

Severe forms of depression affect between 2-5% of the United States' population and up to 20% of the population suffer from milder symptoms (Blazer 2000). Depression can be diagnosed as major depressive disorder (MDD) when a patient displays a number of symptomatic criteria for longer than a two-week period (2000). These symptoms include insomnia or hypersomnia, loss or gain of weight, low energy and feelings of hopelessness. This syndrome can be highly variable and there is no objective diagnostic test for MDD. MDD is a highly heritable psychiatric disorder in which 40-50% of the risk associated with its development is attributed to genetic predisposition (Sanders 1999).

Identifying particular genes that confer vulnerability to depression is complicated by the fact that, like most complex diseases, many genes likely contribute to the development of MDD. The remaining non-genetic risk is also poorly characterized with implications that early childhood trauma, emotional stress, random developmental processes, and even viral infection may contribute to the etiology of depression (Nestler, Barrot et al. 2002). Further investigation of the interaction between genetic predisposition and environmental components are important to both the prevention of the onset of depressive symptoms and to the discovery of novel treatments for MDD.

Traditional approaches to psychiatric drug discovery have yielded few new targets for treatment of MDD. Since novel compounds are currently screened for efficacy with established receptors or known targets, new drugs are at best refinements of classic drugs discovered decades ago. These antidepressant therapies act mainly on serotonin and norepinephrine systems (Lenox 2002). While newer types of antidepressants are better tolerated and have fewer side effects, only 50% of depressed patients respond to current treatments and even then only 35-40% of drug-responsive patients achieve full relief from depressive symptoms (Cassano and Fava 2004).

With the advancement of large-scale gene expression profiling, we have the opportunity to approach target discovery for neuropsychiatric disorders using a "bottom-up" mechanistic approach. Gene expression profiling using microarrays

makes it possible to examine the complex interplay of molecular and physiological pathways. By looking at differential gene expression in brain tissue of patients diagnosed with MDD compared to their age-matched controls, we can develop a better understanding of the gene expression "signature" of MDD while identifying truly novel targets.

Discovery of the role of the FGF system in mood disorders

Our lab, as part of the Pritzker Neuropsychiatric Disorders Research Consortium, aims to discover the neurobiological and genetic underpinnings of psychiatric disorders using large-scale microarray gene expression analysis. To this end, post-mortem brain tissue from individuals diagnosed with MDD and bipolar disorder (BPD) was analyzed for changes in gene expression and compared to tissue of control subjects. This approach resulted in the identification of dysregulation of the fibroblast growth factor (FGF) family gene expression in a number of cortical and subcortical brain regions in MDD (Evans, Choudary et al. 2004; Evans, Choudary et al. ; Turner, Akil et al. 2006; Akil, Evans et al. 2008). While other labs have shown that growth factors are important in antidepressant activity and emotionality (Duman and Monteggia 2006; Kalueff, Avgustinovich et al. 2006; Warner-Schmidt and Duman 2008), this was the first time the FGF family had been implicated for a role in mood disorders. Since our original study, other labs have replicated FGF expression findings and strengthened the

hypothesis that FGF gene dysregulation is involved in MDD (Sibille, Arango et al. 2004; Tochigi, Iwamoto et al. 2008).

While initial studies in our lab have focused on determining the role of the well-studied FGF2 ligand in emotionality (Turner, Gula et al. 2008; Perez, Clinton et al. 2009), FGF9 also appeared to be an attractive target for small molecule inhibition since it was consistently upregulated in MDD. Conversely, FGF2 and FGF receptors were downregulated in MDD. Although little is known about the physiological role of FGF9; its known structure, expression, and functions are reviewed below. We found that FGF2 had antidepressant-like activity and acted as an endogenous inhibitor of anxiety (Turner, Gula et al. 2008). Since FGF9 expression was increased while FGF2 was decreased in MDD, we hypothesized that FGF9 might mediate negative effects on emotionality.

FGF dysregulation was identified in the prefrontal cortex, anterior cingulate, and hippocampus (Evans, Choudary et al. 2004; Evans, Choudary et al. 2004b; Gaughran, Payne et al. 2006). The hippocampus has a well-defined role in learning and memory, and it is also an important regulatory component of the stress response and the hypothalamic-pituitary-adrenal (HPA) axis (Jacobson and Sapolsky 1991; Eichenbaum, Otto et al. 1992). In humans, the hippocampus shows signs of atrophy as a result of elevated glucocorticoids and severe traumatic stress (Jacobson and Sapolsky 1991). Human brain imaging studies have shown that the hippocampus is particularly vulnerable to atrophy or

shrinking in recurrent depressive illness, Cushing's syndrome, post traumatic stress disorder (PTSD), schizophrenia, and aging (Starkman, Gebarski et al. 1992; Bogerts, Lieberman et al. 1993; Golomb, Kluger et al. 1994; Bremner, Randall et al. 1995; Gurvits, Shenton et al. 1996; Sheline, Wang et al. 1996).

In addition to these human findings, a number of animal studies also implicated the hippocampus and have led to the development of the neurotrophic hypothesis of depression and antidepressant action. The neurotrophic hypothesis is a proposed model in which chronic stress decreases hippocampal growth factors and antidepressants increase them (Duman, Heninger et al. 1997; Duman 2004). The alteration of growth factors may mediate the structural damage and reduced neurogenesis in the hippocampus after stress. Additionally, growth factors may mediate the neuroprotective effects of antidepressant treatments (Mallei, Shi et al. 2002; Bachis, Mallei et al. 2008)

Circadian abnormalities are often observed in depressed patients (Germain and Kupfer 2008). It is possible that growth factors may play additional roles in environmental response and maintenance of normal circadian rhythms. Dysregulation of growth factors may lead to disruption in circadian activity and subsequent changes in environmental responses and mood. Alternatively, growth factors may influence circadian disruptions and mood simultaneously.

While growth factors may modulate the hippocampal environment in response to stress and antidepressants, they may also be mediators of circadian activity. For example, BDNF and trkB gene expression showed variation in a 24-hour cycle and have been associated with wakefulness (Bova, Micheli et al. 1998; Berchtold, Oliff et al. 1999). Chronic administration of BDNF can also alter circadian rhythms including locomotor activity (Naert, Ixart et al. 2006). Alteration of environmental awareness or exploration activity due to circadian fluctuations in BDNF and trkB expression, may help to maintain circadian rhythms. Growth factors may alter hippocampal plasticity and may allow for an increased response to environmental changes and adaptive learning.

In order to examine mechanisms underlying growth factor modulation of circadian rhythms and growth factor dysregulation in depression, approximations of symptoms of MDD as well as effective treatments in laboratory animals are essential for the development of new therapeutics. As new targets are identified through microarray studies, animal models are indispensable tools for studying the neuropathology that underlies a target's role in depression. While despair-based models have predictive validity for monoamine-based antidepressants they may not be sufficient to identify therapeutic effects on different targets (Berton and Nestler 2006). However, recent modifications of existing paradigms and development of new tests will help to detect antidepressant actions of compounds acting on a broad range of neural and genetic targets (Cryan, Markou et al. 2002; Hoshaw, Malberg et al. 2005; Krishnan, Han et al. 2007).

While animal models cannot fully recapitulate the symptoms underlying the human disorder, they can provide us with useful information about the interaction of gene expression and behavior.

Central question

Based on the studies mentioned above, I hypothesized that *FGF9 will have negative effects on anxiety-like and depression-like behavior.* In addition*, FGF system expression will show circadian variation in the hippocampus.*

The fibroblast growth factor family

The fibroblast growth factor (FGF) family consists of 22 known ligands and five known receptors in humans. There are six subfamilies of FGF based on differences in homology and phylogeny: FGF1 and FGF2; FGF3, FGF7, FGF10 and FGF22; FGF4, FGF5, and FGF6; FGF8, FGF17, and FGF18; FGF9, FGF16, and FGF20; and FGF19, FGF21, and FGF23 (Itoh and Ornitz 2004). FGF15 cannot be identified in the human genome. FGF11-14, also referred to as fibroblast homologous factors (FHFs), are debated members of the FGF family because they do not bind to FGFRs. They share homology and structure with other FGF ligands, but instead the principal targets of FHFs are intracellular domains of voltage-gated sodium channels (Goldfarb 2005). The FGF ligands

and receptors make up a large and complex family of signaling molecules that are involved in embryonic development, tissue homeostasis and, when inappropriately expressed, are known to cause morphogenetic disorders or cancer (Dailey, Ambrosetti et al. 2005).

Fibroblast growth factors in the central nervous system

Among the many members of the FGF family, only a subset has been identified in the brain. FGF1 and FGF2 are the prototypical ligands of the FGF family, also called acidic and basic FGF respectively, and most of the effects of FGF in the brain are attributed to these ligands. FGF1 is expressed predominantly in neurons (Wilcox and Unnerstall 1991) while FGF2 is expressed in both neurons and glia (Eckenstein, Kuzis et al. 1994; Gomez-Pinilla, Lee et al. 1994; Vaccarino, Schwartz et al. 1999). FGFs play a critical role in CNS development via neurogenesis, axon growth, and differentiation (Molteni, Fumagalli et al. 2001; Vaccarino, Ganat et al. 2001). FGFs are also important to the development and maintenance of neurons (Mufson, Kroin et al. 1999; Perrone-Capano, Da Pozzo et al. 2000) including fate determination (Anderson 1993), migration, and differentiation (Kalcheim 1996).

Three of the FGF receptors expressed in the CNS have been implicated in CNS function. FGFR1 has widespread neuronal expression from early development into adulthood (Asai, Wanaka et al. 1993; Yazaki, Hosoi et al. 1994) and is

abundantly expressed in all hippocampal subfields as well as the cortex (Gonzalez, Berry et al. 1995; Belluardo, Wu et al. 1997). FGFR1 may play a role in cortical lamination and patterning in early development (Shin, Korada et al. 2004). FGFR2 and FGFR3 are found primarily in glia (Asai, Wanaka et al. 1993; Yazaki, Hosoi et al. 1994; Miyake, Hattori et al. 1996) and are weakly expressed in astrocytes of the hippocampus (Weickert, Kittell et al. 2005). While FGFR2 is preferentially expressed in neurons in early development and is primarily found as the IIIb isoform, it is expressed mostly in glia in adulthood as the IIIc isoform. Evidence suggests that FGFR2 plays a presynaptic organizational role in early development then switches to a role in neuroprotection and repair in adulthood consistent with temporal splice variant expression patterns (Alzheimer and Werner 2002; Umemori, Linhoff et al. 2004). FGFR4 is only expressed during the early stages of development and, except in the lateral habenular nucleus, it is undetectable in the adult CNS (Fuhrmann, Kinkl et al. 1999).

A number of FGF ligands have been identified and studied in the CNS. Since most of what is known about the FGF roles in the CNS is attributed to the FGF1 and FGF2 ligands, information about the expression and function of the other FGF family ligands in the CNS is highly fragmented. For example, FGF4 and FGF8 are important for the development of the midbrain-hindbrain boundary of the neural plate via interaction with sonic hedgehog (shh). FGF4 and FGF8 also coordinate the induction of the serotonergic and dopaminergic system (Ye, Shimamura et al. 1998).

FGF9 is also expressed in the CNS and is described in greater detail below. Additionally, another member of the FGF9 family, FGF20, is a neurotrophic factor for rat midbrain dopaminergic neurons (Ohmachi, Mikami et al. 2003). Exogenous treatment of FGF20 and FGF2 of monkey stem cells *in vitro* resulted in differentiation into dopaminergic neurons that, when transplanted, alleviated symptoms of a primate PD disease model (Takagi, Takahashi et al. 2005). Although FGF19 is expressed in the fetal brain (Nishimura, Utsunomiya et al. 1999), it primarily activates FGFR4 (Xie, Holcomb et al. 1999) that is primarily expressed in the habenula (Fuhrmann, Kinkl et al. 1999). Lastly, inhibition of FGF22 or its subfamily members, FGF7 and FGF10, inhibited presynaptic differentiation of mossy fibers contacting granular neurons (Umemori, Linhoff et al. 2004). This fragmented knowledge of ligands implicates FGF in a number of roles in the CNS both in development and adulthood.

Fibroblast growth factor-9

FGF9 (glia-activating factor) was first purified as a heparin-binding, secreted glycoprotein from a cultured human glioma cell line, NMC-G1, and acts as a trophic factor for primary rat glial cells (Naruo, Seko et al. 1993; Matsumoto-Yoshitomi, Seko et al. 1995). Although FGF9 was originally isolated from glioma cells, it is predominantly expressed in neurons. FGF9 mRNA expression is well-

characterized in the human and rat brain (Tagashira, Ozaki et al. 1995; Todo, Kondo et al. 1998).

FGF9 is strongly expressed in many regions of the brain including cerebellar nuclei, brain stem nuclei, and hypothalamic nuclei (Tagashira, Ozaki et al. 1995). FGF9 mRNA signal is moderately strong in the rat hippocampus and cerebral cortex (Tagashira, Ozaki et al. 1995) with very strong expression in the human hippocampus (Todo, Kondo et al. 1998). FGF9 mRNA is also intensely expressed in human and rat spinal motor neurons (Nakamura, Todo et al. 1997). Furthermore, weak glial FGF9 expression is characterized (Nakamura, Todo et al. 1999) and showed marked upregulation in pathological conditions such as Alzheimer's disease and amyotrophic lateral sclerosis (Nakamura, Arima et al. 1998). To this end the therapeutic use of an FGF9 antagonist has been suggested for a number of neurodegenerative diseases (Kanda, Iwasaki et al. 1999; Pataky, Borisoff et al. 2000).

Structural characteristics of fibroblast growth factor-9

All FGFs share a homologous core region of 120-130 amino acids ordered into twelve antiparallel β-strands (β1-β12) flanked by an amino and a carboxyl termini. Crystal structures of FGF1, FGF2, and FGF7 reveal that the common core structure consists of three copies of a four-stranded β-sheet known as the β-trefoil fold (Zhang, Cousens et al. 1991; Zhu, Komiya et al. 1991). The variation

in the N- and C- terminal tails accounts for much of the variation in biological activity of the ligands (Mohammadi, Olsen et al. 2005). The β1-β2 loop and parts of β10 and β12 make up the binding site for heparan sulphate glycosaminoglycan (HSGAG). HSGAG aids in the ligand-receptor binding of FGF-FGFR by binding to both FGF and FGFR simultaneously and stabilizing the protein-protein interaction. HSGAGs also stabilizes FGF against degradation, acts as a storage reservoir for ligand and determines the radius of FGF diffusion (Beenken and Mohammadi 2009).

The crystal structure of FGF9 reveals, like other FGFs, a common core of N- and C-terminal segments outside the beta-trefoil core (Figure 1-1). The N- and C-terminal segments differ in length with unknown functional relevance. FGF1, FGF2, and FGF4 have short C-terminal segments while FGF3, FGF5, and FGF8 have long terminal segments (Plotnikov, Eliseenkova et al. 2001). FGF9 uniquely and readily forms dimers under physiological conditions (K_d = 680nM) (Plotnikov, Eliseenkova et al. 2001). Most of the residues involved in the formation of the FGF9 dimer are also those that correspond to the major receptor-binding site in FGF2 (Venkataraman, Raman et al. 1999). Therefore dimer formation may be an autoinhibitory mechanism of FGF9 action; disassociation must occur for the molecule to be accessible to the receptor. FGF9 dimer interface analysis shows interactions between the N- and C-terminal segments that are described as the driving force behind dimer formation. In FGF1 and FGF2, the region preceding the B-trefoil core is disordered whereas

the N-terminal of FGF9 forms a highly organized α-helix (αN). The FGF9 N-terminal is composed of 17 residues compared to four residues in FGF1 and three residues in FGF2 (Plotnikov, Eliseenkova et al. 2001). The FGF9 dimer mainly consists of hydrophobic contacts but has four hydrogen bonds and two salt bridges in the interaction of the C-terminals of the FGF9 monomers (Hecht, Adar et al. 2001).

FGF9 is a unique member of the FGF family in that it lacks a typical secretory signaling peptide, yet it is still efficiently secreted via a non-cleaved sequence consisting of its first 33 residues (Matsumoto-Yoshitomi, Habashita et al. 1997; Revest, DeMoerlooze et al. 2000). The major difference between the FGF9 structure and those of prototypical FGFs, FGF1 and FGF2, is the β1-β2 and β9-β10 loop conformations. The β1-β2 loop corresponds to a high affinity receptor-binding region consisting of several disjoint tight binding residues. The β1-β2 loop is one residue shorter in FGF9. The β9-β10 loop corresponds to a "low-affinity binding loop" (LAL) that is longer in FGF9 by 4-6 residues than its corresponding loop in FGF1 or FGF2 (Figure 1-2) (Plotnikov, Eliseenkova et al. 2001). The steric bulk of the LAL of FGF9 may affect its interaction with receptors as compared to its counterparts with smaller LAL. FGF12 is the only other FGF ligand with a similar LAL (Taylor 2007). These differences may play a role in the affinity for FGF9 to HSGAGs that are necessary for receptor interaction. The structural differences between FGF9 and prototypical FGFs also account for its receptor binding specificity.

Receptor binding specificity of fibroblast growth factor-9

The five FGF receptors (FGFRs) are a family of tyrosine kinase receptors that consist of three extracellular immunoglobulin domains (D1-D3), a single-pass transmembrane domain and a cytoplasmic tyrosine kinase domain (Mohammadi, Olsen et al. 2005). Alternative splicing, occurring in FGFR1-3 on the C-terminal of the D3 domain, creates initially exclusive isoforms of the receptor (FGFR1-IIIb and FGFR1-IIIc) that have distinct binding specificities (Johnson, Lu et al. 1991). It is known that receptor splice variants provide some of the ligand-binding specificity of FGFs (Ornitz, Xu et al. 1996).

The activation model of FGF signaling begins with FGF binding to HSGAGs, followed by receptor dimerization, autophosporylation and downstream signaling (Ornitz, Yayon et al. 1992; Vainikka, Partanen et al. 1992; Ornitz, Herr et al. 1995). FGF interaction with HSGAGs is well-documented and allows for the formation of distinct complexes that bind with low affinity (K_d= 2X10^{-9}) but with large capacity (10^6 sites/cell) (Yayon, Klagsbrun et al. 1991; Spivak-Kroizman, Lemmon et al. 1994). The crystal structure of a ternary FGF2-FGFR1-heparin complex provides a model by which heparin aids the induction of FGF receptor dimerization (Schlessinger 2000). HSGAGs interact with the HSGAG binding sites of FGF and promote the formation of a 1:1:1 FGF2-FGFR1-heparin complex. Another ternary complex is recruited to the first complex via weak interactions of the first ternary complex with the FGF receptor of the recruited ternary complex. Based on this model, different HSGAG binding motifs with

different sulfation patterns or length may play a role in altering both binding affinities and specific biological activity.

The crystal structure for FGF9 binding to any receptor with HSGAGs has not been determined. However, modeling of the FGF9-FGFR1 interaction by superimposing FGF9 into the FGF2-FGFR1-heparin complex reveals a positively charged groove in heparin binding sites similar to FGF2, but highlights multiple alternative sites for heparin interaction that may affect biological activity (Plotnikov, Hubbard et al. 2000). FGF9 binds preferentially to FGFR2c and FGFR3c receptors (Hecht, Zimmerman et al. 1995; Santos-Ocampo, Colvin et al. 1996; Chellaiah, Yuan et al. 1999). The FGF9 structural differences within the β-trefoil sheet and at the N-terminal sterically hinder FGF9 from engaging with FGFR1c (Plotnikov, Eliseenkova et al. 2001).

Fibroblast growth factor-9 signaling

The five FGF receptors are known to act through two main intracellular pathways, the phospholipase C (PLC) γ1 (also known as FRS1) pathway and the FGFR substrate 2 (FRS2) pathway. The PLCγ pathway activates downstream PKC leading to changes in cytoskeletal organization. The PLCγ pathway also activates the PDK/AKT survival (anti-apoptotic) pathway. The FRS2 pathway activates the Ras/RAF/MAPK (ERKs,P38,JNKs) cascade that leads to transcriptional regulation (Reuss and von Bohlen und Halbach 2003).

Chimeric receptor studies using the cytoplasmic domains of FGFR1, FGFR3 and FGFR4 *in vitro* show that differences in ligand binding do not activate different downstream proteins. Instead the magnitude of the tyrosine kinase activity is the main difference in receptor activation (Raffioni, Thomas et al. 1999). Therefore it is hypothesized that FGFR subtypes drive the same signaling cascades but with different strengths. The mechanisms underlying the diverse and sometimes paradoxical signaling of the FGF family has been reviewed in Dailey, Ambrosetti et al (2005). They concluded that the signaling response is multifaceted and complex. The specific biological response that a cell will deliver will depend on the interplay between the presence or absence of specific FGFs, feedback loops, and crosstalk with other signaling networks. The response will also depend on the availability of the "target" genes to be activated or suppressed in a given cell type. The diversity of the FGF ligands and receptors may be necessary for the activation of varied target cell types and complicated feedback systems.

Transgenic fibroblast growth factor-9 mouse models

While no studies have specifically analyzed the effect of FGF9 knockouts on behavior, the following work summarizes the transgenic studies to date. FGF9 knockout mice exhibited male-to-female sex reversal (Colvin, Green et al. 2001) and died at birth due to lung hypoplasia (Colvin, White et al. 2001). In order to characterize the role of FGF9 in prostate development and tissue homeostatis, an inducible FGF9 knockout mouse was created that encoded wild-type activity

and reverted to a true conditional knockout after recombination induced by Cre recombinase (Lin, Liu et al. 2006). However, no studies besides the characterization of a viable inducible knockout have been published. FGF9 tissue-specific transgenic overexpressers have been developed for skeletal and lung development studies. Increased FGF9 expression in cranial mesenchyme cells led to developmental skull abnormalities and cataracts (Govindarajan and Overbeek 2006). Overexpression of FGF9 in developing lung epithelium resulted in hyperplasia and decreased epithelial branching (White, Xu et al. 2006).

Fibroblast growth factor system and affective behavior

While little is known about the role of FGF9 in affective behavior, our lab has recently characterized the effects of FGF2 in both anxiety-like and depression-like behavior (Turner, Gula et al. 2008; Perez, Clinton et al. 2009). Since FGF2 is one of the better-known family members of the FGF family, the FGF2 link to the neurotrophic hypothesis and its role in mood disorders is more apparent (Newton and Duman 2004; Berton and Nestler 2006; Turner, Calvo et al. 2008).

FGF2 plays a critical role in the development of the brain structure and promotes the proliferation and survival of fetal and postnatal cells cultured from many brain regions (Ray, Peterson et al. 1993) including the hippocampus (Walicke, Cowan et al. 1986; Ray, Peterson et al. 1993). A single subcutaneous injection of FGF2, to postnatal day 1 rodents, increased cell proliferation in the hippocampus and

resulted in a larger hippocampal volume into adulthood (Cheng, Black et al. 2002). Conversely, FGF1 receptor knockouts had decreased hippocampal cell proliferation resulting in permanent atrophy (Ohkubo, Uchida et al. 2004).

FGF2 is also important in the adult brain as evidenced by its role in adult neurogenesis. Intracerebroventricular infusions of FGF2 increased neurogenesis in the subventricular zone of adult rats (Wagner, Black et al. 1999). Furthermore, chronic posterior lateral ventricular injections FGF2 in middle-aged rats increased neurogenesis and enhanced dendritic growth in the subventricular zone and dentate gyrus (Rai, Hattiangady et al. 2007). In support of the hypothesis that FGF2 mediates the effects of antidepressants and subsequent increased neurogenesis following antidepressant treatment, FGF2 expression is increased following antidepressant treatment (Mallei, Shi et al. 2002; Bachis, Mallei et al. 2008).

Our lab has also shown that gene expression of FGF2 and its primary receptor, FGFR1, are decreased by acute social defeat in all sub-regions of the adult rat hippocampus (Turner, Calvo et al. 2008). Our lab has also shown that acute peripheral administration of FGF2 increased anxiety-like behavior (Perez, 2009) and chronic peripheral administration reversed this effect resulting in an anxiolytic response (Perez, Clinton et al. 2009). Furthermore, our lab has shown that basal FGF2 expression is altered in the hippocampus of rats selectively bred to confer reliable differences in vulnerability to anxiety behavior (Stead, Clinton et

al. 2006). Specifically FGF2 gene expression is decreased in low-response to novelty/high-anxiety (LR) rats compared to high-response to novelty/low-anxiety (HR) rats. This data suggests that differences in FGF system expression may change the way that animals interact with their environment. These changes might be directly related to alterations of mood (chapter 3) and might also underlie changes observed in circadian rhythms following FGF2 microinjection (chapter 4).

In our studies of the role of FGF2 in depression-like behavior, we characterized both the effects of FGF2 and the FG loop (FGL) of the neural cell adhesion molecule (NCAM) (Turner, Gula et al. 2008). FGF2 primarily activates FGFR1 but can also bind and activate all four FGF receptors (Reuss and von Bohlen und Halbach 2003). The FGL is the F and G β-strands and the interconnecting loop region of NCAM's second fibronectin type 3 module, and both NCAM (Williams, Furness et al. 1994; Kiselyov, Skladchikova et al. 2003; Christensen, Lauridsen et al. 2006) and FGL (Kiselyov, Soroka et al. 2005) activate FGF receptors. Acute microinjections of both FGF2 and FGL decreased depression-like behavior as evidenced by a decrease in percent time spent immobile on the forced swim test (FST) (Turner, Gula et al. 2008).

Chronic microinjection of FGF2 also produced antidepressant-like effects in the FST and latency to feed as measured in novelty-suppressed feeding test (NST). This result was not confounded by any changes in locomotor activity. FGF2

microinjection also caused a dentate-gyrus specific increase in FGFR1 gene expression 24 hours after FGF2 injection although it did not alter endogenous gene expression of FGF2 (Turner, Gula et al. 2008). While we could not explain the regulation of receptor mRNA, we identified that is possible to observe immediate changes in FGF family regulation.

Circadian rhythm disturbances in depression

Most organisms show a wide range of cyclical activities over a 24-hour period, that we refer to as circadian activity. Circadian activity includes changes in core body temperature, secretion of hormones such as cortisol, and maintenance of the sleep-wake cycle. In humans the sleep-wake cycle is the most obvious daily rhythm (Germain and Kupfer 2008). The superchiasmatic nucleus (SCN) of the hypothalamus is the central pacemaker or the masterclock and can maintain rhythmic activity in the absence of input from any other part of the brain (Inouye and Kawamura 1979). The major output of the SCN is to the paraventricular nucleus (PVN) of the hypothalamus and via a multisynaptic pathway to the pineal gland, where melatonin is synthesized. Melatonin is a human biochemical transducer of the photoperiod to all cells in the body, and it is secreted at night and suppressed during the day (Simonneaux and Ribelayga 2003). The PVN is also the site of cortcotrophin releasing factor (CRF) – secreting neurons, which are part of the hypothalamo-pituitary-adrenal (HPA) axis, that also have a diurnal rhythmicity.

Regulation of the sleep-wake cycle is complex and involves numerous brain areas and pathways (Saper, Scammell et al. 2005). Cortical arousal is mediated by cholinergic and aminergic activity from the pontine tegmentum, locus coereleus, and raphe nuclei. Activity of these wakefulness systems is modulated by orexin, a neuropeptide produced by the lateral hypothalamus (Sakurai, Amemiya et al. 1998; Chemelli, Willie et al. 1999). The endogenous timekeeping system allows for the anticipation and preparation for environmental changes associated with day and night. Most human functions, both physical and mental, display circadian rhythmicity, therefore it is intuitive that disruption of these activities can lead to both physical and mental pathological symptoms.

In healthy human subjects, mood variation over a 24-hour cycle depends on the interaction between the circadian phase and prior wakefulness (Boivin, Czeisler et al. 1997). Not surprisingly, there is a strong association between depression and sleep dysregulation in humans (Wirz-Justice 2006). As many as 90% of depressed patients report impairment of sleep quality (Casper, Redmond et al. 1985) and alterations in sleep architecture were also observed objectively (Kupfer and Foster 1972). Sleep architecture abnormalities consisted of a decreased slow-wave sleep and increased rapid eye moment (REM) in depressed patients. However, it is still not known if changes in sleep quality or quantity lead to neurobiological dysfunction and subsequently a depressed state. Alternatively, alterations of the sleep-wake cycle could be secondary to the onset of depression.

While the sleep-wake cycle is the most obvious circadian rhythm in humans, additional circadian activity disruption has been reported in MDD. Normally, core body temperature peaks in the evening and is at a nadir in the last third of the night. However, elevated nocturnal body temperature is reported in individuals with MDD (Souetre, Salvati et al. 1988; Harding and Duncan 1996) and is normalized with clinical improvement (Avery, Wildschiodtz et al. 1982; Avery, Wildschiodtz et al. 1982). In addition to differences in core body temperature, abnormalities in cortisol secretion rhythms have been revealed in MDD. In normal subjects, cortisol secretion peaks in the morning and progressively declines to a nadir in the evening. In depressed subjects there is an overall increase in cortisol secretion that may disrupt HPA axis function, and the largest increase in cortisol secretion compared to controls is seen at the nadir of the circadian rhythm. There is also an earlier onset of first cortisol secretory episode in depressed subjects (Van Cauter, Leproult et al. 1996).

One chronobiological model, formulated to explain sleep-wake disturbance in MDD, hypothesized that the amount of SWS at night is determined by prior wakefulness (Borbely 1982). The level of sleep propensity during the day and the depth of sleep at night contribute to the “S” process. “S” accumulates during waking hours as the propensity for SWS increases. “S” dissipates at night leading to a decline in SWS. A second process, “C”, is the represents circadian control of sleep propensity, highest at 0300-0500 h and lowest a 1600 h in humans. The tendency to fall asleep depends on the balance of “S” and “C.”

When “S” is equal to “C,” an individual wakes up. It was proposed that the reduction in SWS is the result of impairment in the “S” process where individuals with MDD have decreased accumulation of sleep pressure and reduced dissipation of SWS at night (Borbely 1982). The impairment of “S” during the day can lead to frequent sleep disturbance and early wakening.

In support of this hypothesis, one night sleep deprivation of patients with MDD has a dramatic antidepressant effect. Unfortunately this effect is transient and short-lasting (Wirz-Justice and Van den Hoofdakker 1999). A similar antidepressant effect was observed in rodent models of sleep deprivation in which 24 hours of sleep deprivation increased the swimming activity of rats in the forced swim test (FST) (Lopez-Rodriguez, Kim et al. 2004). This chronobiological model still does not establish the causality of circadian disruption and depression symptoms, but sleep deprivation studies show that mood can be briefly improved by sleep deprivation and correction of the sleep-wake cycle. As the human antidepressant effect is transient, it is possibie that patients with MDD have an inherent circadian dysregulation of the sleep-wake cycle.

Recent studies show that there is a diurnal rhythm to dendritic architecture and spine density in pyramidal neurons in the rat infralimbic cortex (Perez-Cruz, Simon et al. 2009). Since growth factors have gene expression variation in the 24-hour light-dark cycle (Bova, Micheli et al. 1998; Berchtold, Oliff et al. 1999;

Dolci, Montaruli et al. 2003), it is possible that growth factors modulate synaptic plasticity through dendritic architecture and spine formation during a 24-hour cycle. The modulation of synaptic plasticity may underlie response and reactivity to environment that are important components of behavioral activity. Given that chronic microinjection of BDNF interrupted normal circadian activity patterns (Naert, Ixart et al. 2006), it is also possible that alterations of other growth factors' basal expression rhythms may disturb circadian activity patterns. Since we have implicated the FGF family in mood disorders, it is reasonable to ask whether FGF gene expression is regulated over a 24-hour period and if this pattern is altered in MDD. It is possible that the FGF system is responsible for changes in structural plasticity that link stress-induced effects with disrupted biological rhythms involved in the regulation of mood.

In healthy subjects, mood variations across the day are dependent on interactions between circadian phase and duration of prior wakefulness (Boivin, Czeisler et al. 1997). Considering there is such a strong correlation between sleep and mood, it is not surprising that depressed patients, who often complain of sleep disturbances, are profoundly affected by alterations of circadian rhythm. Depressed patients showed sustained activity in brainstem and hypothalamic regions involved in wakefulness compared to healthy subjects (Buysse, Nofzinger et al. 2004). While the neural mechanisms that underlie the cause of these disruptions have not been elucidated, factors that modulate circadian activity may be key to treating circadian disruptions in depressed patients.

Role of the hippocampus in depression and stress response

The hippocampus is a key modulator of hypothalamic-pituitary-adrenal (HPA) axis activation. The HPA axis is a prominent mechanism by which the brain reacts to acute and chronic stress. Neurons in the paraventricular nucleus (PVN) of the hypothalamus secrete corticotropin releasing factor (CRF) that stimulates the synthesis and release of glucocorticoids from the adrenal cortex. Glucocorticoids, cortisol in humans and corticosterone in rats, are well characterized in the pathophysiology of depression (Ursin and Olff 1993).

The hippocampus inhibits the hypothalamic CRF-containing neurons via a polysynaptic circuit. A number of studies highlight the link between depression and HPA axis dysregulation (Feder, Coplan et al. 2004; Wichniak, Brunner et al. 2004; Duval, Mokrani et al. 2006). Most of these studies focus on basal activity of the HPA axis in depressed individuals (Halbreich, Asnis et al. 1985; Lemus, Asnis et al. 1987; Posener, Charles et al. 2004). Fifty to 60% of depressed patients exhibit increased levels of cortisol and loss of feedback inhibition of the HPA axis (Holsboer, Von Bardeleben et al. 1984; Gold, Calabrese et al. 1986).

In addition to modulating the stress reponse, the hippocampus is one the primary sites of adult neurogenesis. The connection between mood disorders and neurogenesis is still being elucidated. It is thought that stress-induced decreases in neurogenesis may be an important factor in eliciting depressive episodes (Duman, Nakagawa et al. 2001; Jacobs 2002; Kempermann and Kronenberg

2003). Animal models of stress show decreased cell survival and disruption of cell proliferation while antidepressants reverse these effects (Czeh, Welt et al. 2002; Malberg and Duman 2003).

While studies have shown that the behavioral effects of antidepressants are dependent on neurogenesis (Santarelli, Saxe et al. 2003), others have shown that neurogenesis effects depression-like activity in only some behavioral measures (David, Samuels et al. 2009). Also, decreased hippocampal volume has been observed in depressed individuals (Sheline 1996; Sheline, Wang et al. 1996; Sheline, Sanghavi et al. 1999) and may be attributed to a decrease in hippocampal neurogenesis (Gould, Tanapat et al. 1998). While the debate still continues on the role of neurogenesis in depression, the functions of the hippocampus in stress and depression may become increasingly important to the treatment of MDD.

Changes in other growth factors in the hippocampus have also been associated with both mood disorders (Duman 2004) and neurogenesis. Previous work has shown that brain-derived neurotrophic factor (BDNF) and vascular endothelial growth factor (VEGF) are responsible for modulating structural plasticity changes resulting from antidepressant treatment. These changes in plasticity have been attributed to neurogenesis in the hippocampus (Shirayama, Chen et al. 2002; Schmidt and Duman 2007; Warner-Schmidt and Duman 2007). Furthermore, infusions of BDNF infusions into the hippocampus and midbrain area produced

an antidepressant-like behavioral effect in rats (Shirayama, Chen et al. 2002). Conversely, transgenic mice with reduced BDNF signaling are insensitive to antidepressants in behavioral tests (Sairanen, Lucas et al. 2005).

These findings and recent findings from our lab support the neurotrophic hypothesis that growth factors play an essential role in hippocampal structural plasticity and subsequent behavioral consequences. It is possible that alterations in FGF family expression observed in the hippocampus of individuals diagnosed with MDD underlie the pathophysiology of the disorder. Further research into the underlying mechanism of FGF action on hippocampal function will be necessary to validate this hypothesis.

Animal models for the assessment of novel antidepressant targets

Animal models of depression are essential tools utilized to reproduce human symptoms of MDD to better understand the disorder's underlying pathophysiology. Given the variability and complexity of symptoms displayed by humans with MDD, it is difficult to demonstrate equivalence between animal behavior and human depression. However, there are a number of established behavioral models that mimic MDD symptoms, cause depression-like and anxiety-like behavioral changes, and respond to antidepressant treatment. Although no one animal model fully encompasses the depressed human

condition, a number of environmental triggers and behavioral endpoints have had predictive value for determining therapeutic antidepressant effects in humans. As the search for novel antidepressants continues, there is concern whether models that have been traditionally used to determine the efficacy of classic antidepressants, tricyclics and monoamine-based therapies, can be used for discovery of new antidepressant compounds. These predictive models are useful for high-throughput screening of target compounds. However, a number of modifications to older models and newer models have emerged that have face validity producing animal behaviors with likeness to human symptoms (Petit-Demouliere, Chenu et al. 2005). These modified and newer animal models allow for validation of antidepressant efficacy outside of tasks that were specifically developed for monoaminergic action. Although the link between stress and depression is not completely understood, human depression is often triggered by traumatic life events in both childhood and adulthood and can be exacerbated by a number of physical and social stressors. Due to their aeteological validity, a number of tests make use of acute or chronic stress to produce a depression-like effect in rats.

The forced swim test (FST) was originally designed as a primary screen for the effect of acute antidepressants (Porsolt, Bertin et al. 1977) and its validity is based primarily on observations seen with the administration of monoamine oxidase inhibitors (MAO-Is) and tricyclic drugs (TCAs). However, additional possibilities for this animal model are being considered due to its increased use

as a neuropharmacological tool. Recent studies have utilized the FST for more than just screening of monaminergic activity. The uncontrollable stress involved in the test has led to the use of the FST as a model of depression. Data supports that acute antidepressant treatment attenuates swim-stress induced corticosterone release in the rat (Baez and Volosin 1994). Also, the fact that electroconvulsive seizures are effective in the test speaks to its ability to pick up broader mechanisms of action (Nestler, Gould et al. 2002).

The FST has been used to validate the effect of neurotrophic factors on depression-like behavior. Brain-derived neurotrophic factor (BDNF) infusion into the midbrain decreased immobility time 70% compared to vehicle controls. Additionally, infusion of BDNF into the ventral tegmental area (VTA) resulted in a 57% shorter latency to immobility compared to vehicle controls (Eisch, Bolanos et al. 2003). Direct injection of BDNF into the VTA has a pro-depressant effect on the FST while BDNF infusion into the midbrain and hippocampus cause an antidepressant-like effect in the FST. A modified FST, consisting of multiple swim tests following the initial 15-minute Day One swim, showed that central administration of either BDNF or IGF-1 caused a persistent antidepressant-like effect in the forced swim test lasting at least six days (Hoshaw, Malberg et al. 2005). In our lab, chronic microinjection of FGF2 into the left lateral ventricle resulted in a significant decrease in percent time spent immobile and an increase in percent time spent swimming (Turner, Gula et al. 2008). While the construct validity of the forced swim test has often been questioned, due to the nature of its

acute and non-ecologically relevant stressor, its predictive value and reliability are still extremely high (Petit-Demouliere, Chenu et al. 2005).

Novelty-supressed feeding (NSF) and novelty-induced hypophagia (NIH) are behavioral paradigms that involve food deprivation and placement of a rat into a novel environment with food. This test is sensitive to chronic antidepressant administration but not to acute administration (Bodnoff, Suranyi-Cadotte et al. 1988; Merali, Brennan et al. 2003; Santarelli, Saxe et al. 2003). Latency to consume a food pellet is used as a measure for anxiety-like behavior. The major problem with NSF is that it is also affected by acute administration of an anxiolytic (Duman and Monteggia 2006), and it is not specific to antidepressants. Although, this test does not test directly for depression-like behavior, high comorbidity rates between depression and anxiety are observed in human patients (Gorman 1996). Of particular importance, it is difficult to see depressant or anxiogenic effects in the NSF since latency to feed in normal rats is generally the full time allotted. Due to this ceiling effect, the NSF test is not a good tool for assessing antidepressant targets that may have anxiogenic or depressionogenic effects.

To address the shortcomings of the NSF model, a modified version of NSF, the NIH model, was developed. In the NIH model, mice are trained to drink liquid containing sweetened condensed milk. Following the training period, the animals are assessed for their latency to drink the sweetened liquid available *ad libitum* in

a novel environment. This model avoids the fixed-food deprivation necessary for NSF and subsequent interference of appetite. Chronic, but not subchronic, antidepressant treatment reduces latency to drink in the novel environment (Dulawa and Hen 2005). This NIH model has mainly been used for mice, but it is very similar to the rat sucrose preference test which measures percent sucrose consumed compared to tap water to assess anhedonia. Preference deficits for the sucrose test exist after two weeks of chronic mild stress and can be reversed by chronic but not subchronic antidepressant treatment (Willner, Towell et al. 1987). A modified version of the NIH model involving sucrose preference may be suitable for assessing anhedonia in rats. Normal unstressed rats will drink high percentages of sucrose solutions therefore providing for room to see an anhedonic effect of antidepressant targets.

The chronic social stress paradigm is a model of social subordination in which an adult male rat (intruder) is introduced into the cage of an unfamiliar, aggressor rat (resident). The intruder is removed as soon as it shows signs of submissive behavior thereby minimizing injury while still creating a stressor. Chronic social stress uses one of the most common human stressors, social conflict, and therefore provides a more realistic model of stress. The model increased anhedonia, increased despair activity, and decreased exploratory behavior in the intruder rat (Rygula, Abumaria et al. 2005). The effects of chronic social stress can be reversed by chronic antidepressant treatment (Rygula, Abumaria et al. 2006).

In the search for new antidepressant therapies, multiple animal models should be used to assess pharmaceutical efficacy. While predictive tests provide a well-validated method correlated with positive effects on human depression, they may fall short of identifying antidepressants with novel mechanisms. Therefore, tests that exhibit face validity may be a good complement when exploring treatments or targets with unknown mechanisms.

Emerging antidepressant treatments

As it is increasingly clear that the pathophysiology of MDD goes far beyond the monaminergic system alone, advances in our understanding of the disorder are guiding the development of novel antidepressant classes. The Sequenced Treatment Alternatives to Depression (STAR*D) clinical trial found that 37% of patients achieved remission with the selective-serotonin reuptake inhibitor (SSRI) citalopram. Only half of the patients who remained depressed following the trial responded to three follow-up treatments involving medication switching and augmentation (Rush, Trivedi et al. 2006). Current treatments take 2-5 weeks to alleviate depression symptoms and show high rates of relapse (Rush, Trivedi et al. 2006). Below, I characterized a number of novel potential targets and their shortcomings. The lack of therapeutic success highlights the need for further investigation of the role of FGFs in mood disorders.

A number of novel targets have emerged from the increased study of physiological abnormalities underlying MDD. Among them are the amino acid neurotransmitters, glutamate and gamma aminobutyric adic (GABA), responsible for the large majority of neurotransmission in the brain. Several studies implicate abnormal glutamatergic activity in the development of MDD (Krystal, Sanacora et al. 2002; Kugaya and Sanacora 2005; Pittaluga, Raiteri et al. 2007). In animal studies, there is evidence that enhanced glutamatergic drive contributes to stress-related neurotoxicity in the hippocampus (Sapolsky 2000).

There is increased evidence from human post-mortem studies showing decreased glial cell numbers and density in a number of brain regions in depressed subjects compared to controls (Kugaya and Sanacora 2005; Rajkowska and Miguel-Hidalgo 2007; Bernard, Kerman et al. 2009). This is important as glia are modulators of both the glutamatergic and GABAergic neurotransmitters systems (Araque and Perea 2004; Santello and Volterra 2009). Clinical evidence showed that antidepressants decreased glutamatergic transmission or indirectly affected *N*-methyl-D-aspartate (NMDA) glutamate receptor function (Feyissa, Chandran et al. 2009; Kucukibrahimoglu, Saygin et al. 2009). Also antidepressants increased GABA levels in cortical regions and decreased GABA deficits in depressed patients (Sanacora, Mason et al. 2002; Sanacora, Mason et al. 2003).

The four potential targets for the glutamate system are the (1) ionotropic glutamate receptors, (2) NMDA receptors, (3) α-amino-3hydroxy-5-methyl-isoxazole-4-propionic acid (AMPA) receptors, and (4) kainite (KA) receptors. AMPA and KA receptors are altered in animal models of depression and in depressed individuals. Additionally, several compounds that alter AMPA and KA signaling result in antidepressant-like activity (Alt, Nisenbaum et al. 2006; Alt, Weiss et al. 2006; Bleakman, Alt et al. 2007; O'Neill and Witkin 2007). However, no clinical data on these compounds is yet available. Thus, compounds that target both NMDA receptors and metabotropic glutamate receptors as well as their clinical efficacy will be described below.

NMDA receptors have gained increased support as novel therapeutic targets for mood disorders due to role of the receptor in learning, neuroplasticity, and neurotoxicity (Pittenger, Sanacora et al. 2007). However, NMDA receptor activation is fairly complex. Synaptic NMDA receptors activate transcription factors, MAPK and CREB, and subsequently increase BNDF expression to promote neuronal survival. Conversely, extrasynaptic NMDA receptor activation increases cell death (Hardingham and Bading 2003; Hardingham 2006). The bi-directional modification of NMDA receptors may be an underlying mechanism for the regulation of synaptic remodeling. Dysregulation of this NMDA receptor signaling and trafficking may contribute to the pathophysiology of mood disorders (Lau and Zukin 2007).

Clinical studies have shown a high correlation between antidepressant efficacy and an antidepressant's ability to modify NMDA receptor binding affinity (Paul, Nowak et al. 1994; Skolnick 1999). Furthermore, clinical studies have also shown that a single dose of the NMDA receptor antagonist, ketamine, caused a significant antidepressant response in depressed individuals. However, most individuals treated with ketamine returned to pre-treatment depression rating scores within five days of the infusion (Berman, Cappiello et al. 2000; Zarate, Singh et al. 2006). While ketamine has potential as a novel therapeutic for the treatment of depression, it produces unwanted side effects such as altered levels of consciousness, perceptual and cognitive disruptions, and hypertension. Additionally, it is not known whether chronic administration of the NMDA receptor antagonist could have detrimental effects on behavior and long-term neuronal survival (Olney 1994; Chen and Lipton 2006).

Another glutamate-modulating drug, riluzole (Rilutek), currently approved for the treament of amyotropic lateral sclerosis (ALS) (Miller, Mitchell et al. 2007), has been implicated in its potential for mood disorder treatment. Riluzole may act as a functional antagonist at GluRs to result in reduction of glutamate release (Benavides, Camelin et al. 1985; Debono, Le Guern et al. 1993). Interestingly, riluzole increased BDNF expression in the hippocampus (Katoh-Semba, Asano et al. 2002). Recent open-label clinical trials have resulted in positive effects on mood disorders (Mathew, Amiel et al. 2005; Zarate, Quiroz et al. 2005; Sanacora, Kendell et al. 2007). However, the total number of patients described

in the trials remains small and no double-blind studies have been described to date. Overall, members of the glutamatergic system have potential as targets for the treatment of depression. While there is much supporting evidence for the role of GABAergic abnormality in both animal studies and in clinical observations (Bateson 2004; Choudary, Molnar et al. 2005; Russell, Carling et al. 2006; Sanacora and Saricicek 2007), benzodiazepines are not effective in the treatment of MDD.

As HPA axis dysregulation has been consistently described in depression research, this system is an obvious target for novel therapeutics (Berger, Krieg et al. 1988; Heuser 1998). Since depressed patients sometimes show excessive cortisol secretion and decreased negative feedback regulation (Ising, Lauer et al. 2005), corticotropin-releasing factor (CRF) emerged as a novel target for antagonism. Transgenic mice overexpressing CRF display depression-like behavior (Stenzel-Poore, Heinrichs et al. 1994). Furthermore, clinical observations find that depressed patients have elevated levels of CRF in their cerebrospinal fluid. These elevated CRF levels can be corrected by antidepressant treatment (Nemeroff, Bissette et al. 1991). However, CRF activates two receptors (Reul and Holsboer 2002), CRF1 and CRF2. CRF2 studies resulted in conflicting observations.

CRF2 knockout mice exhibit increased anxiety-like behavior that can be reversed by a CRF2 agonist (Bale, Contarino et al. 2000). Conversely, central

infusion of a CRF2 antagonist caused decreased anxiety-like behavior (Valdez 2006). Thus, CRF1 is the primary target for antidepressant medication. CRF1 antagonists consistently showed increased antidepressant-like activity in animal models of depression after chronic, but not acute, treatment (Kehne and De Lombaert 2002; Kehne 2007). Additionally, clinical trials investigating the efficacy of R121919, a CRF1 antagonist, found a significant reduction in anxiety and depression scores following administration (Zobel, Nickel et al. 2000; Zorrilla and Koob 2004). Unfortunately, the CRF1 antagonist (Neurocrine, Inc.) also resulted in elevated liver enzymes in two subjects and resulted in discontinuation of the trials. However, more trials using alternate CRF1 antagonists are currently underway.

In addition to CRF1 antagonists, other HPA axis targets are being investigated. Specifically, metyrapone, an inhibitor of cortisol secretion, has been shown to increase expression of hippocampal and cortical BDNF in rats (Rogoz and Legutko 2005). Additionally, clinical trials have found that metyrapone is an effective adjunct therapy in the treatment of MDD and metyrapone was well tolerated in humans (O'Dwyer, Lightman et al. 1995; Jahn, Schick et al. 2004). It accelerated the onset of antidepressant action when used in combination standard serotonergic antidepressants. Despite the positive results of these clinical trials, further studies confirming the use of metyrapone as a stand-alone therapeutic have yet to be reported.

Our lab has also shown that HPA axis dysregulation involving glucocorticoid receptor overexpression in transgenic mice caused a mouse-model of increased emotional lability. These animals have increased anxiety-like and depression-like behavior as well as increased sensitivity to antidepressants (Wei, Lu et al. 2004). Clinical studies show that mifepristone (RU486), a glucocorticoid receptor antagonist, provided improvement in depression-like symptoms in psychotic depression (Belanoff, Rothschild et al. 2002; DeBattista and Belanoff 2006). However, multiple phase III trials for MDD with RU486 failed to reach endpoints of rapid and sustained response, defined as a 50% or greater decrease in Brief Psychiatric Rating Scale (Nihalani and Schwartz 2007).

The melatonin system is another potential target for MDD therapeutics. It is known that melatonin is thought to be a regulator of human sleep/wake cycles (Pandi-Perumal, Zisapel et al. 2005). Melatonin is considered a biochemical transducer that relays photoperiodic information to all cells in the body. Melatonin is secreted at night and suppressed during the day monteleone. Both the concentration and the timing of melatonin release are dysregulated in depression (Rubin, Heist et al. 1992; Wetterberg, Bergiannaki et al. 1999). Administration of melatonin itself does not have an effect on depression in humans (Carman, Post et al. 1976). However, recent studies show that agomelatine, a melatonin receptor and 5HT2C receptor antagonist, have antidepressant-like and anxiolytic effects in animal models of depression. However, it cannot be ruled out that the observed antidepressant effects are simply a result the 5HT2C receptor

antagonist properties of agomelatine (Papp, Gruca et al. 2003; Millan, Brocco et al. 2005). Agomelatine has also shown promising effects on depression in clinical trials, and the melatonin agonist showed a more rapid onset of effect than traditional antidepressants (Kennedy and Emsley 2006; Montgomery and Kasper 2007). While early results are promising, the long-term efficacy and tolerance of agomelatine must still be explored. Melatonin has a number of other targets, including the immune system, which may be adversely affected by prolonged agomelatine administration.

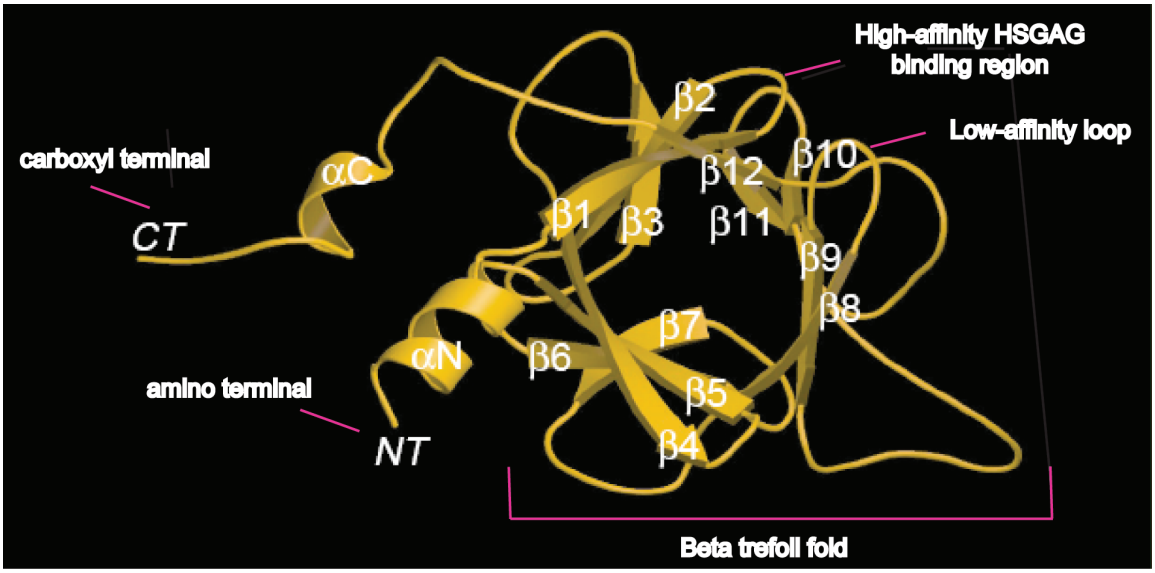
While recent studies, encompassing better models of the pathophysiology of MDD, have resulted in a number of potential targets, the yield of new effective therapeutics has been fairly low. However, developing an arsenal of therapeutics against different targets may eventually allow patients, who are resistant to current therapeutics, the potential for more personalized medicine. The potential for novel neurotrophic targets, particularly FGF, will be discussed throughout the following series of studies.

To this end, the second chapter of this dissertation examines the potential for exogenous administration of FGF9 *in vivo* and identifies important variables to consider when administering growth factors. Following these studies, chapter three characterizes the effect of both acute and chronic administration of FGF9 on depression-like and anxiety-like behavior. In addition, the effect of chronic FGF9 administration and chronic social stress on FGF family expression in the

hippocampus is examined. Finally in chapter four, I examine whether FGF9, FGF2, or FGFR1 have circadian variations in expression throughout a 24-hour light-dark cycle. I also test whether exogenous administration of FGF2 affects circadian activity.

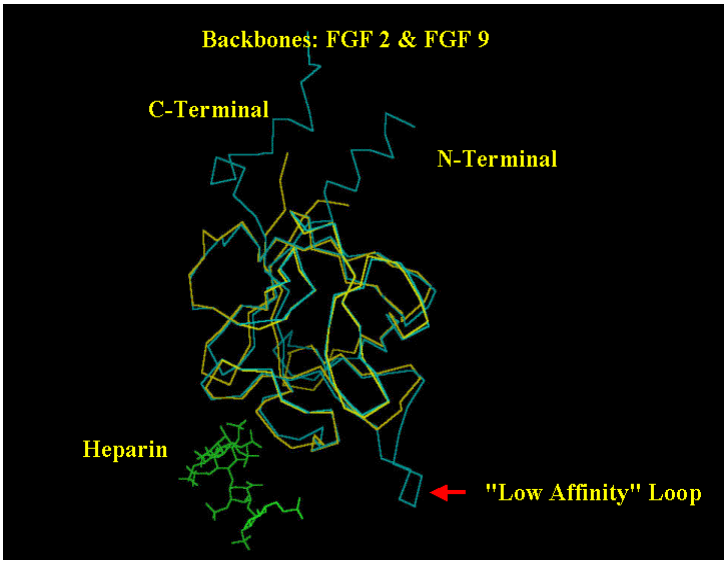
Figures

Figure 1-1. Ribbon structure of FGF9.



The structure of FGF9 consists of twelve antiparallel β-strands flanked by an amino and a carboxyl terminal. The β1- β2 loops and parts of the β10 and β12 loop make up the binding site for heparin sulphate glycosaminoglycan (HSGAGs). HSGAGs aid in the ligand-receptor binding of FGF-FGFR by binding to both simultaneously. The β9- β10 low affinity loops interacts directly with the FGF receptor and accounts for the specificity of FGF9 binding (modified from Plotnikov, 2000).

Figure 1-2. Structural overlay of FGF2 and FGF9.



The overlay of the structural backbones of FGF2 (brown) and FGF9 (blue) highlights that the “low affinity loop” of FGF2 is shorter than that of FGF9. This loop is responsible for the receptor binding specificity of the FGF ligand. This “low-affinity” loop decreases the ability of FGF9 to interact with FGFR1 and FGFR2. FGF9 primarily activates FGFR3-IIIc (modified from Taylor 2007).

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Chapter 2

Critical experimental considerations for determining the effects of fibroblast growth factor-9 on behavior in adult rats

Abstract

In this set of experiments, we examined a number of variables that are crucial to determining the effects of fibroblast growth factor-9 (FGF9) on affective behavior. Animals were injected on post-natal day two (PND2) to determine whether a single early-life subcutaneous (s.c.) injection had long-lasting effects on adult behavior as was observed with FGF2. We observed no significant changes in a battery of adult behaviors. We also tested whether an intraperitoneal (i.p.) injection of FGF9 had an effect on anxiety-like or depression-like behavior. We found no effect on anxiety-like or depression-like behavior following an acute injection via this route of administration. By contrast, we observed that an acute intracerebroventricular (i.c.v.) microinjection of FGF9 increased depression-like behavior during the first half of the light-cycle. The depressionogenic effect was sensitive to time manipulations. Testing during the second-half of the light-cycle revealed an overall decrease in escape activity that may mask the decrease in escape activity observed in the first-half of the light-cycle. We further showed that the increase in depression-like behavior after an acute microinjection of

FGF9 did not persist past 48 hours. Finally, we demonstrated that a novel peptide targeted at the "low-affinity loop" (LAL) of FGF9 did not alter depression-like behavior. These findings provided important information about the route of administration for the subsequent FGF9 studies (described in Chapter 3). Furthermore, the differential effect of timing provided the rationale for assessing rhythmicity of FGF system gene expression (described in Chapter 4). Thus, this set of experiments acted as an experimental framework for the formal studies of the effect of FGF9 on affective behavior and circadian rhythms.

Introduction

Our research group, as part of a larger consortium, was the first to show that fibroblast growth factor family gene expression was dysregulated in post-mortem brain tissue from individuals diagnosed with major depressive disorder (MDD) (Evans, Choudary et al. 2004). Subsequently, other studies have confirmed that FGF2 gene expression was downregulated while FGF9 expression was significantly upregulated in a number of cortical and subcortical brain regions in MDD (Sibille, Arango et al. 2004; Evans, Choudary et al. 2004b; Gaughran, Payne et al. 2006; Tochigi, Iwamoto et al. 2008). Particularly, FGF9 is the single growth factor that is consistently upregulated in multiple brains regions across both Affymetrix and Illumina microarray platforms (Evans, Choudary et al. 2004; Evans, Choudary et al. 2004b). To this end, we have hypothesized that members of the FGF family, especially FGF9, may be potential targets for the therapeutic treatment of mood disorders.

Recently, we have shown that chronic microinjections of FGF2 had antidepressant-like effects on behavior (Turner, Gula et al. 2008). Also, chronic intraperitoneal injections of FGF2 can decrease anxiety-like behavior as measured by increased time spent in the light portion of the light-dark box (Perez, Clinton et al. 2009). While we have previously studied the role of exogenous FGF2 in affective behavior, the following experiments were our first attempts to characterize behavioral effects of exogenous FGF9 on rats. The series of studies parallels early attempts to administer FGF2 *in vivo* (Wagner,

Black et al. 1999; Deguchi, Naito et al. 2000; Wu, Song et al. 2002; Perez, Clinton et al. 2009; Turner, Capriles et al. 2009), and our studies characterized the most effective method for FGF9 delivery *in vivo* to observe effects on affective behavior. In order to understand the effects and timing of FGF9 administration, we tested both persistence of FGF9 effects on affective behavior (Lucki 1997) and manipulated the route of administration and the timing of the injection. These results gave us a better understanding of the variables that were important for subsequent FGF9 studies (Chapters 3 and 4).

Although not much is known about the physiological effects of FGF9, FGF9 is expressed, preferentially in neurons, throughout the human brain in cerebral cortex, hippocampus, motor nuclei of the brain stem, substantia nigra, and Purkinje cell layer (Todo, Kondo et al. 1998). FGF9 also has moderately strong gene expression in the rat hippocampus and cerebral cortex (Tagashira, Ozaki et al. 1995). FGF9 and its subfamily members (FGF16 and FGF20) are unique from other members of the FGF family in that, despite the lack of a typical secretion signal, they are still secreted like members that have a typical signal sequence (Miyamoto, Naruo et al. 1993). Besides MDD, FGF9 expression is upregulated in other brain disorders such as Alzheimer's Disease (Nakamura, Arima et al. 1998) and amyotrophic lateral sclerosis (ALS) (Nakamura, Arima et al. 1998). Furthermore, single nucleotide polymorphisms (SNPs) in FGF20, a member of the FGF9 subfamily, have recently been linked to the increased expression of α-synuclein in Parkinson's disease (PD). FGF20 is currently being

considered as a potential therapeutic target for treatment of PD (Gao, Scott et al. 2008; Wang, van der Walt et al. 2008).

Since FGF9 was consistently upregulated in multiple brain regions of post-mortem tissue of individuals diagnosed with MDD, we hypothesized that FGF9 may be a potential therapeutic target. To this end, we designed a peptide antagonist to the LAL of FGF9. The LAL is primarily responsible for the sequence specifity needed for FGF9 to bind to its primary receptor, FGFR3 (Hecht, Zimmerman et al. 1995). Therefore, the antagonist should decrease FGF9 binding affinity and, subsequently, downstream activation of FGF receptors. In order to characterize the effects of the LAL antagonist *in vivo*, we acutely microinjected rats with multiple doses of the antagonist and measured its effect on depression-like activity.

Material and Methods

Animals

All Sprague-Dawley rats (Charles River Laboratory, MA) 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. The animals were maintained under conditions of constant temperature (21 +/- 2°C)

and a 12-hour/12-hour light-dark cycle with access to food and water *ad libitum*. All animals were allowed one week to acclimate to housing conditions before any experiments were performed. All animals were pair-housed during adulthood with the exception of animals that were cannulated for acute microinjections, they were singly housed post-operatively and throughout the acute injections studies.

Early-life subcutaneous injection of FGF9

Outbred pregnant females (Charles River Laboratory, Wilmington, MA) were housed individually under conditions of constant temperature (21 +/- 2°C) and a 12-hour/12-hour light-dark cycle with access to food and water *ad libitum* throughout their pregnancy, birth, and weaning period. Pups were culled to four male and four females on PND2. Immediately after the culling, animals were injected with either FGF9 (20ng/g in 50µl 0.1M PBS w/ 1% BSA, s.c.; Cell Sciences) or vehicle (0.1M PBS w/ 1% BSA, s.c.) (Turner, Capriles et al. 2009). Group assignments were counterbalanced within each litter. Animals were weaned on PND21 and males were separated and housed two per cage for the remainder of the experiment. The behavior of adult animals, two to three months old, was evaluated with a battery of tests: locomotor activity, elevated plus maze (EPM), forced swim test (FST), and Morris water-maze navigation task (described below). Animals were rested one week between behavioral tests.

Adult intraperitoneal injection of FGF9

Animals (250-300g) were housed two per cage under normal housing conditions. Animals were injected with either FGF9 (5ng/g in 50µl 0.1M PBS w/ 1% BSA, i.p.; Cell Sciences) or vehicle (0.1M PBS w/ 1% BSA, i.p.). Animals were assessed for anxiety-like or depression-like behavior on the EPM or FST (described below), respectively. For the FST, animals received three injections: one immediately after the Day One pre-test swim, one eight hours following the pre-test swim, and one immediately before the Day Two test swim. Animals were rested five days after Day Two of the FST before they were tested on the EPM. The animals were counterbalanced and injected with one injection of FGF9 (5ng/g, i.p.) or vehicle (0.1M PBS w/ 1% BSA, i.p) and immediately tested 15 minutes after the injection.

Adult intracerebroventricular microinjections of FGF9 or LAL peptide antagonist

Rats were anesthetized with isofluorane and were implanted with a guide cannula (22-gauge, Plastics One Inc., VA). The cannula was placed in the left lateral ventricle (coordinates from bregma: AP -1.1; ML +1.3; DV -3.0) using a small-animal stereotaxic instrument. The guide cannula was anchored to the skull using dental cement and was fitted with an obturator. Animals received a microinjection using a 28-gauge injector cannula extending 1.5mm below the tip of the guide. The microinjection cannula was connected by PE-20 tubing to a

Hamilton syringe mounted on a syringe pump (Harvard Apparatus, MA) and rats were microinjected with either recombinant human FGF9 (200ng, i.c.v) (Cell Sciences, MA) or the vehicle (artificial extracellular fluid (aECF), i.c.v) with 100µg/ml bovine serum albumin (Kuhn, Winkler et al. 1997; Cambon, Hansen et al. 2004).

To measure the long-term effects of an acute microinjection of FGF9, animals were injected once after Day One of the FST between 0800 h and 1200 h, and were allowed to wait 48 hours until the next swim on Day Three. To determine whether FGF9 had an effect on the FST from 1300 h – 1700 h, animals were injected immediately after Day One of the FST between 1300 h and 1700 h. Animals were evaluated by a Day Two (24 hours later) swim trial between 0800 h and 1200 h.

For, LAL peptide antagonist studies animals received an injection of the LAL peptide antagonist (YKHUDTGRRYY) (0.5 µg, 5 µg, or 50 µg, i.c.v.) or vehicle (aECF, i.c.v). LAL peptide antagonist study animals were injected once immediately after Day one of the FST between 0800 h and 1200 h and then tested 24 hours later on Day Two. The total volume injected for all microinjection studies was 8µl infused at a rate of 1µl /min in freely moving animals. The injector was left in place for five minutes to allow for diffusion.

Locomotion testing

PND2 animals injected with FGF9 (20ng/g in 50µl 0.1M PBS w/ 1% BSA, s.c) or vehicle (50µl 0.1M PBS w/ 1% BSA, s.c) were allowed to age under normal housing conditions. At two months of age, animals were tested for differences in locomotor activity. The rats were placed in a 43 x 21.5 x 25.5cm acrylic cage with stainless steel floor gridding. Horizontal and vertical (rearing) locomotor activity was monitored every five minutes for one hour by two panels of photocells that recorded beam breaks. For total locomotion, horizontal and vertical components were added together and an average was found for each animal and group. The locomotion-testing rig and motion-recording software were developed in-house at the University of Michigan. All locomotor testing took place between 0800 h and 1200 h.

Morris water-maze navigation task

PND2 animals injected with FGF9 (20ng/g in 50µl 0.1M PBS w/ 1% BSA, s.c) or vehicle (50µl 0.1M PBS w/ 1% BSA, s.c) were allowed to age under normal housing conditions. At three months of age, animals were tested for differences in spatial navigation of the Morris water-maze. The water-maze was a circular tank made from black polyethylene and was 1.5 m in diameter and 0.5 m in height. The tank was filled with lukewarm water and a 10cm Plexiglas circular platform, 1.5cm below the water level, was used as an escape platform. The tank was placed in the middle of a room that contained visual extra-maze cues.

The platform was located at a predetermined direction (north, east, south, or west), and each animal was placed into the tank facing the wall. Escape latency (time between introduction to maze and climbing on the escape platform) was recorded for each animal.

A trial lasted until the animal climbed on the escape platform, or until two minutes had elapsed at which point the animal was placed on the platform. Animals were allowed to stand on the escape platform for 30 seconds following the trial. Testing continued for five days, four trials per day with each trial starting in a different quadrant. Escape latencies were averaged across three trials to yield values for trial blocks. All testing was done in a dimly lit room (~40 lux) between 0800 h and 1200 h.

Forced swim test

Day One of the FST consisted of a fifteen minute pre-test swim and Day Two consisted of a five minute test swim which provides valuable information on the animals' reaction to the second day of stress (Porsolt, Bertin et al. 1977; Porsolt, Deniel et al. 1979). The Day One pre-test swim aids in the development of immobility during the subsequent Day Two test session and increases the sensitivity to antidepressants (Borsini, Lecci et al. 1989). A modified version of the FST was used to test if an acute injection of FGF9 (200 ng, i.c.v.) had a long-lasting effect on depression-like behavior. This modification involved repeated

testing of the same animals on Day Three and Day Six, instead of Day Two (Hoshaw, Malberg et al. 2005). Animals were placed in cylinders filled with water at a depth in which the rat's tail could not touch the bottom with a temperature of 25°C. Water was changed between animals and all sessions were recorded from above by a video camera.

All FST swim sessions took place between 0900 h and 1200 h except in the study to determine whether FST activity can be tested in the second-half of the light-cycle (1300 h to 1700 h). The videotaped activity was scored with the following considerations: (1) swimming consisted of horizontal movement throughout the cylinder, (2) climbing was defined by vertically-directed movement of forepaws against the wall of the cylinder, and (3) immobility was defined as floating or the minimal movement necessary to keep the head above water level. The FST has high predictive validity for antidepressant activity and animals display "despair" behavior as indicated by a decrease in escape behaviors (Lucki 1997). Escape activity was calculated as the percent total duration spent swimming and percent total duration spent climbing. Percent total duration of swimming, climbing, and immobility episodes was scored using The Observer software (Noldus Information Technology, The Netherlands).

Elevated plus-maze

The EPM was used to test the anxiety-like behavior of the rats by using their natural aversion to high open spaces. The maze consists of black Plexiglas with four elevated arms (70cm from the floor, 45cm long, and 12 cm wide). The EPM has two arms enclosed by 45cm-high wall and two open arms arranged in the shape of a cross. At the intersection of the four arms of the maze, a 12 x 12cm square allowed access to all four arms. During the five minute test period, the room was dimly lit (~40 lux), and behavior was 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 facing a closed arm. The tracking system recorded the latency to enter the open arm, the amount of time spent in open and closed arms, and the time spent in the center square. After every animal, the testing apparatus was wiped down with 30% ethanol. Animals that spent a greater percentage of time in the closed arms were considered more anxious while, conversely, animals that spent a greater percentage of time exploring the open arms were considered less anxious.

Statistical analyses

The Morris water-maze navigation experiment using animals injected with FGF9 or vehicle in early-life were analyzed by a two-way repeated measures analysis of variance (ANOVA). The FST experiment using animals that were microinjected

with FGF9 or vehicle, at either 0800 h -1200 h or 1300h -1700 h, was analyzed by a two-way ANOVA. The FST experiment, using animals that were microinjected with different doses of the LAL antagonist, were analyzed by a one-way ANOVA followed by Fisher's least significant difference *post hoc* analysis. All other behavioral data was analyzed by a Student's *t*-test. Data are presented as mean +/- standard error of the mean with statistical significance set at $p < 0.05$.

Results

A single early-life subcutaneous injection of FGF9 had no effect on adult behavior

Animals were injected with FGF9 (20ng/g in 50µl 0.1M PBS w/ 1% BSA, s.c) or vehicle (0.1M PBS w/ 1% BSA, s.c) and were tested for a battery of behaviors. Student's t-test showed no significant difference in locomotor activity including rearing ($t_{(15)} = 0.65$, $p = 0.53$), horizontal movement ($t_{(15)} = 0.90$, $p = 0.38$), and total movement ($t_{(15)} = 0.80$, $p = 0.44$) in animals injected with FGF9 compared to vehicle controls (Figure 2-1A). There were also no significant differences in anxiety-like behavior as evidenced by no dissimilarity in percent time spent on open ($t_{(18)} = 0.73$, $p = 0.48$) or closed arms ($t_{(18)} = 0.46$, $p = 0.65$) of the EPM in animals injected with FGF9 compared to vehicle controls (Figure 2-1B). Furthermore, there was no significant change in depression-like behavior as evidenced by no differences in percent time spent climbing ($t_{(18)} = 0.48$, $p = 0.63$),

swimming ($t_{(18)}$ = 0.64, p = 0.53), or immobile ($t_{(18)}$ = 0.47, p = 0.64) in the FST in animals injected with FGF9 compared to vehicle controls (Figure 2-1C). Finally, analysis by a two-way repeated measures ANOVA showed no significant differences in spatial memory as animals did not differ in their latency time to find the hidden platform in the Morris water-maze navigation task ($F_{(5,68)}$ = 2.02, p = 0.09) for animals injected with FGF9 compared to vehicle controls (Figure 2-1D).

An acute intraperitoneal injection in adult rats had no effect on anxiety-like or depression-like behavior

Animals received an injection of FGF9 (5ng/g in 50µl 0.1M PBS w/ 1% BSA, i.p.) or vehicle (50µl 0.1M PBS w/ 1% BSA, i.p.). Student's t-test showed no significant changes in anxiety-like behavior 15 minutes after an acute injection as shown by the absence of differences in percent time spent on the open arm ($t_{(13)}$ = 1.30, p = 0.22) or closed arm ($t_{(13)}$ = 0.06, p = 0.95) in the EPM in animals injected with FGF9 compared to vehicle controls (Figure 2-2A). There were also no differences in depression-like behavior after an acute injection following Day One of the FST, as evidenced by no group differences in percent time spent climbing ($t_{(13)}$ = 1.5, p = 0.16), swimming ($t_{(13)}$ = 0.91, p = 0.38), or immobile ($t_{(13)}$ = 0.27, p = 0.79) in animals injected with FGF9 compared to vehicle controls (Figure 2-2B).

Acute microinjection of FGF9 increased escape activity in the first-half of the light cycle

Animals were microinjected with FGF9 (200ng, i.c.v.) or vehicle (aECF, i.c.v.). Animals injected and tested in the first-half of the light-cycle (0800 h -1200 h) showed a significant increase in depression-like behavior in the FST as evidenced by a decrease in percent time spent performing escape activity ($F_{(1,20)}$ = 12.89, $p < 0.003$) in animals microinjected with FGF9 compared to vehicle controls (Figure 2-3A). There was a significant decrease in escape activity ($F_{(1,20)}$ = 27.21, $p < 0.001$) observed in vehicle controls tested and injected during the second-half of the light-cycle (1300 h – 1700 h) compared to vehicle controls tested and injected during the first-half of the light-cycle (0800 h - 1200 h) (Figure 2-3A). Animals injected and tested during the second-half of the light-cycle (1300 h -1700 h) showed no significant increase in depression-like behavior as evidenced by no differences in percent time spent climbing ($t_{(14)}$ = 0.67, p = 0.51), swimming ($t_{(14)}$ = 0.54, p = 0.60), or immobile ($t_{(14)}$ = 20.21, p = 0.84) in the FST in animals microinjected with FGF9 compared to vehicle controls (Figure 2-3B).

An acute intracerebroventricular injection of FGF9 did not have lasting effects on depression-like behavior

Animals received a single microinjection of FGF9 (200ng, i.c.v.) or vehicle (aECF, i.c.v.). A swim-trial two days after the Day One pre-test swim in the FST resulted in no significant differences in percent time spent climbing ($t_{(18)}$ = 0.79, p

= 0.44), swimming ($t_{(18)}$ = 0.21, p = 0.65), or immobile ($t_{(18)}$ = 0.14, p = 0.89) on Day Three compared to vehicle controls (Figure 2-4A). A swim-trial five days following the Day One pre-test swim showed no long-lasting effect of FGF9 microinjection on depression-like behavior in the FST as evidenced by no differences in percent time spent climbing ($t_{(1,18)}$ = 0.69, p = 0.50), swimming ($t_{(18)}$ = 0.22, p = 0.83), or immobile ($t_{(18)}$ = 0.32, p = 0.75) on Day Six compared to vehicle controls (aECF, i.c.v.) (Figure 2-4B).

An acute microinjection of a LAL peptide antagonist had no effect on depression-like behavior

Animals were microinjected once with the LAL peptide antagonist (0.5 µg, 5 µg, or 50 µg, i.c.v.) or vehicle (aECF, i.c.v.). There was no overall effect of the LAL peptide antagonist on depression-like activity as evidenced by no dissimilarity in percent time spent climbing ($F_{(3,35)}$ = 2.23, p = 0.10), swimming ($F_{(3,35)}$ = 0.12, p = 0.95), or immobile ($F_{(3,35)}$ = 0.59, p = 0.63) in the FST compared to vehicle controls (aECF, i.c.v.) (Figure 2-5).

Discussion

The results of these studies show a number of negative results that provide the framework for the formal experiments that follow in Chapters 3 and 4. We found that: (1) a single PND2 injection of FGF9 did not alter adult locomotor activity,

anxiety-like behavior, depression-like behavior, or spatial navigation memory, (2) an acute intraperitoneal injection of FGF9 did not alter anxiety-like or depression-like behavior, (3) an acute microinjection of FGF9 had no long-lasting effects on depression-like behavior, (4) an acute microinjection of FGF9 increased depression-like behavior preferentially in the first-half of the light cycle, and (5) an acute microinjection of a peptide antagonist targeted to the low-affinity loop of FGF9 had no effect on depression-like behavior. These findings show that the method and timing of FGF9 injection are important to the evaluation of the effect of FGF9 on affective behavior. The single positive finding in which an acute microinjection of FGF9 increased depression-like behavior in adult rats, indicated that we were in an effective dose range to effect emotional behavior.

In contrast to these results, a single PND2 subcutaneous injection of FGF2 altered adult behavior resulting in a long-lasting increase in cocaine-self administration (Turner, Capriles et al. 2009). A single subcutaneous PND2 injection of FGF2 also resulted in molecular changes as evidenced by altered hippocampal cell proliferation and gene expression (Turner and Inui, unpublished data). Other labs have shown that, a single injection of FGF2 (20ng/g s.c.), facilitated neurogenesis in rats (Cheng, Black et al. 2002), enhanced long-term extinction of fear and reduced reinstatement (Graham and Richardson 2009), and enhanced long-term memory (Graham and Richardson 2009).

However, no changes in adult behavior were observed in animals injected with a single PND2 subcutaneous injection of FGF9 (20ng/g). It is possible that FGF9 requires a higher concentration than FGF2 to observe long-lasting developmental effects. Alternatively, as evidenced by our further peripheral studies, FGF9 may not cross the blood brain barrier (BBB). Finally, we may not have measured the appropriate behavioral or functional endpoint, as we did not assess drug-taking behavior or hippocampal morphology.

An acute peripheral injection of FGF2 increased anxiety-like behavior (Perez 2009), and chronic peripheral FGF2 injections decreased anxiety-like behavior (Perez, Clinton et al. 2009). However, there was no effect of FGF9 on behavior after a peripheral injection of FGF9. While it is known that FGF2 can cross the BBB (Deguchi, Naito et al. 2000; Wu, Song et al. 2002), it is difficult to know how much of the peripherally administered FGF2 is reaching the brain. It is a possibility that FGF9 did not cross the BBB or an effective dose was not used. Therefore, we decided to microinject directly into the left lateral ventricle of the brain. While we are not certain how far FGF9 diffuses after this microinjection, it is likely that a number of brain regions involved in mood disorders are affected by the intracerebroventricular administration of FGF9. Microinjection into the lateral ventricle also minimizes damage caused by directly injecting into the hippocampus. We have preliminary data indicating that direct microinjection of vehicle into the dentate gyrus increased expression of FGF2, FGF9, and FGFR1 (Eren-Kocak E. 2009).

We observed an increase in depression-like behavior following an acute microinjection of FGF9. This result led to the extensive studies described in Chapter 3. Other studies have shown that a single microinjection of other growth factors, insulin-like growth factor-1 (IGF1) and brain-derived neurotrophic factor (BDNF), decreased depression-like behavior for up to five days after the initial Day One swim in the FST (Hoshaw, Malberg et al. 2005). We did not find a persistent increase in depression-like behavior in the FST after an acute injection of FGF9. The half-life of FGF9 in the brain may be shorter than that of other growth factors, including FGF2 (Riva, Molteni et al. 1996). Conversely, increased depression-like behavior may only occur up to 24 hours after microinjection. Regardless of the mechanism, it is important to know that the effect of FGF9 was not long-lasting to inform future experiments.

While trying to determine if splitting groups and testing half in the first-half of the light-cycle and half in the second-half of the light-cycle would be experimentally sound, we observed that there was significant effect of time of day on escape activity in the FST. It is possible FGF9 has differential effects on depression-like behavior during the first-half of the light-cycle than during the second-half of the light-cycle. This possibility led to the formal study of circadian variations of the FGF family in the adult hippocampus discussed in Chapter 4. It is also possible that the decrease in escape activity in vehicle controls during the second-half of the light-cycle is masking the increase in depression-like behavior observed

when animals were tested and injected in the first half of the light-cycle. While others have shown that animals have decreased escape activity in the active period compared to the inactive period (Kelliher, Connor et al. 2000), no one has shown a decrease in escape activity in the second-half of the light-cycle. This timing information is important to keep in mind for all experiments involving the FST for measurement of increased depression-like behavior.

Finally, we synthesized a potential peptide antagonist to the LAL of FGF9. This loop is crucial to the identity of the FGF9 ligand and provides the basis for its preferential activation of FGFR3 (Hecht, Adar et al. 2001). The peptide was developed based on structural targeting and was no verified by *in vitro* studies. While we did not observe an effect of this peptide antagonist on depression-like behavior, this result may suggest that the effect of FGF9 on depression-like behavior is not mediated through activation of FGFR3. FGF9 may be acting as a "physiological antagonist" to FGF2 by direct downregulation of FGFR1 expression or by inhibition of astrocytic differentiation. More work characterizing potent inhibitors of FGF9 *in vitro* must be done before further *in vivo* studies can be tested.

In conclusion, this set of results highlighted the importance of central administration of FGF9 to observe effects on affective behavior in the adult rat. Furthermore, behavioral testing should be consistently performed in the first-half of the light-cycle to detect behavioral effects of FGF9. Finally, targeting the LAL

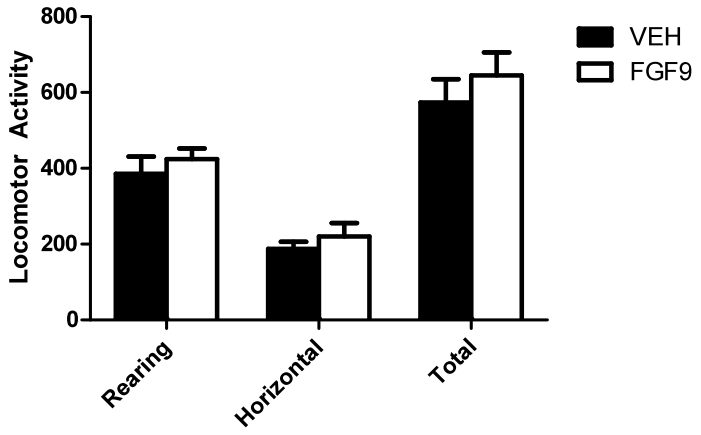
loop of FGF9 did not alter depression-behavior, however targeting other FGF9-specific sequences may lead to development of a novel therapeutic for the treatment of mood disorders.

While a number of the studies in this chapter show negative results, these findings were a logical series of studies to increase understanding of the conditions necessary for detecting FGF9 effects on behavior. Observations of increased depression-like behavior through central microinjections led to a formal series of studies characterizing the exogenous administration of FGF9 (Chapter 3). Observations of time-sensitive behavior led to formal studies of circadian variation of the FGF family in circadian variations of expression and behavior (Chapter 4).

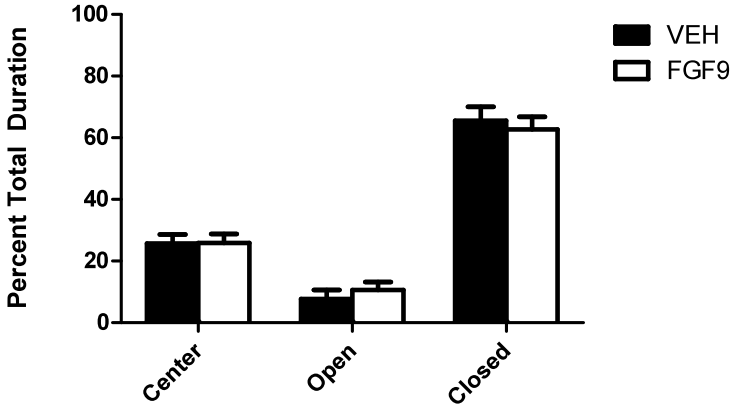
Results Figures

Figure 2-1: An early-life subcutaneous FGF9 injection had no effect on adult behavior.

A.



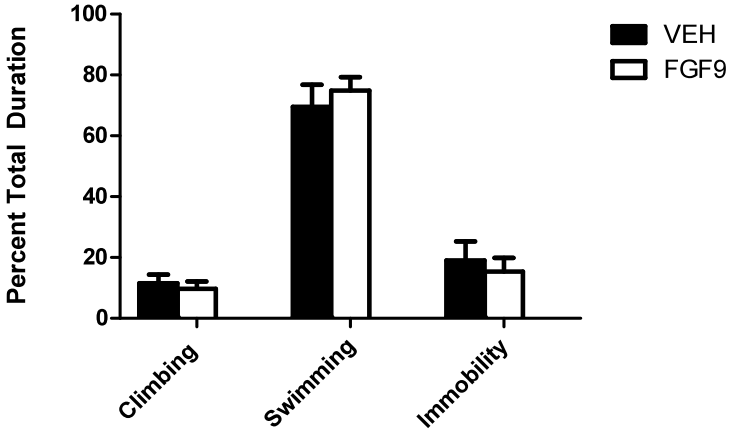
B.



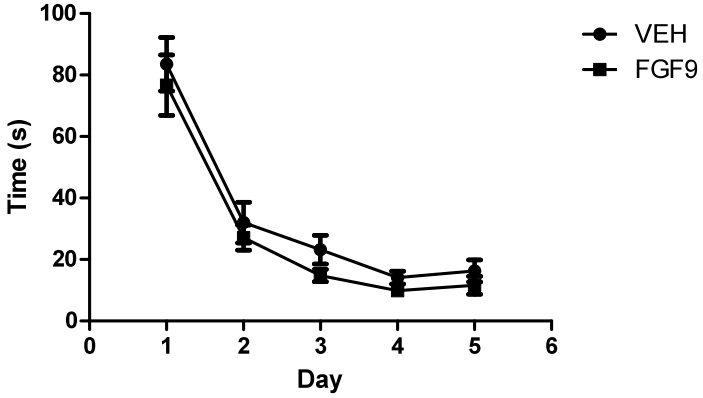
A) An early-life injection of FGF9 (20ng/g, s.c.) had no effect on rearing, horizontal, or total locomotor activity in adult rodents (n= 7-10 animals per group). B) An early-life injection FGF9 (20ng/g, s.c.) had no effect on anxiety-like behavior as shown by no differences in percent time spent in the open or closed arms on the elevated plus-maze compared to vehicle control (n = 10 animals per group).

Figure 2-1 (continued): An early-life subcutaneous FGF9 injection had no effect on adult behavior.

C.



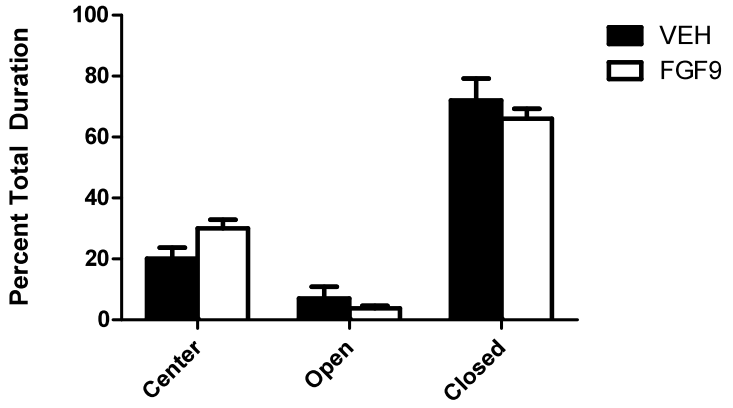
D.



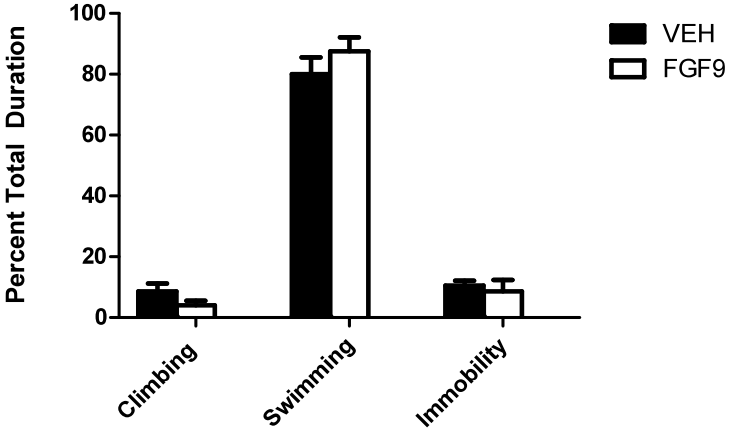
C) An early-life injection of FGF9 (20ng/g, s.c.) had no effect on depression-like behavior in the forced swim test as shown by no differences in percent time spent climbing, swimming, or immobile in the forced swim test (n= 10 animals per group). D) An early-life injection of FGF9 (20ng/g, s.c.) had no effect on spatial memory as shown by no differences in time to platform in the Morris water-maze navigation task compared to vehicle controls (n= 10 animals per group).

Figure 2-2: Intraperitoneal injections of FGF9 had no effect on adult behavior.

A.



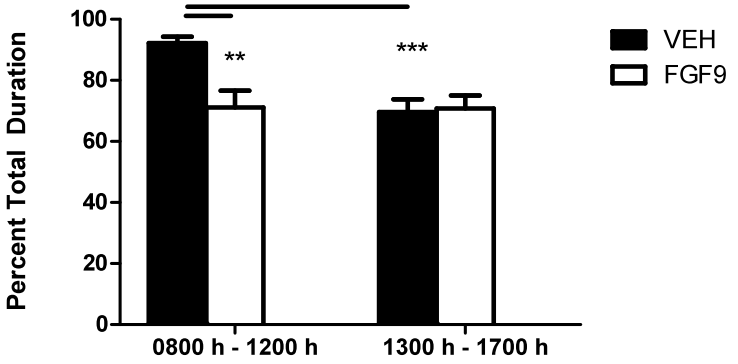
B.



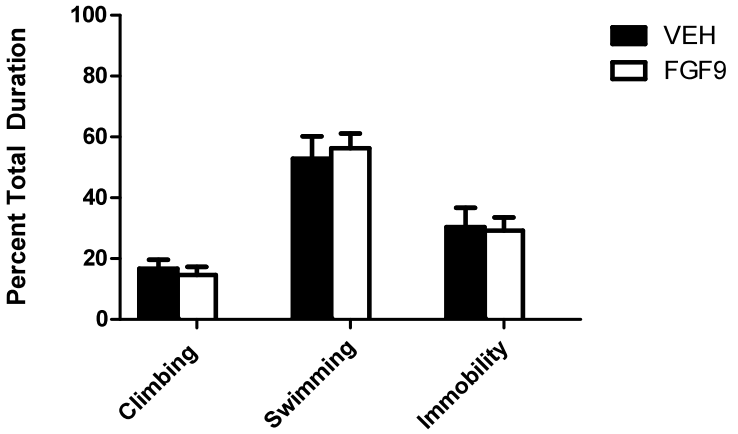
A) An acute injection of FGF9 (5ng/g, i.p.) had no effect on anxiety-like behavior as shown by no differences in percent time spent in the open or closed arms on the elevated plus-maze compared to vehicle controls (n= 7-8 animals/group). B) Three acute injections of FGF9 (5ng/g, i.p.) had no effect on depression-like behavior as shown by no differences in percent time spent climbing, swimming, or immobile in the forced swim test compared to vehicle controls (n= 7-8 animals per group).

Figure 2-3: An acute microinjection of FGF9 administrated during the first-half of the light-cycle increased depression-like behavior. FGF9 administered in the second-half of the light-cycle had no effect on depression-like behavior.

A.



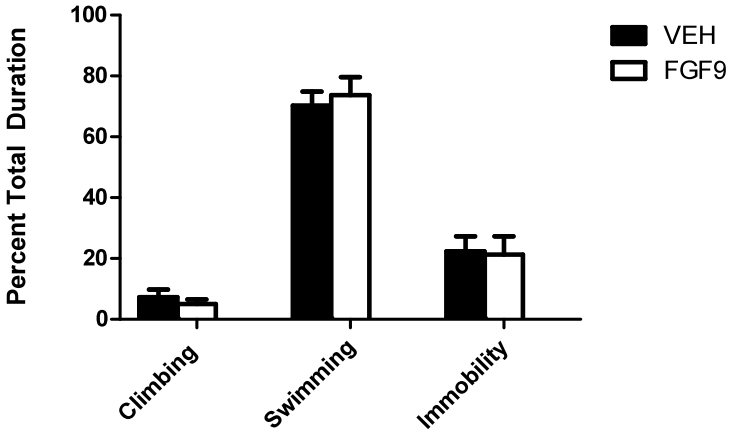
B.



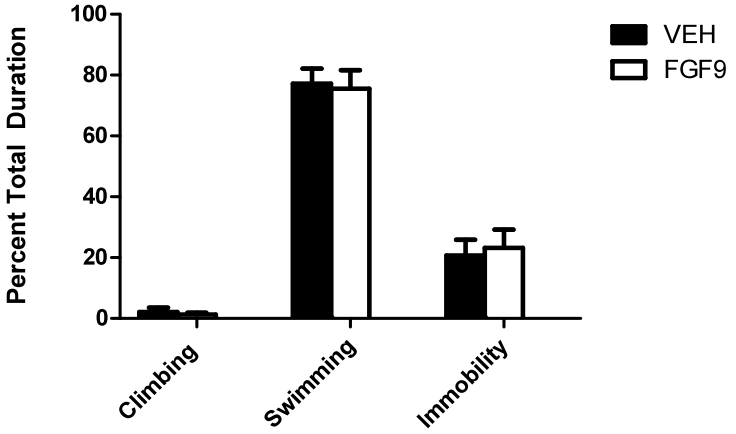
A) A significant decrease in escape activity was observed when FGF9 was microinjected between 0800 h and 1200 h and animals were tested on Day 1 and Day 2 between 0800 h and 1200 h compared to vehicle controls; ** $p < 0.005$, (n= 11 animals per group). A significant decrease was observed in escape activity in vehicle control animals when animals were microinjected and tested between 1300 h and 1700 h compared to vehicle controls (aECF, i.c.v.) tested and microinjected between 0800 h and 1200 h; ***$p < 0.001$ (n= 8-11 animals per group). B) An acute microinjection of FGF9 (200ng, i.c.v.) did not have an effect on depression-like behavior in the forced swim test compared to vehicle controls (aECF, i.c.v.) (n= 8 animals per group).

Figure 2-4: An acute intracerebroventricular injection of FGF9 did not have persistent effects on depression-like behavior.

A.

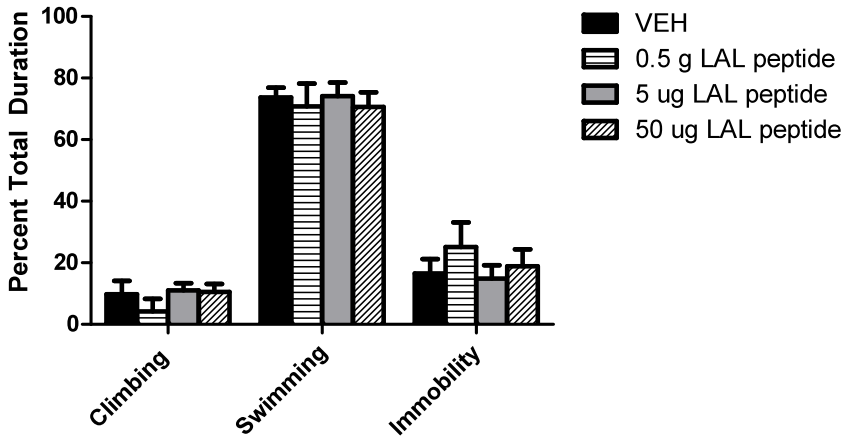


B.



A) An acute microinjection of FGF9 (200ng, i.c.v.) did not result in a persistent increase in depression-like behavior as shown by no differences in percent time spent climbing, swimming, or immobile two days after Day One of the forced swim test compared to vehicle controls (aECF, i.c.v.) (n= 10 animals per group). B) An acute injection of FGF9 (200ng, i.c.v.) did not result in a persistent increase in depression-like behavior as shown by no differences in percent time spent climbing, swimming, or immobile five days after Day One of the forced swim test compared to vehicle controls (aECF, i.c.v.) (n= 10 animals per group).

Figure 2-5: An acute microinjection of a novel peptide designed to target the low-affinity loop (LAL) of FGF9 had no effect on depression-like behavior.



An acute microinjection of the low-affinity loop antagonist (0.5 µg, 5 µg, or 50 µg, i.c.v.) did not have an effect depression-like behavior in the forced swim test compared to vehicle controls (aECF, i.c.v.) (n= 9-10 animals per group).

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Chapter 3

A novel role for fibroblast growth factor-9 in anxiety-like and depression-like behavior

Abstract

Human post-mortem studies have shown that fibroblast growth factor-9 (FGF9) gene expression was upregulated in multiple brain regions in individuals diagnosed with major depressive disorder (MDD). While recent research implicates the fibroblast growth factor (FGF) family in mood disorders, relatively little is known about the role of FGF9 in response to stress or in emotional reactivity. Initially, we investigated whether repeated social stress would alter the expression of FGF9 in the hippocampus. Repeated social stress increased rodent avoidance behavior and increased FGF9 gene expression. We then assessed the behavioral effects of acute FGF9 microinjections. Acute intracerebroventricular (i.c.v.) administration of FGF9 resulted in a dose-dependent decrease in anxiety-like behavior and an increase in depression-like behavior. Using the effective doses from the acute study, we then tested the effect of chronic microinjections on both behavior and alterations of FGF family gene expression in the hippocampus. Chronic microinjections of FGF9 reversed the anxiety-like response seen in the acute microinjection studies, from anxiolytic

to anxiogenic, and maintained the increase in depression-like behavior. Also, chronic microinjections of FGF9 decreased FGFR1 gene expression in the dentate gyrus of the hippocampus. These findings support the role of FGF9 as a a responder to stress, a modulator of affective behavior, and a potential therapeutic target for psychiatric illness.

Introduction

The discovery of molecular signatures and investigation of expression dysregulation in mood disorders may lead to targeted therapeutic treatments. Our lab, as part of a larger consortium, used microarray technology in human post-mortem studies on individuals diagnosed with MDD. We had previously revealed dysregulation of FGF family gene expression in both cortical and subcortical brain regions (Evans, Choudary et al. 2004; Evans, Choudary et al. 2004b; Akil, Evans et al. 2008). Recently, independent efforts have confirmed changes in the FGF system in the post-mortem tissue of depressed individuals (Sibille, Arango et al. 2004; Aston, Jiang et al. 2005; Gaughran, Payne et al. 2006; Tochigi, Iwamoto et al. 2008). Importantly, FGF9 gene expression was consistently upregulated in multiple brain regions of individuals diagnosed with MDD and verified across Affymetrix and Illumina array platforms. In this series of studies, we explore whether: (1) FGF9 expression can be upregulated by repeated social stress (2) FGF9 can act as a modulator of affective behavior, and (3) FGF9 may be a physiological antagonist to FGF2.

It is difficult to determine whether the dysregulation of the FGF family contributes to the development of MDD or is a result of the disease process. In addition to upregulation of FGF9, FGF2 was consistently downregulated in cortical and subcortical brain regions (Evans, Choudary et al. 2004). We have recently investigated the response of FGF2 gene expression to social defeat and the effect of exogenous FGF2 on emotionality to better understand its potential as a

therapeutic agent (Turner, Calvo et al. 2008; Turner, Gula et al. 2008; Perez, Clinton et al. 2009). FGF2 expression was decreased by social defeat, and exogenous administration of FGF2 has antidepressant-like effects in adult rats. However, no previous studies have focused on the function of FGF9 in affective behavior. The consistent upregulation of FGF9 gene expression may provide both a biomarker of MDD as well as a potential molecular target for therapeutic inhibition. While little is known about the role of FGF9 in psychiatric illness, it is well-documented that the FGF family plays an important role in the development (Molteni, Lipska et al. 2001; Vaccarino, Ganat et al. 2001) and maintenance of the CNS (Mufson, Kroin et al. 1999; Perrone-Capano, Da Pozzo et al. 2000). Some of these CNS functions include cell proliferation, differentiation, and neurogenesis (Reuss and von Bohlen und Halbach 2003).

FGF9 is expressed throughout the human and rat brain and is localized preferentially to neurons (Tagashira, Ozaki et al. 1995; Todo, Kondo et al. 1998) although it can be expressed in some oligodendrocyte and astrocyte cell populations (Nakamura, Todo et al. 1997; Colvin, Feldman et al. 1999; Garces, Nishimune et al. 2000). The neuronal expression of FGF9 is thought to support neuronal survival through an autocrine/paracrine system. A great deal is known about the signaling pathways of the FGF family in rats in which the 22 FGF ligands can bind to five different receptors (FGFR1-5) (Reuss and von Bohlen und Halbach 2003). However, much work still needs to be done to understand the specificity and regulation of the signaling process.

Since stressful life events are thought to trigger the onset of depressive episodes, it is hypothesized that animal models of social stressors might lead to a better understanding of the molecular changes occurring in the brain during depression (Kendler, Karkowski et al. 1999). In this study, we employed a repeated social stress paradigm (Miczek 1991; Kabbaj, Evans et al. 2004) to determine the effect of stress on FGF9 expression. Animals exposed to repeated social stress showed increased anhedonia and neuroendocrine responses similar to those of depressed patients (Kudryavtseva, Bakshtanovskaya et al. 1991; Koolhaas, Meerlo et al. 1997). The behavioral and endocrine changes caused by the social stress are often maintained long after the stressful events are over (Koolhaas, Meerlo et al. 1997; Von Frijtag, Reijmers et al. 2000).

In addition to quantifying behavioral measures of depression, we also looked at changes in FGF9 expression in the hippocampus after repeated social stress. The hippocampus is considered key in modulating anxiety-like and depression-like response (Kim and Yoon 1998; McEwen 1999; Kim and Diamond 2002). Previously, we found that FGF2 and FGFR1 mRNA expression was decreased in the hippocampus following acute social defeat (Turner, Calvo et al. 2008). Additionally, there is significant evidence of the impact of stress on the hippocampus as documented by electrophysiological, neuroanatomical, and cellular physiology studies (de Kloet, Joels et al. 2005).

As FGF2 was downregulated and FGF9 was upregulated in post-mortem tissue of patients diagnosed with MDD, it was hypothesized that FGF9 would have opposing behavioral effects in anxiety-like and depression-like behavior in rats. Recently, it has been shown that FGF9 regulated adult neural progenitor cells *in vitro* by inhibiting differentiation of glial fibrillary acid (GFAP)-expressing astrocytes, decreasing oligodendrocyte differentiation, and increasing generation of neural progenitors (Lum, Turbic et al. 2009). By contrast, our lab has also shown that chronic FGF2 treatment increased GFAP-positive astrocyte formation (Perez, Clinton et al. 2009). It is possible that FGF9 is acting as a naturally occurring antagonist to FGF2 by: (1) binding to and sequestering the ligand or (2) binding to the receptor and preventing its response to another molecule. While FGF2 may act as an endogenous inhibitor of anxiety, FGF9 may be mediating the long-term anxiogenic and depression-like symptoms seen in MDD through endogenous antagonism of FGF2.

Materials and Methods

Animals

Adult male Sprague-Dawley rats (Charles River Laboratories, Wilmington, MA), weighing between 220-250g, were housed in pairs on a 12-hour/12-hour light/dark schedule (0700 h lights on), with access to food and water *ad libitum*. Animals were allowed to acclimate to the housing environment for seven days

before any experiments began. Animals used in the microinjection studies were housed in pairs until the surgery and were singly housed after surgery. Housing procedures for the repeated social stress experiments are described below. 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.

Repeated social stress

To determine the regulation of FGF9 gene expression following stress, we used a repeated social stress paradigm consisting of two phases, a training phase lasting four days and a repeated stressor phase lasting ten days. During both phases, Long-Evans male rats (400-450g) were housed with ovariectomized female rats to increase their territorial response and aggressive behavior. During the daily 15-minute social stress episode, Long-Evans females were first removed from the resident cage and an intruder Sprague-Dawley rat (250-300g) was introduced. The intruder was allowed to move freely throughout the cage until an intense physical episode occurred (1-5 minutes). After an initial physical attack, intruders were placed in a protective wire mesh cage (30 x 15 x 15cm) and back into the residents' home cage for the remainder of the 15-minute trial. This paradigm allowed for intense visual, auditory, and olfactory interactions emphasizing the psychosocial component of the stress while maintaining the

physical safety of the intruder rat (Miczek 1991; Kabbaj, Evans et al. 2004). After the 15-minute social stress period, intruders were returned to their home cage and were singly housed. The female Long-Evans rats were returned to the cage of the resident. The intruders and residents were housed in different rooms throughout the entirety of the experiment. During the training phase, four days, Long-Evans residents (n=15) were trained to attack non-experimental Sprague-Dawley intruders and were included when latency to attack was less than one minute. Residents (n=2) that were not included in the experimental group, were used as novel resident targets in the social interaction test. During the repeated stressor phase, ten days, intruders were placed in a cage with a novel resident every day for 15 minutes during the dark phase of the light cycle between 1900 h and 2200 h. Control animals were placed in a novel cage and allowed to move freely for the 15-minute period. Body weights were measured on Day 1 of the repeated social stress and on Day 10 following the last stress period.

Social interaction test

To determine whether repeated social stress caused any behavioral differences in the intruder rats compared to their non-stressed handled controls, we employed a novel measure of social interaction (SI) that was recently shown to identify social avoidance in mice susceptible to social defeat. This social avoidance was long-lasting and reversible by chronic, but not acute, antidepressant treatment (Berton, McClung et al. 2006; Tsankova, Berton et al.

2006). The modified social interaction testing arena consisted of a black Plexiglas open field (100 x 100cm) with boundaries marked for interaction zones and a target field. The movement of the Sprague-Dawley intruder rat was recorded by a video tracking system (Noldus Ethovision). The test consisted of two 5 minute trial periods on consecutive days. Animals that were subjected to repeated social stress were assessed with no resident, novel Long-Evans rat, present on Day 1 and with the resident present on Day 2. On Day 1, the intruder was placed in the center of the testing field with no target. The second trial, 24 hours later, consisted of five minutes during which the intruder rat was placed in the center of the test field with a caged resident present. The interaction ratio was calculated as 100 x (interaction time, target present)/ (interaction time, target absent). Animals with a low social interaction ratio had increased social avoidance behavior.

mRNA *in situ* hybridization

In order to understand the regulation of key FGF ligands and receptors after our experimental procedures, tissue was collected 24 hours following the forced swim test for animals injected chronically with FGF9 or vehicle. For animals subjected to repeated social stress, tissue was collected 24 hours after the second trial of the SI task. All rats were sacrificed by rapid decapitation and their brains were removed, snap-frozen in isopentane, and stored at -80°C until sectioned. Tissue was sectioned at -20°C at 10μm (n=5-6/group), sliced in

series throughout the hippocampus, mounted on Superfrost Plus slides (Fisher Scientific), and returned to -80°C until processed. Sections were taken every 200μm through the hippocampus and *in situ* hybridization methodology and analysis was performed as previously described in detail elsewhere (Kabbaj, Devine et al. 2000). All *in situ* probes were synthesized in our laboratory and exposure times were experimentally determined for optimal signal and are as follows: FGF2 (7 days), FGF9 (14 days), and FGFR1 (7 days). 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). 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 1μg of linearized plasmid, 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, and 1.5μl of T7 or T3 RNA polymerase. Radioactive signals were quantified using computer-assisted optical densitometry software, Scion Image (Scio Corporation, Frederick, MD). Integrated optical densities were determined by outlining a subfield (CA1, CA2, CA3, and dentate gyrus) of the hippocampus on each hemisphere and correcting for background plus 3.5x its SD. Only pixels with grey values exceeding the threshold were included in the analysis. Data from multiple sections per animal were averaged resulting in a mean for each animal and an average was taken for each group.

Acute microinjections

To determine whether FGF9 microinjections had an effect on affective behavior, we performed a dose-response analysis based on similar doses of FGF2 that were known to alter depression-like and anxiety-like behaviors in rats (Turner, 2008; Perez, 2009). Rats were anesthetized with isofluorane and were implanted with a guide cannula (22-gauge, Plastics One Inc., VA). The cannula was placed in the left lateral ventricle (coordinates from bregma: AP -1.1; ML +1.3; DV -3.0) using a small-animal stereotaxic instrument. The guide cannula was anchored to the skull using dental cement and was fitted with an obturator. After five days of recovery, rats were exposed to Day One of the forced swim test (FST) and received a microinjection immediately following the stressor using a 28-gauge injector cannula extending 1.5mm below the tip of the guide. The microinjection cannula was connected by PE-20 tubing to a Hamilton syringe mounted on a syringe pump (Harvard Apparatus, MA) and rats were microinjected with either recombinant human FGF9 (0.2ng, 2ng, 20ng, 200ng, or 2000ng, i.c.v.) (Cell Sciences, MA) or the vehicle (artificial extracellular fluid (aECF), i.c.v) with 100µg/ml bovine serum albumin (Kuhn, Winkler et al. 1997; Cambon, Hansen et al. 2004). The total volume injected was 8µl infused at a rate of 1µl /min in freely moving animals. The injector was left in place for five minutes to allow for diffusion. The rats were then tested 24 hours later on Day Two of the FST. One week later, rats were counterbalanced and received another FGF9 microinjection 15 minutes prior to testing on the elevated plus-maze (EPM).

Chronic microinjections

In order to determine whether chronic administration had a strengthened or differential effect on behavior compared to acute injections, rats were implanted with cannulae as described above and were microinjected daily with FGF9 (20ng, i.c.v.) or vehicle (aECF, i.c.v.) for a total of 18 days. All microinjections were administered between 0800 h and 1200 h. Rats were tested in the EPM (Day 14), for locomotor activity (Day 15), and in the FST (Day 17 and Day 18). All behavioral testing was performed between 0800 h and 1300 h (Kelliher, Connor et al. 2000).

Locomotion testing

After 15 days of chronic microinjections of vehicle (aECF, i.c.v.) or FGF9 (20ng, i.c.v.), locomotor activity was tested to determine if the results seen on behavioral tasks were due to non-specific alterations in locomotor ability. The rats were placed in a 43 x 21.5 x 25.5cm acrylic cage with stainless steel floor gridding. Horizontal and vertical locomotor activity was monitored every five minutes for one hour by two panels of photocells that recorded beam breaks. The horizontal and vertical components were added together and an average was found for each animal and group. The locomotion-testing rig and motion-recording software were developed in-house at the University of Michigan. All locomotor testing took place between 0800 h and 1200 h.

Forced swim test

The FST has high predictive validity for antidepressant activity and animals display “despair” behavior as indicated by a decrease in escape behaviors, swimming and climbing (Lucki 1997). We performed this behavioral measure to determine the effect of FGF9 on depression-like behavior. Day One consists of a 15 minute pre-test swim and Day Two is a five minute test swim which provides valuable information on the animals’ reaction to the second day of stress (Porsolt, Bertin et al. 1977; Porsolt, Deniel et al. 1979). The Day One pre-test swim aids in the development of immobility during the subsequent Day Two test session and increases the sensitivity to antidepressants (Borsini, Lecci et al. 1989).

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°C. Immediately following the Day One pre-test, animals were dried, returned to their home cage, and injected with FGF9 (0.2ng, 2ng, 20ng, 200ng, or 2000ng, i.c.v.) or vehicle (aECF, i.c.v.). Water was changed between animals and all sessions were recorded from above by a video camera. All FST swim sessions took place between 0900 h and 1200 h. The videotaped behaviors were scored by an observer blind to the experimental conditions (Lucki 1997). Swimming consisted of horizontal movement throughout the cylinder. Climbing was defined by vertically-directed movement of forepaws 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 was scored using The Observer software (Noldus Information Technology, The Netherlands).

Elevated plus-maze

The EPM was used to test the anxiety-like behavior of the rat by using their natural aversion to high, open spaces. The maze consists of black Plexiglas with four elevated arms (70cm from the floor, 45cm long, and 12cm wide). The EPM has two arms enclosed by 45cm-high wall and two open arms arranged in the shape of a cross. At the intersection of the four arms of the maze, a 12 x 12cm square platform allowed access to all four arms. 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 facing a closed arm. 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 30% ethanol. Animals that spent a greater percentage of time in the closed arms were considered more anxious while, conversely, animals that spent a greater percentage of time exploring the open arms were considered less anxious.

Statistical Analyses

FGF9 microinjection behavioral studies were analyzed by ANOVAs followed by Fisher's least significant difference *post hoc* analysis and remaining studies were analyzed by a Student's t-test with all data presented as mean +/- standard error of the mean with statistical significance set at $p < 0.05$.

Results

Repeated social stress increased social avoidance behavior and increased FGF9 gene expression

Repeated social stress has been shown to induce a variety of behavioral deficits similar to those seen in human depression (Blanchard, Spencer et al. 1995; Rygula, Abumaria et al. 2005). It is also known that FGF2 and FGFR1 were decreased following an acute social defeat paradigm (Turner, Calvo et al. 2008). Therefore, we tested whether FGF9 expression was increased after repeated social stress, consistent with expression data seen in human post-mortem tissue. Rats subjected to the repeated social stress paradigm (Figure 3-1A) showed significantly increased change in percent body weight, animals that were repeatedly stressed did not gain weight at the same rate as handled controls ($t(22) = 5.8$, $p < 0.001$; Figure 3-1B). Additionally, repeatedly stressed animals showed increased social avoidance behavior (Figure 3-1D) on the social

interaction test ($t(17) = 2.5$, $p < 0.03$; Figure 3-1C) compared to handled controls. There were no significant differences in the FST (data not shown).

The brains of the animals subjected to repeated social stress were harvested and analyzed for FGF9 mRNA transcripts. There was an increase in hippocampal FGF9 mRNA expression following repeated social stress compared to handled controls. This increase occurred through multiple hippocampal subregions: CA1, CA2, CA3, and DG ($t(8) = 2.46$, $p < 0.04$, $t(8) = 3.41$, $p < 0.01$, $t(8) = 3.12$, $p < 0.02$, $t(8) = 3.15$, $p < 0.02$, respectively; Figure 3-2A and 3-2B).

An acute microinjection of FGF9 altered EPM and FST activity in a dose-dependent manner

FGF9 mRNA expression was upregulated in a number of brain regions in post-mortem tissue of patients diagnosed with MDD (Evans, 2004), and chronic FGF2 administration has antidepressant and anxiolytic effects on adult rats (Turner 2008). Thus we decided to test the hypothesis that FGF9 had an opposing effect on affective behavior. We first began by administering a dose-response curve with median doses similar to those in which FGF2 caused behavioral responses in both the FST and the light dark box (Turner, Gula et al. 2008; Perez, Clinton et al. 2009).

There was an overall anxiolytic effect of acute FGF9 administration in the elevated plus-maze with increased percent total duration spent in the open arms

and decreased percent total duration in the closed arms ($F_{(5,58)} = 4.3$, $p < 0.003$; $F_{(5,58)} = 3.3$, $p < 0.02$; Figure 3-3A). *Post hoc* comparisons of each FGF9 dose with vehicle indicated that FGF9 showed an inverted U-shape dose-response curve with a moderate dose ($p < 0.01$ for 20ng) increasing percent time spent in the open arms and decreasing percent time spent in the closed arms ($p < 0.05$ for 20ng FGF9) compared to vehicle controls.

Similarly, there was a main effect of FGF9 administration on performance in the FST. There was an overall depression-like effect of FGF9 administration as evidenced by a decrease in the percent total duration spent swimming and a significant increase in percent total duration spent immobile ($F_{(5,66)} = 3.1$, $p < 0.02$, $F_{(5,66)} = 5.1$, $p < 0.002$, respectively; Figure 3-3B) compared to vehicle controls. *Post-hoc* analysis for dose effects showed that both a 20ng and 200ng microinjection of FGF9 increased depression-like behavior both by decreasing percent time spent swimming ($p < 0.004$ for both 20ng and 200ng) and increasing percent time spent immobile ($p < 0.002$ for both 20ng and 200ng) compared to vehicle controls.

Chronic FGF9 microinjections increased anxiety-like behavior and increased depression-like behavior

As described above, acute FGF9 microinjections resulted in alterations in affective behavior. It is known that an acute injection of an antidepressant

increased anxiety while chronic antidepressant injections decreased anxiety (Bagdy, Graf et al. 2001; Belzung, Le Guisquet et al. 2001; Burghardt, Bush et al. 2007). To that end, we tested whether chronic FGF9 administration continues to have the same effects on both anxiety-like and depression-like behavior. We administered the lowest dose (20ng FGF9) that had an acute behavioral effect on both the EPM and the FST.

Our results indicated that while chronic FGF9 administration continued to increase depression-like behavior, the long-term effects on anxiety-like behavior are reversed. The chronic dose of FGF9 increased anxiety-like behavior on the EPM with FGF9-microinjected animals having a significantly decreased percent total duration spent in the open arms of the EPM ($F_{(1,16)} = 8.1, p < 0.05$; Figure 3-4A) compared to vehicle controls. Consistent with the acute microinjections, there was a decrease in percent total duration spent swimming and an increase in the percent total duration spent immobile ($F_{(1,14)} = 7.3, p < 0.05$, $F_{(1,14)} = 6.6, p < 0.05$, respectively; Figure 3-4B) in animals injected with FGF9 compared to vehicle controls.

Neither acute nor chronic FGF9 microinjections altered locomotor activity during the behavioral test period

In order to rule out non-specific effects of FGF9 on locomotor behavior that could influence activity in the FST or EPM, locomotor activity was monitored 24 hours

after the acute or chronic microinjections (20ng, i.c.v.). The testing took place during the same time that the FST testing took place (0900 h to 1000 h) in a separate set of animals. The acute microinjections resulted in no change in locomotor activity 15 minutes post-injection as measured by the total distance traveled in the EPM ($t(16) = 0.99$, $p = 0.33$; Figure 3-3C). The acute microinjection caused no change in locomotor activity 24 hours after the injection as measured by beam breaks in a locomotion chamber ($t(8) = .03$, $p = 0.98$; Figure 3-3D). Chronic microinjections of FGF9 also had no effect on locomotor activity during the period in which the behavioral testing took place ($t(17) = 0.13$, $p = 0.902$; Figure 3-2C).

Chronic FGF9 microinjections decreased FGFR1 gene expression in the dentate gyrus of the hippocampus

To assess whether the depression-like and anxiety-like effects of chronic FGF9 administration were associated with corresponding FGF family mRNA expression changes in the hippocampus, we tested whether there were changes in FGF2, FGFR1, or FGF9 mRNA transcripts in four regions of the hippocampus (CA1, CA2, CA3, and dentate gyrus). Although chronic FGF9 had no effect on FGF2 or FGF9 gene expression in the hippocampus (data not shown), chronic FGF9 led to a dentate gyrus-specific decrease in FGFR1 mRNA expression ($t(8) = -2.45$, $p < 0.04$; Figures 3-5A and 3-5B).

Discussion

These studies show for the first time that FGF9 altered both anxiety-like and depression-like behavior and is itself modulated by social stress. Specifically, we found that: (1) chronic social stress increased social avoidance behavior in intruder rats and increased the hippocampal expression of FGF9 mRNA, (2) acute administration of FGF9 (multiple doses, i.c.v.) decreased anxiety-like behavior and increased depression-like behavior in a dose-dependent manner, (3) chronic administration of FGF9 (20ng, i.c.v.) increased anxiety-like behavior, reversing the result seen acutely, and increased depression-like behavior, and (4) chronic administration of FGF9 (20ng, i.c.v.) decreased the expression of hippocampal FGFR1 specifically in the dentate gyrus subfield. These findings support the hypothesis that excess exogenous FGF9 protein or endogenous FGF9 gene expression may have negative effects on anxiety-like and depression-like behavior. It is important to note that FGF9 is acting as an endogenous physiological antagonist to FGF2 as evidenced by opposing behavioral and gene expression data. This data suggests that FGF9 may be a potential therapeutic target for peptide inhibition and treatment of MDD.

Repeated social stress is critical to survival of most individuals, and animals who face these conditions are likely to exhibit signs of stress (Kollack-Walker, Watson et al. 1997). In humans, repeated social defeat encounters increase rates of incidence of psychiatric disorders, disease, and even death (Blanchard, Sakai et al. 1993; Kaplan, Asnis et al. 1995; Lemieux and Coe 1995; Gil-Rivas, Fiorentine

et al. 1996). In this experiment we observed a significant increase in percent change of body weight and increased social avoidance behavior compared to handled controls. This evidence supported that increases in FGF9 gene expression are correlated to behavioral and physiological measures of stress response. As gene expression of FGF2 and FGF9 were dysregulated in MDD in opposing directions (Evans, Choudary et al. 2004), expression was also altered in opposing directions after acute social defeat or repeated social stress. The increase in FGF9 gene expression after repeated social stress is consistent with human findings in which FGF9 is increased in MDD. FGF9 may provide a relevant biomarker for mood dysregulation.

While we have no direct evidence of FGF2/FGF9 regulation, we know that FGF2 and FGF9 have opposing actions on GFAP-positive astrocytes. FGF2 increased the presence of astrocytes in the hippocampus and cortex (Perez, 2008; Reuss, 2003) and FGF9 decreased astrocyte differentiation in adult neural progenitor cells (Lum, 2009). While differences in astrocytic differentiation could have an effect on neural differentiation, it is also possible that an FGF9-mediated decrease in astrocytes, which are the primary synthesis sites of FGF2, could lead to a decrease in FGF2 synthesis.

While we have shown that FGF2 has anxiolytic and antidepressant-like effects *in vivo* (Turner, Gula et al. 2008; Perez, Clinton et al. 2009), this is the first study to

show a direct effect of FGF9 on anxiety-like and depression-like behavior. We show that FGF9 treatment is dose-dependent with a U-shape dose-response curve (Calabrese and Baldwin 2003). This pharmacological response is common in ligand-receptor activation in which ligands can be partial agonists at lower to moderate doses and antagonists at high doses. Although it was unexpected that FGF9 would be an acute anxiolytic, it was hypothesized that FGF9 would have a depression-like effect in microinjected animals. However, it is not uncommon for antidepressant treatments to first cause an anxiogenic response and chronically result in anxiolytic behavioral effects (Bagdy, Graf et al. 2001; Belzung, Le Guisquet et al. 2001; Burghardt, Bush et al. 2007). This reversal in effect on anxiety mirrors the results seen with acute and chronic FGF2 microinjection. Acute FGF2 is anxiogenic, while chronic FGF9 is anxiolytic .

It is possible that an initial acute anxiolytic response can result in chronic anxiogenic behavior. This reversal in effect on anxiety, from anxiolytic to anxiogenic, was seen after chronic administration and implicates FGF9 in the increase in anxiety-like behavior. The chronic injection of FGF9 (20ng, i.c.v) continued to increase depression-like behavior in the FST. Since there is significant co-morbidity between clinical anxiety and depression, it is likely that there are conserved mechanisms for behavioral modulation (Gorwood 2004). As an exogenous injection of FGF9 is responsible for a negative effect on both anxiety and depression, it is a logical therapeutic target for small-molecule inhibition for the treatment of MDD. However, further studies into the regulation

of FGF9 would need to be done to determine the exact mechanisms by which it alters affective behavior *in vivo*.

In this study, we show that chronic administration of exogenous FGF9 led to a decrease in FGFR1 gene expression. In contrast, we have shown that chronic administration of FGF2 increased FGFR1 gene expression (Turner, Gula et al. 2008). Interestingly, both of the changes in gene expression were selective to the dentate gyrus of the hippocampus. Other research has shown that sub-region specific knockout of another growth factor, brain-derived neurotrophic factor (BDNF), in the dentate gyrus, specifically attenuated antidepressant activity compared to CA1-specific BNDF knockouts (Adachi, Barrot et al. 2008). The preferential decrease of FGFR1 in dentate gyrus of the hippocampus may lead to decreased FGF2 signaling activity in this region since the FGF2 ligand primarily activates FGFR1 (Ornitz, Xu et al. 1996).

Although FGF9 can induce neurogenesis in adult neural progenitor cells, it mainly increased the number of early neural progenitors (Lum, Turbic et al. 2009). FGF9 also enhanced long-term survival of spinal motor neurons, bulbospinal neurons, and the basal forebrain cholinergic neurons *in vitro* (Kanda, Iwasaki et al. 1999; Pataky, Borisoff et al. 2000). This evidence suggests a maintenance role for FGF9 in which it slows the process of neuronal differentiation. In contrast, FGF2 causes a distinct induction of precursor cells in

the CNS (Vescovi, Reynolds et al. 1993; Shihabuddin, Ray et al. 1997) and promotes neurogenesis *in vivo* in developing (Raballo, Rhee et al. 2000) and adult rats (Yoshimura, Takagi et al. 2001).

Classical antidepressants induce FGF2 expression (Mallei, Shi et al. 2002) and increase neurogenesis (Malberg et al, 2000). Recent studies show conflicting evidence on whether neurogenesis is necessary for antidepressant action (Sahay and Hen, 2008; David et al, 2009). However, there is a prevailing neurotrophic hypothesis which is supported by numerous studies in which the decrease of growth factors increases vulnerability to depression (Duman and Monteggia 2006) while induction of growth factors might be an underlying mechanism of antidepressant action (Berton and Nestler, 2006; Warner-Schmidt and Duman 2008). As FGF9 appears to be acting as a physiological antagonist to FGF2, it may be interfering with the induction of FGF2-mediated cell survival that is correlated with antidepressant activity (Perez, Clinton et al. 2009).

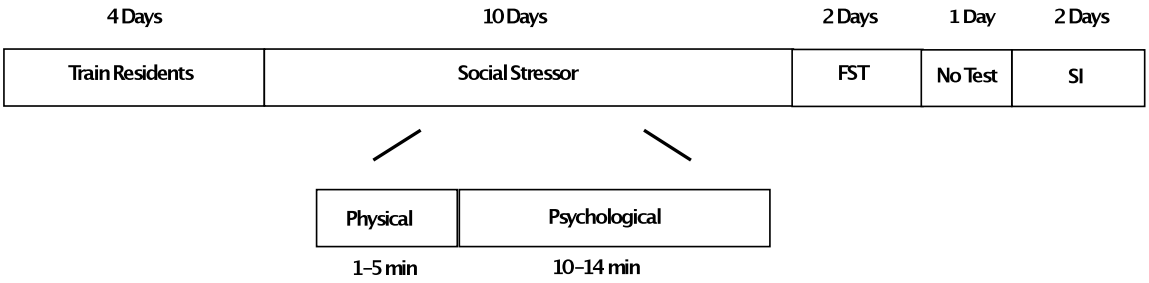
The following evidence supports that FGF9 may be a physiological antagonist to FGF2: (1) repeated social stress upregulated FGF9 gene expression and in contrast social defeat decreased FGF2 gene expression (Turner, Calvo et al. 2008), (2) an acute FGF9 microinjection was anxiolytic and increased depression-like behavior and conversely an acute FGF2 microinjection was anxiogenic and antidepressant-like (Perez, unpublished data), (3) chronic FGF9 microinjections were anxiogenic and increased depression-like behavior while

chronic FGF2 microinjections were anxiolytic and antidepressant-like (Turner, Gula et al. 2008), and (4) chronic FGF9 microinjections decreased hippocampal FGFR1 expression while chronic FGF2 microinjections increased hippocampal FGFR1 gene expression (Turner, Gula et al. 2008). These observations support the hypothesis that FGF9 is acting as a naturally occurring physiological antagonist to FGF2. Overall, increased FGF9 is correlated with both increased stress and negative behavioral consequences. Thus, FGF9 appears to be an ideal candidate for targeted inhibition and a novel therapeutic target for the treatment of mood disorders.

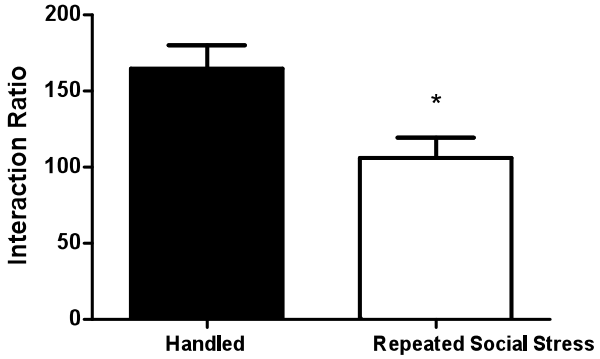
Results Figures

Figure 3-1: Repeated social stress increased percent change in body weight and decreased social interaction.

A.



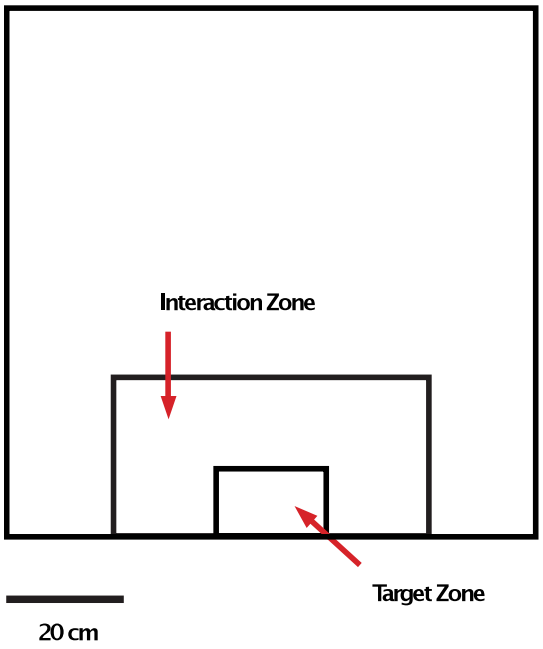
B.



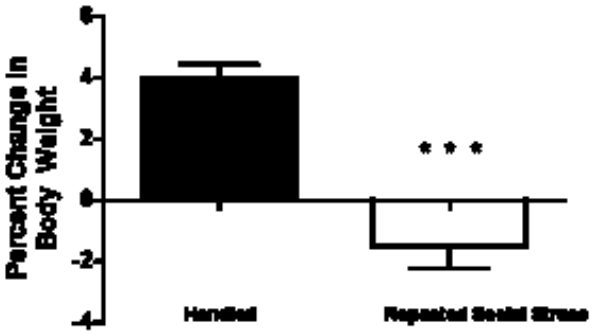
A) Timeline of the repeated social stress experiment. B) Rats subjected to repeated social stress showed increased social avoidance as evidenced by a decrease in the social interaction ratio in the social interaction task compared to handled controls; *p < 0.05 (n= 8-10 animals per group).

Figure 3-1 (continued): Repeated social stress increased percent change in body weight and decreased social interaction.

C.



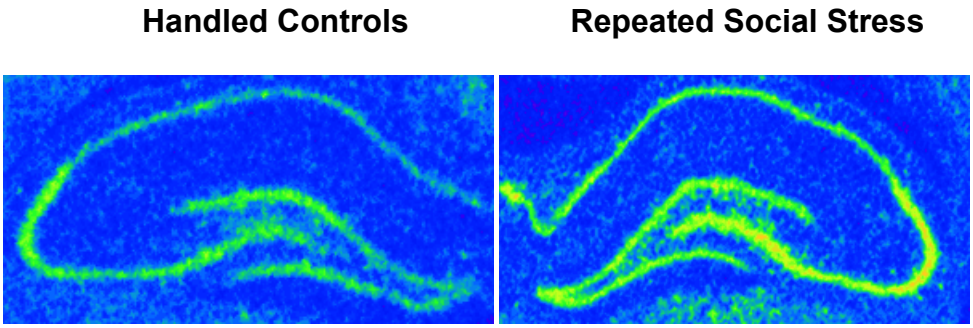
D.



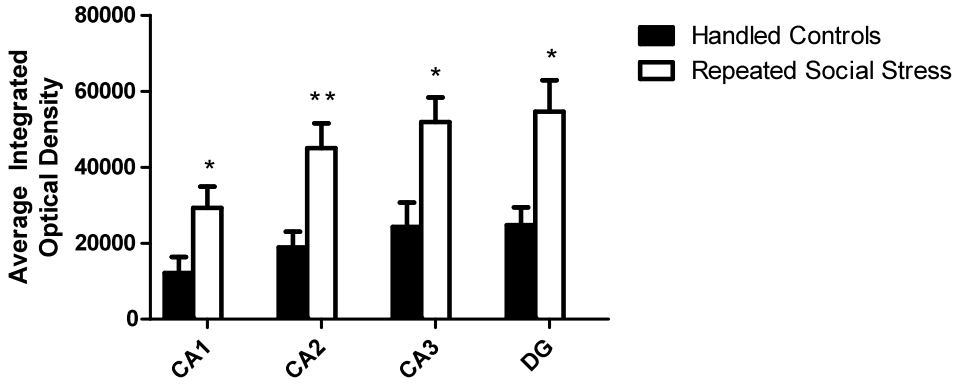
C) Scaled diagram of the interaction arena (100cm X 100cm) including the interaction zone and target area. D) Rats subjected to repeated social stress had a significant percent change in body weight indicative of increased stress compared to vehicle controls; ***p < 0.001 (n= 8-10 animals per group).

Figure 3-2: Repeated social stress increased hippocampal FGF9 gene expression.

A.



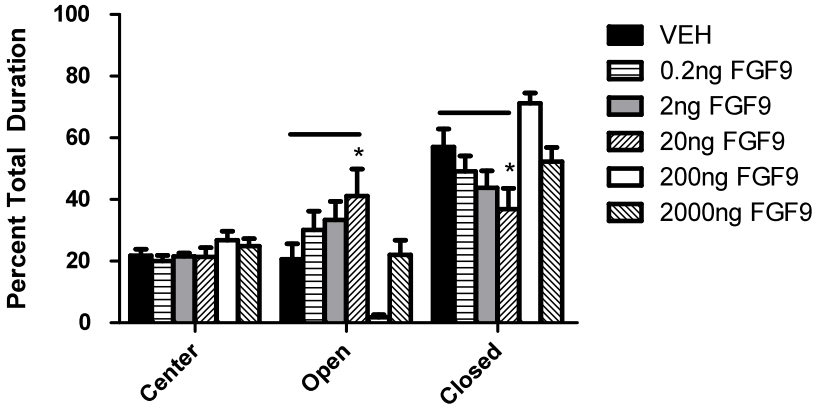
B.



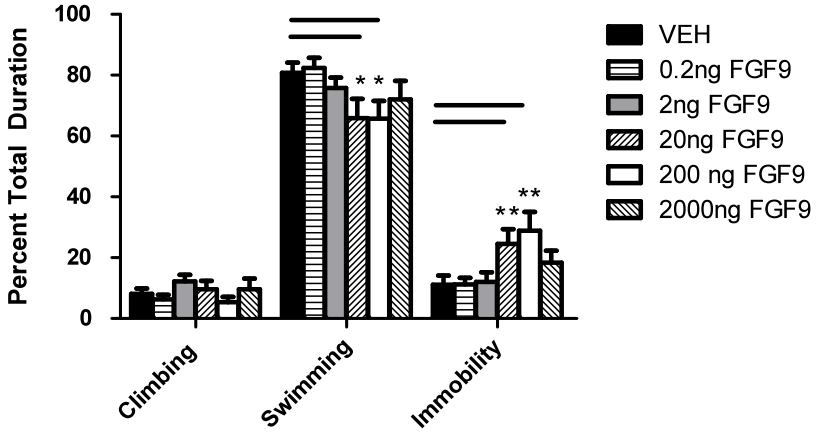
A) Representative mRNA *in situ* hybridization images from handled controls (left) and repeated social stress animals (right). B) Repeated social stress significantly increased FGF9 gene expression in hippocampal subregions: CA1, CA2, CA3, and dentate gyrus;*$p < 0.05$, **$p < 0.01$ (n= 5 animals per group).

Figure 3-3: Acute FGF9 microinjections decreased anxiety-like behavior and increased depression-like behavior.

A.



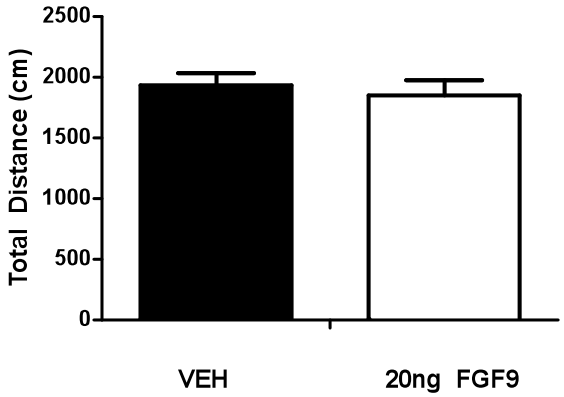
B.



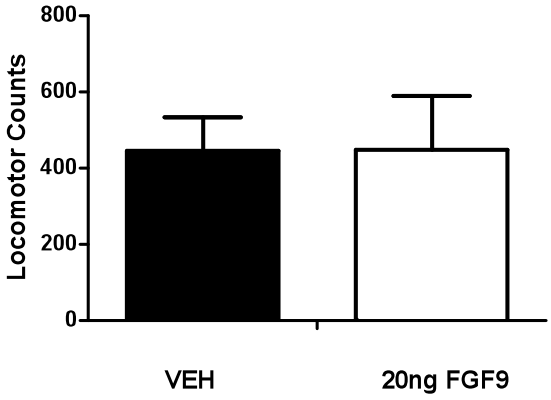
A) An acute microinjection of FGF9 decreased anxiety-like behavior in an inverted-U dose-response curve as shown by a decrease in the percent time spent on the open arm compared to vehicle controls; $^{**}p < 0.01$, $^{*}p < 0.05$ (n= 8-10 animals per group). B) An acute microinjection of FGF9 increased depression-like behavior in a dose-dependent curve as shown by the decrease in percent time spent swimming and increased percent time immobile in the FST compared to vehicle controls (n= 8-10 animals per group).

Figure 3-3 (continued): Acute FGF9 microinjections decreased anxiety-like behavior and increased depression-like behavior.

C.



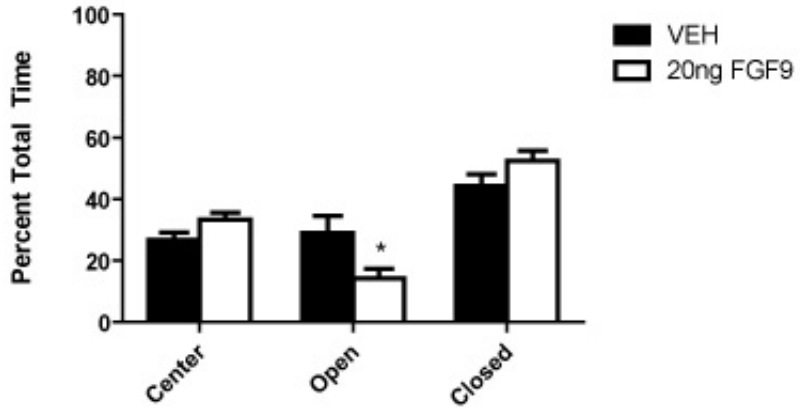
D.



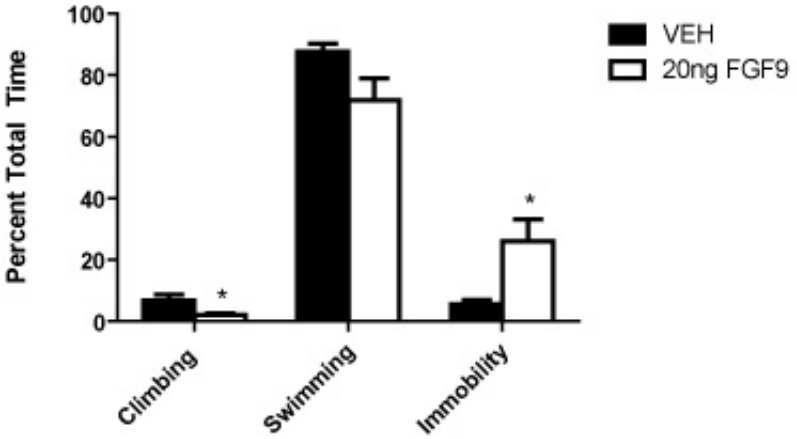
C) Acute microinjections of FGF9 have no effect on locomotor activity 15 minutes following the microinjection (n= 8-10 animals per group). D) Acute microinjections of FGF9 have no effect on locomotor activity 24 hours following the microinjection (n= 5 animals per group).

Figure 3-4: Chronic FGF9 microinjections increased anxiety-like behavior and increased depression-like behavior.

A.



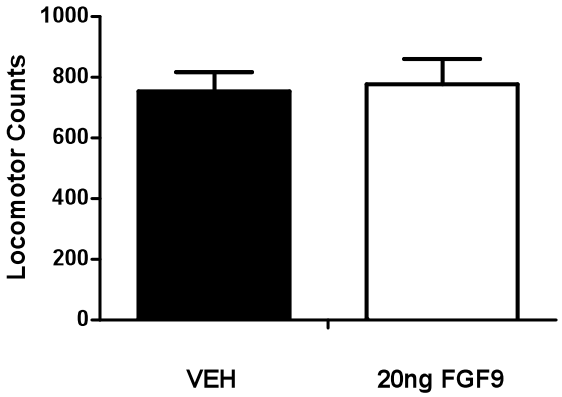
B.



A) Chronic microinjections increased anxiety-like behavior as evidenced by a decrease in the percent time spent on the open arm compared to vehicle controls; *$p < 0.05$ (n= 8-10 animals per group). B) Chronic microinjections increased depression-like behavior as shown by decreased percent time spent swimming and increased percent time spent immobile in the FST compared to vehicle controls; *$p < 0.05$ (n= 8-10 animals per group).

Figure 3-4 (continued): Chronic FGF9 microinjections increased anxiety-like behavior and increased depression-like behavior.

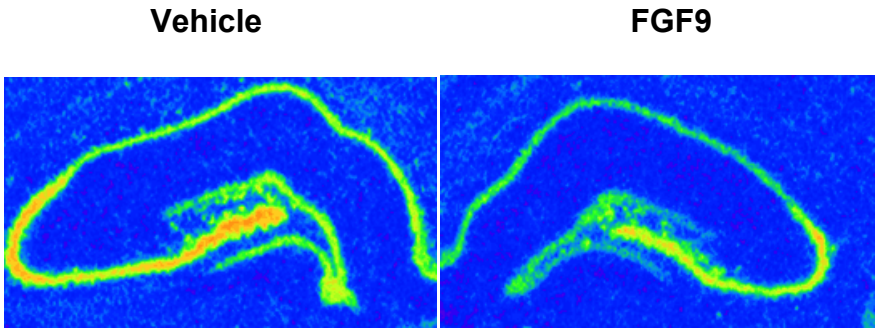
C.



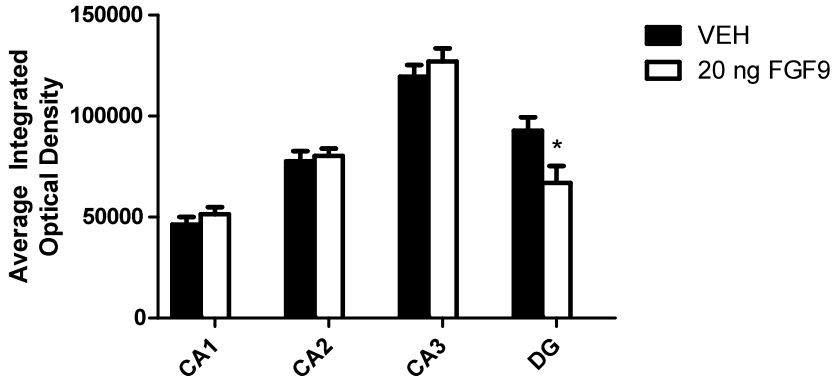
C) Chronic microinjections of FGF9 had no effect on locomotor activity 24 hours following administration (n=10-12 animals per group).

Figure 3-5: Chronic FGF9 microinjections decreased hippocampal FGFR1 gene expression.

A.



B.



A) Representative mRNA *in situ* hybridization images from vehicle controls (left) and chronic FGF9 microinjected animals (right). B) Chronic microinjections for 18 days significantly decreased FGFR1 gene expression in the hippocampal dentate gyrus compared to vehicle controls; *$p < 0.05$ (n= 5 animals per group).

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Chapter 4

Circadian variations of the fibroblast growth factor family in the adult rat hippocampus

Abstract

In the present study, we examined the circadian patterns of fibroblast growth factor-2 (FGF2), fibroblast growth factor-9 (FGF9), and fibroblast growth factor receptor-1 (FGFR1) mRNA expression in the adult rodent hippocampus. Animals were sacrificed at 4-hour intervals throughout a 24-hour light-dark cycle, and the gene expression of FGF2, FGF9, and FGFR1 was evaluated by mRNA *in situ* hybridization. We observed a significant circadian rhythm in FGF2 expression, with a peak at 1600 h (two hours before lights off) and a trough at 0800 h (one hour after lights on). In contrast, FGFR1 mRNA expression peaked at 2400 h and was lowest at 1200 h in a pattern lagging the expression profile of FGF2. FGF9 gene expression showed no significant circadian variation. We observed that the peak in FGF2 expression coincided with the peak in circulating plasma corticosterone in the same experimental animals. In addition to investigating the endogenous fluctuation of FGF2, FGF9, and FGFR1 mRNA expression, we also tested whether a single microinjection of FGF2, administered between 0700 h and 0900 h, could alter circadian locomotor activity. The FGF2

microinjection (200ng, i.c.v.) caused an increase in the dark-phase (active period) locomotor activity compared to vehicle-injected controls (aECF, i.c.v.). These findings provide the first evidence that, in the absence of any experimental manipulation, the expression of FGF2 and FGFR1 undergo circadian oscillations. These fluctuations in mRNA expression could be linked to circulating corticosterone levels and may mediate physiological variations of activity.

Introduction

The discovery of the dysregulation of the FGF system gene expression in post-mortem tissue of individuals diagnosed with major depressive disorder (MDD) (Evans, Choudary et al. 2004; Evans, Choudary et al. 2004b; Akil, Evans et al. 2008) has led to an increased interest in the role of this growth factor family in brain regions associated with mood disorders. In MDD, FGF2 gene expression was downregulated while FGF9 expression was upregulated in cortical and subcortical regions. FGF receptors were also downregulated (Evans, Choudary et al. 2004; Sibille, Arango et al. 2004; Evans, Choudary et al. 2004b; Gaughran, Payne et al. 2006). It is known that FGF2 expression is increased by antidepressants (Mallei, Shi et al. 2002), and FGF2 and FGFR1 expression are decreased following acute social defeat (Turner, Calvo et al. 2008). Chronic administration of FGF2 decreased anxiety-like behavior (Perez, Clinton et al. 2009) and increased antidepressant-like behavior (Turner, Gula et al. 2008). Chronic FGF9 microinjections increased anxiety-like behavior and increased depression-like behavior in adult rodents (Inui, unpublished data). Changes in gene expression of FGF2, FGF9, and FGFR1 as well as exogenous administration of FGF2 and FGF9 can be correlated to behavioral consequences. It is important to consider the endogenous circadian fluctuation of FGF2, FGF9, and FGFR1 gene expression to better understand how their dysregulation may alter behavior. The FGF family may play an important role in both circadian activity and environmental responsivity.

FGF2 has an established role in transcriptional regulation, cellular proliferation and neurogenesis in the adult rat (Kuhn, Winkler et al. 1997; Palmer, Markakis et al. 1999; Jin, Sun et al. 2003; Zhao, Li et al. 2007). FGF2 has moderate levels of expression in the rat hippocampus and its actions are primarily mediated by fibroblast growth factor receptor-1 (FGFR1). In contrast to FGF2, FGFR1 is highly expressed in the rat hippocampus (Gonzalez, Berry et al. 1995). Furthermore, the activation of FGFR1, leads to MAPK and PLCγ activation which alter downstream transcriptional regulation and cytoskeletal organization (Dailey, Ambrosetti et al. 2005). The activation of these two pathways, through FGFR1 signaling, has recently been implicated in the maintenance and self-renewal of adult neural stem cells (Ma, Ponnusamy et al. 2009). FGF9 is also expressed in the rat hippocampus (Tagashira, Ozaki et al. 1995; Todo, Kondo et al. 1998) and is thought to support neuronal survival through an autocrine/paracrine system (Kanda, Iwasaki et al. 1999). FGF9 primarily activates fibroblast growth factor receptor-3 (FGFR3) (Reuss and von Bohlen und Halbach 2003), but it may play a role as a physiological antagonist to FGF2 (Inui, unpublished data).

The discovery of the dysregulation of the FGF system gene expression in post-mortem tissue of individuals diagnosed with major depressive disorder (MDD) (Evans, Choudary et al. 2004; Evans, Choudary et al. 2004b; Akil, Evans et al. 2008) has led to an increased interest in the role of this growth factor family in brain regions associated with mood disorders. In MDD, FGF2 gene expression was downregulated while FGF9 expression was upregulated in cortical and

subcortical regions. FGF receptors were also downregulated (Evans, Choudary et al. 2004; Sibille, Arango et al. 2004; Evans, Choudary et al. 2004b; Gaughran, Payne et al. 2006). It is known that FGF2 expression is increased by antidepressants (Mallei, Shi et al. 2002), and FGF2 and FGFR1 expression are decreased following acute social defeat (Turner, Calvo et al. 2008). Chronic administration of FGF2 decreased anxiety-like behavior (Perez, Clinton et al. 2009) and increased antidepressant-like behavior (Turner, Gula et al. 2008). Chronic FGF9 microinjections increased anxiety-like behavior and increased depression-like behavior in adult rodents (Inui, unpublished data). Changes in expression of FGF2 and FGFR1 as well as exogenous administration of FGF2 can be linked to behavioral consequences. It is important to consider the endogenous circadian fluctuation of FGF2 and FGFR1 gene expression to better understand how their dysregulation may alter behavior.

Since FGF2 supports enhanced cellular production and dendritic growth in the dentate granule cell layer, it may be valuable to consider the relationship of its circadian variations in relationship to those of cell proliferation and synaptic plasticity (Rai, Hattiangady et al. 2007). Adult neurogenesis, cell proliferation, and alteration of hippocampal architecture have been implicated in functional plasticity of neural circuits at the cellular, network, and systems level (Lledo, Alonso et al. 2006). The proliferation and maturation of new neurons and along with increased spine density and arborization in the dentate gyrus have been shown to play a role in certain hippocampal-dependent cognitive abilities

(Lemaire, Aurousseau et al. 1999; Drapeau, Mayo et al. 2003; Leuner, Mendolia-Loffredo et al. 2004).

For decades, we have known of a strong association between depression and sleep dysregulation (Wirz-Justice 2006). Alterations of circadian rhythms in MDD were first described over 40 years ago (Fullerton, Wenzel et al. 1968; Foster and Kupfer 1975; Claghorn, Mathew et al. 1981; Van Cauter and Turek 1986; Germain and Kupfer 2008), and many individuals with MDD complain of sleep-wake cycle abnormalties (Haynes, McQuaid et al. 2006; Monteleone and Maj 2008) . These individuals often have an overall increase in cortisol secretion and a phase advance of the cortisol circadian rhythm (Linkowski, Mendlewicz et al. 1985; Linkowski, Van Cauter et al. 1985). Primary disruption of the sleep-wake cycle could lead to insomnia that may facilitate or exacerbate the depressed state. Thus, alterations of the biological clock at the molecular level could lead to neurobiological dysfunction, such as MDD. Similarly, fluctuations in FGF expression may precipitate some of the activity-related changes underlying mood disorders.

In the present study, we examined the possibility that FGF2, FGF9, and FGFR1 undergo circadian oscillation in the absence of experimental manipulation. Basal expression was quantified at four-hour intervals during the dark-cycle (active period) and light-cycle (inactive period). In order to investigate the endogenous fluctuations of FGF2, FGF9, and FGFR1 gene expression, we used quantitative

mRNA *in situ* hybridization. We also recorded the light-dark cycle oscillation of circulating plasma corticosterone in the same group of animals. To determine if exogenous administration of FGFs could alter the circadian activity cycle of adult rats, we evaluated the locomotor activity of adult rodents for 36 hours following an acute microinjection of FGF2 (200ng, i.c.v.).

Materials and Methods

Animals

All male Sprague-Dawley rats (Charles river Laboratories, Inc., Wilmington, MA), weighing 250g to 300g, were housed in pairs on a 12 hour light, 12 hour dark cycle with lights on at 0700 h . Food and water were available *ad libitum* and the animals were allowed to acclimate to the housing conditions for one week before experiments began. 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.

Experimental Protocol

For the determination of circadian patterns of FGF2, FGF9, and FGFR1 mRNA as well as circulating cortiscosterone levels, 36 animals (6 rats/group) were used.

Animals were sacrificed by rapid decapitation at four-hour intervals for 24 hours starting at 0800 h. Trunk blood was collected into EDTA-coated tubes and kept on ice until centrifugation (1000*g*, 10 minutes at 4 °C). Plasma was separated, frozen on dry ice, and stored at -80°C. Plasma corticosterone was measured using a commercially available radioimmunoassay kit (MP Biomedicals, Solon, OH) according to package instructions. All rats were sacrificed by rapid decapitation and their brains were removed, snap-frozen in isopentane, and stored at -80°C until sectioned. Tissue was sectioned at -20°C at 10μm (n=6/group), sliced in series throughout the hippocampus, mounted on Superfrost Plus slides (Fisher Scientific), and returned to -80°C until processed. All animals at each time point were sacrificed within 45 minutes and safe lights were used during the dark cycle to avoid exposure to light that might alter circadian rhythm.

mRNA *in situ* Hybridization

Tissue sections were taken every 200μm through the rostral hippocampus and *in situ* hybridization methodology and analysis was performed as previously described in detail elsewhere (Kabbaj, Devine et al. 2000). All *in situ* probes were synthesized in our laboratory and exposure times were experimentally determined for optimal signal and are as follows: FGF2 (7 days), FGF9 (14 days) and FGFR1 (7 days). The rat mRNA sequences used for generating probes complimentary to the following RefSeq database numbers: FGF2 (NM_019395, 716-994), FGF9 (NM_012952, 661-880), and FGFR1 (NM_024146, 320-977).

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 1μg of linearized plasmid, 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, and 1.5μl of T7 or T3 RNA polymerase.

Image Analysis

Radioactive signals were quantified using computer-assisted optical densitometry software, Scion Image (Scio Corporation, Frederick, MD). Integrated optical densities were determined by outlining a subfield (CA1, CA2, CA3, and DG) of the hippocampus on each hemisphere and correcting for background plus 3.5x its standard deviation. Only pixels with grey values exceeding the threshold were included in the analysis. Data from multiple sections (10-12 hippocampal measurements per animal) were averaged resulting in a mean for each animal and an average was taken for each group.

Microinjections

Rats were anesthetized with isofluorane and were implanted with a guide cannula (22-gauge, Plastics One Inc., VA). The cannula was placed in the left lateral ventricle (coordinates from bregma: AP -1.1; ML +1.3; DV -3.0) using a small-animal stereotaxic instrument. The guide cannula was anchored to the skull using dental cement and was fitted with an obturator. After five days of recovery, rats received a microinjection, between 0700 h and 0900 h, using a 28-gauge injector cannula extending 1.5mm below the tip of the guide. The microinjection cannula was connected by PE-20 tubing to a Hamilton syringe mounted on a syringe pump (Harvard Apparatus, MA) and rats were microinjected with either recombinant human FGF2 (200ng, i.c.v.) or the vehicle (artificial extracellular fluid (aECF), i.c.v) with 100µg/ml bovine serum albumin (Kuhn, Winkler et al. 1997; Cambon, Hansen et al. 2004; Turner, Gula et al. 2008). The total volume injected was 8µl infused at a rate of 1µl /min in freely moving animals. The injector was left in place for five minutes to allow for diffusion. The rats were placed into locomotion chambers for monitoring immediately after the microinjection.

Locomotion Testing

After an acute microinjection of vehicle (aECF, i.c.v.) or FGF2 (200ng, i.c.v.), locomotor activity was tested to determine whether exogenous FGF2 caused an

alteration in locomotion over a 36-hour period. The rats were placed in a 43 x 21.5 x 25.5cm acrylic cage with normal cage bedding and allowed access to food and water *ad libitum*. Horizontal locomotor activity was monitored every 60 minutes for 36 hours. The results exclude the highly variable locomotor activity during the acclimation to the locomotor box. The horizontal activity was averaged for each group. The locomotion-testing rig and motion-recording software were developed in-house at the University of Michigan.

Statistical Analysis

All mRNA *in situ* hybridization results were analyzed by a general linear model for repeated measures over all hippocampal subregions. An ANOVA was performed to determine the overall hippocampal FGF2, FGF9, and FGFR1 gene expression profiles and corticosterone secretion with Fisher's least statistical difference (LSD) between peak and trough. An ANOVA was performed on the locomotor activity data with Fisher's LSD determined between FGF2 and vehicle-injected animals. Data are expressed as mean +/- standard error of the mean. Significance is assumed at $p < 0.05$.

Results

Circadian variation of FGF2 mRNA in the hippocampus

The largest difference in basal FGF2 mRNA expression was noted between the expression trough at 0800 h and expression peak 1600 h (Figure 4-1A). Analysis of FGF2 mRNA expression data revealed a circadian rhythm with a single peak in mRNA expression (Figure 4-1B). Statistical analysis by one-way ANOVA indicated significant effects of time of day on FGF2 mRNA expression in the hippocampus averaged over all four regions ($F_{(5,26)}$= 3.0, $p < 0.03$). The average hippocampal trough value of FGF2 expression occurred at 0800 h, one hour after lights on, after which levels of FGF2 mRNA rapidly peaked at 1600 h ($p < 0.004$ vs. 0800 h), three hours before lights off. All four regions (DG, CA1, CA2, CA3) quantified had similar expression patterns over the 24-hour light-dark cycle and contributed to the overall expression profile ($F_{(5,26)}$ = 3.4, $p < 0.001$; Figure 4-1C).

Circadian variation of FGFR1 mRNA in the hippocampus

The largest difference in FGFR1 mRNA expression was noted between trough expression at 1200 h and peak expression at 2400 h (Figure 4-2A). Statistical analysis by one-way ANOVA indicated significant effects of time of day on FGFR1 mRNA expression in the hippocampus. The peak value of FGFR1 expression occurred at 2400 h, five hours after lights off, after which average hippocampal FGFR1 expression decreased rapidly with a through at 1200 h, five

hours after lights on ($p < 0.006$ vs. 2400 h; Figure 4-2B). Expression increased between 1200 h and 1600 h but still remained significantly lower than peak FGFR1 expression ($p < 0.04$ vs. 2400 h). CA3 had the most clear pattern of expression whereas CA2 had the least clear pattern ($F_{(5,27)}= 3.0$, $p < 0.03$; Figure 4-2C).

FGF9 mRNA expression showed no circadian variation in the hippocampus

Statistical analysis by one-way ANOVA indicated no significant effects of time of day on FGF9 mRNA expression in the hippocampus (Figure 4-3A). While FGF9 expression does not fluctuate over a 24-h light-dark cycle, the ratio of FGF9 to FGF2 is altered by the variation of FGF2 expression (Figure 4-3B).

The peak in FGF2 gene expression coincided with a rise in circulating plasma corticosterone levels

Circulating corticosterone concentrations exhibited a significant circadian rhythm in rats housed under 12-hour light, 12-hour dark illumination conditions with food available *ad libitum* ($F_{(5,35)} = 7.6$, $p < 0.001$; Figure 4-4). Plasma corticosterone levels were low in the morning (from 0800 h-1200 h), ranging from 21.9 - 41.7pg/ml. Corticosterone levels exhibited an increase from trough expression to reach a peak at the light/dark transition phase, 1600 h (187.6 +/- 16.6 pg/ml, $p < 0.001$ vs. 0800 h) and remained significantly higher than trough expression at

2000 h ($p < 0.002$ vs 0800 h). Subsequently corticosterone levels declined to 113.7 +/- 41.5 pg/ml ($p < 0.02$ vs. 0800h) before returning to trough expression, 21.9 +/- 9.6 pg/ml,at 0800 h (Figure 4-4).

A single FGF2 microinjection increased locomotor activity during the dark-cycle (active period)

An ANOVA showed there was a significant interaction between treatment and locomotor activity ($F_{(1,16)} = 2.7$, $p < 0.05$; Figure 4-5). In response to a single microinjection of FGF2 (200ng, i.c.v.), administered between 0700 h and 0900 h, rats showed a significant increase in locomotor activity compared to vehicle-injected (aECF, i.c.v.) controls during the dark-cycle (active period) and were significantly different at 1900 h, 2000 h, 2200 h, 2300 h and 0300 h ($p < 0.03$, $p < 0.02$, $p < 0.002$, $p < 0.04$ and $p < 0.05$, respectively; Figure 4-5). There were significant differences in activity during the first dark-cycle, however, locomotor changes did not persist into the next cycle. There were no significant differences in locomotor activity during the light-cycle (inactive period).

Discussion

The results of this study provide the first evidence that the gene expression of the FGF family exhibited circadian oscillations in the absence of experimental manipulation. Specifically, we found that: (1) FGF2 has a circadian pattern of

gene expression with a peak at 1600 h and a trough at 0800 h, (2) FGFR1 expression oscillated with a peak at 2400 h and a trough at 1200 h, (3) FGF9 gene expression showed no significant circadian variation over a 24-hour light-dark cycle, (4) the expression peak of FGF2 mRNA corresponded to the peak in circulating corticosterone in the same experimental animals, and (5) an acute microinjection of FGF2, administered between 0700 h and 0900 h increased the locomotor activity of adult rats during the dark-cycle (active period). These findings support the hypothesis that FGF2 and FGFR1 have differences in basal expression across a 24-hour light-dark cycle and that changes in FGF2 availability have an effect on circadian locomotor activity.

Our results indicated that the FGF2 ligand and its primary receptor in the hippocampus display basal circadian fluctuations in mRNA expression. The peak of FGF2 expression, which occurs at the onset of the dark-cycle (active period), precedes the peak in expression of FGFR1. This circadian mRNA expression pattern is also seen in another growth factor, brain-derived neurotrophic factor (BDNF), and its receptor, *trk*B (Bova, Micheli et al. 1998). As it is explained for the BDNF/*trk*B rhythm, it is possible that FGF2 may play a role in regulating its own receptor. Recent findings from our lab show that FGF2 microinjections increased FGFR1 receptor mRNA in the hippocampus (Turner, Gula et al. 2008) supporting the hypothesis that FGF2 induction may regulate FGFR1 expression. Also, it is possible that the induction of transcription of

FGFR1 may require intervening protein synthesis that results in a lag behind the peak expression of FGF2.

Although FGF9 gene expression shows no circadian variation in expression, the ratio of FGF9 to FGF2 expression changes over a 24-hour light-dark cycle. Our ecent studies have shown that microinjections of FGF9 result in behavioral changes in anxiety-like and depression-like behavior that are opposite that seen with exogenous administration of FGF2 (Inui, unpublished data; (Turner, Gula et al. 2008). FGF9 microinjections also decreased the expression of FGFR1 expression in the hippocampus (Inui, unpublished data). The finding that FGF9 is not altered over a 24-hour light-dark cycle, is important to understanding the interplay of FGF2 and FGF9 interaction. The acute increase in depression-like behavior observed in the first-half of the light-cycle (0700 h to 1200 h), was not observed when FGF9 was administered during the second-half of the light-dark cycle (1300 h to 1700 h) (Inui, unpublished data). This time-sensitive alteration in behavioral response may be mediated by an increase in FGF2 expression in the second-half of the light-cycle. Alteration in the ratio of FGF2 and FGF9 may have behavioral consequences. Upregulation of FGF9 expression, as seen in post-mortem tissue of individuals diagnosed with MDD, may lead to a disruption in the natural circadian variation of FGFR1. Therefore, it is important to know that under basal conditions there is no circadian variation in FGF9 mRNA expression in the hippocampus however there is a circadian pattern to the FGF2/FGF9 expression ratio.

In this study, we found that the peak in FGF2 expression coincided with the peak in circulating plasma corticosterone in the same experimental animals. Injections of corticosterone can lead to immediate (six hours post-injection) increases in FGF2 mRNA expression in many regions of the brain, including the hippocampus (Molteni, Fumagalli et al. 2001). Also, early-life exposure to corticosterone causes long-term changes in FGF2 mRNA in the prefrontal cortex and hippocampus (Molteni, Fumagalli et al. 2001). Conversely early-life injections of FGF2 caused long-term behavioral changes in adult rats (Turner, Capriles et al. 2009). While there is a strong link between the expression of FGF2 and corticosterone levels, we do not know if the changes in FGF2 expression result from changes in circulating corticosterone or if FGF2 is a mediator of the cortiscosterone response. Additional experiments involving adrenalectomized animals given steady doses of corticosterone may help determine if the expression of FGF2 is indeed regulated by corticosterone.

Interestingly, the peak expression levels of FGF2 corresponded to the peak in cell proliferation seen in adult rat hippocampus over a 24-h light-dark cycle. It has been shown that cell proliferation was highest in animals sacrificed at 1700 h (the end of the light phase) and at its minimum in animals sacrificed at 0500 h (the end of the dark phase) (Guzman-Marin, Suntsova et al. 2007). Since FGF2 is known to support cell proliferation (Rai, Hattiangady et al. 2007) in the adult rat hippocampus, the synchrony in FGF2 mRNA expression supports the evidence that there is a distinct pattern of cell proliferation throughout the day. It

is thought that increases in cell proliferation may increase synaptic plasticity during an animals' active period and allow for increased informational encoding about the animals' environment (Lledo, Alonso et al. 2006). In addition to supporting cell proliferation, FGF2 can also facilitate long term potentiation (LTP) in the hippocampus (Ishiyama, Saito et al. 1991; Abe, Ishiyama et al. 1992) . An increase in FGF2 and cell proliferation at the onset of the dark-cycle (active period) may lead to increased synaptic plasticity and enhanced behavioral responsiveness to environment stimuli.

In support of the hypothesis that increased FGF2 availability can correspond with alterations in behavior and normal circadian activity, we showed that an acute microinjection administered between 0700 h and 0900 h increased locomotor activity during the dark-cycle (active period). While it has been previously found that exercise can induce the expression of FGF2 in the hippocampus (Gomez-Pinilla, Dao et al. 1997), this is the first time FGF2 has been shown to have an effect on locomotor activity during the dark-cycle (active period). Continuous infusion of another growth factor, BDNF, increased locomotor activity in adult rats (Naert, Ixart et al. 2006). Our lab has also shown that that rats bred for high-response to novelty/low anxiety (HR) have higher FGF2 gene expression (Perez, Clinton et al. 2009) and also show an increase in dark-cycle (active period) activity compared to their low-response to novelty/ high-anxiety littermates (LR) (Clinton and Kerman, unpublished data). It is possible that an increase in FGF2

expression is necessary for increased activity, increased exploration, and in turn a decrease in anxiety-like behavior.

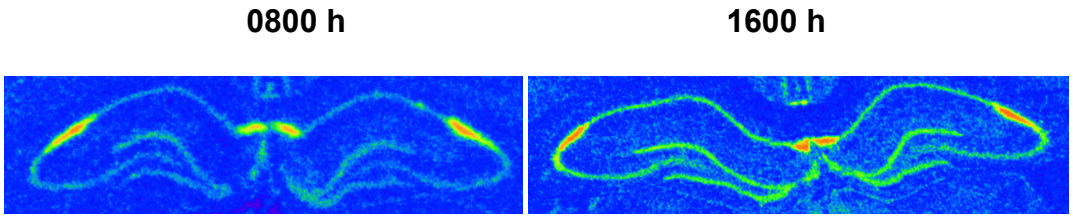
The changes in locomotor activity observed during the dark-cycle (active period) indicate that an increase in FGF2 can lead to alterations in circadian locomotor activity. While we only found a several hour increase in locomotor activity after an acute injection, this finding is supportive of the hypothesis that chronic dysregulation could lead to changes in circadian locomotor activity. It is possible that dysregulation of the FGF family could lead to long-lasting changes in sleep-wake patterns seen in MDD. Little is known about the mechanism underlying the disruptions of sleep patterns in individuals diagnosed with MDD. However, fibroblast growth factor modulation may be a key mediator in circadian locomotor activity and maintenance of appropriate sleep-wake cycles.

In conclusion, our results demonstrated that the expression of FGF2 and FGFR1 in the adult rat hippocampus exhibited circadian variation under basal conditions. Furthermore, the circadian variation of FGF2 and FGFR1 may be directly related to physiological variations in stress hormones. While FGF9 showed no circadian variation during a 24-hour light-dark cycle, the ratio of FGF2 to FGF9 gene expression does change over this period. Thus, the dysregulation of FGF system may lead to disruption in endogenous circadian rhythms, such as locomotor activity and environmental responsivity. These disruptions may be underlying causes of changes in affective behavior related to mood disorders.

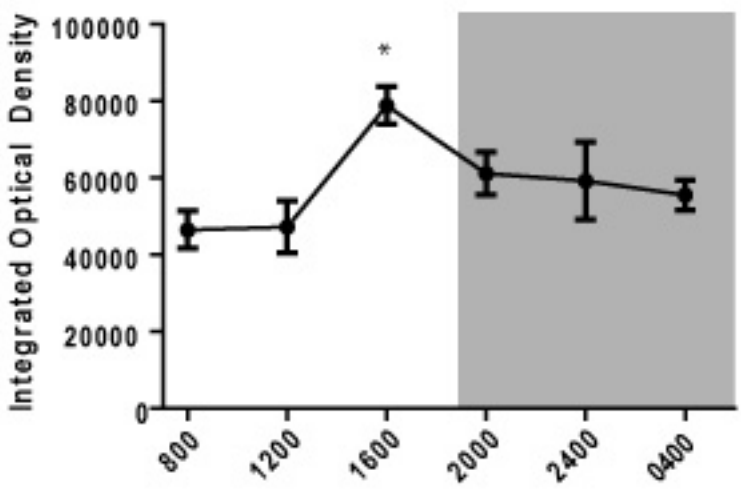
Results Figures

Figure 4-1: FGF2 mRNA circadian variation in the hippocampus.

A.



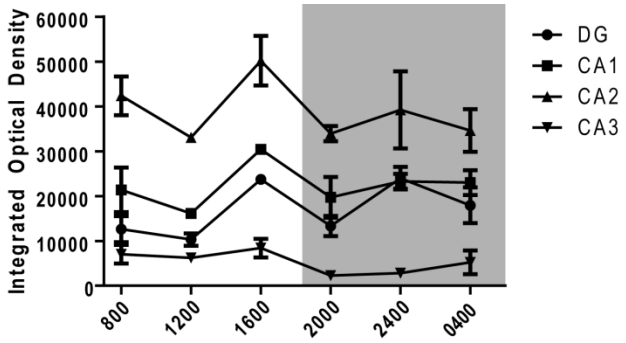
B.



A) Representative FGF2 mRNA *in situ* hybridization images from expression peak (1600 h) and trough (0800 h). B) FGF2 hippocampal gene expression fluctuated over a 24-hour light-dark cycle. FGF2 expression showed peak expression at 1600 h and trough expression at 0800 h; *$p < 0.05$, (n=5-6 animals/group).

Figure 4-1 (continued): FGF2 mRNA circadian variation in the hippocampus.

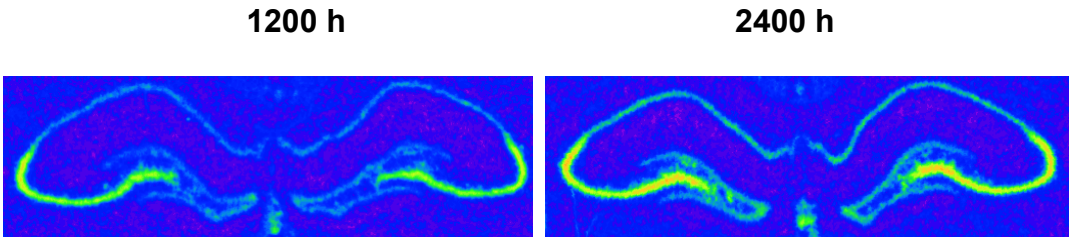
C.



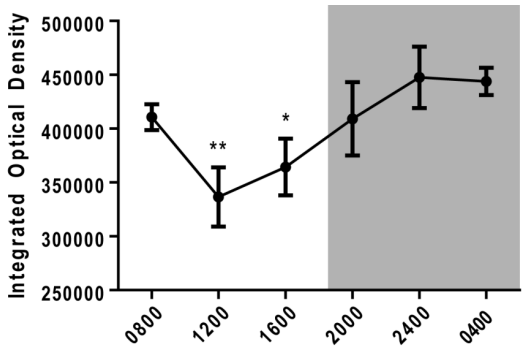
C) All four hippocampal subregions (CA1, CA2, CA3, and DG) contributed to the overall expression pattern of FGF2 over a 24-hour light-dark cycle (5-6 animals/group).

Figure 4-2: FGFR1 mRNA circadian variation in the hippocampus.

A.



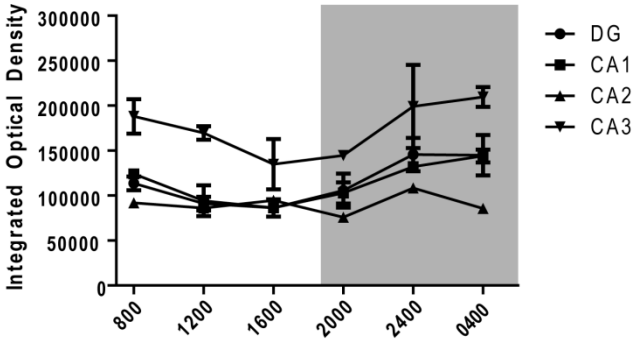
B.



A) Representative FGFR1 mRNA *in situ* hybridization images from expression peak (0400 h) and trough (1200 h). B) FGFR1 hippocampal gene expression fluctuated over a 24-hour light-dark cycle. FGFR1 showed peak expression at 2400 h and trough expression at 1200 h; *p < 0.05, **p < 0.01, (n=5-6 animals/ group).

Figure 4-2 (continued): FGFR1 mRNA circadian variation in the hippocampus.

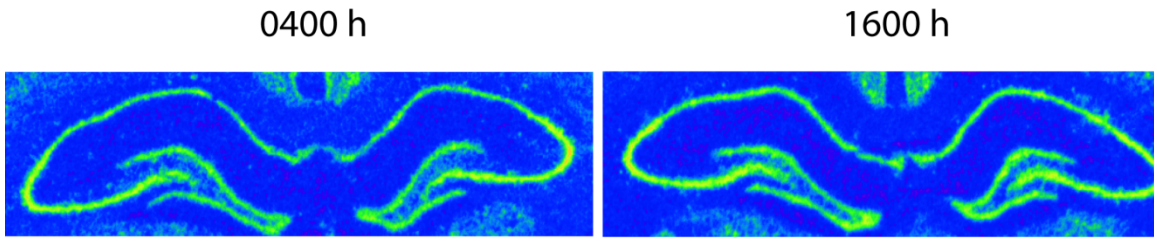
C.



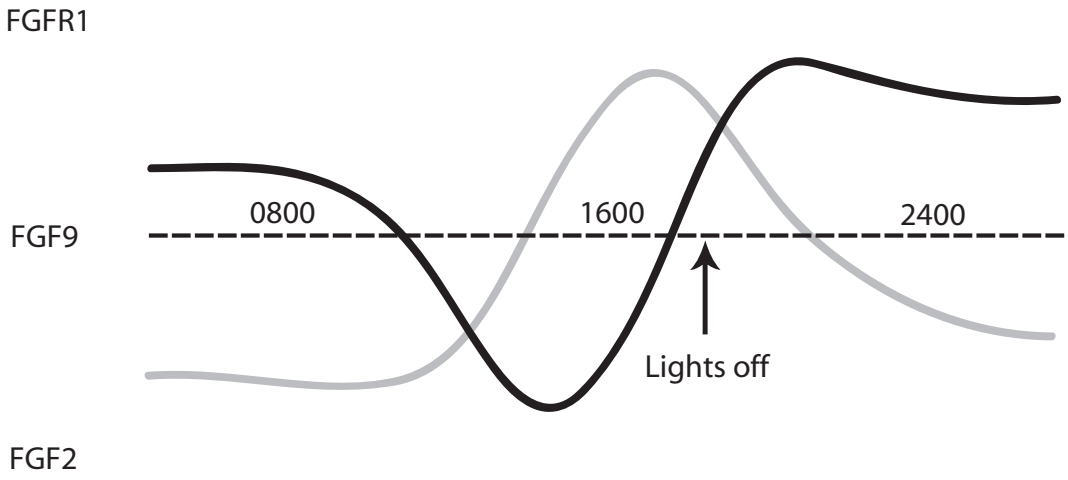
C) All four hippocampal subregions (DG, CA1, CA2, CA3) contributed to the overall expression pattern of FGFR1 over a 24-hr light-dark cycle (5-6 animals/group).

Figure 4-3: FGF9 showed no circadiation variation in hippocampal expression.

A.

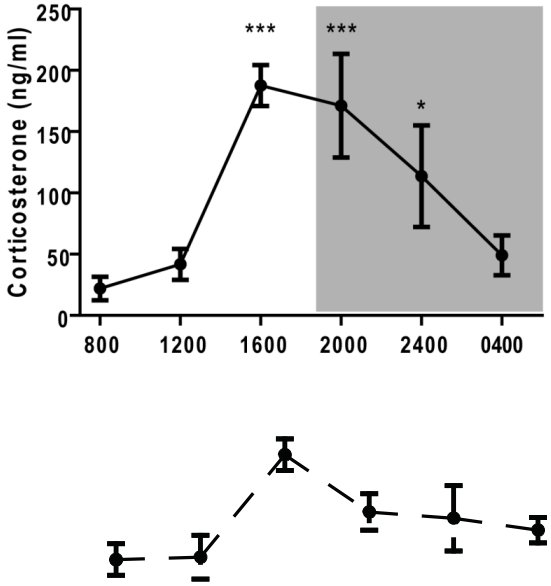


B.



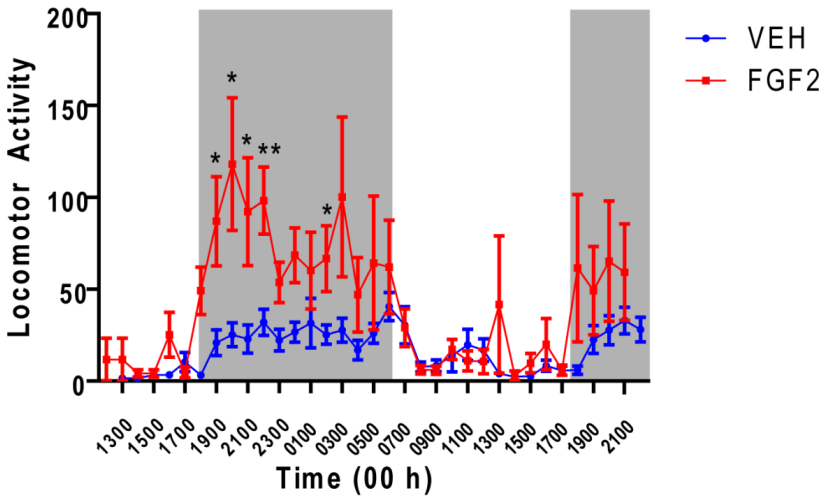
A) Representative FGF9 mRNA *in situ* hybridization images from expression at 0400 h and 1600 h show that there is no significant fluctuation over a 24-hour light-dark cycle. B) A schematic representation of the mRNA expression of FGF2 (gray), FGF9 (dashed), and FGFR1 (black).

Figure 4-4: Circulating plasma corticosterone over a 24-hour light-dark cycle.



Circulating plasma corticosterone exhibited a distinct circadian rhythm with a peak at 1600 h and a trough at 0800 h; *p < 0.05, ***p < 0.001, (n=6-7 animals/ group). FGF2 gene expression peaked when corticosterone secretion peaked (dashed line).

Figure 4-5: FGF2 microinjection increased locomotor activity during the dark-cycle (active period).



A single microinjection of FGF2 (200ng, i.c.v.), administered between 0700 h and 0900 h, increased locomotor activity during the dark-cycle (active period) compared to vehicle-injected controls (aECF, i.c.v.); *p < 0.05, **p < 0.01, (n=5-6 animal/group).

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Chapter 5

Discussion

The current body of work encompasses a series of studies that supports the role of the FGF system in affective behavior and circadian activity. We showed that exogenous microinjections of FGF9 can alter both anxiety-like and depression-like behavior. The effect of FGF9 administration was both dose-dependent and time-sensitive. Acutely, FGF9 decreased anxiety-like activity and increased depression-like activity. However, chronic FGF9 microinjections reversed the effect on anxiety-like behavior, from anxiolytic to anxiogenic, and further increased depression-like behavior. Furthermore, chronic FGF9 microinjections altered FGF system expression in the hippocampus. Specifically, chronic FGF9 injections decreased the expression of endogenous FGFR1 in the dentate gyrus of the hippocampus.

In support of the hypothesis that FGF9 can be modulated by stressors responsible for changing affective behavior, FGF9 gene expression was upregulated by repeated social stress. This stress paradigm concurrently altered gene expression and affective behavior as evidenced by an increase in social

avoidance behavior. Gene expression changes in FGF9 were opposite those observed in previous studies that showed a decrease in FGF2 gene expression following social stress (Turner, Calvo et al. 2008). The changes opposing changes in FGF2 and FGF9 further support the hypothesis that FGF2 and FGF9 are physiological antagonists.

Acutely, FGF2 increased anxiety-like behavior and decreased depression-like behavior. Additionally, chronic FGF2 microinjections reversed the effect on anxiety, from anxiogenic to anxiolytic, while continuing to exhibit antidepressant-like effects. Chronic microinjections of FGF2 increased the expression of FGFR1 in the dentate gyrus of the hippocampus. Acute social defeat also downregulated FGF2 expression (Turner, Calvo et al. 2008). The comparison of these results on affective behavior and expression regulation provides evidence that FGF2 and FGF9 may be physiological antagonists. The mechanisms underlying the opposing effects on affective behavior and on gene expression regulation are still unknown. However, these studies support the hypothesis that FGF9 mediates the negative aspects of affective behavior while FGF2 modulates positive effects on affective behavior. Small-molecule inhibition of FGF9 may sbe an effective approach for novel antidepressant treatment.

In addition to showing that FGF9 has a direct effect on behavior and can be altered by social stress, we also observed that FGF system expression in the hippocampus fluctuates over a 24-hour light-dark cycle. Specifically, the

expression of FGF2 showed peak expression at 1600 h and trough expression at 0800 h. The expression peak of FGF2 coincided with the expression peak of circulating plasma corticosterone levels. This result may indicate that FGF2 is acting in concert with the circadian cycle of corticosterone in the hippocampus. Under basal conditions, increased expression of FGF2 during peak corticosterone secretion may be protective to hippocampal architecture, dendritic spine formation and arborization. Additionally, increased FGF2 may allow for increased synaptic plasticity during the active/exploratory period. Conversely in stressed animals, increased corticosterone secretion and decreased FGF2 expression may lead to negative effects on hippocampal architecture and synaptic plasticity. The expression of FGFR1 peak lagged behind that of FGF2, and it exhibited peak expression at 2400 h and trough expression at 1200 h. While FGF9 showed no distinct changes in gene expression over a 24-hour light-dark cycle, the ratio of FGF2 to FGF9 over the 24-hour period is altered. Therefore, dysregulation of the FGF system may alter the endogenous physiological antagonism of FGF2 and FGF9 and in-turn circadian locomotion/environmental exploratory activity.

An acute microinjection of FGF2, administered between 0700 h and 0900 h, increased locomotor activity during the dark-cycle (active period). Recent studies from our lab show that high-response to novelty/low anxiety rats (HR) have increased expression of FGF2 in the hippocampus. Conversely, low-response to novelty/ high anxiety animals (LR) have decreased hippocampal gene expression

of FGF2. Additionally, HR animals have increased activity during the dark-cycle (Clinton, unpublished data). The FGF2 microinjection studies support the hypothesis that FGF system dysregulation can alter circadian locomotor activity and may play a role in environmental response underlying differences in anxiety. The series of circadian studies showed that FGF2 and FGFR1 gene expression have their own naturally occurring rhythms over a 24-hour period and that circadian locomotor activity can be disrupted by exogenous administration of FGF2.

Taken together these results demonstrate that the FGF system exhibits highly complex regulatory mechanisms in response to stress, circadian cues, and exogenous growth factor manipulations. The dysregulation of the FGF system may underlie changes in affective behavior and alter circadian locomotor activity. It is also possible that changes in circadian locomotor activity/exploratory activity may cause changes in affective behavior and mood.

Role of FGF9 as a regulator of affective behavior

We have observed behavioral responses after administration of intermediate doses of FGF9 (20ng or 200ng, i.c.v.). Initially, FGF9 decreased anxiety-like behaviors and increased depression-like behavior. However, higher doses of FGF9 did not alter affective behavior. It is possible, that at higher doses FGF9 exhibits autoinhibition via self-dimerization, as FGF9 is one of the unique

members of the FGF family that has increased self-interaction (Plotnikov, Eliseenkova et al. 2001). However, the inverted-U shaped pharmacological response is also common when pharmacological agents are receptor agonists at lower concentrations and receptor anatagonists at higher concentrations (Calabrese 2003).

Chronic microinjections of FGF9 reversed the anxiolytic effects observed with acute microinjection of FGF9. The differential response of animals to the same treatment may rely on two separate mechanisms of FGF9 action. The acute response may be a rapid neurotransmitter-like effect while the chronic response may involve istructural and plasticity related changes in the hippocampus. The alteration of FGFR1 expression in the hippocampus following chronic administration with FGF9 supports the hypothesis that FGF9 may induce long-term changes in the hippocampus.

Acute treatment with selective serotonin reuptake inhibitors (SSRIs) increased anxiety-like behavior (Bagdy, Graf et al. 2001; Belzung, Le Guisquet et al. 2001; Burghardt, Bush et al. 2007). The increase in anxiety can be reversed by administration of a 5HT2C agonist while blockade of 5HT1A receptors exacerbates the anxiogenic response (Bagdy, Graf et al. 2001). The anxiety effects have been attributed to the initial decrease in serotonin release at the synaptic cleft. This initial decrease in serotonin transmission could cause increased anxiety. Conversely, chronic treatment leads to the densensitization of

5HT1A autoreceptors and subsequent sensitization of post-synaptic receptors (File, Kenny et al. 2000). It is likely that there is some cross-talk between the monoaminergic systems and FGFs. This is supported by evidence that FGF2 is necessary for behavioral sensitization to drugs of abuse in cooperation with neurotransmitter interaction (Flores, Samaha et al. 2000). Our lab has also shown that early-life FGF2 enhanced the acquisition of cocaine-self-administration in adulthood (Turner, Capriles et al. 2009).

While acute injection of FGF2 increased anxiety, acting similarly to antidepressants, FGF9 initially decreased anxiety. While there is no mechanistic evidence, It is possible that FGF9 is directly mediating and increasing monoamine release at the synaptic cleft resulting in an acute decrease in anxiety. In that respect, the use of methylenendioxyamphetamine (MDMA) as a model for acute action on the monoaminergic system may provide insight into the neurotransmitter-like properties of FGF9. MDMA administration caused an initial increase in 5HT in the pre-synaptic cleft and acutely decreased anxiety-like behavior (Ho, Pawlak et al. 2004; Gudelsky and Yamamoto 2008).

Prolonged administration of SSRIs results in decreased anxiety and decreased depression. However, prolonged administration of MDMA led to decreased serotonergic tone and loss of serotonin transporters (SERT) (Battaglia, Brooks et al. 1988; Battaglia, Yeh et al. 1988). The decrease in serotonergic tone was biphasic, with a rapid acute phase followed by a long delayed phase. In the acute

phase, levels of serotonin recovered to normal in 24 hours whereas long-term loss of serotonergic tone may begin within one week and persist up to one month (Schmidt 1987; Stone, Merchant et al. 1987). Persistent MDMA administration can cause long-term increases in anxiety-like behavior (Morley, Gallate et al. 2001; Fone, Beckett et al. 2002; Gurtman, Morley et al. 2002). In support of the hypothesis that a decrease in serotonergic tone is caused by MDMA, pre-exposure to MDMA decreased the efficacy of antidepressants (Durkin, Prendergast et al. 2008).

FGF2 is increased during antidepressant administration and may be acting in the restructuring of the serotonin system by decreasing post-synaptic monoamine release. FGF9 may acutely increase post-synaptic monoamine release in the hippocampus, thereby decreasing anxiety. Furthermore, long-term consequences of FGF9 may include an overall decrease in monoaminergic tone resulting in increased anxiety and depression. A similar phenomenon has also been observed with amino acid neurotransmission via NMDA receptors. While ketamine, a NMDA receptor antagonist, had acute antidepressant effects *in vivo* (Berman, Cappiello et al. 2000; Zarate, Singh et al. 2006), chronic NMDA receptor antagonism resulted in increased neurotoxicity, cognitive impairment, and even psychosis (Olney 1994; Newcomer, Farber et al. 1999; Young, Jevtovic-Todorovic et al. 2005).

We have examined the differential anxiety-like behavioral response to acute and chronic FGF9 administration. It is possible that short-term decreases in anxiety may be attributed to the cross-talk between FGF and neurotransmitter systems. Furthermore, evidence supports that long-term neurotransmitter activity can lead to long-lasting cellular and behavioral changes. While the FGF system may affect neurotransmitter activity, there is a growing body of evidence that growth factors have independent effects on affective behavior and structural changes in the hippocampus (Berton and Nestler 2006).

Thoughts on the future of growth factors and antidepressant therapy

A possible independent mechanism of growth factor action is encompassed by the emerging neurotrophic hypothesis of depression and antidepressant action (Duman 2004; Berton and Nestler 2006; Duman and Monteggia 2006). This hypothesis was originally based on findings that acute or chronic stress decreased expression of BDNF. Conversely, antidepressants increased the expression of BDNF and reversed the effects of stress on hippocampal morphology (Chen, Dowlatshahi et al. 2001). Stress caused a reduction in dendritic arborization that is reversed by antidepressants (Duman 2004). Additionally, exogenous administration of BDNF increased antidepressant-like activity in learned helplessness tests (Shirayama, Chen et al. 2002).

The FGF system may also be involved in mediating changes in hippocampal morphology during stress. FGF2 may have protective effects in the hippocampus as it is also increased with antidepressant treatment (Mallei, Shi et al. 2002), while FGF9 may exacerbate hippocampal atrophy during stress. More work needs to be done to validate this hypothesis, particularly by looking at changes in hippocampal morphology after administration of growth factors independent of antidepressants. This hypothesis provides an explanation for the long-term independent effects of growth factors on hippocampal morphology and subsequently on affective behavior. However, there is difficulty in targeting growth factor receptors as they are broadly expressed and lead to a complex cascade of signal activation. BDNF also showed poor pharmacokinetic properties in clinical trials of degenerative disease (Pollack and Harper 2002; Pollack and Harper 2002). Therefore, it may be wise to look towards other neurotrophic factors for therapeutic targeting.

FGF9 presents a novel opportunity for the creation of a specific small-molecule antagonist. An FGF9 antagonist may inhibit the negative effects of FGF9 on affective behavior and possibly on hippocampal morphology. Additionally, identification of downstream signaling molecules increased by FGF9 may also provide later-stage targeting elements in the neurotrophic pathways. To date, two downstream targets of neurotrophic factors have been investigated. Direct inhibition of phosphodiesterase 4 (PE4), a molecule that is inhibited by neurotrophins, by rolipram has led to improvement of depression symptoms in

clinical trials but is severely limited by side effects (Fleischhacker, Hinterhuber et al. 1992; Fujimaki, Morinobu et al. 2000; Jeon, Heo et al. 2005). Another downstream target of neurotrophic factor inhibition, glycogen synthase 3 (GSK-3), has shown antidepressant effects in animal models of depression but has not been tested in clinical trials (Gould 2006; Gould, Picchini et al. 2006). Further research into the downstream molecules affected by FGF9 upregulation may lead to increased targets for small-molecule inhibition with decreased side effects.

It is possible that FGF9 has direct action on neurotransmitter systems and independent effects on hippocampal morphology. However, FGF9 may be mediating both its acute neurotransmitter-like and chronic behavioral impairment via direct antagonism of FGF2.

Role of FGF9 as a physiological antagonist of FGF2

As FGF9 consistently altered affective behavior in a manner opposite that seen after administration of FGF2, it is possible that FGF9 is acting as a physiological antagonist to FGF2. It is unlikely that FGF9 is binding to FGFR1, due to the steric hindrance that results in decreased activation of this receptor by FGF9 (Reuss and von Bohlen und Halbach 2003). However, chronic microinjections of FGF9 did decrease the expression of the FGFR1 in the dentate gyrus of the

hippocampus. This in turn would reduce the effects of FGF2-mediated FGFR1 activation in the dentate gyrus.

While the mechanism for FGFR1 reduction remains unknown, FGF9 decreased the differentiation of GFAP-positive astrocytes *in vitro* (Lum, Turbic et al. 2009). Recently, our lab has shown that administration of FGF2 increased the production of GFAP-positive astrocytes (Perez, Clinton et al. 2009). It is possible that the opposing effects of FGF2 and FGF9 in astrocyte formation are structural changes that alter hippocampal function. Loss of glial cells has also been noted in animal models of depression and in depressed patients (Cotter, Mackay et al. 2001; Cotter, Pariante et al. 2001; Sheline 2003; Rajkowska and Miguel-Hidalgo 2007). Additionally, ablation of glia in the prefrontal cortex can lead to depression-like behavior (Banasr and Duman 2008).

Since most of the evidence of FGF2 and FGF9 effects on glial cells is focused on astrocytic mediation, it is important to note that astrocytes are the primary site of synthesis for FGF2. FGF9-mediated decreases in astrocytic differentiation would lead to an overall decrease in FGF2. This may be another mechanism for the differential behavioral responses seen with these ligands. Modulation of astrocytic populations may be an underlying mechanism for fibroblast growth factor-mediated effects.

Astrocytes can modify synaptic transmission and plasticity through modulation of glutamatergic signaling (Verkhratsky, Orkand et al. 1998). Through glutamate uptake astrocytes can modulate the duration of the synaptic current and can protect from potential excitotoxic damage. An FGF9-mediated decrease in astrocytes (Lum, Turbic et al. 2009) could result in increased neurotoxicity. Astrocytes are also involved in the development and remodeling of synaptic functions and the promotion of synapse formation (Pfrieger 2002). Furthermore, astrocytes are responsible for the conversion of l-serine to d-serine (Wolosker, Blackshaw et al. 1999), and d-serine is necessary for the coactivation of NMDA receptors (Johnson and Ascher 1987). Regulation of the amount of d-serine may in-turn affect NMDA receptor activation.

As described above, acute administration of NMDA receptor antagonist, ketamine, has antidepressant-like effects (Berman, Cappiello et al. 2000; Zarate, Singh et al. 2006). However, chronic antagonism may lead to neurotoxicity (Newcomer, Farber et al. 1999). Chronic suppression of astrocytic differentiation, by increased exogenous FGF9 or endogenous FGF9 expression, could lead to long-term decreases in synaptic plasticity and subsequently negative behavioral consequences. Conversely, FGF2- induced increases in astrocytic proliferation (Perez, Clinton et al. 2009) could mediate the neuroprotective properties and subsequent positive effects on affective behavior.

Potential for FGF9 as a novel antidepressant target

While the potential peptide antagonist to the "low-affinity loop" of FGF9 may not have altered depression-like behavior in the experiment described in chapter two, FGF9 still has potential for inhibition. It is possible that the potential peptide antagonist may require higher concentrations for efficacy or may have more robust activity on different molecular or behavioral endpoints. However, further *in vitro* assays are necessary for the validation of a peptide antagonist or possibly a small molecule inhibitor for FGF9. Alternatively, sequence-specific knockdown by RNA interference *in vivo* may provide a biologically relevant approach to ligand regulation (Pardridge 2007). Additionally, antibody neutralization is another alternative to determine the effects of FGF9 activity blockade (Tao, Black et al. 1997). While these methods of inhibition will help validate FGF9 as a target for mood disorders, they would not be ideal therapeutics.

To date, BDNF and other growth factors have shown poor pharmacokinetic properties and low efficacy in degenerative disease patients (Pollack and Harper 2002). Additionally, development of small-molecule agonists of neurotrophic receptors has been unsuccessful. FGF2, BDNF, and other growth factors require increased expression or increased receptor activation for positive effects on depression-like and anxiety-like behavior. Conversely, FGF9 presents a novel target for specific inhibition to decrease its negative effects on affective behavior. This decrease in FGF9 activity could be achieved by blocking ligand-receptor binding by targeting critical binding sequences. Alternatively, blocking

the receptor site with a non-activating molecule could prevent FGF receptor signaling. However blocking the receptor would block all ligand activation and not just that of FGF9. While further research is necessary to validate effective peptide antagonists or small-molecule inhibitors, FGF9 provides a unique neurotrophic target for the treatment of mood disorders. Decreased FGF9 activity may lead to an improvement in depression symptoms and decreased anxiety.

Ultimately, complete inhibition of FGF9 activity may not be ideal. FGF9 may act as a physiological antagonist to FGF2 for a regulatory purpose. While a brief relief from the physiological antagonism may boost FGF2 activity, increased synaptic plasticity and antidepressant properties, long-term increases in FGF2 expression may also be detrimental. FGF9 inhibition may be more of a selective rather than chronic treatment. For example, electroconvulsive therapy increases growth factor expression and improves mood and is used as a “jump-start” in patients that are unresponsive to standard antidepressant medication.

FGF system as a mediator of circadian activity

While initial studies involving the timing of FGF9 administration led us to investigate the basal circadian expression of the FGF2 system, this series of studies supports the hypothesis that FGF2 dysregulation may alter basal circadian activity amplitude and subsequently sleep-wake cycles. We showed

that the administration of FGF9 acutely decreased escape behavior when administered in the first-half of the light-cycle. However, this result was not replicated when animals were injected and tested in the second-half of the light-cycle. Endogenous circadian-mediated decreases in overall escape activity may have masked the decrease in escape activity caused by FGF9 microinjections (Kelliher, Connor et al. 2000). However, it is also possible that FGF9 activity was diminished by the increased expression of FGF2 that occurs during the second-half of the light-cycle. The ratio of FGF2 to FGF9 at a given time-point may determine the effect of the ligands on synaptic plasticity and on affective behavior.

Importantly, FGF2 gene expression and circulating plasma corticosterone were increased at the onset of the active period. Since exogenous corticosterone and acute stress can both increase FGF2 expression (Molteni, Fumagalli et al. 2001), it is possible that FGF2 modulating the circadian effects of corticosterone in the hippocampus. FGF2 expression may be elevated while corticosterone secretion is in order to protect against architectural damage caused by elevated glucorticoids. This simultanous increase of FGF2 expression and corticosterone may be one mechanism for increasing environmental responsivity and synaptic plasticity. Further investigation into the effect of circadian corticosterone regulation of FGF2 expression is necessary to determine the involvement of FGF2 expression dysregulation in the disruption of sleep-wake cycles. The role

of the FGF system in circadian locomotor activity may be another mechanism by which FGF dysregulation can underlie the pathophysiology of depression.

In support of this dissertation recent studies from our lab and others show support the role of the FGF system in affective behavior (Molteni, Fumagalli et al. 2001; Molteni, Lipska et al. 2001; Fumagalli, Bedogni et al. 2005; Riva, Molteni et al. 2005; Akil, Evans et al. 2008; Turner, Calvo et al. 2008; Turner, Gula et al. 2008; Perez, Clinton et al. 2009). Additionally recent work has implicated the FGF family in drug abuse behavior (Fumagalli, Pasquale et al. 2006; Fumagalli, Di Pasquale et al. 2008; Turner, Flagel et al. 2008; Fumagalli, Franchi et al. 2009; Turner, Capriles et al. 2009) which is important as depression is a behavioral sequelae of addiction (Shaffer, LaPlante et al. 2004). The above discussion revisits the findings supporting the novel role for FGF9 in affective behavior as well as the involvement of the FGF system in the amplitude of circadian locomotor activity. The present studies support the hypothesis that FGF9 may mediate the long-term negative aspects of depression. Additionally, the dysregulation in FGF system expression may underlie pathophysiological changes in depression that lead to disruptions of normal circadian activity.

Experimental Limitations

Microinjections of FGF9 into the lateral ventricle of the brain did not allow us to characterize the direct effects of FGF9 on the hippocampus. As described

below, central microinjections may alter multiple brain regions associated with behavior. Therefore the observed results are a sum of the effect observed over multiple brains region. As the FGF system is expressed diffusely throughout many brain regions, it is difficult to quantify gene expression in regions other than the hippocampus, such as the nucleus accumbens, ventral tegmental area, and amygdala. However, further assessment of the FGF system in these regions is necessary to fully understand the dysregulation of the FGF system in mood disorders. Site-specific injections into these regions may elucidate the action of FGF9 in regards to affective behavior.

Additionally, the above studies focused on the dysregulation of gene expression through mRNA analysis. However, we showed no evidence of protein-level changes in the hippocampus. It is possible that alterations in mRNA transcription do not necessarily correlate to changes in protein expression. As therapeutics for FGF9 antagonism would focus on the protein-level inhibition, it is important to characterize both changes and protein and post-translational modifications of FGF9.

As FGF2 has been shown to increase astrocytes, increase cell proliferation, and support neurogenesis in adult rats, these mechanisms were not explored in the above studies. As FGF9 has behavioral effects opposite those seen with FGF2 administration, we would hypothesize that FGF9 would decrease astrocytes, decrease cell proliferation, and inhibit adult neurogenesis. While it is important to

identify the mechanism of FGF9, it is often difficult to observe differences in basal changes of adult cell proliferation. As adult rodents have a fairly low basal level of neurogenesis and cell proliferation, observing a decrease in these hippocampal changes will be a challenge.

While our circadian studies focused on basal gene expression fluctuations of FGF system in the hippocampus, we did not identify disruptions in these gene expression rhythms following a stressor. We would hypothesize that a stressor, such as repeated social stress, would disrupt the regulation of FGF system expression in the hippocampus and subsequent locomotor behavior. It may also be informative to test locomotor response to novelty after FGF2 microinjection and during the dark-period in order to determine if exogenous FGF2 increases an animals' environmental responsivity. We also did not show if the fluctuations of FGF2 and FGFR1 are the result of circadian corticosterone regulation. Further studies, using adrenalectomized rats could determine if the changes in FGF system expression are corticosterone-dependent. Additionally adrenalectomized rats could be administered steady corticosterone treatment to look at the effect on FGF system expression when there is present but not fluctuating corticosterone secretion.

We have evidence that rats bred for high response to novelty (HR) have higher FGF2 gene expression in the hippocampus (Perez, Clinton et al. 2009) and have increased locomotor activity during the active period (Clinton, unpublished).

However, we did not show that animals bred for low response to novelty have an increase in FGF9 gene expression in the hippocampus. This information would support our hypothesis that the expression of FGF2 and FGF9, as well as the ratio of expression, has an effect on environmental responsivity. Additionally, we also have evidence that neonatal FGF2 administration can increase cocaine self-administration in rats, but we have not tested this hypothesis in animals microinjected with FGF9.

Future Directions

There are incremental steps for the further characterization of FGF9 as an anti-depressant target. Additionally, there are larger experimental leaps that can be made to develop novel therapeutics for peptide antagonism or small-molecule inhibition of FGF9. While the FGF system is a good target for emerging therapeutic studies, we must continue to develop a greater understanding of the pathophysiology of depression by increased mechanistic probing. Additionally, research characterizing the post-translational modifications caused by environmental influence may provide further insight into the underpinnings of the interaction between environment and depression. Finally, analysis of individual variances in response to antidepressant treatment may also allow for more effective treatment of patients diagnosed with MDD.

The behavioral characterization of FGF9 inducible knockout mice may further validate the role of FGF9 in affective behavior. Additionally, current techniques allow for selective knockout of your inducible gene of interest in specific brain regions using inducible RNA interference (Hitz, Wurst et al. 2007). This selective FGF9 knockout could further elucidate the role of the hippocampal FGF9 and FGF2 in affective behavior. As exogenous microinjection of FGF9 was directed into the lateral ventricle, FGF9 may have been mediating effects through other brain regions important to mood regulation. Recent studies have shown that different brain regions can have opposing influences on depression-like behavior.

Specifically, addition of exogenous BDNF to the hippocampus or posterior mid-brain nuclei attenuated depression-related phenotypes (Shirayama, Chen et al. 2002). However, intra-ventral tegmental area (VTA) infusions of BDNF resulted in increased depression-like effects (Eisch, Bolanos et al. 2003). As depression is marked by anhedonia, or lack of reward, it is suggested the dopaminergic cells in the VTA and their terminal fields in the nucleus accumbens (NAc) mediate the reinforcing effects of drugs of abuse, food, and sex (Koob 1998; Bassareo and Di Chiara 1999; Everitt, Parkinson et al. 1999). Dyresgulation of the reward pathways may also contribute to the pathopysiology of depression (Di Chiara, Tanda et al. 1999; Gambarana, Tolu et al. 2001; Nestler, Barrot et al. 2002). Additionally, other molecules implicated in depression, CREB and S-adenosyl-L-methionine, also have differential effects in the hippocampus compared to the VTA-NAc (Nibuya, Nestler et al. 1996; Genedani, Saltini et al. 2001; Barrot,

Olivier et al. 2002). It is important to fully understand the overall effects of FGF9 administration to multiple brain regions to avoid unwanted side-effects on mood.

While a structural analysis-based approach to peptide design, we did not observe any changes in depression-like behavior after administration of the LAL peptide. While *in vivo* experiments present the best approximation of the translational value of a peptide antagonist, it is difficult to optimize in this way. As sophisticated high-throughput cell based assays are available for *in vitro* assessment, advanced tools exist for rational screening of potential therapeutic compounds (Uttamchandani and Yao 2008; An and Tolliday 2009). However, the assay would have to assess a downstream molecule of FGF9 activation and given the complexity of the signaling system this approach will be challenging.

Recent investigations of epigenetic mechanisms underlying the pathophysiology of depression attempt to explain high discordance rates between monozygotic twins, the differences among inbred rats, the chronic relapse nature of the illness, and the increased prevalence of depression in females (Mill and Petronis 2007). Post-translational modification may be a mechanism by which environmental experiences can alter gene expression in the absence of DNA sequence modification in the brain. Epigenetic changes have been implicated in a number of psychiatric disorders (Tsankova, Renthal et al. 2007); however, depression research has focused on two main chromatin-modifying processes. Histone acetylation, which leads to transcriptional activation and decondensed chromatin,

may be a key mechanism for antidepressant action (Tsankova, Kumar et al. 2004) . Increased histone acetylation of the BDNF promoter is required for the antidepressant actions of imipramine to reverse the negative behavioral effects of social defeat (Tsankova, Kumar et al. 2004; Tsankova, Berton et al. 2006). Additionally, histone deacetylase (HDAC) inhibition showed increased antidepressant-like activity in the social defeat paradigm (Tsankova, Berton et al. 2006; Schroeder, Lin et al. 2007).

While histone acetylation can be altered by experience and modulate gene expression, DNA methylation can also be modified by environmental factors (Jaenisch and Bird 2003). The methylation of the glucocorticoid receptor (GR) promoter was enhanced when mothers provided inadequate maternal care characterized by licking and grooming behavior (Weaver, Cervoni et al. 2004). This alteration resulted in decreased binding of transcriptional enhancers that regulate GR gene expression. Early-life molecular imprinting increased anxiety-like behaviors and persisted into adulthood (Weaver, Cervoni et al. 2004). The long-lasting effects of epigenetic modifications may provide an explanation for why childhood trauma increases the risk for mood disorders in adulthood (Heim and Nemeroff 2001; McGowan, Sasaki et al. 2009). Modification of histones can facilitate or inhibit the access of associated proteins necessary for regulation of gene expression. Further investigation of epigenetic modification in response to environmental factors may lead to an increased understanding of the interaction between gene expression and experience.

While epigenetic modifications may undermine individual genetic polymorphisms by modifying genetic promoters (Mill and Petronis 2007), there is still reported value in the assessment of individual differences. As we hope to eventually realize the potential of pharmacogenomics and pharmacokinetics, further research into individual responses to treatment and genetic characterizations is necessary. This genetic information may provide information for effective antidepressant treatments and minimized side effects.

To date, most antidepressant pharmacogenomic studies have focused on candidate genes in the monoaminergic pathways. The strongest findings of individual differences in depression, from clinical trials with more than two thousand patients, are as follows: (1) FK506-binding protein (FKBP5), a glucocorticoid-receptor-regulating co-chaperone, polymorphism is associated with a beneficial antidepressant response (Binder, Salyakina et al. 2004; Lekman, Laje et al. 2008), (2) 5-hydroxytryotamine receptor 2A (HTR2A), a serotonin receptor that mediates the effects of atypical antipsychotics, polymorphism is associated with outcome of antidepressant treatments and antidepressant side effects (Sato, Yoshida et al. 2002; McMahon, Buervenich et al. 2006; Suzuki, Sawamura et al. 2006), and (3) kainic- acid-type glutamate receptor KA1 (GRIK4), a receptor responsible for fast-acting gluatamatergic neurotransmission may be associated with treatment-resistant dependence (Paddock, Laje et al. 2007). Further analysis of genetic polymorphisms from both antidepressant responders and non-responders may provide evidence of

both treatment selection and potential novel genetic targets. Understanding the interaction between genetic predisposition and environmental factors is of paramount importance for rational drug design and personalized treatment of mood disorders.

In conclusion this dissertation provides support for the hypothesis that *FGF9 has negative effects on anxiety-like and depression-like behavior.* In addition, FGF system expression shows daily variation in the hippocampus and changes in FGF2 can alter daily locomotor activity. *Dysregulation of the FGF system may alter environmental responsivity and in-turn affective behavior.*

Conclusions

Specifically, we observed that: (1) early-life and peripheral administration of FGF9 did not alter adult behavior, (2) acute microinjection of FGF9 resulted in anxiolytic and depressionogenic behavior in adult rats, (3) chronic microinjections of FGF9 were anxiogenic and depressionogenic in adult rats, (4) chronic administration of FGF9 decreased the dentate gyrus gene expression of the FGFR1 receptor, (5) repeated social stress increased social avoidance and the hippocampal gene expression of FGF9, (6) both FGF2 and FGFR1 gene expression exhibited hippocampal variation over a 24-hour light-dark cycle, (7) an acute microinjection of FGF2 can alter the amplitude of circadian locomotor activity. These studies were the first to show that FGF9 modulated affective

behavior and was altered by increased stress. Additionally, this is the first time that the daily fluctuation of the FGF system over a 24-hour light-dark cycle and its ability to alter circadian activity were observed.

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