An Unexpected Role for Fibroblast Growth Factor-2 in modulating Anxiety and Hippocampal Stem Cells

by

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Dedication

To my beloved wife; “Te amo, es por ti que puedo lograr cualquier cosa en esta vida, y es por ti, Rodolfo y nuestra nueva bebita que tengo tanta pasion y ganas de seguir luchando”

To my beloved mother; “Ivonne, es de ti de quien nacio mi pasion por la busqueda”

Mis hermanos “Gabriel, Miguel y Victor los amo, y agradezco su apoyo”.

Papi; “Aunque no me lo digas se que estas orgulloso de mi, me amas y me admiras por que yo a ti tambien”.

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Abstract

Strong evidence supports the role of genetic endowment conferring either vulnerability or protection to mood disorders. However, environmental factors are also known to play a critical modulating role in such vulnerability. Recent reports revealed decreased levels of Fibroblast Growth Factor-2 (FGF-2) gene expression in several post-mortem brain regions of subjects with a history of major depression. One of the regions showing profound alterations was the hippocampus of these severely depressed subjects. These reports implicated FGF-2 in depression, however they did do not address whether the observed dysregulation in FGF-2 expression represents a predisposing factor to the illness or a consequence of the disease process. Given that altered anxiety is observed in mood disorders such as depression we examined the potential contribution of FGF-2 in two genetically distinct groups of rats selectively bred to differ dramatically in their response to novelty and to anxiety-provoking conditions (HRs= Low Anxiety/High Response to Novelty vs. LRs= High Anxiety/Low Response to Novelty). We demonstrate that the Low-Anxious HRs have significantly elevated levels of hippocampal FGF-2 mRNA relative to the High-Anxious LR's, and that there exists a highly significant inverse correlation between FGF-2 levels and anxiety behavior. Interestingly, FGF-2 expression is modulatable by environmental factors that alter anxiety and enhance

neurogenesis-- thus environmental complexity (EC) reduces anxiety behavior induces FGF-2 expression and promotes neurogenesis in the hippocampus, particularly in the High Anxious LR's. Moreover, a 3-week treatment regimen of administered FGF-2 is highly effective at blunting anxiety behavior, specifically in the High Anxious LR's. This anxiolytic effect is accompanied by an increase in the survival of hippocampal adult stem cells, both neurons and astrocytes, again specifically in the LR's. Furthermore, we show that the impact of EC on hippocampal cell genesis is dependent on the FGF system as FGF blockade disrupted the effects of EC on increasing cell proliferation and new cell survival. This suggests that hippocampal cell genesis might contribute to modulating the anxiolytic effects of FGF-2 and EC. Taken together, these findings implicate hippocampal FGF-2 as a novel modulator of anxiety behavior and underscore its potential as a new target for the treatment of mood and anxiety disorders.

Chapter 1

Introduction

It is increasingly evident that behavior, including emotional responsiveness, is controlled by the interplay between genetic predisposition and the impact of the environment on the individual. While genetic endowment may confer either vulnerability or protection towards a high level of emotional reactivity and stress responsiveness, environmental factors are also thought to play a critical modulating role. In particular, early life stress is known to alter emotionality later in life. Moreover, acute psychosocial stress (so-called life events) is often the trigger for episodes of severe mood disorders. However, less is known about the *protective* consequences of the environment and how they might interact with genetic vulnerability to anxiety, a behavior often seen altered in mood disorders.

Emotionality refers to the unique constellation and magnitude of the endocrine, neural and behavioral responses of an organism in response to environmental stimuli that have valence, be it negative or positive. The hippocampus along with its well-known function in learning and memory (Squire, 1992, Jarrard, 1993) is one of the key brain structures that modulate these stress responses via its negative feedback regulation (Jacobson and Sapolsky, 1991). Exposure to sustained stress increases anxiety-like behavior and can lead to various

structural and synaptic plasticity changes in the hippocampus (Kim and Yoon, 1998) (Sapolsky, 2003), including dendritic remodeling and disruption of proliferation and survival of adult neurogenesis (McEwen, 2001). Such changes are in part thought to underlie the neural mechanisms associated with altered cognitive and emotional behaviors seen in mood disorders (Duman, 2002, McEwen, 2005).

As mentioned above, genetic as well as environmental factors are thought to modulate emotionality. Genetic influences on emotionality have been evident in the selectively bred HR (High Responder) and LR (Low Responder) animals. HR-LR animals show characteristic differences in emotional responsiveness to novelty where HR animals show higher locomotor response to a novel environment as opposed to LRs, which display limited response to novelty. These differences in response to novelty have previously been shown to predict propensity to drug-taking behavior, where HRs learn to self-administer drug of abuse more readily than LRs, including cocaine and amphetamine (Piazza et al., 1990). HR-LR animals also show predictable differences in anxiety-like behavior, where HRs display low anxiety relative to the highly anxious LRs (Kabbaj et al., 2000). Recently, the genetic contribution brought by these behavioral phenotypes was demonstrated as selective breeding showed these behavioral phenotypes being highly heritable (Stead et al., 2006). Thus selective breeding of HRs and LRs has been shown to confer differences in vulnerability to anxiety-like behavior.

Findings by our laboratory support the role of the hippocampus as a key structure modulating differences in propensity to anxiety-like behavior between HR-LR. Specifically, HR animals show lower levels of glucocorticoid receptor GR expression in the hippocampus relative to LRs. These differences in hippocampal GR are thought to modulate in part such differences in anxiety as direct microinjections with a GR antagonist into the hippocampus of LRs reduces their anxiety to that of an HR (Kabbaj et al., 2000).

Conversely, HR-LR differences in emotionality are also subject to differential propensity to stress impact as social isolation confers an anxiogenic effect in HRs without affecting LRs (Kabbaj et al., 2000). Similarly, restraint stress causes HR animals to behave like LR animals. This suggests that stressful experiences may also differentially affect the typical HR-LR anxiety responses. Moreover, differences in HR-LR behavioral responses to environmental stimulation also extend to differences in neural responses as observed by immediate early gene expression.

Changes in immediate early gene expression in response to stress or environmental stimulation are thought to precede short and long-term changes resulting from environmental stimulation (Kabbaj and Akil, 2001). Furthermore, growth factor gene expression changes resulting from environmental stimulation are thought to initiate changes in structural plasticity (Rampon et al., 2000) (McClung and Nestler, 2008). As mentioned above the hippocampus is one the

key brain structures showing high responsiveness to stress and environmental stimulation. Such responsiveness has been shown to result in structural plasticity changes including changes in dendritic arborization, synaptogenesis and neurogenesis.

Growth factors are thought to mediate in part most of these structural plasticity changes occurring in the brain (Filus and Rybakowski, 2005, McClung and Nestler, 2008). These include changes in neurogenesis associated with VEGF expression as well as changes in dendritic remodeling in response to FGF-2 (Cao et al., 2004, Rai et al., 2007). Moreover, the role of growth factors in structural plasticity in the hippocampus has been extended by their genetic contribution, observed in animals showing overexpression or deletion of growth factor genes (Govindarajan et al., 2006) (Raballo et al., 2000, Korada et al., 2002).

As mentioned above, HR and LR animals show heritable differences in emotionality and differences in response to environmental stimulation. Given that growth factors have been singled out as key players modulating structural plasticity resulting from genetic contribution and experience, it is reasonable to suspect that differences in HR-LR anxiety-like behavior and response to environmental stimulation may relate to differences in growth factor expression.

Previous work has mostly focused on growth factors such as BDNF and VEGF in modulating structural plasticity changes resulting from antidepressant treatment. Specifically, changes in anxiety and depression-like behavior have been attributed to changes in neurogenesis brought by VEGF and BDNF (Shirayama et al., 2002) (Warner-Schmidt and Duman, 2007) (Schmidt and Duman, 2007). However, work in our group has led to a novel set of target growth factors that may also participate in modulating differences in anxiety seen in HR and LR.

Previous microarray studies performed by a consortium of researchers that includes our laboratory (the Pritzker Consortium) have revealed decreased levels of Fibroblast Growth Factor-2 (FGF-2) gene expression in several post-mortem brain regions of subjects with a history of major depression (Evans et al., 2004). These differences in FGF-2 transcripts seemed to partially reverse with antidepressant treatment. Furthermore, recent, unpublished observations have shown that the hippocampus of these severely depressed subjects shows the most profound alterations.

While evidence implicating FGF-2 in emotional behavior is limited, studies suggest that experiences that affect emotional behavior influence FGF-2 gene expression. For example; rats that were raised by mothers who demonstrated better care for their pups, showed higher protein levels of FGF-2 and enhanced survival of adult-born neurons (Bredy et al., 2003). In turn, rats that received increased maternal care show reduced endocrine stress responses and better

adaptive behaviors towards environmental challenges (Francis and Meaney, 1999, Menard et al., 2004). Interestingly, the deficits in hippocampal development resulting from decreased maternal care have been shown to be rescued by environmental complexity (EC) (Bredy et al., 2004). EC has also been shown to reverse the behavioral, neurogenic and endocrine deficits resulting from prenatal restraint stress (Morley-Fletcher et al., 2003, Laviola et al., 2004). While these reports generally support the role of EC in modulating emotional behavior, it remains to be determined whether EC increases FGF-2 expression in the adult hippocampus. Furthermore, prenatal stress has been shown to reduce both neurogenesis (Coe et al., 2003), and levels of FGF-2 in the hippocampus of adult rats (Molteni et al., 2001), EC reverses the effects of stress on neurogenesis (Morley-Fletcher et al., 2003, Laviola et al., 2004). Given that FGF-2 enhances neurogenesis on mature adults and during early development, one might predict that the reversal effects of EC may in part be due to a rescue of basal levels FGF-2.

Given that anxiety is one of the strong hallmarks of vulnerability to depression in humans, it is reasonable to consider that experiences known to reduce anxiety and enhance neurogenesis may help elucidate the role of FGF-2 in emotionality. Moreover as HR and LR animals show basal differences in emotionality (e.g anxiety-like behavior), which are subject to experiential influence, our studies will examine the potential role of FGF-2 on emotionality where the genetic and experiential contributions are taken into account.

Fibroblast Growth Factors

FGF-2 is a member of the FGF gene family composed of 24 proteins that have multiple functions including development of the nervous system and angiogenesis (Eckenstein et al., 1991). FGF-2 and other FGF ligands are classified as family members based on a central domain conserved region of about 120 amino acids which bind heparin (Faham et al., 1996). The typical genomic structure of this family is made up of three coding exons ranging from 5kb to 100kb, where exon one contains the start codon. The family can also be subdivided into three groups. One subgroup; FGF 1, 2, 9, 16 and 20 lack an NH-2 terminal signal sequence but they are still transported to the extracellular environment, while FGF 11- FGF 14 also lack this signal and remain intracellular. The remaining FGF subgroup uses the endoplasmic reticulum-golgi secretory pathway to be transported extracellularly.

The complexity produced by the diversity of family member ligands includes different isoforms resulting in some members such as FGF-2 and FGF-3, which have additional 5' transcribed sequences from upstream AUG starting codons (Arnaud et al., 1999). These isofoms are said to confer different functions to the protein and affect affinity for the FGF receptors. For example amino terminal extensions of bFGF or FGF-2 have been hypothesized to promote nuclear targeting of the protein, thereby regulating different functional responses within the cells (Bikfalvi et al., 1998). On the other hand, several factors contain a single start codon (Gaughran et al.), but exon one is subdivided into two to four different

subexons by alternative splicing. Others use alternative 5'exons, which will confer them with alternative amino terminals. These transcriptional variations may also have some implications for receptor affinity and tissue specificity, which ultimately may translate to different functional activity.

Fibroblast Growth Factors; Structural Characteristics of FGF-2

FGF-2 has been crystallized and characterized structurally by (Zhu et al., 1991). Its structure is important to discuss since its interaction with FGF receptors is made up of a terciary complex that also includes interactions with heparan sulfate, which can also affect cell activity response. Several isofoms of FGF 2 ranging from 18 kDa to 24 kDa have been identified. The crystal structure of the 18kDA isoform was determined in the presence and absence of heparan sulfate by (Faham et al., 1996). FGF 2 is composed of 12 antiparallel β-sheets within the conserved core domain organized as a pyramidal structure of three groups of 4 β-sheets. Beta strands 10 and 11 contain several basic residues that form the heparin-binding site, which provide protection against denaturing agents (Schlessinger et al., 2000). Heparan sulfate which is found throughout all the mammalian tissues, contrary to heparin, will have a role in modulating FGF2 function and distribution by binding FGF-2 as soon as it is secreted from cells. This in turn will affect ligand–receptor binding interaction.

Fibroblast Growth Factor-2; Receptors

In the adult, FGF-2 shows prominent expression in the cytoplasm as well as in the nucleus of astrocytes throughout the brain, whereas neuronal expression is almost exclusive to the hippocampus (Woodward et al., 1992). Several isoforms of FGF 2 ranging from 18 kD to 34 kD have been identified (Nugent and Iozzo, 2000). FGF-2 exerts its function by interacting with four receptor types with varying affinity depending on ligand and receptor isoforms (Ornitz, 2000, Reuss and von Bohlen und Halbach, 2003). These receptors are trans-membrane glycoproteins containing three Ig-like loops (I-II-III) in the extracellular domain and a split tyrosine kinase domain. Three of the FGF receptors, FGFR1, FGFR2 and FGFR3, are expressed in the brain (Eckenstein, 1994), while FGFR1 is most abundantly expressed in the hippocampus (Belluardo et al., 1997). There are two binding sights from where bFGF can interact with the FGF receptors. This gives a chance for one FGF-2 molecule to bind to two different receptors at the same time or activate a single receptor within two different locations, (Kan et al., 1993). This same group has also shown that FGF receptor 1 contains heparan sulfate binding sites, and have suggested that this interaction functions to enhance FGF2 affinity to receptors (Kan et al., 1999). Affinity for receptors by FGF-2 and other family members is modulated by different factors such as receptor isoforms and the types of heparan sulfate proteoglycans on the cell surface, (Guimond and Turnbull, 1999), (Lin et al., 1999), (Ornitz et al., 1996), (Spivak-Kroizman et al., 1994). The Ig-III loop is the site of specificity for the receptor, (Johnson et al., 1991) and it is where isoforms are formed, hatl enhance or decrease the affinity

of the receptors 1,2 and 3 for any given FGF. These isoforms (IIIb or IIIc) are formed through alternative splicing of two exons that code for the carboxyl terminal half of the loop III. FGF binding affinity studies have been done in cell culture using Baf 3 cells (Ornitz et al., 1996). The results suggest that FGF-2 has a higher affinity for FGF receptors 1IIIc and 3 IIIc and FGF receptor 4 while showing very low affinity for isoforms of receptor 2 IIIb and 3 IIIb.

Fibroblast Growth Factor-2; Anatomy

The expression of FGF-2 in the adult brain has been observed by immunohistochemistry showing FGF-2 most prominently expressed in astrocytes throughout the whole CNS including the hippocampus and the neocortex (Woodward et al 1992). The only reported immunoreactivity of FGF-2 on neurons was in the hippocampal formation. FGF 2 expression was found in the cytoplasm as well as in the nucleus of both astrocytes and neurons. In the rat brain FGF-2 is widely expressed across regions with most prominent expression in astrocytes. Anatomical mapping shows FGF-2 synthesizing cells showing high co-localization with FGF-2 protein expression (Gonzalez et al. 1995). Thus most astrocytes synthesize FGF-2 whereas FGFr1 is mostly neuronal. Interestingly, FGF-2 mRNA shows low abundance in the dentate gyrus, whereas protein expression is very high in abundance. FGF-2 expression in the hippocampus is much higher in the CA2 with lower levels of expression observed in the CA1 and CA3. This suggests that FGF-2 action must be relatively localized within each hippocampal subregion as its synthesis in astrocytes must impact FGFr1

neuronal cells located in the hippocampus. On the other hand it is also possible that FGF-2 synthesized within the hippocampus could be acting outside of this region. This is particularly evident in regions such as the amygdala where FGF-2 mRNA expression is almost absent, yet high protein expression is observed. Moreover, the amygdala shows high levels of FGFr1 expression suggesting that FGF-2 stemming from other regions should be acting in these receptors. This is consistent with reports showing FGF-2 as a diffuseable protein. Moreover, FGF receptors are mostly expressed within axonal fibers, thus most of FGF-2 activity within the receptors should stem from secreted FGF-2 as oligodendrocytes do not synthesize FGF-2. Finally although most of the FGF receptors are expressed in the white matter only FGFr1 is expressed in neuronal populations, with high levels of expression found in the hippocampus including the dentate gyrus and the CA3 subregions.

Fibroblast Growth Factor-2; Cell signaling

Signaling cascades by FGF receptor activation have been determined and hypothesized to involve mitogenic activation, but the target molecular pathways for specific functions have yet to be determined. These signals depend on receptor isoforms as well as glycoprotein interaction within the cell surface, as previously mentioned. Binding of FGF-2 will activate the receptor, which will cause dimerization and autophosphorylation by specific tyrosine residues within the receptor. Then binding of phospholipase C to phosphotyrosine residues will mediate signal, through hydrolysis of phosphatidylinositol to inositol-3-phophate

and dyacilglycerol (DAG) leading to Ca+ release and subsequent activation of PKC. Another signal transduction pathway involving FGF receptor activation is through binding of FGF substrate receptor 2 (FRS2) and/or SHC. These will stay bound to tyrosine residues within the receptor and bind the Grb2-SOS complex activating RAS. In turn Ras recruits RaF-1, which will then phosphorylate MEK, followed by phosphorylation of MAPK by MEK, resulting in activation and phosphorylation of transcription factors. Both of these signaling cascades have been attributed to FGF-2 function. However many factors mentioned above may affect which cell signaling cascades regulate FGF-2 functional responses at different times.

Fibroblast Growth Factor-2; Development

Basic FGF or FGF-2 has been shown to play an important role during embryonic development of the neocortex and hippocampus of mice (Vaccarino et al., 1999, Raballo et al., 2000, Cheng et al., 2002) however its enhancing proliferative effects on neurons have not been extensively studied in the adult brain. Expression of FGF-2 and its receptors FGFr 1,2 and 3 during development is temporally regulated within the ventricular zone (VZ), which is the area from where inducing signals for neocortical progenitors occur reviewed by (Ford-Perriss et al., 2001). The progenitors cells within the VZ express high levels of bFGF whereas migrating cells once they leave the VZ do not. FGF-2 is expressed early during neurogenesis, becoming almost absent by the end, when other growth factors such as FGF-7 and FGF-8 increase expression (reviewed by

(Dono, 2003). Most studies on FGF-2's role in development have focused on neocortical progenitor cell cultures whereas its mitogenic and survival effects have been documented by (Murphy et al., 1990), (Cavanagh et al., 1997). Moreover, (Palmer et al., 1995) (Palmer et al., 1999) showed the neurogenic effects of bFGF in vitro from stem cells of adult neocortex an area where only glial proliferate during adulthood.

Furthermore studies done by (Korada et al., 2002) showed that FGF-2 knockout mice had a decrease of 40% in cortical glutamatergic pyramidal neurons and a reduction in the size of their cell bodies, with no effects observed in the hippocampus. These effects were restricted to frontal and parietal cortex. Furthermore, FGF-2 injected at Embryonic day 15.5 to null mutants results in an 18% and 87% increase in the volume and in total number of neurons, respectively in the adult cerebral cortex (Vaccarino et al., 1999). Finally studies have also demonstrated the role of bFGF in cholinergic sprouting and axonal remodeling after entorhinal cortex lesions, (Ramirez et al., 1999) (Fagan et al., 1997). Taken together these reports exemplify the variety of responses and actions FGF-2 provides throughout the brain during development. However, this does not address the variety of neurogenic responses FGF-2 could bring about in the postnatal brain.

FGF-2 has mostly been recognized for its mitogenic and survival effects on adult stem cell cultures derived from numerous areas of the central nervous system

(Palmer et al., 1999). These include areas such as the striatum, neocortex, and subventricular zone, which have shown proliferative responses from exposure to bFGF, inducing neurons and glia. However, these neurogenic effects have only been reported *in vivo* during certain developmental periods. For example single peripheral injections of FGF-2 have been shown to enhance hippocampal neurogenesis in young rodents, however these effects were not observed in the adult (Wagner et al., 1999). It is possible that repeated injections might be required for such effects to take place, since repeated exogenous administration of FGF-2 rescues age related decline of hippocampal neurogenesis in mice and rats (Jin et al., 2003)

Neurogenesis; Modulation by Environmental Factors

Neurogenesis refers to neuronal birth, differentiation, and short-term survival of new neurons. Neurogenesis occurs well into adulthood in certain areas of the brain, including the subventricular zone and the dentate gyrus of the hippocampus. In the adult rat hippocampus, neurogenesis occurs in the subgranular zone located at the border of the granule cell layer and hilus. New neurons in the dentate gyrus migrate into the granule cell layer, extend axons into the mossy fiber pathway, make synaptic contacts with targets in CA3, and express neuronal markers (van Praag et al., 2002).

It is estimated that in the rat hippocampus the number of granule neurons added to the granule cell layer of the dentate gyrus within 1 month is 6% of the total

granule cell population, representing 28% and 62% of the afferent and efferent neuronal populations respectively (Cameron and McKay, 2001). This represents a massive cell turnover, which makes this structure particularly sensitive to experience-dependent changes. However, it also renders the dentate gyrus particularly susceptible to environmental factors, which could alter hippocampal structure and function. Indeed, exposure to negative regulators of hippocampal neurogenesis such as stress result in reduced performance on hippocampus-dependent learning tasks (Luine et al., 1994, Bodnoff et al., 1995, Endo et al., 1996, Krugers et al., 1997, Shors et al., 2001), while positive regulators such as exercise and environmental complexity correlate with improved performance (Kempermann et al., 1997, Luine et al., 1998, van Praag et al., 1999a).

It has been shown that when rodents are housed in an enriched environment there is an increased in the numbers of new neurons in the dentate gyrus (Kempermann et al., 1997, Nilsson et al., 1999). This increase in number of both neurons and glia has been attributed to an enhanced survival of new cells as opposed to proliferation (Nilsson et al., 1999). Moreover, an increase in hippocampal volume has been seen after exposure to a complex environment. This suggests that adult neurogenesis facilitates the increase in hippocampal volume and that such increase in neurogenesis may help individuals cope better with novelty and complexity (Kempermann et al., 1997, Kempermann et al., 1998).

Similarly an increase in neurogenesis is observed in response to hippocampus-dependent learning whereas no increase is observed following learning tasks that do not require the hippocampus (Gould et al., 1999, Ambrogini et al., 2000). This has been suggested to result form increased survival of new cells as opposed to proliferation, as most untrained animals see a reduction in neurogenesis within two weeks (Cameron et al., 1993), whereas animals living in a complex environment or trained in hippocampus-dependent learning tasks show reduced pyknosis and increased neurogenesis in the hippocampal subgranular zone (Young et al., 1999) (Gould et al., 2000).

Similarly, exercise increased neurogenesis in mice (van Praag et al., 1999a, van Praag et al., 1999b, van Praag et al., 2002), while enhancing hippocampal LTP, suggests that the integration of these new cells may have an active participation in synaptic plasticity. Indeed it has been show that new cells born in the dentate gyrus show similar functional properties to those seen in mature neurons (van Praag et al., 2002). Interestingly, running has been shown to have antidepressant-like effects in rodents and humans which could therefore be mediated in part by increased hippocampal neurogenesis (Hill et al., 1993, Ernst et al., 2006). This leads me to speculate that environmental complexity could perhaps have similar antidepressant effects as a result of increased neurogenesis, whereas stress could lead to an increase in vulnerability to mood disorders as a result of decreased neurogenesis.

In the adult hippocampus stress inhibits neurogenesis, and impairs learning and anxiety (Kim and Diamond, 2002). In rats, odor of a natural predator suppresses proliferation in the dentate gyrus (Tanapat et al., 2001). Similarly, in the marmoset stress provoked by introduction of a resident intruder decreases neurogenesis in the dentate gyrus (Gould et al., 1998).

Stress and depression are associated with morphological changes in many brain regions including the hippocampus (Drevets, 2000, Rajkowska, 2000). For example, decreased hippocampal volume has been shown in patients suffering from depression (Sheline et al., 1996, Sheline et al., 1999) and post-traumatic stress disorder (PTSD) (Bremner et al., 1995, Gurvits et al., 1996, Bremner et al., 1997). Recently, similar findings were shown in rats where hippocampal volume was negatively correlated with Hippocampal volume (Kalisch et al., 2006). This reduction in volume has been attributed to a decrease in neurogenesis (Gould et al., 1998). Furthermore, studies have indicated that reduced hippocampal volume may predispose to, as opposed to result from, affective disorder as seen in twin studies from combat patients suffering from PTSD (Gilbertson et al., 2002). Taken together these findings along with the reports above, suggest that both experience and genetic propensity interact to produce the vulnerability to mood disorders with associated structural plasticity in the hippocampus.

In fact it has been shown that prenatal stress predisposes adults to depression in humans and rats (Dugovic et al., 1999, Watson et al., 1999). In support of the

role of neurogenesis in such vulnerability (Lemaire et al., 2000) demonstrated that prenatal stress induces a life-long reduction in neurogenesis in the dentate gyrus of rats.

While stress and depression may reduce hippocampal neurogenesis, possibly contributing to reduced hippocampal volume, proliferation is increased after chronic administration of antidepressants including SSRIs, (Malberg et al., 2000). Antidepressants increase the rate of hippocampal neurogenesis over a period of two to three weeks of administration. This is consistent with the time in takes for antidepressants to show beneficial effects on mood disorders. This suggests that increased adult neurogenesis may be a possible mediator of antidepressant action in humans (reviewed by (Duman et al., 2001, Manji et al., 2001, Jacobs, 2002).

The effects of antidepressants on neurogenesis have been extensively studied in animal models. Increases were observed in the rat following administration of several classes of antidepressants, while non-antidepressant agents had no effect on neurogenesis (Malberg et al., 2000). Furthermore, combined antidepressant treatment with stress in tree shrews and rats reverse the effects of stress on decreasing proliferation in the adult dentate gyrus, (Czeh et al., 2001) (Malberg and Duman, 2003).

As a result it has been suggested that stress-induced decrease in neurogenesis may be an important factor in eliciting depressive episodes (Duman et al., 2001, Jacobs, 2002, Kempermann and Kronenberg, 2003). Reports supporting this hypothesis have shown that animal model of stress disrupt cell proliferation and cell survival, while antidepressants reverse such effects (Czeh et al., 2002, Malberg and Duman, 2003). Furthermore, it has been shown that the behavioral effects of antidepressants are dependent on neurogenesis in the hippocampus (Santarelli et al., 2003). Reports also show that depressed subjects have decreased hippocampal volume when compared to normal subjects, whereas treated depressed subjects do not show such deficits (Sheline et al., 1996, Sheline et al., 1999). These differences in hippocampal volume could in part be due to differences in neurogenesis brought by increased amounts of stress. While the above reported data suggest that decreased neurogenesis may underlie the behavioral deficits of depression (van Praag et al., 2000), the neural mechanisms responsible for decreased neurogenesis are yet to be determined.

It has been well documented in animal models that stress has detrimental effects on emotional behavior and decreases hippocampal neurogenesis (Gould et al., 1997, Tanapat et al., 1998, Malberg and Duman, 2003, Mirescu et al., 2004). Moreover, the effects of such stressful experiences are reversed by antidepressant treatment (Malberg and Duman, 2003). Since antidepressants represent an effective treatment strategy, their mechanisms of action have been used as a point of departure for understanding the etiology of mood disorders

(Jacobs, 2002). Thus, increased adult neurogenesis has been suggested as one of the mechanisms by which antidepressants exert their beneficial effects on behavioral measures of anxiety and depression-like behavior (Santarelli et al., 2003). The convergence of evidence, with stress decreasing and antidepressants increasing neurogenesis has led to the hypothesis that mood disorders may be related to decreased levels of neurogenesis in the hippocampus. However, much remains to be done to fully determine whether decreased neurogenesis underlies the emotional distress seen in depression. Moreover, it is important to examine the interplay between neurogenesis and individual differences in emotionality, and especially the impact of experience on these processes, without relying exclusively on a treatment based-hypothesis (Grossman et al., 2003). Non-aversive experiences such as Environmental Complexity (EC) show similar effects to antidepressants, including reducing anxiety and increasing neurogenesis (Kempermann et al., 1997, Benaroya-Milshtein et al., 2004). Understanding the mechanisms of action of EC will provide an insight into the neural mechanisms whereby certain types of environmental experience can enhance neurogenesis and modulate emotional behavior.

Environmental Complexity

Environmental complexity refers to housing conditions facilitating sensory, exercise and social stimulation provided by the addition of more animal, toys and obstacles in the housing environment (van Praag et al., 2000). Animals living

under such housing conditions have shown many structural and behavioral plasticity effects relative to animals living on standard housing conditions. These effects seem to underlie the mechanisms of action of the potential beneficial effects of experience, given that EC has been shown to ameliorate the effects of animal models of neurological disorders such as Alzheimer's (AD), Parkinson's and Fragile X among others (Nithianantharajah and Hannan, 2006).

As mentioned above, EC has been shown to have a variety of effects from behavioral to cellular and molecular (van Praag et al., 2000). Initial observations made by Hebb in the 1940's documented that, rats taken from the laboratory roaming freely at his house displayed behavioral improvements relative to littermates left at the laboratory. Later on in the 1960's, Rosenzweig and colleagues showed that animals living in a more complex environment showed increased brain weight (Bennett et al., 1969). More recent studies have shown EC to increase dendritic branching and length as well as increase the number of spines and synapses (Volkmar and Greenough, 1972, Greenough and Volkmar, 1973). These findings although reported to occur in various areas of the brain such as cortex have been shown to occur in the hippocampus as well (Diamond et al., 1976). Specifically, dendritic density was shown to be increased in the dentate gyrus of enriched rats relative to controls (Juraska et al., 1985). Similarly, an increase in the number of dendritic spines and dendritic density has also been reported in the CA3 region of the hippocampus in response to living in a more complex environment (Altschuler, 1979). These initial anatomical

findings preceded what has now become a well-documented paradigm for studying experience dependent plasticity. Moreover the power of such experience to alter dendritic remodeling is thought to exemplify the ability of the brain for self-repair and adapt to change. Although the mechanisms by which such changes occur are yet to be understood, evidence suggests that EC alters the expression of plasticity-related genes including growth factors, which could participate in the structural and synaptic plasticity changes occurring in the brain (van Praag et al., 2000).

In the hippocampus specifically structural plasticity changes in response to EC have been associated with increase in growth factors such as NGF (Mohammed et al., 1990). Moreover, besides changing the expression of genes EC has also been known to alter hippocampal dentate gyrus synaptic transmission and plasticity related events such as increase EPSPs (Green and Greenough, 1986). These changes go in parallel with findings showing increase field potentials found in the hippocampus of rats housed in a complex environment (Sharp et al., 1985).

Most of these changes occurring in the hippocampus have been attributed to the positive impact of EC on learning and memory. Moreover the fact that EC impacts electrophysiological properties of the hippocampus suggest that changes in LTP may be occurring to animals housed under EC (Duffy et al., 2001). Thus given that LTP is a well accepted model for learning and memory (Bliss and

Collingridge, 1993) it is prudent to hypothesize that changes in LTP occurring in the dentate gyrus of rats (Bronzino et al., 1994) may help explain in part the improvement in learning and memory occurring in rats exposed to EC.

The initial studies have more recently been followed by studies showing the neurogenic effects of EC (Kempermann et al., 1997). Such increases in neurogenesis in the dentate gyrus of the hippocampus have in part been attributed to increases in gene expression of growth factors such as VEGF, NGF and BDNF (Young et al., 1999, Cao et al., 2004, Zhu et al., 2006). Interestingly, behavioral changes in response to EC have also been attributed to increased expression of growth factors after EC (Zhu et al., 2006). Specifically improvements in learning and memory have been related to increases in VEGF after EC (Cao et al., 2004). Moreover, changes in behaviors altered in mood disorder such as anxiety have also been shown to be improved upon living in a more complex environment (Benaroya-Milshtein et al., 2004). Given the inherent ability of EC in promoting cellular and behavioral plasticity in the intact brain, EC has gained more attention as a potential tool for studying the recovery of brain disorders (Laviola et al., 2008).

Transgenic mouse models have demonstrated that EC has the ability to reverse adverse effects of several neurological disorders. For example EC housing ameliorated the spatial memory deficits and delayed the onset of symptoms in an animal model of Huntington's disease (Hockly et al., 2002, Glass et al., 2004).

Interestingly, epidemiological results found that a more stimulating environment improved physical, mental and social functioning in human Huntington's disease patients (Sullivan et al., 2001). Furthermore, transgenic mouse models containing the human APOE alleles found in Alzheimer's disease patients have also shown improvement in working memory after housing under EC (Levi et al., 2003). These behavioral changes were accompanied by an increase in NGF and synaptophysin in the hippocampus, suggesting that behavioral changes could result from changes in neural plasticity. The notion that EC may interact with genetic manipulations which model disease implies that experience interacts profoundly with genetics in altering vulnerability towards disease. This suggests that psychiatric disorders, which are known to have a strong genetic component, are subject to experiential manipulation thus altering their degree of onset.

Psychiatric disorders present an extreme challenge given the inherent complexity of gene-environment interactions affecting vulnerability to such disorders. Although animal models of psychiatric disease present advantages they explicitly rely on drug treatment hypothesis for elucidating their characterization. For these reasons it is important to adopt models that encompass the contribution of experience and genetic propensity. Such models of mood disorders may provide a better understanding of the neurobiological underpinnings of such disorders. To this end I have adopted the use of the High Responder (HR) Low Responder (LR) model characterized by individual differences in anxiety-like behavior in conjunction with EC. This model should produce a better understanding of the

contribution of both experience and genetic predisposition on vulnerability towards mood disorders.

High Responder (HR) and Low Responder (LR) Model

The HR and LR model has previously been described as a model of individual differences in emotionality. Early studies carried out by Piazza and co-workers demonstrated a relationship between a behavioral response to a novel environment and propensity to self-administer drugs of abuse (Piazza et al., 1989). When outbred rats are exposed to the mild stress of a novel environment high responder (HR) animals exhibit high rates of locomotion while Low Responders (LR) exhibit low rates of locomotion (Piazza et al., 1989). These two groups of animals differ in their drug seeking behavior as HR rats learn to self-administer psychostimulants faster than LR rats (Dellu et al., 1996, Marinelli and White, 2000).

Findings show the HR-LR phenotype predicts differences in response to drugs of abuse as HR rats exhibit a heightened behavioral sensitization to amphetamine. Reports have also found a positive correlation between locomotor response to novelty and the magnitude of sensitization to psychostimulants (Hooks et al., 1991). Moreover, differences in the acquisition of self-administration have been reported between HR and LRs. For example, HR rats learn to self-administer amphetamine more quickly than LR rats (Piazza et al., 1989, Piazza et al., 1990). These differences have also been observed in cocaine self-administration as HR

learn to self administer low and high doses of cocaine more rapidly than LRs (Kabbaj et al., 2001). It is noteworthy that once LRs achieve a stable pattern of self-administration their drug-taking behavior becomes indistinguishable from that of an HR. Thus the HR-LR model has certainly become suitable for examining individual differences in the initial part of drug-taking behavior.

Current work is aimed at determining the neural correlates underlying the basal differences between HR and LR. Such findings could render an understanding of the mechanisms that contribute to the vulnerability of drug abuse. Initial work focused on differences in Dopamine (DA) responses, with HR showing greater DA response relative to LR (Piazza et al., 1991). These findings included basal and stress related DA responses (Rouge-Pont et al., 1993) which are modulatable by glucocorticoids (Piazza et al., 1996). Interestingly, HRs show a more profound release of glucocorticoids in response to stress (Kabbaj et al., 2000).

However, more recent work by our laboratory has highlighted the importance of other neural mechanisms contributing to the phenotypic differences. Specifically our laboratory has rendered the importance of stress molecules associated with HR-LR differences, as stress is an inherent component of the novelty response and a critical factor in vulnerability to drug taking behavior. Findings along these lines demonstrated HR-LR differences in the expression of several stress related

genes such as CRH in the amygdala and hypothalamus, and glucocorticoid receptor in the hippocampus (Kabbaj et al., 2000).

Anatomical profiling using immediate early gene expression of c-fos has also enabled the characterization of neural activation in HR-LR in response to mild stressors. Relative to LRs, HRs show low expression of c-fos in the CA1 region of the hippocampus, while showing high expression in the olfactory area, orbital cortex, cingulate cortex, dorsal striatum and the paraventricular nucleus of the hypothalamus (Kabbaj and Akil, 2001). Given that c-fos is a transacting factor that alters the expression of other genes, it seems possible that HR-LR might show different long-term responses to stress as evidenced by their different immediate early gene expression response in different anatomical regions. Thus, experiences may render different neural consequences in HRs and LRs, which could translate into differences in emotional reactivity in both phenotypes. Indeed it has been shown that stress differentially alters HR and LR drug-taking behavior. Thus while HRs typically self-administer drugs more readily relative to LRs, after social defeat stress the scenario is dramatically different as HR animals appear inhibited (Kabbaj et al., 2001). Thus, repeated social defeat stress, which is known to enhance drug-taking behavior, results in a transient inhibition of self-administration in HRs followed by a return to normal drug talking behavior. On the other hand, LR animals, which typically show little response to drugs, respond in the opposite manner by showing a dramatic increase in self-administration thus becoming indistinguishable from HRs. Taken together the

HR-LR model reliably demonstrates that stressful experiences may differentially impact individual drug taking behavior. Moreover, the differential neural responses observed in HRs and LRs in response to mild stress suggest that different neural mechanisms may underlie the propensity for drug taking behavior; *where in some animals (LR) drugs are seek in response to stress, whereas in others drug-seeking is due to their propensity for novelty response*.

Although the initial characterization of these phenotypes was revealed in the context of vulnerability towards drug taking behavior, more recently the HR-LR model has provided insight on the neural correlates associated with emotional stress responsiveness to the environment (Piazza et al., 1989, Dellu et al., 1996). Our laboratory has shown that these differences in locomotor response to novelty also predict differences in anxiety-like behavior (Kabbaj et al., 2000) (Kabbaj and Akil, 2001), with HR animals exhibiting lower levels of anxiety behavior in the light-dark box test and Elevated Plus Maze (EPM) relative to LRs. Thus, HR animals seem to display high exploration on novel environments that may be considered mildly stressful. Moreover, these behavioral differences are also accompanied by differences in corticosterone stress responses where HRs exhibit a more prolonged corticosterone response. These different endocrine responses are attributed to differences in the negative feedback regulation of the HPA axis as HR animals show lower levels of GR expression in the hippocampus relative to LRs. However, it is important to note that such basal differences in anxiety-like behavior are also subject to experiential influence, as seen earlier

with drug taking behavior. For example if HRs are socially isolated their behavior becomes indistinguishable from that of an LR thus showing a more inhibited anxious-like phenotype (Kabbaj et al., 2000). Similarly, when a stressor is imposed such as in restraint stress the low anxiety HR behavioral phenotype disappears. Thus, these results imply that basal HR-LR differences in anxiety-like behavior are also modulated by experiences that may lead to differential changes in gene expression. Such experiences in turn may translate into changes in the expression of genes associated with neural plasticity such as FGF-2.

Finally, while much of this literature has relied on outbred animals, our laboratory has embarked on an ongoing selective breeding project (now in the 19th generation). This selective breeding project has demonstrated that these behavioral phenotypes are highly heritable. The knowledge gained from our selectively bred HRs and LRs has shown that novelty response is a genetically heritable trait, which can reliably predict individual differences in anxiety-like behavior. This provides an advantage for ascertaining neural correlates associated with HR-LR differences in anxiety-like behavior. Furthermore the HR-LR selective breeding project provides a model profiting heavily from genetic homogeneity, thus enabling us to better study differences in the propensity towards anxiety-like behavior.

Elucidating the mechanisms by which experience can interact with genetic predisposition to control emotional reactivity is fundamental for understanding normal behavior and for explaining the etiology of mood disorders. Clearly, adverse stressful experience has been identified as one of the main epigenetic factors leading to the onset of mood disorders (Lopez et al., 1999). As a result much effort has focused on the impact of stress on brain structure and function as well as on emotional responsiveness. However, most of these studies have focused on stressors that are aversive, uncontrollable and generally negative for the animal. Less is known about the impact of other environmental manipulations on emotionality, including changes in the environment that might be considered either positive or at least more complex in their valence such as in EC. This dissertation focuses on this latter class of environmental experience and examines the potential role of FGF-2 in this process. Based on reports mentioned above I hypothesize that; ***High levels of hippocampal FGF-2 brought by genetic inheritance or environmental complexity contribute to reduce anxiety-like behavior.***

To this end, in my first chapter we examined the link between changes in hipocampal FGF-2 gene expression, anxiety behavior and hippocampal cell genesis observed after environmental complexity. These initial studies investigated whether changes in hippocampal cell genesis and anxiety behavior seen after EC were dependent on the FGF system activity. Following these studies chapter 2 examined the direct role of the endogenous hippocampal FGF

system on anxiety behavior by examining the impact of an FGF antagonist on anxiety. In addition I also investigated the specific role of FGF-2 on anxiety by looking at its impact on selectively bred HR-LR animals, a model characterized by genetically inherited differences in anxiety-like behavior. Finally, we conclude this dissertation by examining the role of FGF-2 in mediating the gene-environment interaction on vulnerability to anxiety. Specifically I investigated how FGF-2 can serve as a protective agent on individual vulnerability to anxiety as brought by genetic endowment, as seen in HR-LR, or from environmental influence, as seen after EC. Finally, I end this thesis project by investigating the role of hippocampal cell genesis as a potential mechanism mediating the influence of FGF-2 on vulnerability to anxiety behavior.

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Chapter 2

FGF-2 is required for the beneficial effects of Environmental Complexity on hippocampal cell genesis and anxiety

Abstract

Emotionality refers to the unique constellation and magnitude of the endocrine, neural and behavioral responses of an organism when exposed to environmental stimuli that have valence be it negative or positive. The hippocampus is one of the key brain structures that modulate these responses. Exposure to sustained stress increases anxiety-like behavior and can lead to various structural changes in the hippocampus, including disruption of adult neurogenesis. In contrast Environmental Complexity (EC), a behavioral paradigm involving communally housed animals exposed to various objects and toys, has been shown to enhance neurogenesis and reduce anxiety-like behavior. Previous findings showing decreased levels of FGF-2 expression in brains of postmortem subjects that suffered from major depression have implicated the FGF system as a novel target modulating vulnerability to mood disorders. However, the extent of such findings has yet to be linked to behavioral measures of emotionality. Given that altered anxiety is observed in mood disorders such as depression we examined the potential contribution of FGF-2 on the anxiolytic and neurogenic effects of

EC. Our results showed that EC increased FGF-2 gene expression and neurogenesis in the hippocampus. EC reduced anxiety-like behavior in the novelty suppressed feeding test, extending previous findings that used other measures of anxiety behavior. Interestingly, when animals were administered with an FGF receptor antagonist during exposure to EC we saw a disruption in the anxiolytic effects of EC. These effects were paralleled by changes in hippocampal cell genesis as FGF blockade disrupted the effects of EC on increasing cell proliferation and new cell survival. Taken together our results suggest that hippocampal FGF-2 is required for the anxiolytic effects of EC, and that neurogenesis might contribute to modulating the anxiolytic effects of EC.

Introduction

While genetic endowment may confer either vulnerability or protection towards a high level of emotional reactivity and stress responsiveness, environmental factors are also thought to play a critical modulating role. In particular, stressful experiences are known to alter emotionality, and are often recognized as the trigger for episodes of severe mood disorders. However, less is known about the protective consequences of the environment and experience on emotionality. One particular paradigm well known for showing the protective consequences of the environment on emotionality is Environmental Complexity (EC). Specifically, EC has been shown to reduce anxiety behavior (Benaroya-Milshtein et al., 2004). Moreover, EC has been reported to improve learning and memory and enhance hippocampal neurogenesis, (Nilsson et al., 1999) a phenomenon regarded as a key mechanism by which antidepressants exert their behavioral effects on animal models of mood disorders (Santarelli et al., 2003).

It has been well documented that stress has detrimental effects on emotional behavior and decreases hippocampal neurogenesis (Gould et al., 1997, Tanapat et al., 1998, Malberg and Duman, 2003, Mirescu et al., 2004). Moreover, the effects of such stressful experiences are reversed by antidepressant treatment (Malberg and Duman, 2003). Since antidepressants represent an effective treatment strategy, their mechanisms of action have been used as a point of departure for understanding the etiology of mood disorders (Jacobs, 2002). Thus, increased adult neurogenesis has been suggested as one of the

mechanisms by which antidepressants exert their beneficial effects on behavioral measures of anxiety (Santarelli et al., 2003).

Studies on the neural and behavioral effects of antidepressants have mostly focused on Brain Derived Neurotrophic Factor (BDNF) (Duman, 1998, Shirayama et al., 2002, Duman, 2004). However, recent studies involving our research group have shown a down-regulation of FGF-2 in the brains of post mortem subjects with major depression (Evans et al., 2004). Moreover, reports have also shown that antidepressant treatment increases FGF-2 expression in the rat hippocampus (Mallei et al., 2002). These findings led us to the hypothesis that decreased levels of FGF-2 may be involved in the pathophysiology of severe depression and the closely associated increase in anxiety, and that antidepressants and anxiolytic drugs may exert their effects by increasing FGF-2 (Turner et al., 2006, Akil et al., 2008).

FGF-2 is a member of the FGF gene family composed of 24 proteins that have multiple functions including development of the nervous system and angiogenesis (Eckenstein et al., 1991). In the adult, FGF-2 shows prominent expression in astrocytes throughout the brain, whereas neuronal expression is almost exclusive to the hippocampus (Woodward et al., 1992). FGF-2 exerts its function by interacting with four receptor types with varying affinity depending on ligand and receptor isoforms (Ornitz, 2000, Reuss and von Bohlen und Halbach, 2003). These receptors are trans-membrane glycoproteins containing three Ig-like loops (I-II-III) in the extracellular domain and a split tyrosine kinase domain.

Three of the FGF receptors, FGFR1, FGFR2 and FGFR3, are expressed in the brain (Eckenstein, 1994), while FGFR1 is most abundantly expressed in the hippocampus (Belluardo et al., 1997). These receptors are expressed in both neurons and glial and may participate in glial and neuronal signaling interactions that may have important implications during development as FGFR1 has previously been shown to be required for hippocampal growth (Ohkubo et al., 2004).

FGF-2 has mostly been recognized for its mitogenic and survival effects on adult stem cell cultures derived from numerous areas of the central nervous system (Palmer et al., 1999). However, these neurogenic effects have only been reported *in vivo* during certain developmental periods. For example single peripheral injections of FGF-2 have been shown to enhance hippocampal neurogenesis in young rodents, yet these effects were not observed in the adult (Wagner et al., 1999). However, repeated exogenous administration with FGF-2 has been shown to rescue age related declines of hippocampal neurogenesis (Rai et al., 2007).

While evidence implicating FGF-2 in emotional behavior is limited, studies suggest that at least during development, there may be an important interplay between emotional experiences and FGF2 expression. For example; rats that were raised by mothers who exhibited better care for their pups showed higher protein levels of FGF-2 and enhanced survival of neurogenesis during adulthood

(Bredy et al., 2003). Since rats that receive increased maternal care show reduced endocrine stress responses and better adaptive behaviors towards environmental challenges (Francis and Meaney, 1999, Menard et al., 2004), this might suggest an association between higher FGF-2 levels and lower anxiety behavior. Conversely, prenatal stress has been shown to reduce both basal levels of FGF-2 and neurogenesis (Coe et al., 2003) in the hippocampus of adult rats (Molteni et al., 2001). Interestingly, the behavioral and endocrine deficits resulting from prenatal restraint stress as well as the associated decrease in neurogenesis have been reversed by EC (Morley-Fletcher et al., 2003, Laviola et al., 2004). Similarly, deficits in hippocampal development resulting from decreased maternal care have been shown to be rescued by EC (Bredy et al., 2004). Together, these studies suggest that during development, both positive and negative environmental factors (stress, maternal care, environmental enrichment) can exert lifelong impact on both emotionality and hippocampal function, including neurogenesis, in the adult animal. They also suggest that some of these effects are associated with altered levels of FGF-2 during development. However, they do not firmly establish a causal role of FGF-2 in mediating environmental influences on emotional reactivity.

However, it remains unclear whether FGF2 is relevant only during a certain developmental time window or whether it plays a key role during adulthood in encoding environmental influences and mediating changes in emotional responses. While the human postmortem studies uncovered a decrease in FGF-

2 in severe depression, it was difficult to ascertain whether this was due to a genetic predisposition that was present throughout the patient's life, the impact of the illness itself, or some combination of factors.

Thus, in this chapter I asked whether a non-aversive experience that can decrease anxiety in the adult animal (EC) can also modulate FGF-2 expression. Moreover, we directly test the idea that this alteration in FGF-2 expression is not simply a byproduct of the environmental change, but plays a causative role in the associated change in anxiety behavior.

Materials and Methods

Animals

Adult Male Sprague-Dawley rats were obtained from our in-house breeding colony at the Molecular and Behavioral Neuroscience Institute (MBNI) where we have maintained the selectively bred HR-LR lines for over 18 generations. Adult rats (300g-400g) around 2 months old were housed two animals per cage (one HR, one LR per cage to balance out potential intergroup variance) after locomotor screening under a 12 hr light/dark cycle (lights on at 6:00 am) with food and water available *ad libitum.* Animals were allowed to acclimate to the housing conditions for at least 7 days prior to any experiments. 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 (UCUCA) at the University of Michigan.

Locomotion Testing

At postnatal day 55-60, adult male rats were screened for locomotor response to a novel environment by placing them in a standard size (43×21.5×24.5) clear acrylic cage in a different room from where the animals had been housed. Locomotor activity was monitored every 5 minutes for 1 hour by two panels of photocells connected to a computer. The first panel of three photocells was placed at ground level to record horizontal locomotion, with the second panel of five photocells located near the top of the cage to determine rearing behavior. The locomotion testing rig and motion recording software were created in-house at the University of Michigan. Locomotion activity was tested between 9:00 and 11:30 am. Final locomotion scores were determined by summing horizontal and rearing activities.

Environmental Complexity

Rats were housed for 21 days in 3 × 3 × 3 ft stainless steel cages which contained different toys, obstacle courses, and enriching stimuli. Every day, animals were exposed to a set up of toys and sensory stimuli as part of their housing environment.

Novelty Suppressed Feeding Test

Rats were food deprived for 18 hrs prior to testing. On the next morning, animals were placed in an open field for a maximum of 5 minutes with a food pellet placed in the middle of the open field, and the latency to grab and start eating the food pellet was used as a measure of anxiety.

mRNA *in situ* Hybridization

At the conclusion of each experiment, rats were sacrificed by rapid decapitation, and their brains were removed, snap frozen, and stored at -80 ºC. Brains were cryostat sectioned at -20°C at 20µm and sliced in series throughout the hippocampus, mounted on Super Frost Plus slides (FisherScientific) and stored at -80°C until processed. *In situ* hybridization methodology has been previously described in detail elsewhere (Kabbaj et al., 2000). Sections taken every 100µm were fixed in 4% paraformaldehyde at room temperature for 1 h, and then washed three times in 2X SSC. Slides were processed in a solution containing acetic anhydride (0.25%) in triethanolamine (0.1 M, pH 8.0) for 10 min, rinsed in distilled water, dehydrated through graded ethyl alcohols (50, 75, 85, 95 and 100%) and then air dried, all at room temperature. After air-drying, the sections were hybridized with a ^{35}S-labeled cRNA probe for FGF-2. The sequences of rat mRNA used for generating probes of genes are complementary to the following RefSeq database nos FGF2 (NM_019305, 716-994), and the probe was synthesized in our laboratory. 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 FGF-2 probe was labeled in a reaction mixture consisting of 1 µg of linearized plasmid, 1X transcription buffer (Epicenter Technologies, Madison, WI), 125 µCi of 35S-labeled-UTP, 125 µCi of 35S-CTP, 150 µM ATP and GTP, 12.5 µM dithiothreitol, 0.5 ml of RNase inhibitor, and 1.5 µl of T3 RNA polymerase. The reactions were incubated for 120 min at 37ºC, and then 1 µl of DNAse (RNAse free) was added to the reaction to incubate for another 15 min at room temperature. The labeled probes were purified using Micro Bio-Spin P-30 Tris Spin Columns (Bio-Rad Laboratories), then diluted in hybridization buffer (containing 50% formamide, 10% dextran sulfate, 3XSSC, 50 mM sodium phosphate buffer, pH 7.4, 1XDenhardt's solution, 0.1 mg/ml yeast tRNA, and 10 mM dithiothreitol) to yield 106 dpm/70 µl. A cover slip with 70 µl of diluted riboprobe was placed on each slide. Slides were placed in a humidified box with filter paper saturated with 50% formamide buffer, and incubated overnight at 55°C. Following overnight incubation, the coverslips were removed, rinsed, and washed twice in 2X SSC for 5 min each. The sections were treated for 1 h in RNAase A solution (20 µg/ml in Tris buffer containing 0.5 M NaCl, pH 8) at 37°C. Following treatment, the sections were washed in increasingly stringent solutions of SSC, 2X, 1X and 0.5X, for 5 min each. The slides were then incubated for 1 h in 0.1X SSC at 65°C. Finally the sections were rinsed with water and dehydrated through graded alcohols, air-dried, and exposed to a Kodak XAR film (Eastman Kodak, Rochester, NY, USA). Exposure time of 7 days was experimentally determined to maximize signal for the FGF-2 probe. The films were developed (Kodak D-19;

Eastman Kodak, Rochester, NY, USA), and brain section images were captured from film with a CCD camera (TM-745, Pulnix) using MCID and relative optical densities were determined for each section. Radioactive signals were quantified using computer-assisted optical densitometry software (Scion Image Beta 4.03; Scion Corporation, Frederick, MD). Integrated densities were found by outlining the region of interest from both hemispheres. Optical density measurements were corrected for background, and the signal threshold was defined as the mean gray value of background plus 3.5× its standard deviation. Only pixels with gray values exceeding the above-defined threshold were included in the analysis. Optical density measurements were taken for 4 subregions of the hippocampus (hippocampal fields CA1-CA3, and the dentate gyrus) from the left and right sides of the brain. Data from multiple sections per animal were averaged resulting in a mean integrated optical density value for each animal and then averaged for each group.

Surgeries and Microinjections

Rats were anesthetized using isoflurane before surgery and a single cannulae (22-gauge, Plastics One Inc., VA. USA) was implanted into the left lateral ventricle (coordinates from bregma: AP -.9; ML +1.3; DV -1.8) using a stereotaxic apparatus. The guide cannulae were anchored to the skull and fitted with an obdurator. Obdurators were removed and 28-gauge injector cannulae were inserted extending 1.5mm below the tip of the guide. The cannulae were connected by PE-20 tubing to a Hamilton syringe mounted on a syringe pump

(Harvard Apparatus, MA. USA). Rats were microinjected with 100nM dose of the FGF receptor antagonist PD173074 (Pfizer) dissolved in artificial cerebrospinal fluid (aCSF) or vehicle three times a week for 21 days throughout the duration of the EC training period. Total volume of 5ul was infused at a rate of 1.0µl/min, and the injector was left in place for an additional 2 min to allow diffusion of the drug. Animals were subjected to behavioral testing 24 hours after the last injection. Rats were killed by decapitation and the brains were snap frozen in isopentane after behavioral testing.

BrdU/ Ki67 Immunohistochemistry

To assess the effect of EC on hippocampal new cell survival, rats were injected with Bromodeoxyuridne (BrdU) (Calbiochem) for two days prior to the start of training paradigm at a dose of 200mg/kg dissolved in saline. Twenty-four hours post BrdU labeling animals underwent 21 days of EC before being exposed to behavioral testing after which they were sacrificed by decapitation and brains were snap frozen. For determining the rate of cell proliferation we performed immunohistochemical labeling of Ki-67, which is an endogenous marker of ongoing cell proliferation. For BrdU and Ki67 imunohistochemistry a series of every 8 sections was cut throughout the entire extent of the hippocampus at 30 μm and slide mounted. For Ki-67 DAB staining, sections were postfixed in 4% paraformaldehyde for 1hr, followed by a 45 minute incubation in 10mM sodium citrate at 90°C. Sections were then rinsed with PBS and washed in 0.3% peroxide followed by blocking with BSA containing 1% goat serum and 0.05%

Triton X-100. Subsequently sections were incubated overnight with rabbit polyclonal anti-ki67 (University of Michigan) 1:40000 in BSA. After PBS washes sections were then incubated in biotinylated goat anti rabbit secondary antibody 1:1000, (Vector labs) followed by Avidin/Biotin complex (Vectastain Elite ABC kit) and subsequent DAB reaction for visualization of signal. For DAB staining of BrdU, sections were postfixed in 4% Paraformaldehyde for 1hr, rinsed in PBS and washed in 0.3% peroxide. Sections were then incubated in 50% formamide-2X SSC at 65°C for 2 hours followed by two 5 minute rinses in 2X SSC. Slides were then placed for 30 minutes in 2N HCL at 37°C and 10 minutes in 0.1M Boric Acid at room temperature, followed by rinsing in PBS and blocked with BSA containing 1% goat serum and 0.05% Triton X-100. Sections were incubated overnight at room temperature with rat monoclonal anti-BrdU (Accurate) 1:1000 in BSA. After PBS washes sections were then incubated in biotinylated goat anti rat (Vector labs) secondary antibody 1:1000 followed by Avidin/Biotin complex amplification (Vectastain Elite ABC kit) and subsequent DAB reaction for visualization of signal. Cresyl violet staining was performed for both immuno stains and sections were subsequently dehydrated through graded alcohols followed by immersion in xylene and then coverslipped with Permount® mounting medium.

Cell Counting

For quantification of DAB stained Ki-67 and BrdU cells, slides were initially coded and the code was not broken until counts were analyzed to assure that a blind

observer performed cell counts. To estimate the total number of cells we followed a modified unbiased stereological procedure used by (Malberg and Duman, 2003) where the total number of cells counted per animal was multiplied by the reciprocal of the sampling factor. BrdU and Ki67 cells were counted in the granule cell layer and SGZ of the hippocampus on a light microscope under 63X objective. Cells were included in SGZ counts if the cell was near the SGZ or touching the SGZ and was excluded if the cell was more than two cell diameters from the SGZ.

Statistical Analysis

Behavioral studies and anatomical studies were analyzed by Student t-tests or ANOVAs followed by Fishers PLSD post-hoc test comparisons. All data are presented as mean $\pm$ standard error. Statistical significance was assumed at $p<0.05$.

Results

Environmental Complexity increases FGF-2 gene expression in the hippocampus and reduces anxiety like behavior.

To begin to elucidate whether hippocampal FGF-2 expression responds to experiences known to reduce anxiety such as Environmental Complexity (EC) we examined hippocampal FGF-2 gene expression in animals that were exposed to EC or standard housing conditions. I focused on the hippocampus as FGF-2 shows prominent neuronal expression in this area, and since enhanced

hippocampal neurogenesis has been observed after EC. As seen in **Figure 2-1**, EC training results in an overall significant increase in FGF-2 expression in the dentate gyrus, [$t_{(21)}$=4.53, p<0.001], CA3 region, [$T_{(21)}$=3.5, p<0.01], and CA1 region, [$t_{(21)}$=5.6, p<0.0001] whereas no significant effects where observed in the CA2 region [$t_{(21)}$=0.23, p=0.82]. Furthermore, as seen in **Figure 2-2.**, EC decreased anxiety as shown by a decrease in the latency to feed on the novelty suppressed feeding test [$t_{(20)}$=-2.2, p<.05]. These results were specific to the novelty arena as no differences were observed in home cage feeding [$T_{(20)}$=1.6, p>.05]

Environmental Complexity increases cell proliferation and new cell survival.

Although EC had previously been linked to an increase in neurogenesis we wanted to verify whether such changes paralleled the increase in FGF-2 expression seen earlier in response to EC on our experimental conditions. As seen in **Figure 2-3.**, EC exposure results in a significant increase in cell proliferation in the dentate gyrus as shown by an increase in the number of Ki67 labeled cells, [$t_{(15)}$=2.8, p<0.05]. Moreover, there was also an increase in the survival of newly born cells in the dentate gyrus as measured by the number of Brd-U labeled cells that had incorporated BrdU over 3 weeks earlier [$t_{(15)}$=3.7, p<0.01].

FGF receptor antagonism blocks the effects of Environmental Complexity on increasing cell proliferation and survival.

Given that EC led to a significant increase in FGF-2 gene expression in the dentate gyrus and enhanced cell genesis, we asked whether FGF-2 played a causative role in the change in neurogenesis. Thus, we used chronic treatment with an FGF receptor antagonist to test whether it was sufficient to block the effects of EC on increasing cell genesis in the hippocampus.

As shown in **Figure 2-4.**, under vehicle conditions, EC results in a significant increase in cell proliferation and cell survival as shown by an increase in the number of Ki67 [$F_{(1,8)}$=5.8 p<0.05] and BrdU cells [$F_{(1,8)}$=10.6, p<0.01] respectively. On the other hand when animals were treated with an FGF receptor antagonist, EC failed to significantly increase cell proliferation [$F_{(1,8),}$=2.2, p=0.20] and new cell survival [$F_{(1,8)}$=2.1, p=0.20]. These results suggest that FGF receptor activation is required for EC to induce an increase in cell genesis in the dentate gyrus of the hippocampus and that the FGF system might contribute to the neurogenic effects of EC.

FGF receptor antagonism blocks the anxiolytic effects of Environmental Complexity.

Given that we had seen that EC resulted in an increase in FGF-2 expression and a decrease in anxiety we aimed to determine whether FGF receptor activation was required for EC to reduce anxiety. For these experiments we used an FGF receptor antagonist PD173074 (Pfizer), which was injected 3 times a week

throughout the duration of the EC training period. As seen earlier in our results and in part A. of **Figure 2-5.,** under normal conditions EC animals respond with a significant decrease in anxiety as shown by a decrease in the latency to feed [$T_{(20)}$=-2.2, p<0.05]. On the other hand, when EC animals are treated with an FGF antagonist this significant change in anxiety behavior in lost. Thus, FGF receptor blockade results in a disruption of the anxiolytic effects of EC [$T_{(16)}$=0.8, p>.05]. Taken together these results suggest that FGF receptor activation is required for EC to reduce anxiety-like behavior.

Discussion

The present study evaluated the potential role of FGF-2 to modulate the effects of experience on anxiety and neurogenesis. Using enrichment as a model of an environmental manipulation that is construed as neuroprotective, we determined whether FGF-2 was impacted by experience in the adult brain, and whether in turn this growth factor played a direct role in experience-dependent modulation of anxiety-like behavior and hippocampal neurogenesis. As previously reported, our demonstrate show that exposing animals to a more complex environment, as opposed to standard housing conditions, results in a decrease in anxiety-like behavior. Moreover, it increases the generation of new hippocampal cells by enhancing cell proliferation and cell survival. Importantly, we demonstrated that the exposure to a complex environment was accompanied by a significant increase in FGF-2 gene expression in the hippocampus. It was therefore reasonable to ask whether this increase in FGF-2 expression played a role in the

behavioral and/or neurogenesis alterations resulting from exposure to EC. This was tested by administering an FGF receptor antagonist to block the effect of the rise in endogenous FGF-2, and to determine whether this would prevent the neural and behavioral effects of EC. Our findings suggest that this treatment produced a blockade on the effects of EC in cell proliferation and cell survival. Moreover, treatment with the FGF receptor antagonist resulted in the disruption of the anxiolytic effects of EC. Taken together our results suggest that FGF-2 plays a key role as a neural modulator of the protective effects of experience on anxiety and hippocampal cell genesis.

The ability of EC to enhance neurogenesis has been reported previously (Kempermann et al., 1997) (Nilsson et al., 1999), and has been regarded as part of an overall beneficial response of hippocampal circuitry to this treatment (van Praag et al., 2000). Changes in cell genesis in the hippocampus have led to intense debate about their relevance to overall hippocampal function, particularly as it relates to the underlying mechanisms of mood disorders (Duman and Monteggia, 2006). For example stress, which is regarded as a robust trigger of depressive episodes has been shown to decrease proliferation and survival of adult stem cells (Gould et al., 1997), whereas antidepressant treatment has been shown to increase neurogenesis (Malberg et al., 2000). Furthermore, neurogenesis has been proposed as being critical to the behavioral effects of antidepressants (Santarelli et al., 2003). These reports along with others have prompted the hypothesis that decreased neurogenesis might in part be the

underlying mechanism of affective disorders (Warner-Schmidt and Duman, 2006). Nevertheless, these studies are largely correlational as it is difficult to manipulate the rate of cell birth, cell survival or cell differentiation in the hippocampus in a selective manner, and ascertain the functional impact on any given behavior. However, there is great advantage in determining some of the triggers of neurogenesis, and being able to modulate them and study their impact on behavior.

Growth factors such as FGF-2 have been shown to respond to antidepressant treatments (Mallei et al., 2002) and shown to be differentially down regulated in post mortem brains of depressed subjects (Evans et al., 2004). Furthermore, FGF-2 has been shown to enhance neurogenesis in the hippocampus when administered exogenously (Wagner et al., 1999, Rai et al., 2007). Thus, it is reasonable to hypothesize that FGF-2 might be one of the factors that might modulate the rate of neurogenesis. However, the role of endogenous FGF-2 in the adult hippocampus had not been extensively studied, either as a modulator of neurogenesis or as a regulator of emotional responsiveness.

Our initial results support the notion that increased hippocampal FGF-2 gene expression induced by EC could participate in the modulation of cell proliferation and cell survival. This was further supported by our second set of experiments, which showed that when animals were administered concomitantly with an FGF receptor antagonist during exposure to EC, the increased proliferation and

survival response was partially disrupted. These results support the role of FGF-2 as a modulator of EC's effect on increasing cell genesis. This is supported by findings showing the same FGF receptor antagonist compound blocking the increase cell proliferation responses of FGF-2 in vitro (Skaper et al., 2000). Furthermore, they point to FGF-2 as being necessary for EC to increase hippocampal cell genesis.

Although our results point to FGF-2 as being a key player in modulating EC's effects on increasing cell genesis it is difficult to ascertain whether the antagonist treatment reflects a blockade in FGF-2 activity. Although this drug has been shown to be specific for FGF receptors (Bansal et al., 2003), this compound does not necessarily discriminate on which FGF receptor it acts, as it binds to the tyrosine kinase domain of all FGF receptors (Mohammadi et al., 1998). This suggests that this compound has the potential to act on many different FGF receptor isoforms, including those, which show high and low affinity for FGF-2 binding and activation. Three of the four FGF receptors are expressed in the brain, each with at least 2 isoforms identified, thus a diverse number of receptors could be targeted. Furthermore, many more FGF ligands are expressed in the brain all with different binding affinity and efficiency response to each of the receptors (Ornitz et al., 1996). This makes for a plethora of FGF ligand receptor interacting combinations that could be targeted and disrupted in response to the antagonist. Conversely it is known that FGF receptor 1 is most abundantly expressed in the hippocampus whereas FGFr2 and FGFr3 show a more diffuse

expression within the white matter (Belluardo et al., 1997). Furthermore, as we saw FGF-2 increases significantly in the hippocampus in response to EC and both FGF-2 and FGFr1 have been shown to have a key role in hippocampal neurogenesis (Ohkubo et al., 2004) (Cheng et al., 2002). Finally, reports suggest that FGF-2 seems to have a preferential efficiency response in proliferation when interacting with FGF receptor 1 as opposed to FGFr2 and FGFr3 (Reuss and von Bohlen und Halbach, 2003) (Ornitz et al., 1996). Thus while it is possible to suspect that the effects of the antagonist could be attributed to other FGF ligands our results strongly implicate FGF-2 as mediator of the effects of EC on increasing cell genesis.

Our results also point to FGF-2 as an active mediator of the effects of EC on decreasing anxiety-like behavior. This is supported by evidence showing the FGF receptor antagonist blocking the anxiolytic effects of EC on the novelty suppressed feeding test. As seen in our results under baseline conditions EC strongly reduces anxiety, whereas in the presence of an FGF receptor antagonist treatment this difference disappears. These results strongly implicate the FGF system as an important player in the anxiolytic effects of EC.

Although other studies have shown other growth factors such as VEGF and BDNF modulating emotional behavior (Shirayama et al., 2002, Warner-Schmidt and Duman, 2007) this is the first time the FGF system is implicated in experience dependent modulation of emotional behavior. FGF-2 has previously

not been linked to anxiety, however, FGF-2 is well known for responding with changes in gene expression particularly in the hippocampus in answer to experiences and pharmacological treatments known to modulate anxiety-like behavior. For example hippocampal FGF-2 gene and protein expression increases in response to chronic antidepressants (Mallei et al., 2002) and acute treatments with diazepam (Gomez-Pinilla et al., 2000) both of which are known to reduce anxiety-like behavior. On the other hand FGF-2 gene expression in the hippocampus is also decreased after anxiogenic experiences including social defeat (Turner et al., 2008), in prenatal stress (Fumagalli et al., 2005) and decrease maternal care (Caldji et al., 1998, Bredy et al., 2003). Interestingly, EC has been shown to reverse the anxiogenic effects of maternal separation (Francis et al., 2002). However, it is yet to be determined whether such changes are due to increased FGF-2 expression in the hippocampus. Given our findings along with the previous reports we hypothesize that FGF-2 is an essential mediator of experience dependent anxiety behavior.

The mechanism by which FGF-2 may modulate experience dependent anxiety is yet to be determined. One hypothesis of depression states that neurotrophic factors and neurogenesis are critical players in mediating the behavioral responses of antidepressants and suggests that growth factors mediate the antidepressant behavioral responses by increasing neurogenesis in the hippocampus (Schmidt and Duman, 2007). In support of this hypothesis, there are data showing FGF-2 expression increased in the hippocampus after antidepressant treatment (Mallei et al., 2002) as well as evidence showing FGF-2

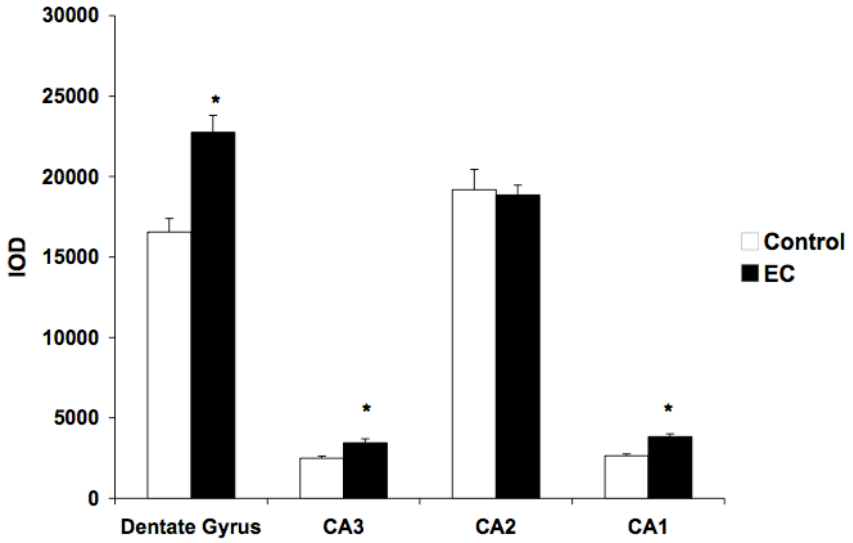
positively modulating hippocampal neurogenesis (Pieper et al., 2005). Given that EC shows similar effects on both increasing neurogenesis and reducing anxiety, we hypothesized that EC could work via the same mechanism. The likelihood that FGF-2 mediates the anxiolytic effects of EC via neurogenesis is supported by our findings, which show an FGF receptor antagonist to blocking the increase in cell genesis after EC, while blocking the anxiolytic effects of EC. However, our results do not rule out the possibility that FGF-2 may act by other mechanisms of plasticity such as increasing dendritic arborization given that EC has been shown to reduce anxiety independent of neurogenesis (Meshi et al., 2006) and FGF-2 has been shown before to promote dendrite outgrowth in the hippocampus (Rai et al., 2007). This would be especially plausible given that not only has EC previously been shown to promote such plasticity in several brain areas including the hippocampus, but other aversive experiences which increase anxiety such as stress (Wood et al., 2008) have been shown to have opposite effects (Watanabe et al., 1992).

In conclusion we present for the first time that the FGF system is an important modulator of the impact of EC on decreasing anxiety and hippocampal cell genesis. These results support the role of the FGF system as a modulator of experience dependent anxiety-like behavior pointing to FGF-2 as a potential candidate mediating EC's impact on anxiety. Although, our results suggest that EC requires FGF receptor activity for its anxiolytic impact there cannot be a direct comparison between the baseline and antagonist treatment conditions in the

current study as both results stem from different experiments. Thus, further chapters in this dissertation will use other measures of anxiety to corroborate the anxiolytic impact of EC and the specific role of FGF-2 in such effects both acutely and chronically. Moreover, we will also evaluate how individual differences in anxiety like behavior relate to hippocampal FGF-2 expression and how this affects the impact of EC.

Results Figures

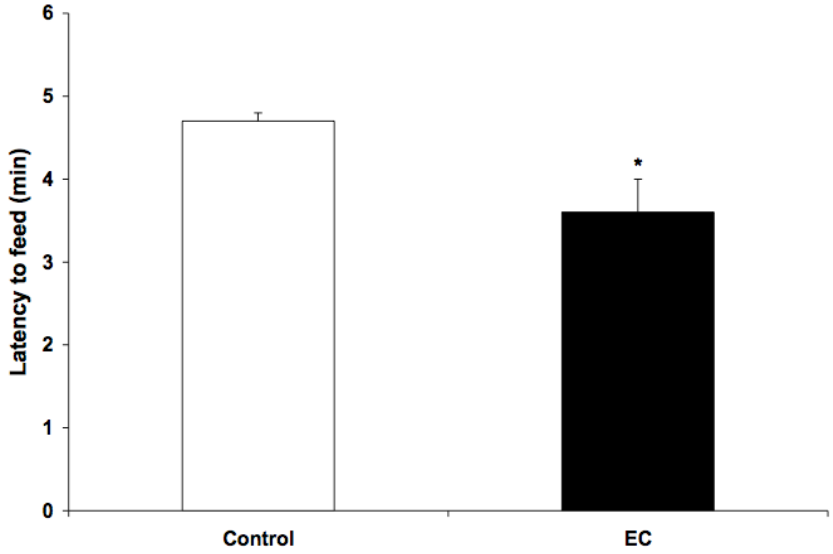
Figure 2-1: Environmental Complexity increases FGF-2 gene expression in the hippocampus.



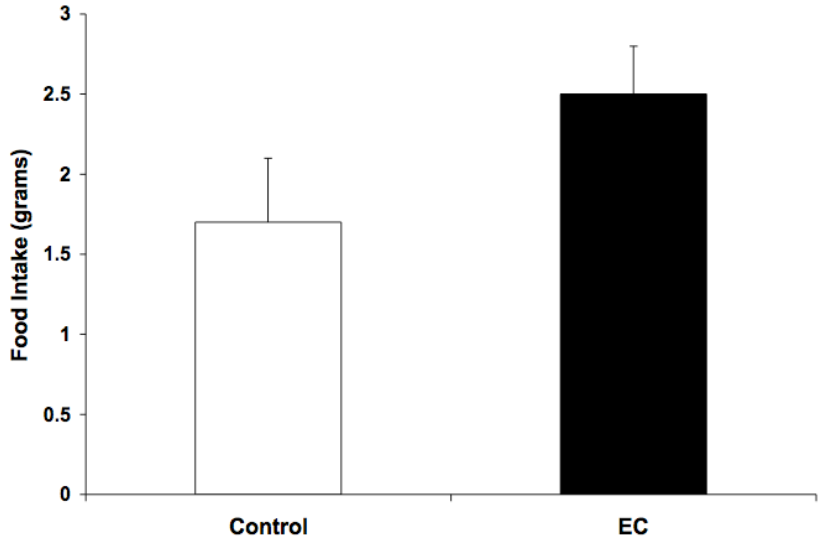
EC significantly increased FGF-2 expression in the Dentate Gyrus, [$T_{(21)}$=4.53, p<0.001], CA3 region, [$T_{(21)}$=3.5, p<0.01], and CA1 region, [$T_{(21)}$=5.6, p<0.0001] whereas no significant effects where observed in the CA2 region [$T_{(21)}$=0.23, p=0.82](n=11-12 per group).

Figure 2-2: Environmental Complexity decreases anxiety-like behavior

A.



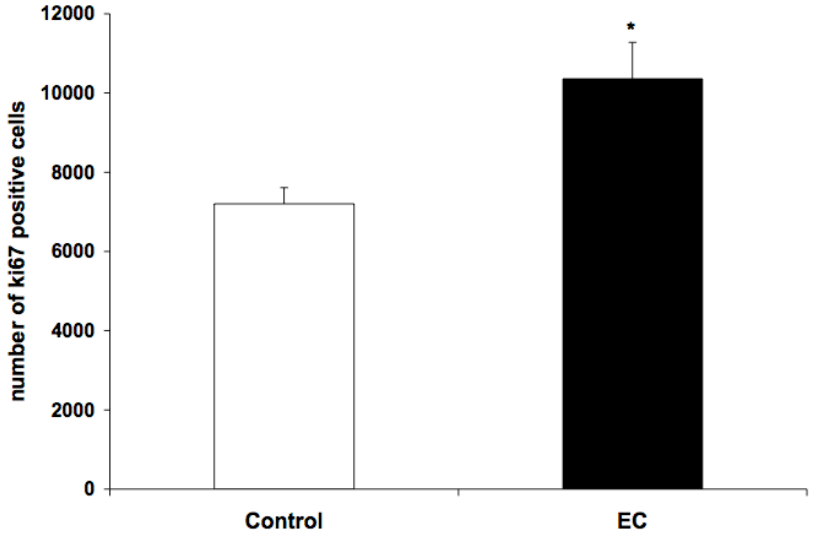
B.



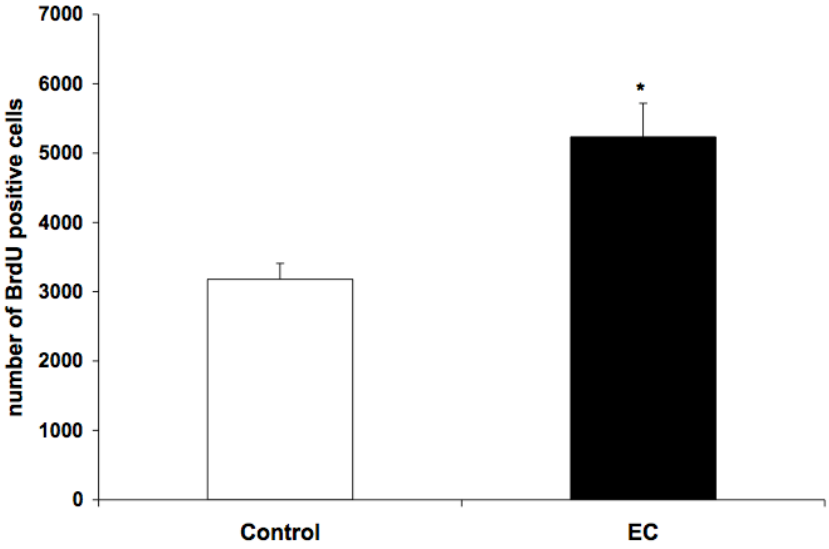
A) EC training decreases anxiety-like behavior as shown by a decrease in the latency to feed on the novelty suppressed feeding test [$T_{(20)}$=-2.2, p<0.05]. B) These results were specific to the novelty arena as no differences were observed in home cage feeding [$T_{(20)}$=1.6, p>0.05] (n=10-12 per group).

Figure 2-3: Environmental Complexity increases hippocampal cell genesis

A.



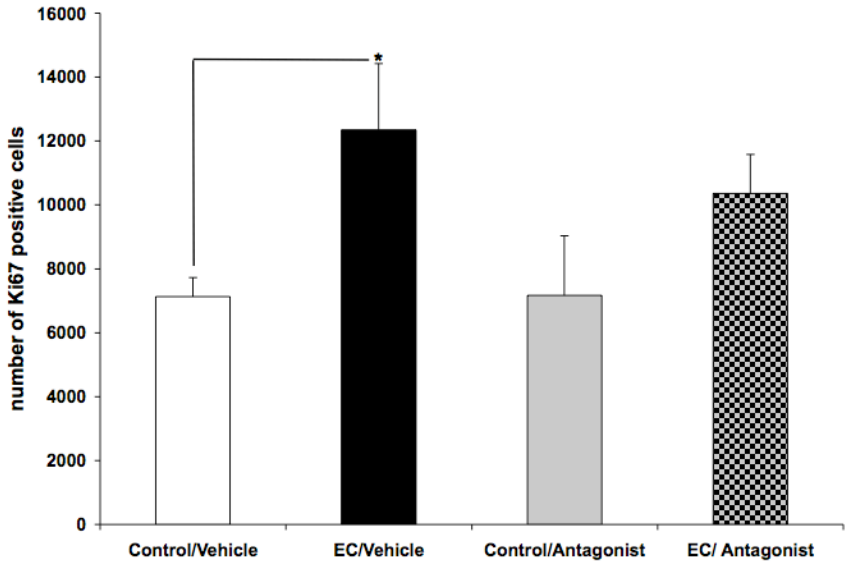
B.



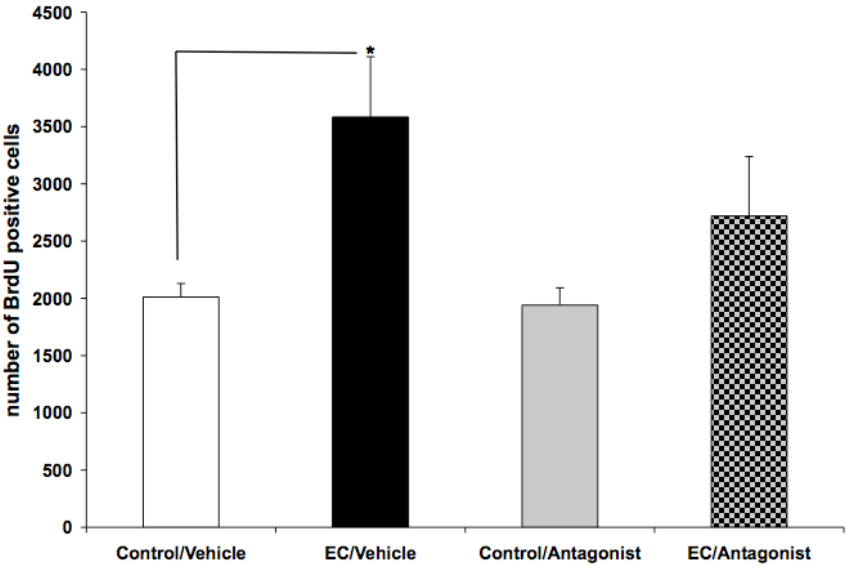
A) EC significantly increases cell proliferation in the Dentate Gyrus as shown by an increase in the number of Ki67 labeled cells, [$T_{(15)}$=2.8, p<0.05]. B) EC also increase survival of newly born cells in the dentate gyrus as measured by the number of Brd-U labeled cells [$T_{(15)}$=3.7, p<0.01]. (n=8-9 per group).

Figure 2-4: FGF receptor antagonism blocks the effects of Environmental Complexity on increasing cell proliferation and survival

A.

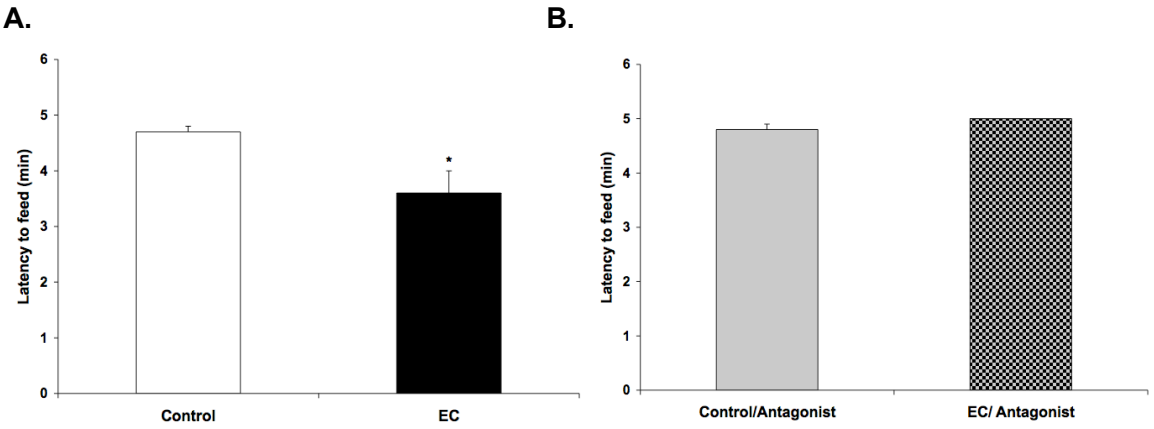


B.



A) EC results in a significant increase in cell proliferation as shown by an increase in the number of Ki67 cells [$F_{(1,8)}$=5.8, p<0.05], however such effects are lost when EC animals are treated chronically with an FGF receptor antagonist [$F_{(1,8)}$=2.2, p=0.20]. B) Similarly, EC's significant effects on increasing new cell survival [$F_{(1,8)}$=10.6, p<.01] where lost with concurrent treatment of an FGF receptor antagonist [$F_{(1,8)}$=2.1 p=0.20] (n=5 per group)

Figure 2-5: FGF receptor antagonism blocks the anxiolytic effects of Environmental Complexity



Under basal conditions EC animals respond with a significant decrease in anxiety as shown by a decrease in the latency to feed [$T_{(20)}$=-2.2, p<0.05]. On the other hand, when EC animals are treated with an FGF antagonist this significant change in anxiety behavior in lost. Thus, FGF receptor blockade results in a disruption of the anxiolytic effects of EC [$T_{(16)}$=.8, p>0.05] (n=8-10 per group)

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Chapter 3

Hippocampal Fibroblast Growth Factor System Modulates Anxiety-like Behavior Acutely: Effects in Selectively Bred Lines of Rats with Differing Anxiety Behavior

Abstract

The fibroblast growth factor (FGF) system, along with other growth factors, has been implicated in the pathophysiology of psychiatric illness including depression. Moreover, growth factors such as BDNF have been shown to reduce depressive-like behavior, while increasing anxiety behavior. To further characterize the role of the FGF system on anxiety, we tested the effects of FGF-2 in the High Responder (HR), Low Responder (LR) model of individual differences in emotionality, were HR animals show decreased anxiety like behavior compared to LR in the Elevated Plus-Maze (EPM). We also tested the effects of FGF receptor blockade on anxiety via hippocampal microinjections of the PD173074 FGF receptor antagonist. Acute administration of FGF-2 resulted in an overall increase in anxiety, and this was differentially observed in HR animals as demonstrated by their decreased time spent in the open arm of the EPM. Furthermore, the role of the hippocampal FGF system in enhancing anxiety acutely was supported by the anxiolytic effect of the FGF receptor

antagonist PD173074 upon direct microinjection in the hippocampus. The anxiolytic effect of FGF receptor blockade was evidenced by increased time spent in the center of the Open Field as well as time spent in the open arms of the EPM. Taken together our results demonstrate a complex role of hippocampal FGF-2 in regulating anxiety-like behavior.

Introduction

FGF-2 is a member of the FGF gene family composed of 24 proteins that have multiple functions including angiogenesis and nervous system development (Eckenstein et al., 1991). In the adult, FGF-2 shows prominent expression in astrocytes throughout the brain, whereas neuronal expression is almost exclusive to the hippocampus (Woodward et al., 1992). Several isoforms of FGF 2 ranging from 18 kD to 34 kD have been identified (Nugent and Iozzo, 2000). FGF-2 exerts its functions by interacting with four receptor types with varying affinity depending on ligand and receptor isoforms (Ornitz, 2000, Reuss and von Bohlen und Halbach, 2003). These receptors are trans-membrane glycoproteins containing three Ig-like loops (I-II-III) in the extracellular domain and a split tyrosine kinase domain. Three of the FGF receptors, FGFR1, FGFR2 and FGFR3, are expressed in the brain (Eckenstein, 1994), while FGFR1 is most abundantly expressed in the hippocampus (Belluardo et al., 1997).

A current hypothesis proposes that a deficiency in growth factors is a key contributor to the pathophysiology of mood disorders (Duman and Monteggia, 2006). While previous work had focused on BDNF, findings by our research group first demonstrated a down-regulation of several members of the FGF family, including FGF-2 in postmortem brains of subjects that had suffered from major depression (Evans et al., 2004). We have recently extended these human findings to animals and demonstrated a down regulation of hippocampal FGF-2 in a social stress model of depression in the rat (Turner et al., 2008a).

While our group has established the potential role of the FGF system in mood disorders, most studies on antidepressants have mostly focused on the role of BDNF as a mediator of the long-term effects of these drugs on behavior and mood (Duman, 1998, Shirayama et al., 2002, Duman, 2004). However, reports have also shown that antidepressant treatment increases FGF-2 expression in rat hippocampus (Mallei et al., 2002). These findings suggest that decreased levels of FGF-2 may be involved in the pathophysiology or the course of severe depression, and that antidepressants may exert their effects by increasing FGF-2. Furthermore, while BDNF has been shown to display antidepressant effects in animal models of depression, (Shirayama et al., 2002) recent findings from our laboratory show that FGF-2 also exerts antidepressant effects (Turner et al., 2008b).

While this body of evidence points to a role of endogenous FGF-2 in exerting antidepressant effects, it also raises an interesting dilemma: stimuli that lead to increased anxiety such as stress, have also been linked to enhanced FGF-2 expression. For example increased hippocampal FGF-2 has been shown in response to acute restraint stress (Molteni et al., 2001, Fumagalli et al., 2005). Furthermore, exposure to acute predator stress has resulted in increased levels of FGF-2 expression as measured by microarray in PVG hooded rats, which display high anxiety behavior (Wang et al., 2003). Moreover, manipulations of corticosterone levels have been shown to modulate FGF-2 expression in the hippocampus. Specifically, adrenalectomy resulted in decreased FGF-2

expression, whereas corticosterone replacement rescued these effects suggesting that corticosterone modulates levels of FGF-2 expression in the hippocampus (Molteni et al., 2001). Furthermore, exogenous corticosterone has been shown to directly impact and increase FGF-2 expression in the hippocampus while corticosterone synthesis inhibition blocked the stress-induced FGF-2 response (Frank et al., 2007).

There are some lines of evidence that can help reconcile these two disparate findings regarding the relationship between FGF-2 and emotional responses. Given the antidepressant effects of FGF-2 noted above, and the increase in FGF-2 triggered by classical antidepressant drugs, it is important to note that several antidepressants have been known to increase anxiety during the early phases of treatment (Bagdy et al., 2001). Interestingly, while BDNF over-expressing mice display an antidepressant-like behavioral phenotype, they also exhibit an increase in anxiety behavior (Govindarajan et al., 2006). This supports the notion that growth factors may exert antidepressant properties but their effects may also extend to enhance anxiety behavior, at least in the short term, in a fashion similar to that of antidepressants. While the antidepressant role of FGF-2 has been described (Turner et al., 2008b), its role in modulating anxiety has yet to be explored. Thus, the current experiments were aimed at exploring the potential impact of hippocampal FGF-2 on anxiety behavior after acute administration.

While the current studies will explore the potential effects of acute FGF-2 on anxiety behavior it is important to also evaluate the chronic effects of FGF-2. This is important, as acute treatments with selective serotonin reuptake inhibitors (SSRIs) and other antidepressants are known to be anxiogenic (Bagdy et al., 2001), whereas their antidepressants effects extend to reduce anxiety after chronic treatment (Dulawa et al., 2004). Thus, it is possible that treatments with FGF-2 may have a similar response as antidepressants on measures of anxiety, where acute administration could serve anxiogenic effects whereas chronically they may serve to reduce anxiety.

However, in light of this potential discrepancy, in this chapter we first focus on investigating the acute effects of FGF system manipulations on anxiety. Specifically we first tested the impact of an FGF antagonist on anxiety acutely as previous results from Chapter 2 suggested that FGF receptor activation is required for environmental complexity to decrease anxiety. Finally, we followed these studies with acute treatments with FGF-2 in order to test whether FGF-2 specifically, altered anxiety given that in our previous chapter hippocampal FGF-2 seemed to correlate with decrease anxiety as a result of EC. Thus, our current experiments were aimed at testing whether FGF-2 has a direct role in modulating anxiety-like behavior.

Materials and Methods

Animals

Male Sprague-Dawley rats (375-450g) were selectively bred based on their locomotor response to novelty at our in-house colony at the Molecular and Behavioral Neuroscience Institute (MBNI). Adult rats (85-90 day old) were housed two animals per cage after locomotor screening (were an HR animal was always paired with an LR animal under a 12 hr light/dark cycle with food and water available *ad libitum.* *A*nimals were allowed to acclimate to the housing conditions for 7 days prior to any experiments. 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 of the animal ethics committee at the University of Michigan.

Locomotion Testing

For each generation of breeding, naïve animals were handled for three consecutive days prior to testing to familiarize them with the investigator, then screened for locomotor response to a novel environment by placing them in a standard size (43×21.5×24.5) clear acrylic cage in a different room from where the animals had been housed. Locomotor activity was monitored every 5 minutes for 1 hour by two panels of photocells connected to a computer. The first panel of three photocells was placed at ground level to record horizontal locomotion, with the second panel of five photocells located near the top of the cage to determine rearing behavior. The locomotion testing rig and motion

recording software were created in-house at the University of Michigan. Locomotion activity was tested between 9.00 and 11.30 am. Final locomotion scores were determined by summing horizontal and rearing activities.

Peripheral FGF-2 Injections

Rats were administered FGF2 (1ng/g, 10ng/g, or 20ng/g i.p.) or vehicle (0.1M PBS with .1% BSA). Animals were tested for anxiety-like behavior 8 hrs after injection on the Elevated Plus Maze (EPM) test, as it has been shown previously that brain levels of FGF2 peak 8 hours post peripheral injections of bFGF (Wagner et al.1999).

Surgery and Microinjections

Rats were anesthetized using isoflurane before surgery and bilateral guide cannulae (22-gauge, Plastics One Inc., VA. USA) were implanted into the dorsal hippocampus (coordinates from bregma: AP:-5.0 ML:+3.5 DV:-2.1) using a stereotaxic apparatus. The guide cannulae were anchored to the skull and fitted with an obdurator. Seven days after surgery, the obdurators were removed and 28-gauge injector cannulae were inserted extending 1.5mm below the tip of the guides. The cannulae were connected by PE-20 tubing to a Hamilton syringe mounted on a syringe pump (Harvard Apparatus, MA. USA). LR rats were microinjected with PD173074 (Pfizer) at 1nM, 10nM and 100nM, doses dissolved in artificial extracellular fluid (aECF). Total volume of 1ul was infused at a rate of

1.0µl/min, and injectors were left in place for an additional 2 min to allow diffusion of the drug. Animals were then subjected to behavioral testing within 5 minutes of injection. Rats were killed by decapitation and the brains were snap frozen in isopentane after behavioral testing. The brains were sliced and cresyl violet staining was performed to verify the placement of the microinjection cannulae.

Elevated Plus Maze (EPM)

Rats were tested 5 min after the microinjection or 8 hours post peripheral injections on the elevated plus maze, which was constructed of black Plexiglas, with four elevated arms (70 cm from the floor, 45 cm long, and 12 cm wide). The arms were arranged in a cross, with two opposite arms enclosed by 45-cm-high walls, and the other two arms open. At the intersection of the open and closed arms, there was a central 12×12 cm square platform giving access to all arms. The test room was dimly lit (approximately 40 lux), and behavior was monitored using a computerized videotracking system (Noldus Ethovision, Leesburg, VA). At the beginning of the 5 minutes test, each rat was placed in the central square facing a closed arm. The computerized tracking system recorded the latency to first enter the open arm, the amount of time spent in the open arm, closed arm, or center square over the course of the 5 minutes test. Behavior testing was performed between 8.00 and 11.30 am.

Open Field

The open field maze was a 150×150×50 cm^3 white Plexiglas box with the floor marked into 16 equals 37.5 cm^2 squares. Testing was conducted under dim light (40 lux) and recorded using a computerized videotracking system (Noldus Ethovision, Leesburg, VA). The experiment was started by placing the rat into one corner of the open field. The computerized tracking system recorded the amount of time spent in the center, periphery, or corner of the test apparatus over the course of the 5 minutes test. Behavior testing was performed between 8.00 and 11.30 am.

Statistical Analyses

Behavioral studies were analyzed by T-test or Analysis of Variance. Data are presented as mean $\pm$ SEM and significance was assumed at $P<0.05$.

Results

Hippocampal FGF receptor blockade decreases Anxiety-like Behavior

To begin examining the role of the FGF system on anxiety we first aimed to determine the effects of acute FGF receptor blockade on anxiety-like behavior. For these experiments we targeted the dorsal hippocampus due to its high density of neuronal FGF receptor expression and given that the hippocampus has previously been implicated in modulating individual differences in anxiety behavior as seen in HR and LR. As seen in **Figure 3-1.,** hippocampal microinjections with the FGF receptor antagonist PD173074 resulted in an overall

decrease in anxiety behavior on the EPM as shown by an increase in the percent time spent in the open arm [$t_{(13)}$= 3.1, p<0.01] and a decrease in the latency to enter the open arm, [$t_{(13)}$= -.2.5, p<0.05]. Moreover, we also evaluated the effects of the FGF receptor antagonist PD173074 on the Open Field test, where we also observed a decrease in anxiety as seen by an increase in the percentage of time spent in the center [$t_{(10)}$=3.5, p<0.01] and a decrease time spent in the corners [$t_{(10)}$= -2.3, p<0.05]. Finally, we also evaluated the extent of the effects of the FGF receptor antagonist on the EPM to determine whether such effects varied with doses. As seen in **Figure 3-2.,** we found that the effects of the PD173074 compound on decreasing anxiety are partially dose dependent with anxiolytic effects progressively increasing with increasing doses. Specifically, we saw that the middle dose at 10nM and highest dose at 100nM concentration were the most effective at increasing the percentage of time spent in the open arm compared to vehicle controls, (p<0.01).

Peripheral FGF-2 Increases Anxiety-like Behavior

Given that we saw an anxiolytic effect in response to hippocampal FGF receptor blockade we wanted to distinguish whether acute FGF-2 specifically mediates the FGF system's impact on anxiety behavior in the EPM. To this end we administered FGF-2 or vehicle peripherally (i.p) in HR and LR to test whether this impact was also dependent on individual differences in anxiety-like behavior. As seen in **Figure 3-3.,** acute treatment with FGF-2 results in an overall significant increase in anxiety-like behavior. Overall FGF-2 treated animals show an

increase in the latency to enter the open arms [$F_{(3,33)}$=4.7, p<0.05]. Moreover the anxiogenic effects of FGF-2 were also evident as shown by an overall decrease in the percent time spent in the open arms [$F_{(3,33)}$=5.6, p<0.05] and a decrease in the percent of open arm entries [$F_{(3,33)}$=5.2, p<0.05]. We also saw a significant interaction effect of treatment and phenotype where Fisher's PLSD post hoc tests revealed that FGF-2 treated HR animals showed a differential decrease in the percent time spent in the open arms relative to vehicle treated control HRs (p<.01).

Following our initial results where FGF-2 resulted in enhancement of anxiety-like behavior an acute dose response study was performed to determine whether the anxiogenic effects of FGF-2 observed on the EPM where dose dependent. For these purposes FGF-2 was injected at 1ng/g, 10ng/g and 20ng/g to HR and LR animals eight hours prior to testing on the EPM. As seen in **Figure 3-4.,** we saw an overall effect of phenotype [$F_{(7,57)}$=4.7, p<0.001] where HR animals show less anxiety behavior relative to LRs as seen by an overall higher percentage of time spent in the open arms of the EPM. Furthermore, there was an interaction effect of treatment where Fisher's PLSD post hoc tests revealed that FGF-2 results in a differential increase in anxiety at the 10ng/g dose relative to the vehicle treated animals as shown by a decrease in the percentage of time spent in the open arm (p<0.05). These results show that FGF-2 shows a dynamic U-shaped curve effect on anxiety-like behavior on the EPM as no differences in anxiety were observed at the lower (1ng/g) and higher (20ng/g) doses. Moreover, as seen in

our initial experiments there was an interaction effect of phenotype and treatment [$F_{(7,57)}$=5.1, p<0.01]. Fisher's PLSD post hoc tests revealed that FGF-2 at the 10ng/g dose differentially increases anxiety in HRs relative to the vehicle treated HRs as shown by a decrease in the percentage of time spent in the open arms (p<0.0001).

Discussion

The present study demonstrates for the first time the direct role of the hippocampal FGF system in modulating anxiety behavior. Our results show that acute exogenous treatment with FGF-2 increases overall anxiety like behavior, with anxiogenic effects being stronger in Selectively Bred HR animals, which normally display lower anxiety relative to Selectively Bred high anxiety LRs (Stead et al., 2006). Moreover, effects of FGF-2 on anxiety displayed a U-shaped curve as shown by the anxiogenic effects of the middle dose and lack of effects from lower and higher doses. Finally, in support of the modulatory role of the endogenous FGF system on anxiety-like behavior, intra-hippocampal blockade of FGF receptor decreased anxiety in the EPM and Open Field Test. Taken together the results presented above implicate the hippocampal FGF system as key a modulator of anxiety behavior and suggest that acutely FGF-2 increases anxiety like behavior.

Our findings showed acute peripheral treatments with FGF-2 resulting in an overall increase in anxiety in the EPM as shown by an increase in the latency to

enter the open arm, a decrease in the percent time spent in the open arms and percent of open arm entries. Furthermore, our results show that FGF-2 differentially enhanced HR's anxiety behavior as shown by a selective decrease in the percent of time spent in the open arms. Although our results failed to show significant differential effects in the additional measures of anxiety such as the latency to enter open arm and open arm entries, our results due point to a trend for the anxiogenic effects of FGF-2 to be differential in HR's. For example, while our results do point to an overall effect of FGF-2 in increasing anxiety, they show that HRs display a three-fold change in the latency to enter the open arm compared to a two-fold change in LRs. Furthermore, the trend on the differential impact of FGF-2 treatment in HRs is even more evident in the percent of open arm entries as HRs show a 37% percent decrease compared to LRs, which show a 17% change. Taken together these results suggest that FGF-2 is more effective at increasing anxiety in animals that naturally display lower anxiety such as the HRs. On the other hand its anxiogenic impact on LR animals, which naturally display high anxiety is more moderate. These differences in response to FGF-2 could be related to a floor effect of the LR phenotype on anxiety or to existing neurobiological differences between HRs and LRs related to levels of FGF-2 expression in the hippocampus.

While our results support the notion that FGF-2 modulates anxiety-like behavior, our findings with the use of the FGF receptor antagonist specifically point to the hippocampal FGF system as a key player in maintaining an endogenous tone of

anxiety. As presented above, intrahippocampal treatment with an FGF receptor antagonist resulted in decrease anxiety as shown by a decrease in the latency to enter the open arms and an increase in the percent of time spent in the open arms. Furthermore, the anxiolytic effects of FGF receptor blockade were also observed in the Open Field test as shown by a decrease in the percent of time spent in corners and an increase in the percent of time spent in the center. Finally, our results show that FGF blockade effects on reducing anxiety are dependent on dose. Doses of 10nM and 100nM, which have previously shown to block FGF-2 mediated effects on proliferation (Bansal et al., 2003), showed significant differences on decreasing anxiety, while a lower dose of 1nM was not effective at altering anxiety behavior. These results confirm that hippocampal FGF receptor activity is required for maintaining an endogenous tone on anxiety behavior.

The present study presents two major findings for the first time; acute treatment with FGF-2 increases anxiety-like behavior and hippocampal FGF receptor blockade reduces anxiety, thus directly implicating a novel function for this growth factor in modulating emotional behavior. Although these findings represent a new function for hippocampal FGF-2 by regulating emotionality, previous reports also support the role of growth factors in being active modulators of emotional behavior. Specifically, transgenic mice overexpressing BDNF show higher anxiety behavior relative to wildtype (Govindarajan et al., 2006). However, these animals overexpressed BDNF in the entire forebrain while

its functional relevance to anxiety was regarded as being modulated by the amygdala.

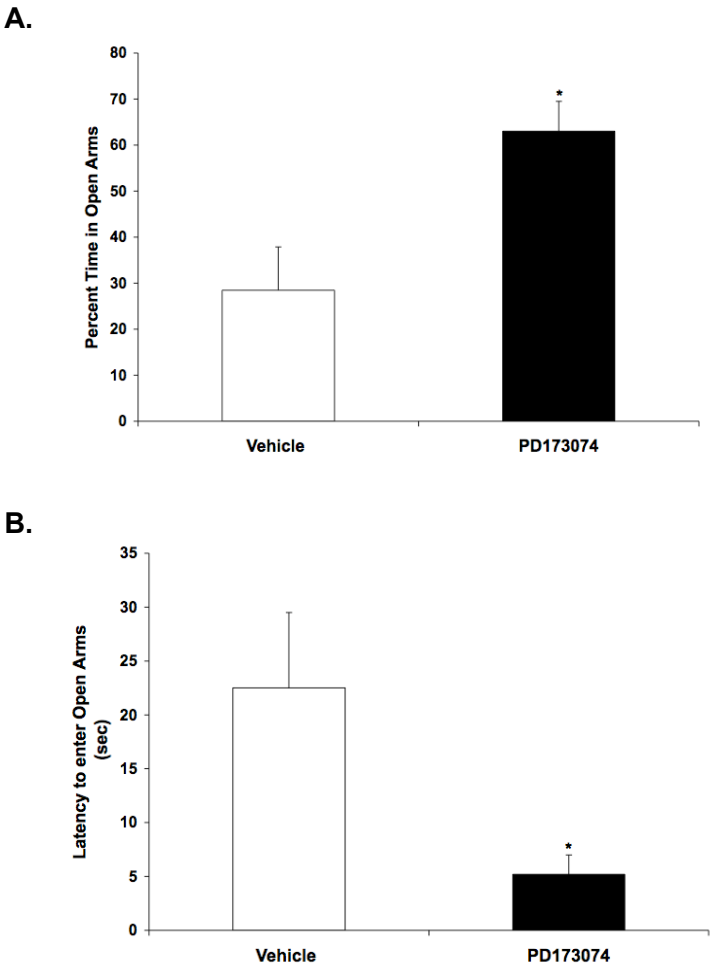
Conversely, our results point to the hippocampus as being an active modulator of FGF mediated anxiety behavior. Although most reports point to the amygdala as being the key structure responsible for modulating anxiety, several reports do support the role of the hippocampus in anxiety behavior. It has been suggested that the hippocampal lesions produce anxiolytic effects through behavioral disinhibition (Bannerman et al., 2002, Bannerman et al., 2004). These reports include anxiolytic effects on tests of anxiety such as the novelty suppressed feeding test, and the Elevated Plus maze (Bannerman et al., 2002). Thus our results support the notion that the hippocampus is an active player in the modulation of anxiety and further elaborate on one specific molecule, FGF-2, as being a key modulator.

Finally while FGF-2 acutely was shown to be anxiogenic, it is important to consider that opposite effects may occur in response to chronic treatment. As previously mentioned acute treatment with SSRI antidepressants increases anxiety (Bagdy et al., 2001) whereas chronically they are anxiolytic (Dulawa et al., 2004). Therefore, it is plausible that FGF-2 may have similar effects on anxiety given that FGF-2 increases in response to chronic antidepressant treatment (Mallei et al., 2002). Moreover, FGF-2 itself has previously been shown to have antidepressant-like effects (Turner et al., 2008b). Thus, FGF-2 seems to

show a therapeutic profile equal to SSRI antidepressants, where it reduces depressive like behavior, but not without first increasing anxiety acutely. This leads us to suspect that FGF-2 may have anxiolytic effects after chronic treatment. In support of this notion are the findings from our previous chapter showing EC increasing FGF-2, while requiring FGF receptor activation for its anxiolytic effects. Consequently, the following chapter will specifically test whether chronic FGF-2 reduces anxiety behavior. Moreover, we will further test the extent to which the HR and LR anxiety phenotypes are important factors in mediating such effects.

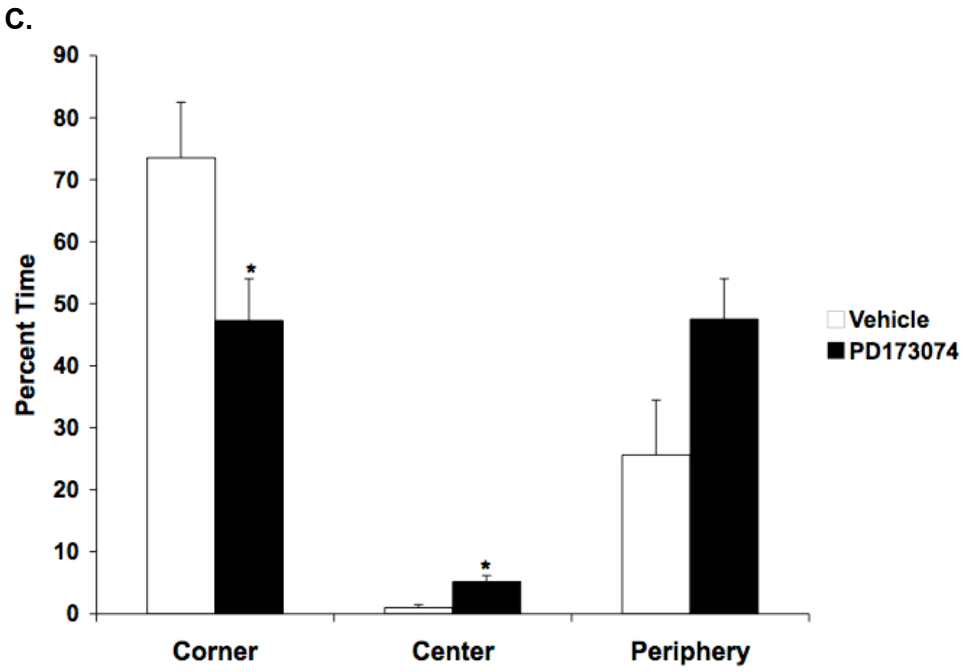
Results Figures

Figure 3-1: Hippocampal FGF receptor blockade decreases anxiety-like behavior



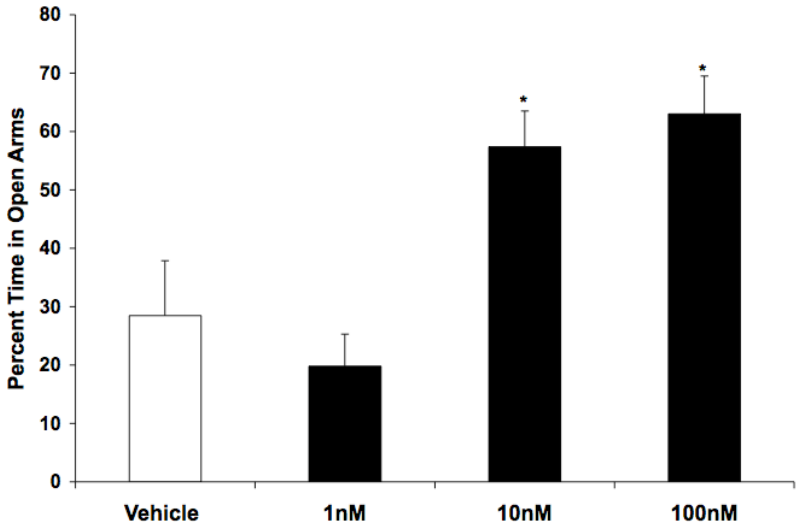
A) FGF receptor antagonist PD173074 decreases anxiety on the EPM as shown by an increase in the percent of time spent in the open arm [$T_{(13)}$= 3.1, p<0.01 and B) a decrease in the latency to enter the open arm, [$T_{(13)}$= -.2.5, p<0.05]. (n=7 per group)

Hippocampal FGF receptor blockade decreases anxiety-like behavior



C) FGF receptor antagonist PD173074 decreases anxiety on the Open Field as shown by an increase in the percentage of time spent in the center [$T_{(10)}$=3.5, p<0.01] and a decrease time spent in the corners [$T_{(10)}$= -2.3, p<0.05] (n=6 per group).

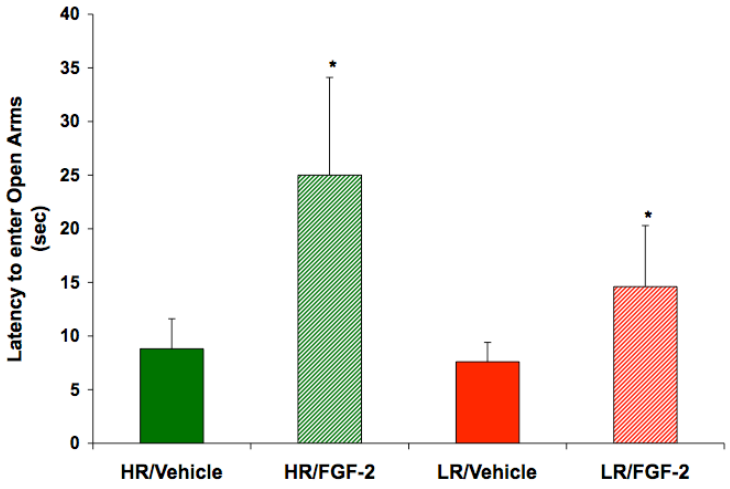
Figure 3-2: Hippocampal FGF receptor blockade decreases anxiety-like behavior



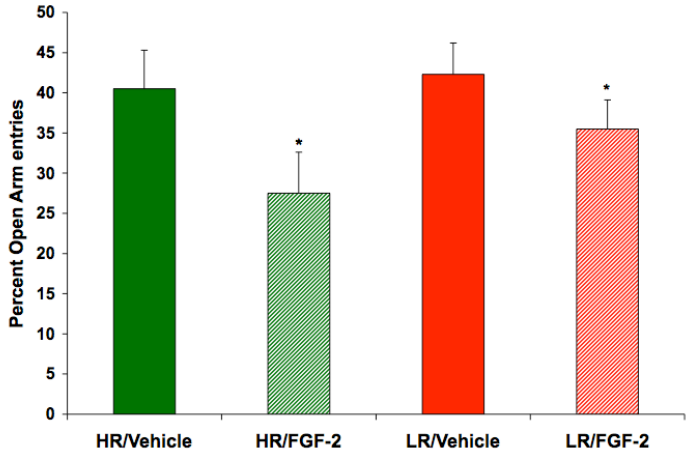
FGF receptor blockade reduces anxiety. FGF receptor antagonist PD173074 decreases anxiety in a dose dependent manner were the middle dose at 10nM and the highest doses at 100nM concentration were both equally effective at increasing the percentage of time spent in the open arm compared to vehicle controls, (p<0.01) (n=7 per group).

Figure 3-3: FGF-2 Increases anxiety-like behavior

A.



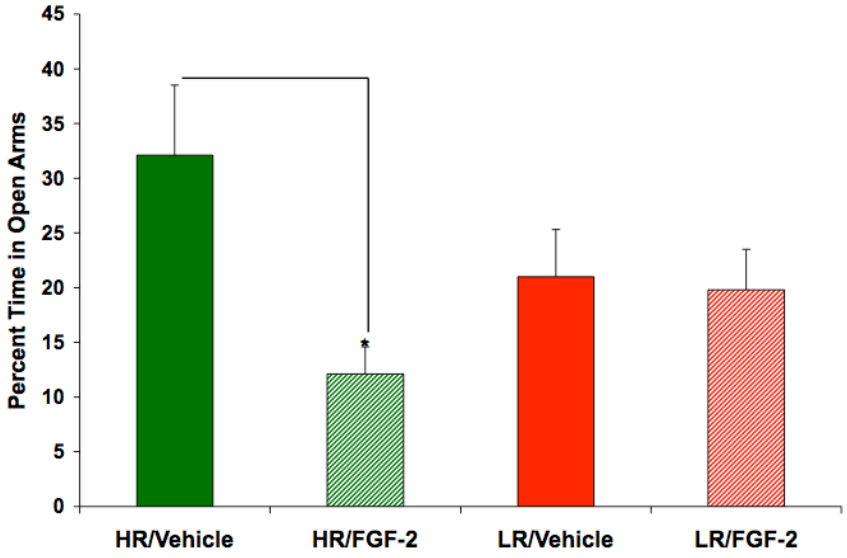
B.



A) FGF-2 significantly increases anxiety-like behavior as shown by an overall increase in the latency to enter the open arms) [$F_{(3,33)}$=4.7, p<0.05]. B) FGF-2 anxiogenic effects are also observed as an overall decrease in the percent of open arm entries [$F_{(3,33)}$=5.2, p<0.05]. n=9-11 per group)

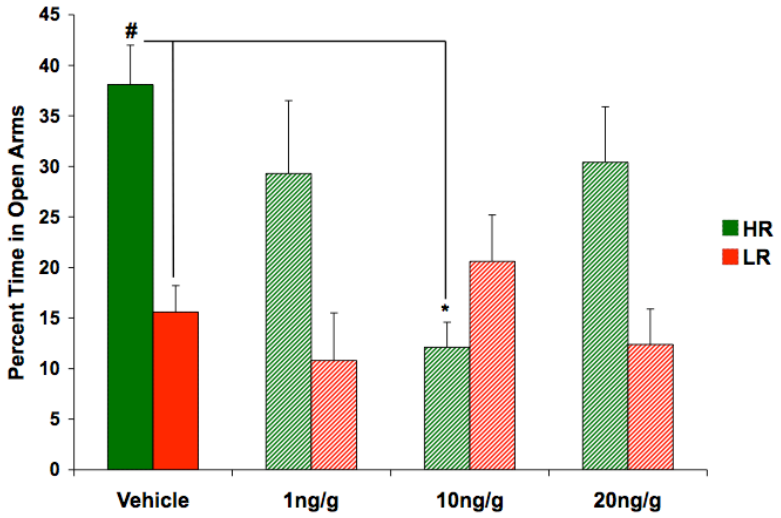
FGF-2 Increases anxiety-like behavior

C.



C) FGF-2 resulted in an overall decrease in the percent of time spent in the open arms [$F_{(3,33)}$=5.6, p<0.05]. A significant interaction of phenotype and treatment was also observed as FGF-2 resulted in a differential decrease in the percentage of time spent in the open arms in HR animals (p<0.01).

Figure 3-4: FGF-2 increases anxiety in a dose dependent manner in HR animals



FGF-2 shows a dynamic U-shaped curve effect on anxiety-like behavior on the EPM. FGF-2 differentially increases anxiety at the 10ng/g dose relative to the vehicle treated animals as shown by a decrease in the percentage of time spent in the open arm ($p<0.05$) as no effects were seen at higher or lower doses. HR animals show less anxiety behavior relative LRs as seen by an overall higher percentage of time spent in the open arms of the EPM [$F_{(1,57)}=4.7$, $p<0.001$]. There was also an interaction effect of phenotype and treatment [$F_{(7,57)}=5.1$, $p<0.01$], where Fisher's PLSD post show FGF-2 at the 10ng/g dose differentially increasing anxiety in HRs relative to the vehicle treated HRs as shown by a decrease in the percentage of time spent in the open arms ($p<0.0001$) (n=7-9 per group)

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Chapter 4

A New Role for FGF-2 as an Endogenous Inhibitor of Anxiety

Abstract

Human postmortem studies have demonstrated that FGF-2 expression is decreased in the brain of severely depressed individuals. It remained unclear, however, whether this is a consequence of the illness or whether FGF-2 plays a primary role in the control of mood and emotions. In this series of studies, we first asked whether endogenous FGF-2 expression correlates with spontaneous anxiety, a trait associated with vulnerability to severe mood disorders in humans. This was tested in two genetically distinct groups of rats selectively bred to differ dramatically in their response to novelty and to anxiety-provoking conditions (HRs= Low Anxiety/High Response to Novelty vs. LRs= High Anxiety/Low Response to Novelty). We demonstrated that the Low-Anxiety HRs have significantly elevated levels of hippocampal FGF2 mRNA relative to the High-Anxious LRs, and that there exists a highly significant inverse correlation between FGF-2 levels and anxiety behavior. We then demonstrated that FGF-2 expression is modulatable by environmental factors that alter anxiety, thus environmental complexity (EC) reduced anxiety behavior and induced FGF-2

expression in hippocampus, particularly in the High Anxious LRs. Finally, we directly tested the role of FGF-2 as an anxiolytic and show that a 3-week treatment regimen of peripherally administered FGF-2 was highly effective at blunting anxiety behavior, specifically in the high anxiety LRs. This treatmen effect was accompanied by an increase in survival of hippocampal adult stem cells, both neurons and astrocytes, again specifically in the LRs. Taken together, these findings implicate hippocampal FGF-2 as a novel modulator of anxiety behavior and underscore its potential as a new target for treatment of mood and anxiety disorders.

Introduction

The molecular factors that impart either vulnerability or resilience to mood and anxiety disorders remain elusive in spite of active efforts to elucidate their genetic bases. Gene expression profiling in postmortem human brains represents a complementary approach that can uncover molecular changes associated with these disorders. Expression profiling of the frontal cortex of severely depressed individuals revealed that the fibroblast growth factor (FGF) system was the most significantly altered family of molecules relative to controls (Evans et al., 2004). This work has since been extended to other brain regions, including the hippocampus and amygdala and has shown a consistent decrease of FGF2 mRNA and FGF-R2 mRNA in most brain regions examined (Akil et al., 2008). In particular, the decrease in FGF-2 mRNA has been independently documented in the hippocampus (Gaughran et al.), supporting the association between FGF system dysregulation and mood disorders.

However, it is difficult to distinguish whether the observed differences represent predisposing factors to the illness or are secondary to the disease process. We therefore undertook a series of studies using animal models to ask whether members of the FGF families can indeed modulate affective behavior, and whether they may constitute predisposing factors for individual differences in emotional reactivity. Here we focused on the role of the FGF system in anxiety-like behavior, given that anxiety is one of the hallmarks of depression, that there is significant co-morbidity between anxiety and major depression and that there

is mounting evidence that the two disorders may be closely associated due to a common etiology or risk factors as reviewed by, (Gorwood, 2004). We focused on FGF-2 because it is one of the 22 FGF family members that exhibit the most consistent changes in major depression, as well as being one of the best studied FGF's in the central nervous system. FGF-2 exerts its function by interacting with four receptors (Ornitz, 2000, Reuss and von Bohlen und Halbach, 2003), three of the which, FGFR1, FGFR2 and FGFR3, are expressed in the brain (Eckenstein, 1994), with FGFR1 being most abundantly expressed in the hippocampus (Belluardo et al., 1997). This growth factor is critical in the development of the mammalian brain (Raballo et al., 2000), is known to modulate hippocampal neurogenesis in the developing brain (Wagner et al., 1999) and has been implicated in adult neurogenesis (Pieper et al., 2005) (Palmer et al., 1995)

In order to test the possible role of FGF-2 in affective behavior, we relied on an animal model in which we used a genetic selection strategy in Sprague-Dawley rats to enhance basal differences in novelty-seeking and spontaneous anxiety behaviors (Stead et al., 2006). Thus, after several rounds of breeding, the selectively bred line of High Responders (HR) exhibits significantly greater exploration of a novel environment relative to oubtred Sprague-Dawley rats, whereas the selectively bred line of Low Responders (LR) exhibits significantly lower exploration than outbred animals, as well as dramatic differences from the selectively bred HRs. As importantly, the two groups display consistent and profound differences in all tests of spontaneous anxiety, with HR's exhibiting low

and LR's exhibiting high anxiety behavior. Beyond these basal differences due to genetic background, we also manipulated environmental conditions, by enriching the rats' environment and increasing its complexity (EC) and asked whether this manipulation impacted anxiety behavior in the two selectively bred lines. This model then tested both the possible genetic differences in FGF-2 and the environmental modulation of its expression. Given the results from these endogenous studies, we went on to administer exogenous FGF-2 in order to directly test its potential role in modulating anxiety behavior in the two groups of rats and ascertain the correlates of this treatment on the proliferation and survival of adult newborn cells in the hippocampus.

Materials and Methods

Animals

Adult male Sprague-Dawley rats were obtained from our in-house breeding colony at the Molecular and Behavioral Neuroscience Institute (MBNI) where we have maintained the selectively-bred HR-LR lines for over 19 generations. HR-LR lines were selectively bred based on their differences in exploratory response to novelty a characteristic initially used to predict individual differences in drug taking behavior (Piazza et al., 1989). We recently published a description of our breeding strategy and an initial behavioral characterization of the HR-LR differences in anxiety-like behavior (Stead et al., 2006). We have also shown that this behavior is likely genetic as it shows little change when animals are cross-fostered to a mother from the opposing line (Clinton et al., 2007). An

additional set of outbred Sprague-Dawley rats obtained from Charles River was used to provide an intermediate phenotype and determine the correlation of anxiety behavior and FGF-2 expression in the hippocampus. Adult rats (300g-400g) around 2 months old were housed two animals per cage (one HR, one LR per cage) after locomotor screening under a 12 hr light/dark cycle (lights on at 6:00 am) with food and water available *ad libitum*. Animals were allowed to acclimate to the housing conditions for at least 7 days prior to any experiments. 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 (UCUCA) at the University of Michigan.

Locomotion Testing

At postnatal day 55-60, adult male rats from our selective breeding colony were screened for locomotor response to a novel environment by placing them in a standard size (43×21.5×24.5) clear acrylic cage in a different room from where the animals had been housed. Locomotor activity was monitored every 5 minutes for 1 hour by two panels of photocells connected to a computer. The first panel of three photocells was placed at ground level to record horizontal locomotion, with the second panel of five photocells located near the top of the cage to determine rearing behavior. The locomotion testing rig and motion recording software were created in-house at the University of Michigan. Locomotion activity was tested between 9:00 and 11:30 am. Final locomotion scores were determined by

summing horizontal and rearing activities verifying the differential acivity reposne to novelty between selectively bred HRs and LRs. (see appendix 4-3)

Light-dark box (LDB) anxiety test

The LDB apparatus is a 30 × 60 × 30-cm Plexiglas shuttle box with a translucent cover. The floor is composed of stainless steel bars suspended above corncob bedding. Each box is divided into two equal-sized compartments by a wall with a 12 cm-wide open door. One compartment is painted white and brightly illuminated, and the other is painted black with very dim light. Time spent in each compartment is monitored by rows of five photocells located 2.5 cm above the grid floor of each compartment, and total time spent in each compartment is recorded with a microprocessor. Animals that spend less time in the illuminated compartment and more time in the dark compartment are classified as showing greater anxiety behavior.

Elevated Plus Maze (EPM)

The elevated plus maze is constructed of black Plexiglas, with four elevated arms (70 cm from the floor, 45 cm long, and 12 cm wide). The arms are arranged in a cross, with two opposite arms enclosed by 45-cm-high walls, and the other two arms open. At the intersection of the open and closed arms, there is a central 12×12 cm square platform giving access to all arms. The test room is dimly lit (approximately 40 lux), and behavior is monitored using a computerized video tracking system (Noldus Ethovision, Leesburg, VA). At the beginning of the

5 minutes test, each rat is placed in the central square facing a closed arm. The computerized tracking system records the latency to first enter the open arm, the amount of time spent in the open arm, closed arm, and center square over the course of the 5 minutes test. Behavior testing is performed between 8:00 and 11:30 am.

Environmental Complexity

To increase the complexity of the environment, rats were housed for 21 days in 3 × 3 × 3 ft stainless steel cages which contain different toys, obstacle courses, and enriching stimuli. The number of toys is increased over the course of the 3 weeks of EC. Thus, every day, animals are exposed to novel sensory stimuli and increased opportunities for exploration as part of their housing environment.

Chronic FGF-2

The Chronic FGF-2 regimen involved the administration of either FGF-2 (5 ng/g, i.p) or vehicle (0.1M PBS with 0.1% BSA) every day for three weeks. Systemic injections with this dose have previously been shown to alter neurogenesis (Wagner et al., 1999). The three-week treatment period is used to match the duration of the EC housing period**.** Animals are then tested for anxiety measures starting one day after the last injection.

mRNA *in situ* Hybridization

At the conclusion of each experiment, rats were sacrificed by rapid decapitation, and their brains removed, snap frozen in isopentane, and stored at -80 ºC. Brains were cryostat sectioned at -20°C at 20μm for the EC studies or 10μm for the basal HR-LR studies and sliced in series throughout the hippocampus, mounted on Super Frost Plus slides (Fisher Scientific) and stored at -80°C until processed. *In situ* hybridization methodology has been previously described in detail elsewhere (Kabbaj et al., 2000). The FGF-2 probe was labeled in a reaction mixture consisting of 1μg of linearized plasmid, 1X transcription buffer (Epicenter Technologies, Madison, WI), 125 μCi of 35S-labeled-UTP, 125 μCi of 35S-CTP, 150 μM ATP and GTP, 12.5mM dithiothreitol, 1 μl of RNase inhibitor, and 1.5 μl of T3 RNA polymerase. Radioactive signals were quantified using computer-assisted optical densitometry software (Scion Image Beta 4.03; Scion Corporation, Frederick, MD). Integrated densities were determined by outlining the region of interest from both hemispheres. Optical density measurements were corrected for background, and the signal threshold defined as the mean gray value of background plus 3.5X its standard deviation. Only pixels with gray values exceeding the above-defined threshold are included in the analysis. In the present studies, optical density measurements were taken for 4 subregions of the hippocampus (hippocampus fields CA1-CA3, and the dentate gyrus) from the left and right sides of the brain. Data from multiple sections per animal were averaged resulting in a mean integrated optical density value for each animal and then averaged for each group.

BrdU/Ki67 Immunohistochemistry

To assess the effect of FGF-2 on hippocampal new cell survival, rats were injected with BrdU (Calbiochem) once daily for two days prior to the start of treatment paradigm at a dose of 200mg/kg dissolved in saline. Twenty-four hours post BrdU labeling animals underwent 21 days of FGF-2 treatment before being exposed to behavior testing after which they were sacrificed by decapitation and brain were snap frozen. For determining the rate of cell proliferation we performed immunohistochemical labeling of Ki-67, which is an endogenous marker of ongoing cell proliferation. For BrdU and Ki67 immunohistochemistry a series of every 8 sections was cut throughout the entire extent of the hippocampus at 30μm and slide mounted. For Ki-67 DAB staining, sections were postfixed in 4% paraformaldehyde for 1hr, followed by a 45 minute incubation in 10mM Sodium Citrate at 90°C. Sections were then rinsed with PBS and washed in 0.3% peroxide followed by blocking with BSA containing 1% goat serum and 0.05% Triton X-100. Subsequently sections were incubated overnight with rabbit polyclonal anti-ki67 (University of Michigan) 1:40000 in BSA. After PBS washes sections were incubated in biotinylated goat anti rabbit secondary antibody 1:1000, (Vector labs) followed by avidin/biotin complex (Vectastain Elite ABC kit) and subsequent DAB reaction for visualization of signal. For DAB staining of BrdU, sections were postfixed in 4% paraformaldehyde for 1hr, rinsed in PBS and washed in 0.3% peroxide. Sections were then incubated in 50% formamide-2X SSC at 65°C for 2 hours followed by two 5 minute rinses in 2X SSC. Slides are then placed for 30 minutes in 2N HCL at 37°C and 10 minutes

in 0.1M boric Acid at room temperature, followed by rinsing in PBS and blocked with BSA containing 1% goat serum and 0.05% Triton X-100. Sections were incubated overnight at room temperature with rat monoclonal anti-BrdU (Accurate) 1:1000 in BSA. After PBS washes sections were then incubated in biotinylated goat anti rat (Vector labs) secondary antibody 1:1000 followed by Avidin/Biotin complex amplification (Vectastain Elite ABC kit) and subsequent DAB reaction for visualization of signal. Cresyl violet staining was performed for both immuno-stains and sections are subsequently dehydrated through graded alcohols followed by immersion in xylene and then coverslipped with Permount® mounting medium. For Fluorescent triple labeling we used 1:1000 dilution of rat anti-BrdU in combination with 1:1000 dilution of mouse monoclonal anti-GFAP (Chemicon). For NeuN labeling we use a primary monoclonal mouse anti-NeuN antibody tagged with an Alexa 488 fluor (Chemicon). For fluorescent secondary antibodies we use Alexa 594 goat anti-rat and Alexa 647 goat anti-mouse (Invitrogen).

Cell Counting

For quantification of DAB stained Ki-67 and BrdU cells, slides were initially coded and the code was not broken until counts were analyzed to ensure that a blind observer performed all cell counts. To estimate the total number of cells we followed a modified unbiased stereological procedure used by (Malberg and Duman, 2003) whereby the total number of cells counted per animal was multiplied by the reciprocal of the sampling factor. BrdU and Ki67 cells were

counted on a light microscope under 63X objective in the granule cell layer and subgranular zone (SGZ) of the hippocampus located at the border of the granule cell layer. Cells were included in SGZ counts if the cell is near the SGZ or touching the SGZ and excluded if the cell is more than two cell diameters from the SGZ. In triple labeling experiments at least 30 BrdU cells were examined per subject to determine the percentage of BrdU positive cells that co-label with NeuN or GFAP using a laser scanning confocal microscope. Laser scans of 0.5μm serial Z-section planes were visualized using a 63x objective to determine the Neuronal or Glial differentiation of BrdU positive cells. To obtain an estimate of the total number of new neurons and new glial cells in the hippocampus of each animal, we used the percent of BrdU+ cells co-labeled with NeuN, GFAP or neither and multiplied it by the total number of BrdU+ cells for each animal.

Statistical Analyses

Behavioral studies and anatomical studies were analyzed by ANOVAs followed by Fishers PLSD post-hoc comparisons. Baseline HR-LR in situ hybridization studies were analyzed by Students t-test. Pearson correlation test was used was used to evaluate relationship between hippocampal FGF-2 mRNA expression and behavioral anxiety measure. All data are presented as mean +/- standard error. Statistical significance is assumed at $p<0.05$.

Results

FGF-2 is decreased in the Hippocampus of High Anxious LR animals.

To elucidate the role of FGF-2 in modulating anxiety behavior, we examined basal levels of FGF-2 gene expression in the selectively bred HR and LR animals, which have been previously shown to display basal differences in anxiety behavior. We specifically began examining the hippocampus as this region has been previously shown to be the primary target region modulating HR-LR differences in anxiety-like behavior and is a region where FGF-2 shows prominent neuronal expression, and exhibits dysregulation in human depressed subjects. As seen in **Figure 4-1 A** and **B**, results show that the high anxiety LR animals have lower basal levels of FGF-2 expression in the dentate gyrus [$t_{(16)}$=2.58, p<0.05] and CA3 [$t_{(16)}$=2.12, p<0.05] relative to the less anxious HR animals. No significant differences between HRs and LRs were observed in the CA1 or CA2.

In addition to the selectively bred lines, we studied, outbred Sprague-Dawley rats that show the full range of distribution of anxiety-like behavior. We tested them on the EPM for the assessment of anxiety behavior, and then measured their resting FGF-2 mRNA levels. We observed a significant positive correlation between the levels of FGF-2 expression in the CA2 region of the hippocampus and the amount of time spent in the open arm [*R ^2= 0.59*, p<0.01] **Figure 4-1 C**, suggesting that elevated resting levels of FGF-2 message may be related to low

levels of anxiety behavior. While we did not observe significant differences in FGF-2 expression in the CA2 region between HRs and LRs our results in the outbred rats parallel an observed tendency for HRs showing higher levels of FGF-2 gene expression. It is possible that significant differences were not observed as a result of saturation of exposure during our In situ hybridization experiments with HRs and LRs. This is a result of high density of FGF-2 expression in the CA2 region, which is greater than anywhere else in the hippocampal formation.

Environmental Complexity differentially reduces anxiety like behavior in LR animals.

Given that EC has been previously shown to influence emotionality by reducing anxiety (Benaroya-Milshtein et al., 2004), we aimed at determining whether this manipulation would have differential effects in the HR versus LR animals, given their basal differences in anxiety behavior. This behavioral study was also a prelude to assessing the impact of this environmental manipulation on FGF2 expression in the two lines of rats. To test the effects of EC on anxiety behavior in HR and LR animals we used the Light Dark Box (LDB) test and Elevated Plus Maze (EPM) test as both of these tests have previously been used to characterize the behavioral differences between these phenotypic groups.

As seen in **Figure 4-2.,** there was a significant main effect of EC resulting in an overall reduction of anxiety-like behavior in both tests. In the LDB test there was

a significant main effect of EC on decreasing anxiety-like behavior, as evidenced by an overall increase in the percent time spent in the illuminated compartment [$F_{(1,35)}$=6.02, p<.05]. Interestingly, while HR animals spent an overall higher percentage of their time in the Illuminated Compartment relative to LR's [$F_{(1,35)}$=7.7, p<0.01], this was primarily due to the basal differences (i.e. under Control, non-EC conditions) and not the EC condition. This is confirmed by the finding of a significant interaction effect between HR/LR phenotype and treatment (Control vs. EC) [$F_{(1,35)}$=6.8, p<0.05]. Post-hoc tests revealed a differential effect of EC on LR, with LR EC animals displaying significantly less anxiety behavior than LR control animals (p<0.01). Thus, the EC manipulation abolished the differences in spontaneous anxiety between HR and LR animals, showing that in the Light-Dark test, EC has a selective impact in alleviating the anxiety behavior of the High Anxious LRs. In the EPM, as expected, there was a significant main effect of phenotype; whereby HR animals displayed lower anxiety behavior compared to LR animals as measured by the percent open arm entries [$F_{(1,52)}$=8.1, p<0.01]. EC resulted in an increase in the percentage of open arm entries [$F_{(1,52)}$=20.0, p<0.0001] indicating the anxiolytic impact of this manipulation on both groups in the context to the EPM.

Environmental Complexity differentially increases FGF-2 expression in LR animals.

Our previous results indicated that HR animals have higher basal levels of FGF-2 expression in the hippocampus and display lower anxiety relative to LR animals.

Given the finding that EC reduced anxiety behavior more consistently in the LR animals, we asked whether EC resulted in increased FGF-2 expression in the hippocampus, and whether this effect was differential across the two selectively bred lines of rats.

Our results in **Figure 4-3.,** show that EC training results in an overall significant increase in FGF-2 expression in the dentate gyrus, [$F_{(1,19)}$=23.3, p<0.001], and CA3 region, [$F_{(1,19)}$=16.7, p=0.001]. Moreover, there was an interaction effect of EC housing and phenotypic group on FGF-2 expression in the dentate gyrus, [$F_{(1,19)}$=6.17, p<0.05] and in the CA3 region [$F_{(1,19)}$=6.4, p<0.05]. Post-hoc analysis revealed a differential effect on LR EC animals as compared to LR controls within dentate gyrus (p<.0001) and CA3 region (p<0.001). Thus, the high anxious LR's showed a greater impact of EC on both their anxiety behavior and FGF2 expression in the hippocampus, leading us to hypothesize that FGF2 plays a role in mediating the decrease in anxiety that results from exposure to a complex environment.

FGF-2 differentially reduces anxiety-like behavior in LR animals.

Given the results above showing that both genetic endowment and environmental conditions modulate hippocampal FGF-2 expression, with higher levels being correlated with reduced anxiety behavior, we set out to directly test the hypothesis that FGF-2 is a key regulator of anxiety behavior. We therefore

administered FGF-2 chronically and asked whether it would alter measures of anxiety behavior in both the high anxious LR's and the low anxious HRs.

As illustrated in **Figure 4-4.**, our results show the expected difference in the HR/LR phenotype in the LDB test. Importantly, they reveal an overall anxiolytic effect of FGF2 treatment. Thus, HR animals overall spent more time in the illuminated compartment of the LDB [$F_{(1,46)}$=38.5, p<0.0001]. Moreover, FGF-2 treatment resulted in an overall increase in the percent of time spent in the Illuminated Compartment of the LDB, [$F_{(1,46)}$=5.9, p<0.05]. More importantly, there was an interaction effect of treatment with phenotype [$F_{(1,46)}$=5.6, p<0.05]. Post-hoc analysis revealed FGF-2 treatment having a significant anxiolytic effect in FGF-2 treated LRs relative to vehicle treated LRs (p<0.01), whereas no significant effect of FGF2 treatment was observed in the HR rats.

A similar pattern was seen in the EPM. Thus, a significant interaction effect of chronic FGF-2 treatment x phenotype was observed on measures of anxiety behavior in the EPM, including percent of open arm entries [$F_{(1,46)}$=7.2, p<0.01] and percent of time spent in the open arm [$F_{(1,46)}$=6.1, p<0.05]. Further post-hoc analysis revealed that LR animals differentially benefited from the anxiolytic effects of chronic FGF-2 treatment, whereas HR animals did not. Thus, FGF2-treated LRs showed a significant increase in the percent of open arm entries (p<0.01) and in the percent of time spent in open arms (p<0.01) relative to

vehicle treated LRs. No significant effects were seen when comparing these measures in FGF2-treated versus vehicle-treated HRs.

FGF-2 differentially increases new cell survival in LR animals.

Given that EC is well known for increasing neurogenesis and that it produced a significant increase in FGF-2 gene expression in the dentate gyrus, it was reasonable to hypothesize that increase in neurogenesis post EC may be mediated at least in part by FGF-2. Indeed, exogenous treatment with FGF-2 has been shown to increase neurogenesis in the dentate gyrus of the hippocampus (Wagner et al., 1999, Pieper et al., 2005). Our specific question was whether the treatment protocol that we used, chronic peripheral FGF-2 administration, would alter hippocampal neurogenesis and if it would do so in a manner that correlates with the alterations in anxiety behavior—i.e. more markedly in the LR rats that are more responsive to the treatment.

As shown in **Figure 4-5.,** HR and LR animals show a non-significant tendency to exhibit basal differences in number of BrdU labeled cells (2739 +/- 295 in HR and 2061 +/-127 in LR) (p=.08). Chronic treatment with FGF-2 results in a significant overall increase in new cell survival in the Dentate Gyrus as shown by an increase in the number of Brd-U labeled cells [$F_{(1,16)}$=18.5, p<0.001]. Interestingly, chronic administration of FGF-2 produced little change in the total number of BrdU cells in HR's (3270 +/- 306, an 18% increase). By contrast FGF-2 produced a substantial increase in the total number of BrdU labeled cells in the

LR rats (up to 3802 +/- 286) representing a 46% increase. Thus, there was a significant interaction of treatment x phenotype [$F_{(1,16)}$=5.2, p<0.05].

It is noteworthy that these results where independent of an increase in cell proliferation, as we observed no differences between HR and LR in proliferation as chronic FGF-2 failed to increase the number of Ki67 labeled cells [$F_{(1,16)}$=1.1 p>0.05]. Thus, it appears that HR and LR lines exhibit a tendency towards a basal differences in the survival of adult born cells, with the more anxiety prone LR's showing lower rates of survival. Thus, FGF-2 treatment increases cell survival only in the LR animals, in conjunction with their more consistent decrease in anxiety behavior.

FGF-2 differentially alters differentiation of adult stem cells in the hippocampus of LR animals.

Given the differential increase in cell survival in LRs in response to FGF-2, we evaluated the pattern of differentiation of the surviving BrdU labeled cells by obtaining an estimate of the total number of neurons and astrocytes generated in the hippocampal dentate gyrus as a function of treatment with FGF-2.

Statistical analysis of the data summarized in **Table 4-1** shows several main effects as well as interaction between HR/LR phenotype and the impact of FGF-2 treatment on the various stem cell populations. Beyond the tendency for the basal differences in total number of BrdU labeled cells described above, HR and

LR animals exhibit basal differences in the *proportions* of cells where BrdU is co-labeled with NeuN, GFAP or neither (**Figure 4-6 A &C).** In particular, relative to LRs, HRs show a higher number of new astrocytes generated in the dentate gyrus across both vehicle and FGF-2 conditions [$F_{(1,16)}$=27.1, p<0.0001] and this is particularly due to the basal difference in newly born astrocytes (235 $\pm$ 25 in HR vs. 62 $\pm$ 4 in LR) as shown by post-hoc analysis (p<0.0001) .

FGF-2 treatment results in an overall increase in the number of new astrocytes [$F_{(1,16)}$=33.1, p<0.0001] and new neurons [$F_{(1,16)}$=20.2, p<0.001], but the impact is different as a function of the HR/LR phenotype. Thus, there were significant interaction effects in the number of newly generated neurons [$F_{(1,16)}$=9.7, p<0.01], astrocytes [$F_{(1,16)}$=9.6, p<0.01], as well as on undifferentiated cells [$F_{(1,16)}$=9.5, p<.01].

As noted above, in the HR animals, chronic administration of FGF-2 produced little change in the total number of BrdU+ cells, and **Table 4-1.,** and **Figure 4-6B.,** show that this held true especially for neurons (13% increase) with a slightly greater increase in astrocytes (23%). Interestingly, the small population of BrdU labeled cells that was co-labeled with neither marker increased the most post FGF-2 (52%; p<0.01) in the HR population. Such differential increase resulted in a disruption of the basal differences between HRs and LRs as under vehicle conditions LRs show a higher number of undifferentiated cells (p<0.01) relative to vehicle HRs.

By contrast, in the LR animals, FGF-2 produce a substantial increase in BrdU labeled cells, and **Table 4-1.,** and **Figure 4-6B.,** show that this represented a doubling of neurons (201% increase over basal; $p<0.0001$), and a remarkable four-fold increase in astrocytes (398% over basal; $p<0.0001$). The population of BrdU labeled cells that was co-labeled with neither marker decreased slightly (12%) in LRs. Thus, as depicted in **Figure 4-6D,** the relative proportions of neurons, astrocytes and "neither" was altered by FGF-2 particularly in the LRs, with what appears to be a relative proportional increase in astrocytes at the expense of uncommitted cells.

Discussion

The present study establishes for the first time a role for hippocampal FGF-2 in modulating anxiety behavior, and suggests a mechanism for this role via the modulation of hippocampal neurogenesis. Our findings are as follows: a) selectively bred HR animals, which naturally show lower anxiety-like behavior compared to selectively bred LR's, exhibit higher basal levels of hippocampal FGF-2 relative to their LR counterparts. Moreover, in an outbred population, higher levels of FGF-2 mRNA predict lower anxiety behavior; b) Exposure to an environmental manipulation which increases complexity of the physical surrounds of an animal, decreases anxiety behavior and leads to increased expression of FGF-2 mRNA. The impact of EC on FGF-2 hippocampal expression was seen selectively in the more anxious LR animals, who also exhibited a more consistent decrease in anxiety behavior post EC as manifested in both the EPM and the LDB; c) chronic administration of exogenous FGF-2

decreases anxiety behavior and this is seen only in selectively bred LR's ; d) chronic administration of exogenous FGF-2 increases new cell survival in the hippocampus without altering proliferation. This is associated with in an overall increase in neurogenesis and glial genesis. Once again, this effect is seen primarily in the LR animals. These results strongly implicate hippocampal FGF-2 as a modulator of anxiety behavior. Moreover, they show that this modulation can take place as a result of a genetic predisposition (HR express more FGF-2 basally than do LR's) but that environmental factors, specifically an enriched environment, can also induce it, essentially compensating for the genetic vulnerability in the more anxiety-prone animals. Finally, this body of work suggests that treatment with exogenous FGF-2 or its analogues, or more broadly altering the FGF system, may benefit individuals prone to high anxiety.

While FGF2 had not been directly implicated in the control of anxiety, several strands of evidence give this view face validity. First, as, indicated above, we and others have found that several FGF family members including FGF-2 are decreased in the brains of post mortem subjects suffering from major depression (Evans et al., 2004),(Gaughran et al., 2006, Akil et al., 2008). Moreover, recent animal studies from our group and others support its relevance to depression, as FGF-2 is decreased in social defeat, an animal model of depression (Turner et al., 2006) and both FGF-2 and related molecules function as antidepressants in the Porsolt Swim Test (Turner et al., 2008b). Moreover, chronic classical antidepressant drugs have been shown to increase FGF-2 expression (Mallei et

al., 2002) and to enhance neurogenesis (Malberg et al., 2000). Thus, this body of work is consonant with the current hypothesis that a deficiency in growth factors represents a vulnerability factor for mood disorders (Duman and Monteggia, 2006) and that the induction of growth factors is critical to the effectiveness of antidepressant drugs (Warner-Schmidt and Duman, 2007), along with the possibility that neurogenesis plays a critical role in antidepressant action (Santarelli et al., 2003). Taken together, these findings suggest that decreased levels of FGF-2 may be involved in the pathophysiology of depression, and that antidepressants may exert their neurogenic effects by increasing FGF-2. However, in spite of the close co-morbidity between anxiety disorders and major depresive disorders, much less has been done to establish the role of FGF-2 in modulating anxiety. More importantly, the findings here suggest that the impact of FGF-2 is not only seen under pathological conditions but in the context of natural variation in anxiety-like behavior and that it is modulated by relatively mild environmental changes (e.g increasing complexity).

The differential impact of Environmental Complexity on FGF-2 and anxiety in LRs suggest that some experiences may only benefit certain individuals that have a high genetic propensity for anxiety like that observed in the selectively bred LRs (Stead et al., 2006). Given the evidence showing FGF-2 decreasing anxiety, it is possible that HRs did not show less anxiety in the LDB given the lack of beneficial increase in FGF-2 in response to EC. This could potentially be related to a ceiling effect that may have precluded the HR's increase in hippocampal

FGF-2. Although, no differential impact on anxiety in LRs was observed on the EPM in response to EC, our results do point to LRs being favored. Normally, LRs show 25% percent open arm entries whereas in response to EC this number increases to 47%, representing almost a two-fold decrease in anxiety. On the other hand, HRs low anxiety performance went from 41% to 55%, thus resembling a more modest change in anxiety behavior which could alternatively be explained by increased expression of BDNF or VEGF in response to EC (Young et al., 1999) (Cao et al., 2004).

In support of the role of FGF-2 as a modulator of anxiety, our results indicate that chronic exogenous FGF-2 treatment is sufficient for reducing anxiety in LRs. This novel role for FGF-2 is supported by anxiolitic treatments increasing FGF-2 expression in the hippocampus (Gomez-Pinilla et al., 2000, Mallei et al., 2002) and by our findings showing EC increasing FGF-2 expression and reducing anxiety in LRs. They also expand on the potential role of endogenous FGF-2 in modulating experience dependent anxiety behavior. In support of this notion social defeat decreases FGF-2 expression in the hippocampus (Turner et al., 2008a) and increases vulnerability to anxiety (Kinsey et al., 2007). Furthermore, rats suffering low maternal care during development show low levels of hippocampal FGF-2 (Bredy et al., 2003) and high anxiety (Caldji et al., 1998). However it is unknown whether such differences in anxiety are due to differences in hippocampal FGF-2.

Our results also demonstrate that FGF-2 increases neurogenesis by enhancing new cell survival selectively in LRs. These findings are consonant with previous findings showing exogenous FGF-2 salvaging cell survival deficits (Rai et al., 2007). Interestingly, effects of FGF-2 on survival were independent of changes in cell proliferation. These results parallel findings showing decrease hippocampal cell survival with no changes in cell proliferation after social defeat (Thomas et al., 2007). They also expand on reports showing decrease FGF-2 and low cell survival in the hippocampus of rats suffering from low maternal care (Bredy et al., 2003) and support our findings showing LRs with a tendency towards low cell survival. Taken together our results suggest that LR's low levels of hippocampal FGF-2 expression may lend high vulnerability to low cell survival which could in turn lead to a decrease in the generation of new neurons and new astrocytes.

In the adult, FGF-2 shows prominent expression in astrocytes throughout the brain, whereas neuronal expression is almost exclusive to the hippocampus (Woodward et al.). Although FGF-2 has mostly been related to neurogenesis, FGF-2 has also been noted to modulate the number of glial cells in the hippocampus (Cheng et al., 2002). This is consistent with our findings showing an increase in both glial genesis and neurogenesis. However, it is worth noting that FGF-2 showed a larger increase in glial genesis as seen by a four-fold increase compared to a two-fold increase in neurogenesis. This suggests that FGF-2 shows a preferential increase in glial genesis.

While we are not aware of FGF-2 being directly linked to hippocampal astrocyte differentiation, several findings have shown it modulating the expression of GFAP in the cortex (Reuss et al., 2003) and to synergistically participate with cilliary neurotrophic factor (CNTF) in promoting astrocyte differentiation (Song and Ghosh, 2004). Furthermore, phenotypic analysis of BrdU+ cells support the role of FGF-2 in astrocyte differentiation as HR animals show an overall higher pattern of glial differentiation relative to LRs. Interestingly, observed differences in astrocyte differentiation between control HR and LR were lost after FGF-2 treatment. This suggests that FGF-2 modulates the differentiation of new cells generated in the hippocampus translating into an enhancement of astrocytes.

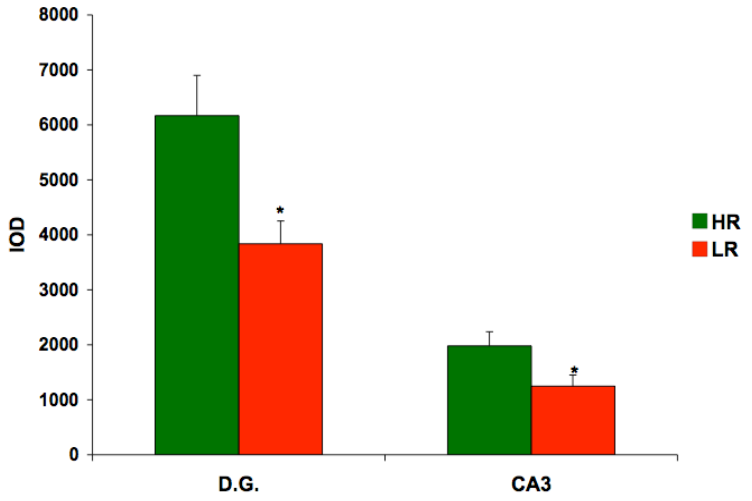
In conclusion our results suggest that FGF-2 plays a role in modulating anxiety behavior by increasing the rate of cell survival and in turn promoting the generation of new astocytes and neurons. This is supported by our data showing LR animals, which display increased anxiety behavior as having lower basal levels of FGF-2 relative to the less anxious HR. However, when LR's endogenous levels of FGF-2 are increased either by EC or by exogenous treatment they respond with less anxiety lending their behavior become similar to that of an HR animal. Moreover, FGF-2 anxiolytic effects were accompanied by increased new cell survival that in turn promoted the preferential increase in new astrocytes. This again made the LR's resemble that of an HR phenotype as HR show a higher number of newly generated astrocytes. Thus the results above suggest that low levels of hippocampal FGF-2 expression in LRs contribute to

low survival rates of newly generated astrocytes, making them more prone to high anxiety behavior. Taken together the present study supports the role of FGF-2 as a novel mediator of anxiety-like behavior and a potential novel treatment target for assisting individuals showing high anxiety behavior.

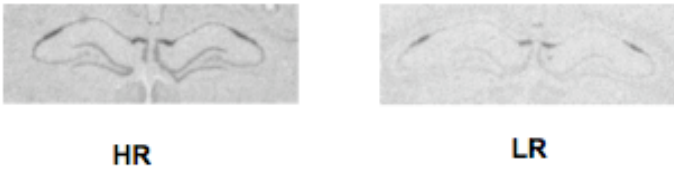
Results Figures

Figure 4-1: FGF-2 is decreased in the Hippocampus of LRs

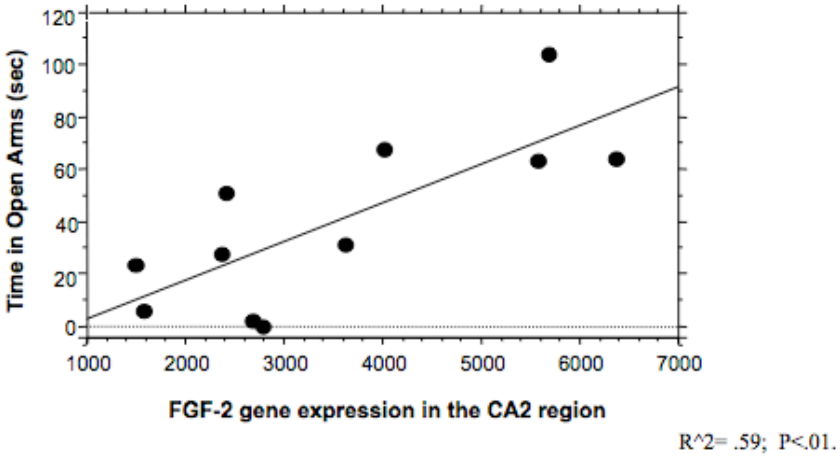
A.



B.

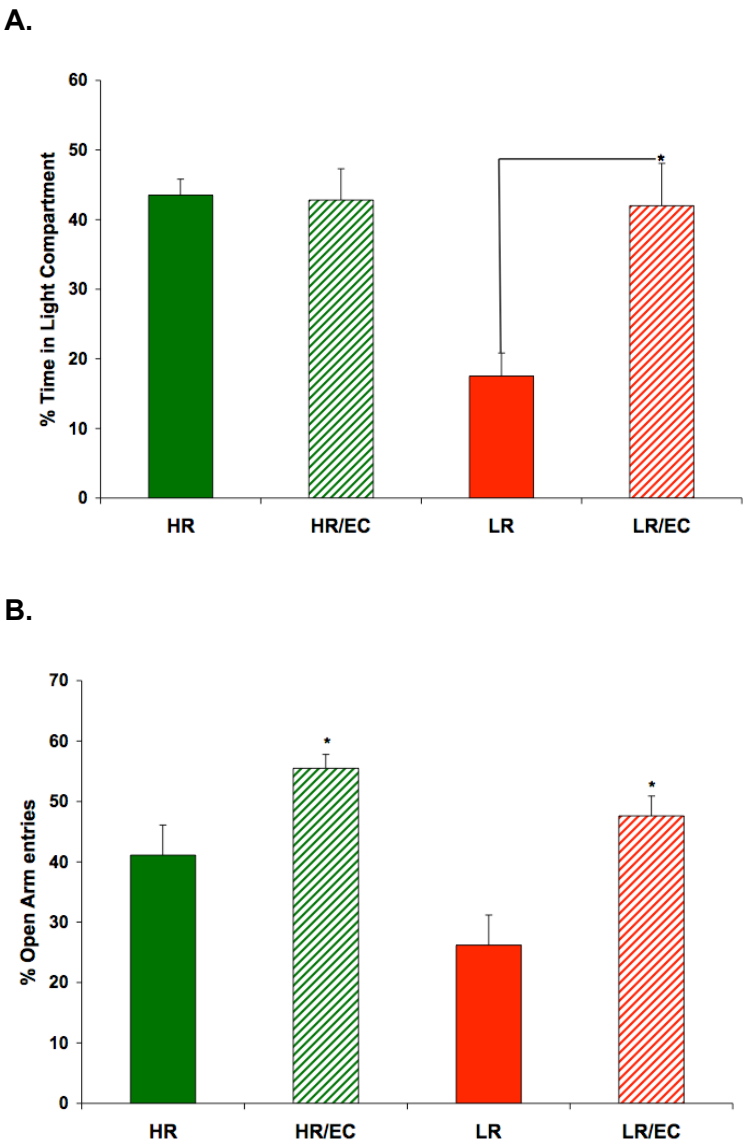


C.



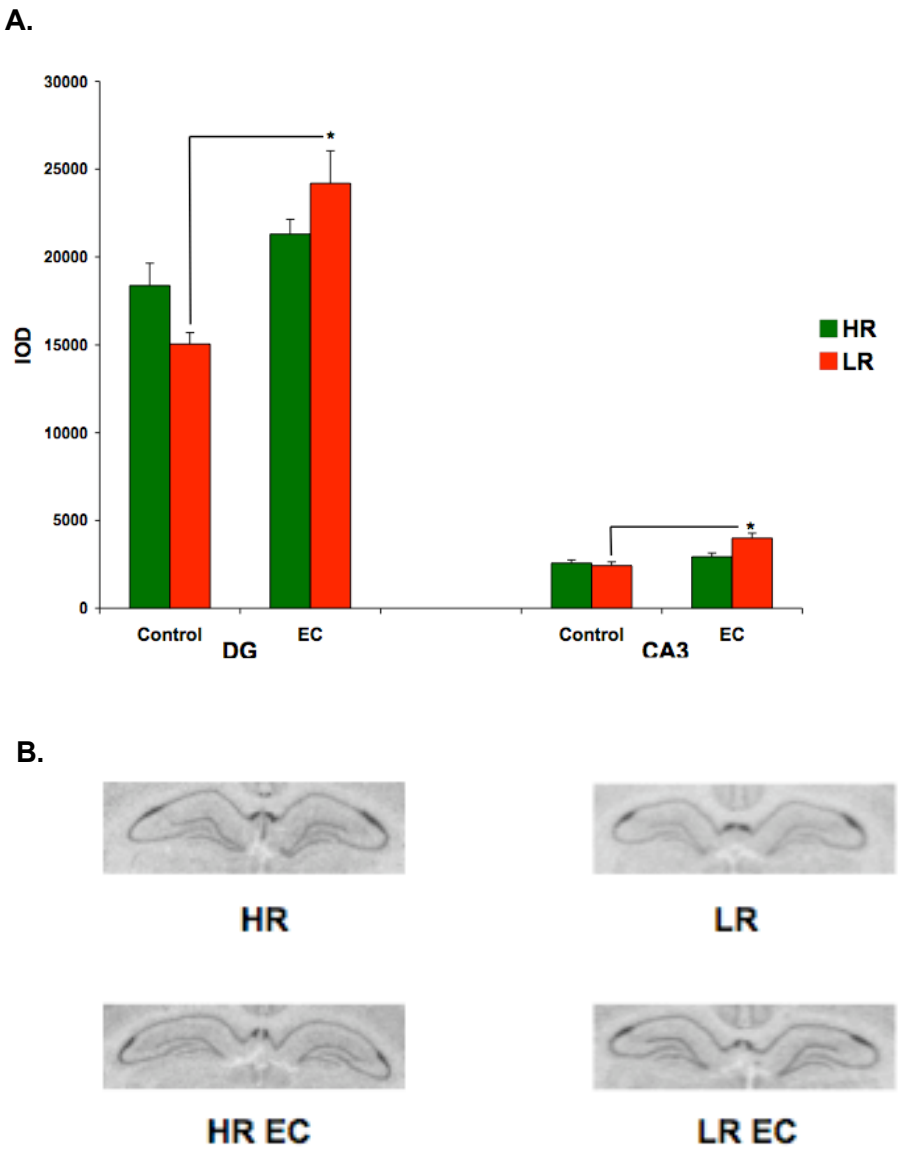
A) LR animals show decreased FGF-2 expression in the Dentae Gyrus [$t_{(16)}$=2.58, p<0.05] and CA3 regions of the hippocampus [$t_{(16)}$=2.12, p<0.05] (n=9-10 per group). B) Representative images from HR and LR animals showing basal FGF-2 expression in the hippocampus. C) Correlation of FGF-2 expression in the hippocampal CA2 region and time spent in the open arm [R^2=0.53, (p<0.01] (n=11).

Figure 4-2: Environmental Complexity differentially reduces anxiety-like behavior in LR animals



A) Overall HR animals displayed lower anxiety behavior compared to LR animals as measured by their increase in the percent time spent in the Illuminated Compartment [$F_{(1,35)}$=7.7, p<0.01]. There was also a significant main effect of EC on decreasing anxiety-like behavior as shown by an increase in the percent time spent in the Illuminated Compartment [$F_{(1,35)}$=6.02, p<.05]. There was also an interaction on the time spent in the illuminated Compartment [$F_{(1,35)}$=6.8, p<0.05]. Post-hoc tests revealed a differential effect on LR EC trained versus LR control animals (p<0.01) (n=13-15 per group). B) There was also a main effect of phenotype in the EPM as HR animals showed an higher percent of open arm entries [$F_{(1,52)}$=8.1, p<0.01]. Main effects of EC in the EPM also show an overall increase in the percentage of open arm entries [$F_{(1,52)}$=20.0, p<0.0001].

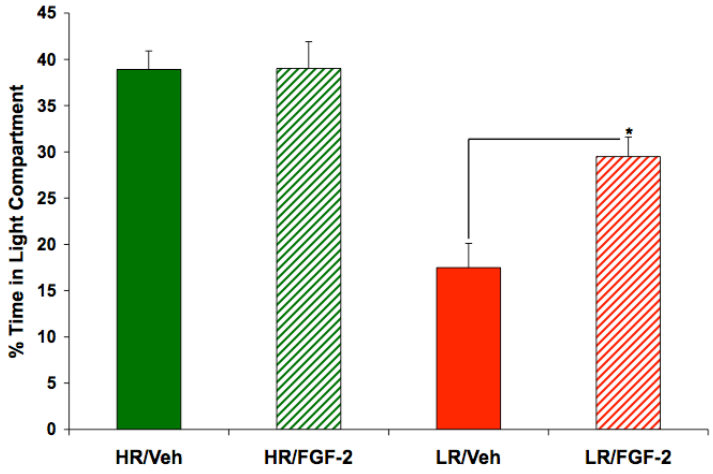
Figure 4-3: Environmental Complexity differentially increases Hippocampal FGF-2 expression in LRs.



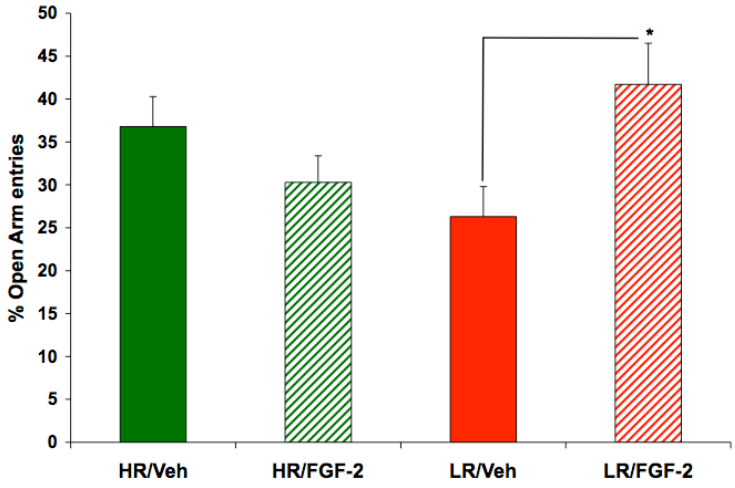
A) EC results in an overall significant increase in FGF-2 expression in the dentate gyrus, [$F_{(1,19)}$=23.3, p<0.001], and CA3 region, [$F_{(1,19)}$=16.7, p<0.0001]. There was also an interaction effect of EC housing and phenotypic group on FGF-2 expression in the dentate gyrus, [$F_{(1,19)}$=6.17, p<0.05] and CA3 region [$F_{(1,19)}$=6.4, p<0.05]. Post-hoc revealed a differential effect on LR EC animals as compared to LR controls within dentate gyrus (p<0.0001) and CA3 region (p<0.001). (B) Representative images of FGF-2 expression in the hippocampus of HR and LR animals after control and EC housing (n=5-6 per group).

Figure 4-4: FGF-2 differentially reduces anxiety-like behavior in LRs

A.



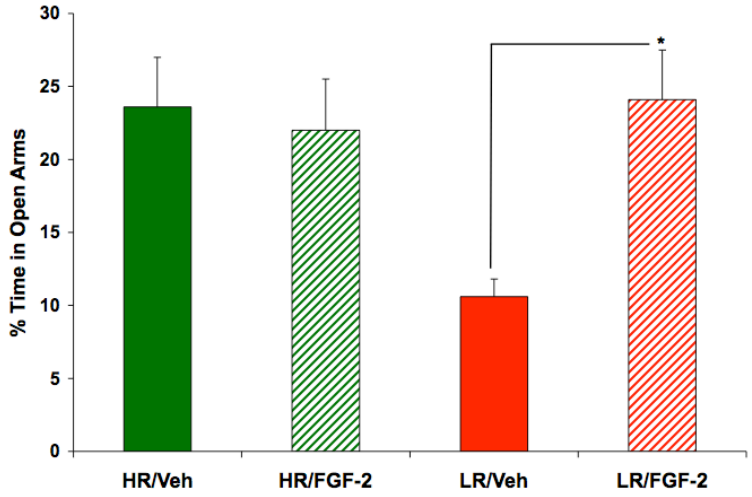
B.



A) Overall main anxiolytic effects of phenotypes and treatment were observed in the LDB, as HR [$F_{(1,46)}$=38.5, p<0.0001] and FGF-2 treated animals [$F_{(1,46)}$=5.9, p<0.05] spent more time in the illuminated compartment. Interaction effects of treatment and phenotype where also observed in the LDB [$F_{(1,46)}$=5.6, p<0.05]. Post-hoc analysis revealed FGF-2 treated LRs showing less anxiety relative to vehicle treated LRs (p<.01). B) There was an interaction of FGF-2 treatment and phenotype group on reducing anxiety as measured by an increase in the percent of open arm entries in the EPM [$F_{(1,46)}$=7.2, p<0.01] and posthoc analysis revealed effects in LR FGF-2 treated animals relative to LR vehicle controls (p<.01) (n=11-15 per group).

FGF-2 differentially reduces anxiety-like behavior in LRs

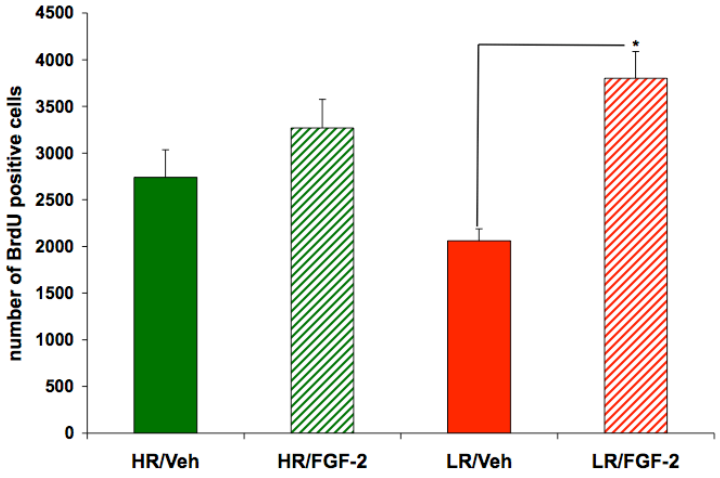
C.



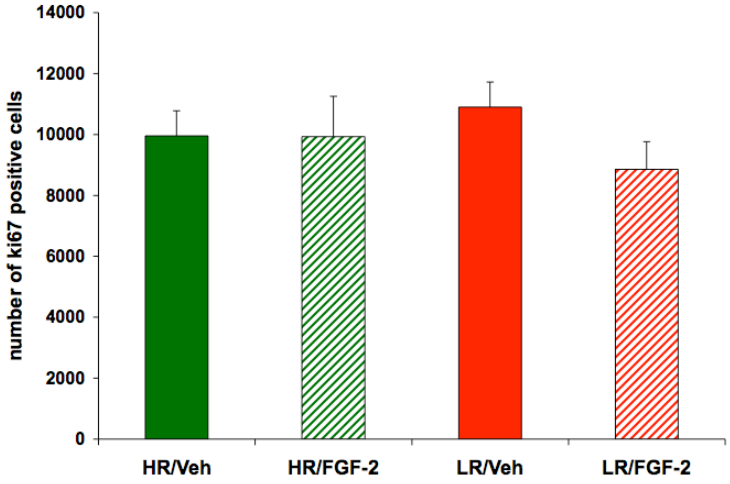
C) Interaction effects were also observed in the percent of time spent in the open arm [$F_{(1,46)}$=6.1, p<0.05]. Analysis by post hoc revealed LR FGF-2 treated animals differentially showing an increase in the percent time in open arms (p<0.01) (n=11-15 per group).

Figure 4-5: FGF-2 diferentially increases new cell survival

A.



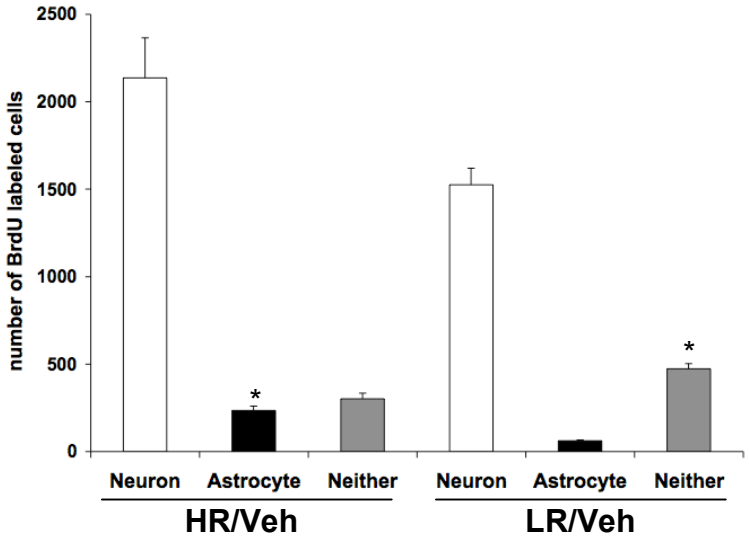
B.



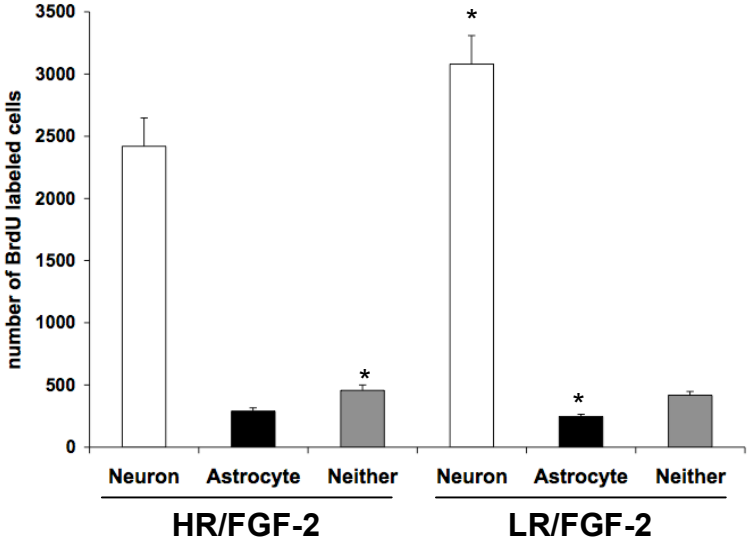
A) Chronic FGF-2 results in an overall significant increase in new cell survival in the dentate gyrus as shown by an increase in the number of Brd-U labeled cells [$F_{(1,16)}$=18.5, p<.001]. Furthermore there was an interaction of treatment and phenotype group [$F_{(1,16)}$=5.2, p<.05], as LR FGF-2 treated animals showed an increase in cell survival relative to LR vehicle treated animals (p<.001) B) No significant effects were observed in cell proliferation as chronic FGF-2 failed to increase the number of Ki67 labeled cells [$F_{(1,16)}$=1.1 p>.05], (n=5 per group).

Figure 4-6:FGF-2 differentially alters cell differentiation

A.



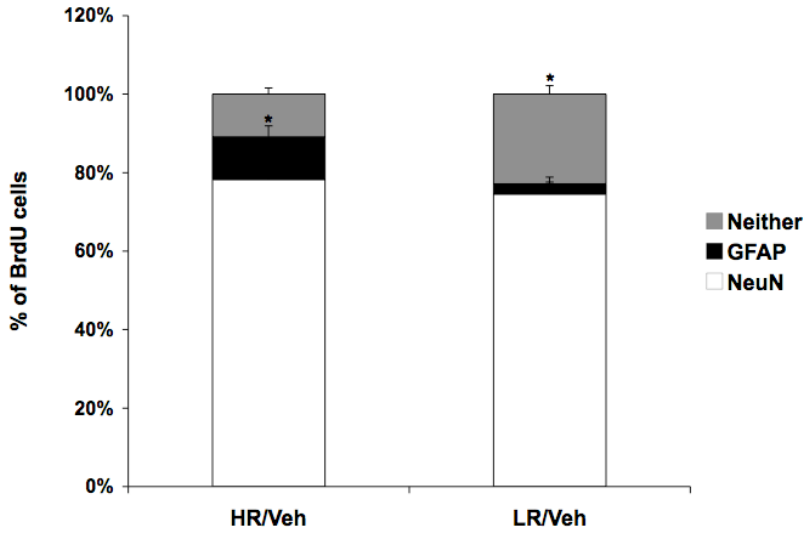
B.



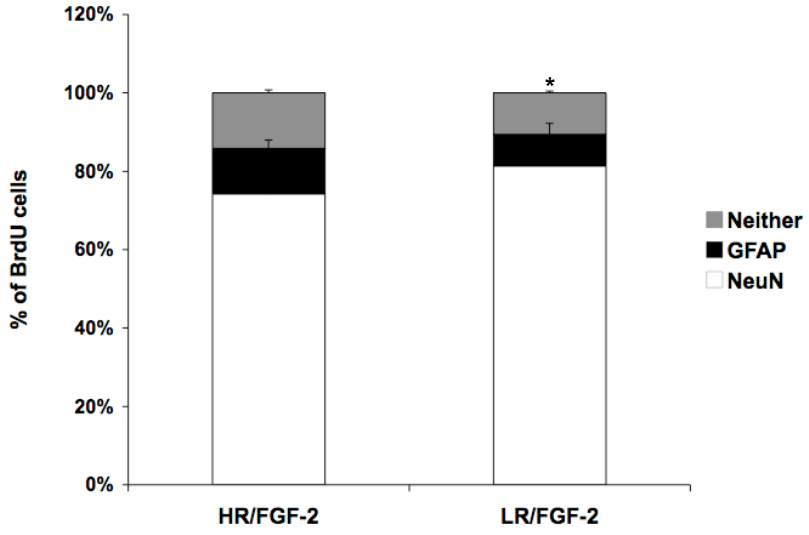
A) Overall main effects showed HR animals showing higher number of BrdU+ astrocytes relative to LRs [$F_{(1,16)}$=27.1, p<0.0001]. An interaction effect was also observed on the number of undifferentiated cells [$F_{(1,16)}$=9.5, p<0.01] with post-hoc tests revealing that under vehicle conditions LRs show a higher number of undifferentiated cells (p<0.01) relative to vehicle HRs. B) Upon FGF-2 treatment HR animals showed a differential increase in the number of undifferentiated cells (p<.01) eliminating basal differences between HRs and LRs on the number of undifferentiated cells. Overall main effects showed FGF-2 treated animals showing an increase in the number of new neurons [$F_{(1,16)}$=20.2, p<0.001] and astrocytes [$F_{(1,16)}$=33.1, p<.0001], with significant interactions observed in the number of new neurons and astrocytes respectively [$F_{(1,16)}$=9.7, p<0.01] [$F_{(1,16)}$=9.6, p<0.01]. Post-hoc analysis revealed that FGF-2 treatment differentially increased the number of neurons (p<.0001) and new astrocytes (p<.0001) in LRs (n=5 per group).

FGF-2 differentially alters the pattern of cell differentiation

C.



D.



C) HR animals showed a greater proportion of astrocyte differentiation [$F_{(1,16)}$= 5.77, p<0.05] whereas LRs show an overall increase in the percent of undifferentiated BrdU labeled cells [$F_{(1,16)}$= 7.73, p<0.05]. D) Overall FGF-2 treatment effects in the percent of undifferentiated cells [$F_{(1,16)}$= 9.1, p<0.05] and its interaction [$F_{(1,16)}$= 26.9, p<0.001] resulted in a disruption of HR/LR differences in astrocyte and undifferentiated cells. Post-hoc revealed FGF-2 treatment decreased the percent of undifferentiated cells selectively in LRs (p<.0001) (n=5 per group).

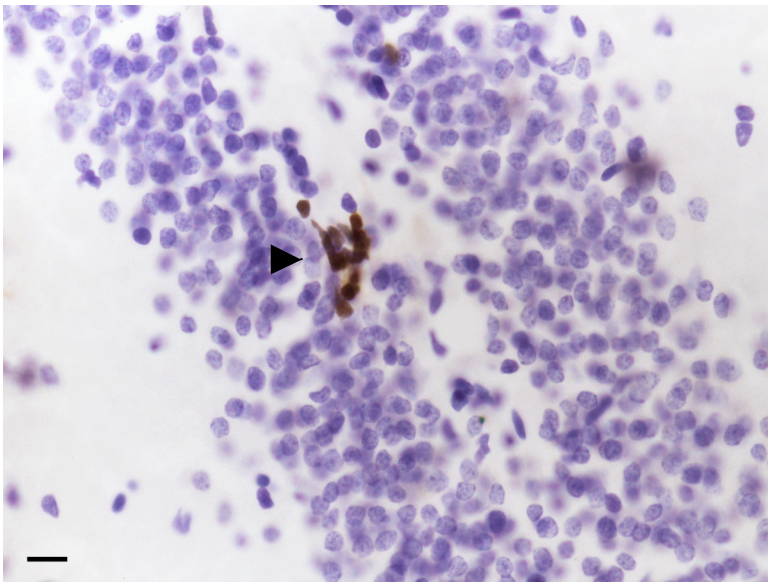
Table 4-1: FGF-2 differentially alters cell differentiation in HR and LR animals

	HR/Veh	HR/FGF2	HR-FGF/ HR-Veh	LR/Veh	LR/FGF2	LR-FGF/ LR-Veh
Neuron	2136 (230)	2420 (226)	113%	1525 (94)	3079 (231)*	201%
Astrocyte	235 (25)	290 (27)	123%	62 (4)	246 (19)*	398.00%
Neither	301 (33)	458 (43)*	152%	474 (29)	418 (31)	88.18%

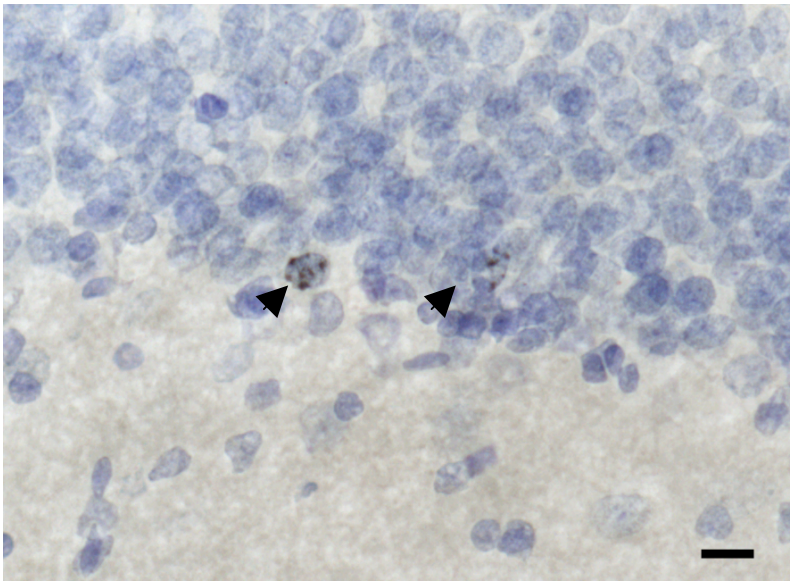
Table 4-1 shows the total number of BrdU+ neurons, astrocytes and undifferentiated cells along with their standard error of the mean in parenthesis. Overall main effects showed HR animals showing higher number of BrdU+ astrocytes relative to LRs [$F(1,16)=27.1$, $p<0.0001$]. An interaction effect was also observed on the number of undifferentiated BrdU+ cells [$F(1,16)=9.5$, $p<0.01$] with post-hoc tests revealing that under vehicle conditions LRs show a higher number of undifferentiated cells ($p<0.01$) relative to vehicle HRs. Upon FGF-2 treatment HR animals showed a differential increase in the number of undifferentiated cells ($p<.01$) eliminating basal differences between HRs and LRs on the number of undifferentiated cells. Overall main effects showed FGF-2 treated animals showing an increase in the number of BrdU+ neurons [$F(1,16)=20.2$, $p<0.001$] and astrocytes [$F(1,16)=33.1$, $p<.0001$], with significant interactions observed in the number of BrdU+ neurons and astrocytes respectively [$F(1,16)=9.7$, $p<0.01$] [$F(1,16)=9.6$, $p<0.01$]. Post-hoc analysis revealed that FGF-2 treatment differentially increased the number of new neurons ($p<.0001$) and astrocytes ($p<.0001$) in LRs ($n=5$ per group).

Appendix 4-1: Representative images of Ki67 and BrdU labeled cells

A.



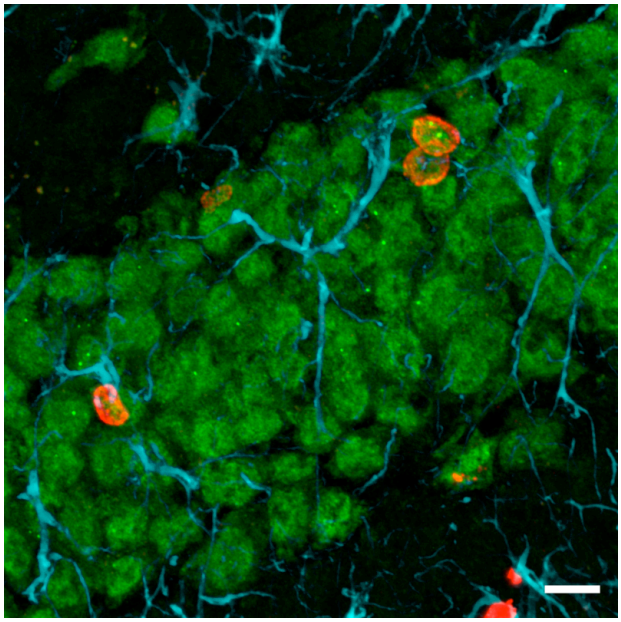
B.



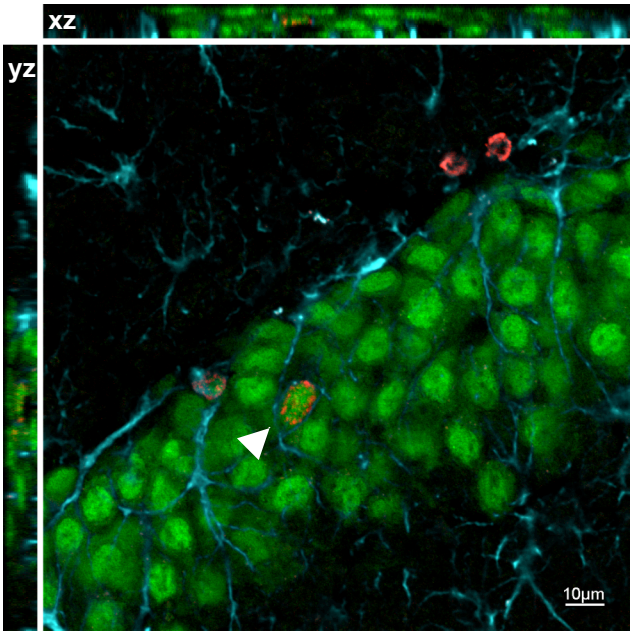
A) Representative image of a cluster of ki67 labeled cells marked with arrowheads. (Scale bar 20 μm.) B) Representative image of BrdU labeled cells marked with arrowheads. (Scale bar 10μm).

Appendix 4-2: Representative images of neurogenesis and glial genesis

A.

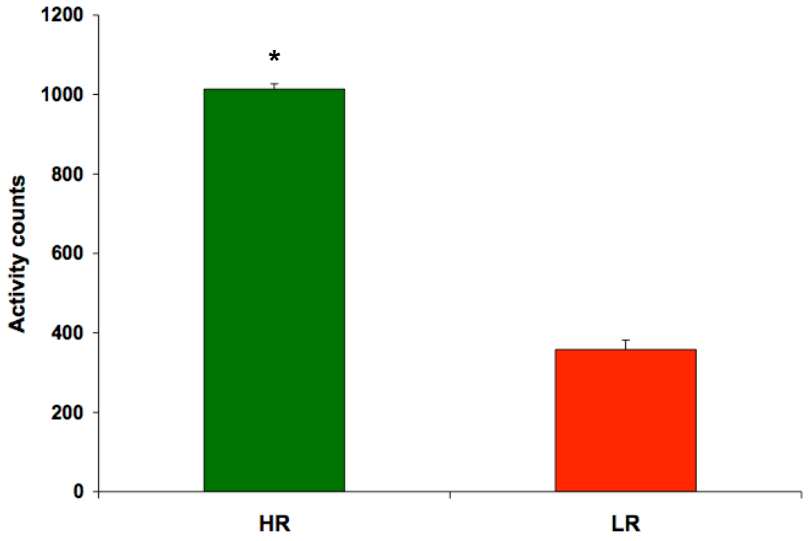


B.



A) Representative confocal images of BrdU/NeuN co-labelled cells and BrdU/ GFAP co-labeled cells (scale bar 10μm). red labeling are BrdU positive cells, blue labeling are GFAP positive cells and green are NeuN positive cells. B) Arrowhead points to BrdU/NeuN co-labeled verified in xy,/xz axis axis.

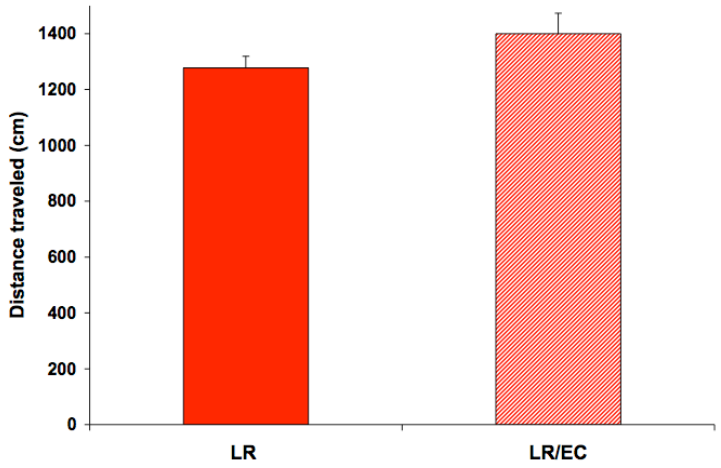
Appendix 4-3: Differences in locomotor response to novelty in selectively bred HR and LR animals.



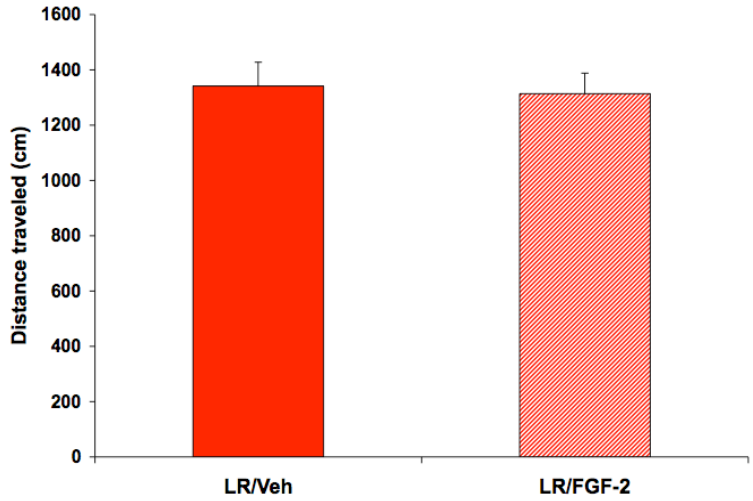
Selectively bred HR and LR animals show reliable differences in locomotor response to novelty [$t_{(136)}$=522.1, p<0.0001].

Appendix 4-4: Differences in anxiety response to EC and FGF-2 are not related to changes in overall activity.

A.



B.



A) Selectively bred LR animals show no significant differences in general activity response to EC on the EPM [$t_{(9)}$=4.09, p=0.07]. B) FGF-2 does not impact activity in the EPM [$t_{(25)}$=0.64, p=0.81].

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Chapter 5

Discussion

The present dissertation encompasses a series of studies demonstrating a novel role for endogenous hippocampal FGF-2 as a mediator of anxiety-like behavior. In support of this concept, we show that an experience such as housing in a complex environment, construed to be neuroprotective, reduces anxiety while requiring hippocampal FGF receptor activation for such effects. Moreover, FGF-2's role in reducing vulnerability to anxiety was further validated in animals showing genetic propensity to high anxiety. Specifically, LR animals, which are genetically predisposed to exhibit high anxiety behavior, have low basal levels of hippocampal FGF-2 gene expression whereas their counterparts, the low anxiety HRs, show high basal levels of hippocampal FGF-2. Importantly, upon exposure to EC, FGF-2 expression in the hippocampus of LRs becomes significantly increased as their anxiety levels become reduced. Moreover, when the high anxiety LRs where treated chronically with exogenous FGF-2, their anxiety behavior was reduced to a level comparable to that of HRs, mirroring the impact of EC on these selectively bred animals. Thus, LR's appear to be genetically "FGF2 deficient" and more anxiety-prone, and treatments including direct

administration of *exogenous FGF2* or exposure to a manipulation that induces *endogenous FGF2* reverse the anxiety phenotype. Taken together these results demonstrate for the first time that *FGF-2 serves as endogenous modulator of anxiety behavior, and that its effects are partially determined by genetic factors but are readily modulatable by experience.*

Interestingly, FGF-2's anxiolytic impact after chronic treatment stands in contrast to its anxiogenic impact upon acute treatment. Thus, acute treatment with FGF-2 increased anxiety-like behavior with particularly strong effects in the HR animals. This contradictory dilemma harks back to a profile of action often seen upon acute treatments with antidepressants (Bagdy et al., 2001). Initially, some antidepressant treatment regimens lead to acute anxiogenic effects before presenting their beneficial effects on depression and anxiety after two to three weeks of treatment (Dulawa et al., 2004). This same the scenario occurred in our results whereby acute FGF-2 treatment was anxiogenic, whereas chronic FGF-2 treatment exhibited the anxiolytic response elicited by antidepressants and EC. This is consistent with other findings from our laboratory that showed that FGF-2 also has antidepressant effects (Turner et al., 2008b). Our results also suggest that FGF-2 may be acting in a neurotransmitter-like fashion, or at least mediating its behavioral effects by modulating neurotransmitter systems. Furthermore, they suggest that chronic FGF-2 may be mediating its anxiolytic impact by modulating structural plasticity, as has been proposed for chronic antidepressants such as SSRIs.

In support of this notion, our results show chronic FGF-2 increasing hippocampal cell genesis, by means of increasing cell survival and promoting the generation of new neurons and especially new astrocytes. Similarly, HRs, which naturally show high levels of hippocampal FGF-2 gene expression show a tendency for high levels of hippocampal new cell survival while showing significantly more newly generated astrocytes. These results parallel effects seen after EC, wherein increases in cell proliferation and cell survival were observed. This led to the hypothesis that FGF-2 may be mediating the cell survival effects seen after EC. This hypothesis was tested and supported by the studies described in Chapter 2, which demonstrated that an FGF antagonist injected into the hippocampus disrupts the effects of EC on increasing hippocampal cell genesis. However, both proliferation and cell survival were disrupted by the antagonist, which blocks the effects of all the FGF's that impact on hippocampal receptors. *This indicates that while FGF-2 may be mediating the cell survival effects seen after EC, other FGF ligands may be participating in the modulation of EC's impact on cell proliferation*.

The fact that FGF-2 increases hippocampal cell genesis and reduces anxiety presents an interesting scenario. Thus, as mentioned above, chronic antidepressant treatment increases hippocampal FGF-2 gene expression (Mallei et al., 2002) and hippocampal cell genesis (Malberg et al., 2000) while reducing anxiety. Similarly, EC increases neurogenesis reduces anxiety and increases hippocampal FGF-2 gene expression. *This suggests that the long-term*

behavioral impact of both EC and antidepressants may rely on FGF-2, and that this growth factor exerts its effects on behavior, at least in part, by altering hippocampal cell genesis.

The combination of using the HR-LR model and altering environmental complexity offers an excellent opportunity to assess both the genetic and the environmental factors critical in modulating vulnerability to mood disorders. Furthermore, these paradigms allowed the use of non-pharmacological strategies to demonstrate the role of *endogenous FGF-2,* and uncovering its function in modulating anxiety behavior. Key elements that have emerged in common with antidepressant mechanisms of action include alterations in expression levels of growth factors and enhanced cell genesis in the adult hippocampus. Thus, a deficiency in growth factor function has been hypothesized to increase vulnerability to clinical depression, and antidepressants have been proposed to mediate their behavioral effects by increasing growth factor support, which in turn can alter neural plasticity including enhancement of neurogenesis. Indeed this appears to be the case for FGF-2, as its expression levels are low in the brains of severely depressed individuals, and this decrease is less marked in subjects who had received SSRI treatment (Evans et al., 2004) (Gaughran et al., 2006). The current results led us to hypothesize that *a similar set of mechanisms may be mediating vulnerability to anxiety*, an affective response that is closely linked to depression. Consequently, the results of this dissertation led to the following extension of the previous human and animal studies: *Endogenous hippocampal*

FGF-2 decreases vulnerability to anxiety. It does so, at least in part, by altering hippocampal neuroplasticity, increasing survival of adult stem cells and particularly the generation of new astrocytes, which may in turn provide better support for general hippocampal function.

The above synopsis of the results presented in this project serves as a prelude to the discussion and general conclusions of this dissertation.

Role of FGF-2 as Regulator of Anxiety

As part of the results presented in this project I report that FGF-2 presents seemingly contradictory effects on anxiety-like behavior. In particular, during acute administration FGF-2 increases anxiety behavior, a result consistent with our findings showing that acute FGF receptor antagonism in the hippocampus reduces anxiety. However, these acute results stand in contrast to our findings that chronic FGF-2 treatment leads to decreased anxiety, and this is accompanied by increased survival of newly born cells in the adult hippocampus. While I propose that both of these effects are mediated by the hippocampus, our results present a puzzling discrepancy that we aim to explain with two distinct mechanism of action: *acute effects of FGF-2 which involve immediate neurotransmitter-like action, and chronic effects of FGF-2 which involves structural plasticity-related changes within the hippocampal circuitry*. Furthermore, I will also discuss how the HR and LR trait differentially interacts

with the mechanisms of action of FGF-2 in modulating anxiety acutely and chronically.

While the acute anxiogenic effects of FGF-2 may raise questions about its potential for long term treatment of anxiety and depression, it is worth mentioning that a similar response pattern is observed with antidepressant treatments. Thus, acute treatment with selective serotonin reuptake inhibitors (SSRIs) has been shown to cause acute anxiogenic effects (Bagdy et al., 2001), while it is well known that chronic antidepressants reduce anxiety (Dulawa et al., 2004). Taking the antidepressant treatment paradigm as a model, we can draw parallels to arrive at possible mechanistic explanations of the acute versus chronic behavioral responses to FGF-2. On the other hand, alternate mechanisms linking FGF-2 to acute stress responses may also provide a viable mechanistic explanation of the acute anxiety responses.

It is known that effective treatment regimens with SSRIs require chronic administration in order to see improvements in behavioral responses in humans. Thus most of the literature has focused on the neural plasticity mechanisms involving the effects of antidepressants on reversing behavioral responses reminiscent of depression such as anxiety. One of the most studied and perhaps most widely accepted neural mechanism suggested to mediate antidepressant action is hippocampal cell genesis, as antidepressants such as SSRIs are well known to increase neurogenesis (Malberg et al., 2000). Interestingly,

hippocampal FGF-2 expression is increased in response to chronic antidepressants (Mallei et al., 2002). Furthermore, previous reports showed FGF-2 enhancing cell proliferation and cell survival (Wagner et al., 1999) (Rai et al., 2007).

Thus, there is mounting evidence pointing to a possible role of FGF-2 in mediating the hippocampal cell genesis response seen after antidepressant treatment. Our results in Chapter 3 support this idea as chronic FGF-2 increased hippocampal new cell survival. Furthermore, we showed that experiences reducing anxiety such as EC require FGF-2 to increase cell genesis. On the other hand experiences known to negatively impact anxiety, which serve as depression models, such as social defeat, (Kinsey et al., 2007) have been shown to decrease FGF-2 expression in the hippocampus (Turner et al., 2008a). Furthermore, social defeat also decreases hippocampal new cell survival (Thomas et al., 2007). Given that the effects of antidepressants on modulating anxiety take two to three weeks to be observed, it is reasonable to suggest that changes in hippocampal neurogenesis could in part be the underlying mechanism of such changes in behavior. Furthermore, the relevance of FGF-2 in reducing anxiety added to its positive impact on hippocampal cell genesis leads us to hypothesize that chronic FGF-2 modulates anxiety in part via an enhancement of hippocampal cell genesis.

While the evidence linking FGF-2 to potential plasticity mechanisms reducing anxiety under chronic conditions is considerable, sparse reports also implicate this growth factor in modulating anxiety behavior under acute conditions. For example, previous studies show an increase of hippocampal FGF-2 gene expression in response to anxiogenic stimuli, such as acute restraint (Fumagalli et al., 2005). Moreover, corticosterone administration up-regulates FGF-2 in the hippocampus (Molteni et al., 2001) and stress-mediated increases in hippocampal FGF-2 require corticosterone responses (Frank et al., 2007). These reports suggest that hippocampal FGF-2 may participate in modulating acute stress related behavioral responses and that such acute modulation works in concert with modulators of anxiety behavior such as glucocorticoids (Wei et al., 2004).

FGF-2's acute anxiogenic behavioral effects may well result from an endogenous neural stress response within the hippocampal circuitry. Corticosterone provides an alerting and regulatory response to the organism when encountering threatening environmental or physiological stimuli (Wei et al., 2004). This, in turn, triggers an up-regulation of FGF-2 expression, as the FGF-2 gene predominantly responds to glucocorticoid receptor (GR) (Molteni et al., 2001). *We hypothesize that this induction of FGF-2 by glucocorticoids may provide an endogenous protective and adaptive response to highly stressful stimuli*. FGF-2 is known to be a necessary player in neurotrophic and neurogenic support after injury (Yoshimura et al., 2001), and it may play a similar neurotrophic and neurogenic

support role after stressful conditions, preparing the hippocampal circuitry for coping with imminent changes occurring in the organism's external environment. Thus FGF-2 may provide an endogenous trophic support in response to stressors such as novelty or anxiogenic stimuli. Thus stress may induce FGF-2 (this can also be the stress of an enriched environment), which could initially trigger an anxiogenic response. In turn FGF-2 induces astrocytes, which contain more FGF-2 that can also be protective and lead to resilience against anxiety after long term treatment.

The notion that FGF-2 may serve to protect the neural circuitry when an organism is confronted with an environmental threat is exemplified in FGF-2's prevention of excitotoxic cell death (Mattson et al., 1995) via inactivation of NMDA receptors on hippocampal neurons (Boxer et al., 1999). Interestingly, glucocorticoids reduce hippocampal cell proliferation via NMDA receptor activation (Cameron et al., 1995). On the other hand, glucocorticoids increase hippocampal FGF-2. Thus the inactivation of NMDA receptors by FGF-2 could be seen as part of a *negative feedback response* regulating stress-related neural activity. FGF-2's inactivation of NMDA receptors may constitute a cellular protective response within the hippocampus, resulting from acute stress conditions. In turn the anxiety behavioral response elicited by FGF-2 may help preserve the subsequent survival of new cells by limiting the exposure of the individual to additional threatening stimuli. This endogenous mechanism would help counteract or reduce the deleterious effects of glucocorticoids and NMDA

activity such as the decrease in cell proliferation that results in response to stressors (Gould et al., 1997).

While the current studies did not directly test the idea that acute stress is both anxiogenic and induces FGF-2, the impact of acute stress on FGF-2 expression has been previously demonstrated (Molteni et al., 2001). We suggest that the direct injection of FGF-2 into the hippocampus mimics the later stages of this proposed process—it generates an acute anxiety response while setting into motion the neuroprotective mechanisms that become evident upon repeated administration of the growth factor.

Alternatively, the mechanism underlying the differential pattern of anxiety after acute and chronic FGF-2, may well be related to a neurotransmitter-like modulation of anxiety similar to that of serotonin. This is particularly appealing given that acute injections with 5-HT1A receptor agonists directly into the hippocampus increase anxiety (File and Gonzalez, 1996). Furthermore, chronic treatment with SSRIs antidepressants also reduces anxiety (Dulawa et al., 2004). This change in behavioral effects from being anxiogenic acutely to being anxiolytic chronically has been attributed to changes in serotonergic transmission activity. Initially drugs acting on somatodendritic 5HT1A autoreceptors directly such as agonists or indirectly such as SSRIs lead to a decrease in the release of serotonin at the synaptic cleft in the terminal projection areas. By contrast, chronic treatment with such drugs leads to a desensitization of 5HT1A

somatodendritic autoreceptors and subsequent disinhibition (File et al., 2000), lending to a sensitization of postsynaptic receptors, thereby decreasing anxiety. Given that FGF-2 follows a similar pattern from acutely being anxiogenic to chronically being anxiolytic, we reason that perhaps a mechanism of action of "*cross-talk"* between the FGF-2 and the serotonin system could be taking place. Furthermore, the fact that FGF-2 increases in response to chronic SSRI treatment supports the notion that FGF-2 may be acting in the synaptic restructuring of the serotonin system (Mallei et al., 2002). Thus, acutely FGF-2 may enhance anxiety by enhancing the response brought by decrease in post-synaptic serotonergic release in the hippocampus. On the other hand chronic treatment with FGF-2 may promote a sensitization of hippocampal postsynaptic 5HT1A receptors, which may contribute to reduce anxiety. This is especially plausible given that FGF-2 has previously been shown to be required for behavioral sensitization to drugs of abuse and it has been suggested that it does so in concert with neurotransmitters (Flores and Stewart, 2000). These changes in serotonin receptor sensitization may in turn provide a venue for increasing cell genesis in the hippocampus seen in response to chronic antidepressants (Malberg et al., 2000).

Differential responses to FGF-2 in HR and LR

When presenting our results relating to the acute anxiogenic and chronic anxiolytic responses to FGF-2 we observed that these effects showed strong

interactions with the HR-LR phenotype. Specifically, under acute administration, FGF-2 had a differential anxiogenic response in HR animals converting the low anxiety HR animal into a high anxiety LR-like phenotype. On the other hand, when FGF-2 was injected chronically, the typically high anxiety behavior of LRs changed to resemble that of an HR animal. This result was particularly intriguing as HR animals show higher basal levels of FGF-2 relative to the high anxiety LRs. *Thus, chronic FGF-2 administration appears to rescue the low levels of FGF-2 in the hippocampus of the LR animals, rendering them HR-like in terms of anxiety behavior.*

Thus, it appears that low levels of hippocampal FGF-2, as seen in LRs, may enhance vulnerability towards high anxiety-like behavior. Conversely, high levels of FGF-2 in the hippocampus can decrease the vulnerability to anxiety, be it by genetic endowment as seen in HRs or by chronic administration as seen in the LRs repeatedly treated with FGF2. Moreover, this same set of mechanisms can be invoked in conjunction with environmental complexity, a treatment that led to a differential increase in FGF-2 in LRs in addition to a differential decrease in their anxiety-like behavior. This further supports the view that the high anxiety behavior seen in LRs could be attributed to low levels of FGF-2. *Thus, EC may provide an alternative approach to decreasing vulnerability to anxiety-like behavior by increasing hippocampal FGF-2 in animals such as LRs that are basally deficient in the expression of this growth factor.*

By contrast, the acute anxiogenic effect of FGF-2 in HR's, along with its lack of anxiolytic effectiveness when administered chronically to these same animals, suggest that FGF2 may not be a universally effective strategy for reducing anxiety behavior. In HRs the support of FGF- 2 seems to be built as part of their genetic endowment and inducing or delivering more FGF-2 may perhaps "saturate" the system. Thus, acutely, FGF2 readily elicits in HR's the anxiety response discussed above, whereas chronically FGF2 appears ineffective in altering anxiety behavior in these animals—they appear "treatment resistant". It is therefore reasonable to suggest that *an optimal level of FGF-2 is required for effectiveness in controlling anxiety behavior. This level is achieved under basal conditions by the HRs and is only reached by LR's under enriched conditions such as EC.*

While this may be the case under basal conditions, it is conceivable that, under stressful conditions, FGF-2 treatment might prove beneficial even to the HR animals. It should be noted that in response to strong stressors such as social defeat, HR's behavior comes to resemble that of LR's (Kabbaj et al., 2001). Indeed, certain stressors have a stronger impact on HRs than they do on LR's. Remarkably these same manipulations decrease hippocampal FGF-2 expression (Kabbaj et al., 2001). Thus it is conceivable that the differential impact of stress in HRs might be associated with an increased requirement for FGF-2. In other words, under normal circumstances HRs exhibit low vulnerability to anxiety behavior, in part due to high hippocampal levels of FGF-2. However, HR animals

are highly reactive to their environment, and a chronic stressor may both lead to profound behavioral changes and deplete the stores of FGF2 in the HRs. Thus, it would be critical to determine whether manipulations such as social defeat have a particularly strong impact on FGF2 expression in the HR animals, and whether they would now profit from FGF2 treatment at least as much, or possibly more than, the LR animals.

This scenario is plausible as antidepressant treatment in humans is only effective when clinically needed, whereas its administration in a non-depressant individual may not prove beneficial and may carry untoward side effects, such as increased anxiety. This is important because when addressing vulnerability it is imperative to take contextual conditions into account as well as individual genetic endowment. Thus, the FGF-2 treatment strategy for anxiety may be beneficial to LRs under normal circumstances whereas in HRs the treatment may be effective only under stressful conditions. One particular piece of evidence to support such an idea is that while HR and LR differ on basal levels of anxiety under normal conditions, when exposed to stronger stressors the typical behavioral responses seen in HRs become more LR-like (Kabbaj et al., 2001). It is possible that under such circumstances we might see FGF-2 having an anxiolytic response in HRs and perhaps that a chronic treatment might be a viable alternative.

Finally, it is worth mentioning that under normal conditions laboratory animals such as HRs and LRs live in environments of reduced complexity and have

severe limitations of physical activity compared to their wild counterparts. The above studies showing increased neurogenesis and reduced anxiety following FGF-2 and EC could therefore be viewed as a decrease in neurogenesis and an increase in anxiety following environmental deprivation (Gould et al., 2000). Similarly, it could be viewed that HRs are less affected by environmental deprivation as opposed to LRs, and exposure to EC rescues the stressful impact of living in such deprived conditions. Thus the apparent genetic differences between HRs and LRs may not necessarily account for the basal differences in FGF-2 expression, but in response to the environmental deprivation. It could be that environmental deprivation has a bigger toll on LRs, that translates to a decrease in FGF-2 expression in the hippocampus, which in turn results in an increase in anxiety. Furthermore it could be that such effects of environmental deprivation were normalized by FGF-2 and EC in LRs whereas no correction was necessary in HRs as they might be more resilient to the negative impact of environmental deprivation.

In conclusion, FGF-2 has different effects in HRs and LRs on anxiety behavior. Although the selectively bred HR and LR animals provide an excellent model of genetic vulnerability to anxiety, it is also appealing to have these animals as a potential pharmacogenomics model. This is viable to the extent seen in our results with FGF-2 as well as in previous studies where HR and LR animals showed a differential response profile to antidepressants (Jama et al., 2008). It is also critical to assess their responsiveness under appropriate contextual

conditions, for example examining treatment responses under basal versus stressful conditions. Such an approach would provide a more complete characterization of the differential treatment responsiveness of HR and LR animals.

The hippocampus as a mediator of anxiety-like behavior

Our results suggest a novel role for the FGF system in the modulation of anxiety-like behavior that may be mediated by the hippocampus. The hippocampus has mostly been studied for its role in learning and memory (Scoville and Milner, 2000), and we recognize that the hippocampus has not particularly been considered as a key region responsible for modulating anxiety-like behavior. However, recent evidence suggests that the hippocampus may play a functional role in modulating anxiety-like behavior. For example, hippocampal lesions and direct intra-hippocampal pharmacological treatments have been shown to alter anxiety-like behavior (Bannerman et al., 2004, Engin and Treit, 2008). In the following section, we will focus our attention on the role of the hippocampus in modulating anxiety-like behavior.

The fact that hippocampal FGF-2 modulates anxiety-like behavior was initially supported by our findings that showed HR-LR basal differences in FGF-2 expression in the hippocampus. HRs, which typically exhibit low levels of anxiety-like behavior, displayed higher expression of FGF-2 in the hippocampus than

LRs. Moreover, hippocampal FGF-2 increased in response to EC, which we showed to be anxiolytic, supporting the supposition that high expression of endogenous FGF-2 in the hippocampus results in a phenotype with decreased anxiety-like behavior. Finally, FGF-2 itself reduced anxiety-like behavior further validating its ability to modulate anxiety-like behavior. However, these results were correlational, as FGF-2 was not shown to directly act on the hippocampus.

Nonetheless, one particular set of results, which unequivocally implicated the hippocampus in anxiety-like behavior, was the effects of direct microinjections of an FGF receptor antagonist. In this set of studies, we saw that intra-hippocampal microinjections with an FGF receptor antagonist significantly reduced anxiety-like behavior. These results are in agreement with the anxiogenic effects observed following systemic FGF-2 injections and suggest that the hippocampus may play a functional role in regulating anxiety-like behavior. Furthermore, the microinjection study also pointed to the specific importance of the hippocampus in modulating individual differences in anxiety-like behavior. Here, we saw that the acute anxiogenic effects of FGF-2 were more prominent in HRs, whereas the chronic anxiolytic effects of FGF-2 were observed in LRs.

The role of the hippocampus in modulating individual differences in anxiety-like behavior has previously been shown in the HR an LR model (Kabbaj et al., 2000). Specifically, HR and LR animals have been shown to exhibit differences in hippocampal glucocorticoid receptor (GR) expression. In this example, LRs

displayed higher expression of GR relative to HRs. These differences in hippocampal GR expression are, in part, responsible for the HR-LR differences in anxiety as hippocampal microinjections with a GR antagonist have been shown to disrupt individual differences. Furthermore, several models known to show individual differences in anxiety-like behavior have yielded results reminiscent of hippocampal differences found between HR and LR animals. For example, offspring of dams that exhibited decreased maternal behavior show increased anxiety-like behavior relative to offspring of mothers that exhibited greater maternal attention (Caldji et al., 1998). Although these differences are not necessarily dependent on differences in the hippocampus, there is evidence that adult offspring of mothers that exhibited differences in maternal behavior display differences in hippocampal gene expression and morphology similar to those observed between HR and LR animals. To this end, animals with reduced anxiety-like behavior and greater maternal behavior showed higher levels of FGF-2 and higher rates of cell survival in the hippocampus (Bredy et al., 2003). Taken together, the results presented in this dissertation along with a few previous studies suggest that the hippocampus is a strong modulator of individual differences in anxiety-like behavior.

Although the functional relevance of the hippocampus in anxiety-like behavior is still controversial, several reports support this function in addition to its primary role in learning and memory. Previously, lesion studies have shown the hippocampus to be involved in anxiety-like behavior. For example, hippocampal

lesions resulted in decreased anxiety-like behavior (Bannerman et al., 2002). Interestingly, evidence supports an anatomical segregation within the hippocampus, whereby the ventral hippocampus is predominantly involved in anxiety modulation and the dorsal hippocampus is predominantly involved in learning (Bannerman et al., 2004).

Studies have demonstrated that ventral lesions decrease anxiety-like behavior with no effects observed in the dorsal hippocampus (Bannerman et al., 2003). Specifically, anxiolytic effects of lesions to the ventral hippocampus have been demonstrated in a series of tests of anxiety-like behavior including the novelty-suppressed feeding test, the light-dark box test, the social interaction test and the elevated-plus maze. On the other hand, when the dorsal hippocampus was lesioned, deficits were observed in spatial learning and memory without any observable effects on anxiety-like behavior. Interestingly, ventral hippocampal lesions did not affect spatial learning. Accordingly, several authors have attributed a double dissociation of the effects of ventral and dorsal hippocampal lesions, where the dorsal hippocampus participates on spatial learning and working memory tasks and the ventral hippocampus has a preferential role in anxiety-like behavior (Bannerman et al., 2004).

Although the reports mentioned above support the notion that the ventral hippocampus is involved in the modulation of anxiety, it is important to mention that such conclusions go beyond anxiety, and extend their findings to fear

learning. As several of the above-mentioned results suggest that the ventral hippocampus modulates anxiety-like behavior, further results also suggest that ventral hippocampal lesions reduce freezing behavior after cued and contextual fear conditioning (McHugh et al., 2004). Thus, it is important to make a distinction between fear conditioning and anxiety-like behavior and acknowledge that studies often include fear conditioning as a measure of anxiety even though fear conditioning has a learning component. In this dissertation, none of the measures of anxiety used involved learning, as the anxiety tests were chosen to be representative of spontaneous anxiety. Furthermore, previous findings that ventral hippocampal lesions altered freezing on cued and contextual fear conditioning supports the notion that an interaction with the amygdala is crucial for modulation of fear and anxiety. Thus, the ventral hippocampal modulation of both anxiety and fear has an intricate circuitry associated with the amygdala, especially given that connections between the hippocampus and amygdala are via the ventral hippocampus (Ishikawa and Nakamura, 2006). This is particularly important as lesions within the ventral hippocampal subregion may well be affecting input and outuput connections that can actively participate in complex interactions between the amygdala and cortical regions (Ishikawa and Nakamura, 2003).

Although fear and anxiety seemingly go hand in hand, it is important to distinguish between the two in order to determine the role of the ventral hippocampus in anxiety-like behavior, as the distinction is of utmost importance

to review in this dissertation. "Fear refers to a phasic response to explicit conditioned aversive cues whereas anxiety is a more tonic response to diffuse unconditioned aversive cues or situation" (Bannerman et al., 2004). Although the above-mentioned reports suggest a favored role for the ventral hippocampus in anxiety-like behavior, it is more appropriate to state that the ventral hippocampus participates in the modulation or inhibition of fear associated responses that may also be behaviorally present on measures of anxiety. Such assumptions are supported by studies which suggest a role for the hippocampus in "behavioral inhibition" as defined by an adaptive process whereby an organism suppresses behaviors that are not appropriate when confronted with mildly stressful stimuli (Bannerman et al., 2004).

The evidence above citing the ventral hippocampus as a key component of the hippocampal modulation of anxiety-like behavior appears to be in conflict with the results presented in this dissertation given that administration of an FGF antagonist into the dorsal hippocampus reduced anxiety-like behavior in both the elevated-plus maze and open field test. However, it is worth noting that we did not verify whether injections into the ventral hippocampus would have similar or more pronounced anxiolytic effects. Nonetheless, the majority of the literature supports the preferential role of the dorsal hippocampus in spatial learning and memory (Bannerman et al., 2003). However, there is substantial evidence supporting the role of the dorsal hippocampus in various measures of anxiety-like behavior. The role of the dorsal hippocampus in mediating anxiety-like behavior,

is supported by studies showing direct microinjections having profound effects on both increasing and reducing anxiety-like behavior that varies by neurotransmitter system (Engin and Treit, 2008). One such recent finding reported that direct microinjection of substance P into the dorsal but not the ventral hippocampus had anxiolytic effects on the several anxiety tests (Carvalho et al., 2008). Moreover, dorsal hippocampal activation of the GABAregic system with several agonists resulted in anxiolytic effects (Rezayat et al., 2005). While benzodiazepine antagonists are able to block the anxiolytic effects of GABA-A agonists, no effects were observed when the compounds were microinjected alone. This suggests that although GABA modulates anxiety in the dorsal hippocampus, GABA-A receptors do not maintain a tonic modulation of anxiety-like behavior in the dorsal hippocampus. Similarly, as presented earlier in this chapter, other neurotransmitters systems, such as the serotonergic and cholinergic systems, have been shown to actively play a role in the modulation of the impact of the dorsal hippocampus on anxiety-like behavior. Specifically, dorsal intra-hippocampal microinjections with 5-HT1A agonists, such 8-OH-DPAT, showed an anxiolytic effect. Moreover, indirect acetylcholine agonists, such as the acetylcholinesterase inhibitor physostigmine, also showed anxiolytic effects following microinjection into the dorsal hippocampus (Engin and Treit, 2007).

Although we attribute endogenous FGF-2 mRNA differences in the hippocampus to differences in anxiety it is important to note that other anatomical regions may

also be participating in such behavior. Moreover, it is possible that the effect of the FGF-2 protein may extend to other input and output regions connecting to the hippocampus.

As mentioned earlier, FGF-2 is widely expressed in the rat brain including other regions associated with anxiety-like behavior such as amygdala, locus coeriliues, dorsal raphe and medial prefrontal cortex. Anatomical mapping of FGF-2 has shown that FGF-2 synthesizing cells show colocalization with FGF-2 protein expression in areas such as the hippocampus (Gonzalez et al. 1995). However, it has been shown that FGF-2 mRNA shows low abundance in the dentate gyrus, whereas protein expression is very high in abundance. Importantly, though the expression of FGF-2 and FGFr1 in the hippocampus is much higher in the CA2 and CA3 respectively. Thus given that FGF-2 is a diffuseable protein it is possible that FGF-2 could also be acting outside the dentate gyrus. This is highly probable given that FGF receptors are mostly expressed within axonal fibers and that the dentate gyrus itself show low protein expression of FGF receptors. Thus it is plausible that FGF-2 synthesize in the dentate gyrus could be acting in other sub-regions of the hippocampus or other anatomical locations within the brain. This is particularly possible for regions in which FGF-2 synthesizing cells are found in low abundance such as the amygdala and dorsal raphe. Thus such regions may require the synthesis of FGF-2 stemming from the hippocampus. However, in support of our effects of chronic FGF-2 on neurogenesis and anxiety FGFr1 mRNA is highly expressed in the dentate gyrus.

Although these findings do not parallel the low protein expression of this receptor it is well known that FGFr1 is required for hippocampal neurogenesis (Ohkubo et al 2004).

Taken together, these reports further support the role of the hippocampus as a key modulator of anxiety-like behavior and expand on the possibility that FGF-2 may be acting in concert with several transmitters systems within the hippocampal circuitry to modulate anxiety-like behavior. Furthermore, the hippocampus may be the site whereby chronic FGF-2 can modulate anxiety-like behavior. Finally, the anxiolytic effect of FGF-2 may be related to the effect of FGF-2 on cell genesis. Differences in cell genesis following FGF-2 favor an increase in cell survival followed by a preferential increase in glial cell differentiation.

Differential impact of FGF-2 on hippocampal cell genesis

Chronic FGF-2 increased new cell survival selectively in LRs. These findings are consistent with previous findings that showed exogenous FGF-2 rescued deficits in cell survival in middle aged animals (Rai et al., 2007). The fact that LRs showed lower cell survival than HRs is also consistent with reports of decreased FGF-2 and decreased cell survival in the adult hippocampus of offspring of mothers with reduced maternal behavior (Bredy et al., 2003). Taken together, LR's low expression of hippocampal FGF-2 may be associated with lower cell

survival which could, in turn, lead to decreased cell genesis, particularly in the production of astrocytes.

The impact of FGF-2 on cell genesis has mostly been associated with neurogenesis; however, our results showed that chronic FGF-2 could preferentially modulate the number of glial cells in the hippocampus. Following FGF-2 treatment, there was a four-fold increase in the production of new astrocytes compared to a two-fold increase in neurogenesis. These results are consistent with FGF-2 knockout animals exhibiting a reduction in both glial genesis and neurogenesis (Cheng et al., 2002). Although there were a significantly lower number of BrdU labeled astrocytes in LRs, FGF2 promoted the differentiation of both neurons and astrocytes and preferentially increased glial genesis in LRs. Thus, FGF-2 preferentially promoted glial differentiation in LRs.

Phenotypic analysis of BrdU+ cells in vehicle-treated animals supports the role of FGF-2 in astrocyte differentiation, as HR animals showed an overall higher pattern of glial differentiation of BrdU+ cells relative to LRs. Interestingly, HR and LR control animals exhibited differences in astrocyte differentiation. However, treatment with FGF-2 ameliorated these baseline differences in astrocyte differentiation. To our knowledge, FGF-2 has not previously been directly linked to astrocyte differentiation; however, several findings have shown FGF-2 to modulate the expression of GFAP in the cortex (Reuss et al., 2003) and to synergistically participate with CNTF in promoting astrocyte differentiation (Song

and Ghosh, 2004). This suggests that FGF-2 may modulate differentiation of newly-generated cells in the hippocampus, and this modulation may translate into an enhancement of GFAP-expressing astrocytes.

Several reasons could explain why FGF-2 showed a preference to promote the differentiation of GFAP-positive BrdU cells. As mentioned above, FGF-2 may participate in a regulatory mechanism that supports the promotion of glial fate determination. Additionally, FGF-2 may also be promoting an increase in the GFAP-expressing primary Type B progenitor cell pool (Zheng et al., 2004) (Doetsch, 2003)

The subgranular zone of the dentate gyrus contains two types of dividing cells that give rise to new neurons. The slowly dividing Type B cells that express the intermediate filament GFAP, and the Type D precursor cells that express markers of differentiating neurons (Doetsch, 2003). The duration of our experimental design enabled us to see the terminal differentiation of BrdU labeled cells. Here, neuronal differentiation was corroborated with NeuN co-labeling, and an increase in the astrocyte differentiation was verified with GFAP co-labeling. However, the apparent increase in astrocyte differentiation may actually reflect an increase in the GFAP expressing Type B cell pool and not necessarily the number of astrocytes. The majority of cells generated in the dentate gyrus are neurons resulting from the Type B and Type D stem cell pool.

The GFAP-expressing progenitors reside within the subgranular zone and can be further categorized into two subtypes as follows: radial glial-like cells and horizontal cells. Radial cells extend prominent processes along the granule cell layer as well smaller processes at the base of the subgranular zone. The horizontal cells lie at the base of the subgranular zone and lack radial processes (Ma et al., 2005). The radial GFAP astrocytes are sometimes referred also as the Type 1 progenitors and act as the primary slowly dividing progenitors that give rise to the Type D progeny which ultimately become new neurons (Doetsch, 2003). These radial GFAP astrocytes cells interact extensively with clusters of type D cells which snuggle themselves within the radial processes and migrate their way into the granule cell layer. Based on our data, it is plausible that chronic FGF-2 may have enhanced the ability of the Type B GFAP-expressing cells to self-renew and thereby increased the stem cell pool in LR animals (Doetsch et al., 2002).

The pattern of division of Type B cells *in vivo* is unknown; hence, it is not clear whether type B cells divide symmetrically, asymmetrically or both. However, factors such as FGF-2 and epidermal growth factor (EGF) are thought to be essential constituents of the stem cell niche where GFAP-expressing Type B cells are thought to maintain the structural milieu. Interestingly, FGF-2 and FGF receptors are thought to be required for proliferation of Type B cells (Zheng et al., 2004). As mentioned above, growth factors are known to have positive effects on cell proliferation and self-renewal, as well as significantly impact cell fate

determination (Palmer et al., 1999). Thus, FGF-2 could act by promoting the self-renewal of Type B cells along with promoting cell survival. This, in turn, would lead to an increase in GFAP-labeled dividing cells as seen in LRs.

Although the above scenario presents an alternative explanation to the preferential increase in the number of GFAP-positive cells, some cells could have become typical or horizontal astrocytes, whereas other cells could have become Type B cells. Thus, an increase in GFAP-expressing BrdU cells could be merely one of the responses observed after FGF-2 treatment.

There is scarce evidence to suggest that FGF-2 has an impact on type B cells in the hippocampus. More than likely, multiple cells responded to FGF-2. Although this has not yet been shown *in vivo* with FGF-2, there is evidence that another growth factor, EGF, has distinct effects on proliferation and differentiation on Type B and Type C cells in the subventricular zone.

The subventricular zone stem cell categorization presents some similarities to the dentate gyrus neural stem cells. In the subventricular zone (SVZ), Type B cells are GFAP-expressing slowly dividing cells, whereas as the Type C cells are transit amplifying cells similar to the type D cells in the hippocampus. The Type D cells ultimately become type A neuroblasts. EGF resulted in an increase in type C cell self-renewal due to an increase in proliferation and a stop in neuroblast production (Doetsch et al., 2002). Thus, EGF both increased proliferation and

had a negative effect on the production of new neurons. Although this was not the case in our results because the increase in GFAP cells was not at the expense of new neurons, it presents a scenario where specific cell types respond to growth factors in characteristic ways to regulate differentiation.

In a different example with a growth factor similar to FGF-2, platelet-derived growth factor (PDGF) had a direct impact on Type B cells of the SVZ. Specifically, type B cells express the PDGF receptor and respond with increased proliferation upon ligand binding and result in a differentiation pool composed mostly of oligodendrocytes (Hart et al., 1989). This is interesting because, as mentioned previously, the Type B neural stem cells give rise to Type C or Type D cells and the result is usually the generation of new neurons. Thus, growth factors seem to play a regulatory role in neural stem cell differentiation in a manner that is cell-type specific, as well as growth factor specific whereby certain stimuli may promote glial or neuronal differentiation or neither. Finally, growth factors may regulate the balance of new neurons generated by promoting the differentiation of glial cells, by promoting self-renewal or by stopping the differentiation of neuroblasts.

The multi-faceted ability of growth factors is particularly applicable given that HR animals responded with an increase in non-differentiated cells. In HRs, FGF-2 provided the stem cell niche with an excess of FGF-2, which ultimately led to an arrest in the differentiation of new neurons without affecting the survival of new

cells. That is, FGF-2 perhaps may have provided the ingredients necessary for new cell survival. FGF-2 may also have activated Type B cells or other molecules within the niche to respond in manner that was not conducive to the generation of new neurons similar to the above-mentioned example with EGF in the SVZ.

Another remaining question is how LR animals may have benefited from an increase in new GFAP-expressing cells and a decrease in the number of undifferentiated cells. Perhaps FGF-2 activated a quiescent stem cell niche in LRs that resulted from low levels of FGF-2. The prolonged low levels of FGF-2 in the subgranular zone may have inhibited the self-renewal or proliferation of Type B cells, which in turn, may have prevented the generation of new cells. It is plausible that, upon treatment with FGF-2, the niche may have responded in a such that new GFAP Type B cells were recruited in order to promote the generation of new neurons (Song et al., 2002).

An alternative scenario could also help explain how the deficit in GFAP-expressing BrdU labeled cells was rescued upon FGF-2 treatment in LRs. During the aging process, the overall levels of FGF-2 in the dentate gyrus decrease dramatically. Furthermore, a decrease in the rate of cell survival in the dentate gyrus has also been reported (Shetty et al., 2005). Moreover, aging rats suffer from delayed neuronal differentiation of newly generated cells in the dentate gyrus (Rao et al., 2005). This delayed differentiation of neurogenesis

has been attributed to the decrease in the levels of FGF-2 in the dentate gyrus. Interestingly, the deficits in the levels of FGF-2 in the dentate gyrus have been explained by a decrease in the number of FGF-2 synthesizing astrocytes (Shetty et al., 2005). Thus, it is plausible that a similar phenomenon may be occurring in the dentate gyrus of LRs, where we have observed low levels of FGF-2. The decrease in FGF-2 observed in LRs could be negatively impacting the differentiation of newly generated cells by slowing the progress of differentiation thus leading to a higher number of undifferentiated cells under basal conditions. Consequently, treatment with FGF-2 may have restored the deficits in cell survival and the progress of differentiation leading to more neurons and astrocytes.

This reestablishment of an active stem cell niche coupled with the recruitment of Type B cells via treatment with FGF-2 could help explain the increase in new cell genesis in LR animals. I hypothesize that such restoration could lead to an increase in neurons and astrocytes within the hippocampal dentate gyrus. This increase in new hippocampal cells could potentially contribute to a decrease in anxiety-like behavior in LR animals.

These ideas present an interesting question of whether FGF-2 itself is prompting neurogenesis or whether an increase in astrocyte cells synthesizing FGF-2 are doing so. Our hypothesis is that it is a combination of both so that; FGF-2 induces astrocytes, and in turn astrocytes synthesize FGF-2 in a self-amplifying

the process. The chronic treatment with FGF-2 provided an increase in the survival of FGF-2 synthesizing astrocytes, which in turn provide the support for Type B cells to divide and promote the survival of new neurons. Thus the effects of chronic FGF-2 are two-fold one direct and one indirect. The direct impact of chronic FGF-2 is to promote the survival of astrocytes cells synthesizing FGF-2. Upon increase availability of such astrocytes newly dividing cells are recruited and promoted to differentiate into neurons by the FGF-2 that has been synthesized from the newly survived astrocytes. Such mechanisms provides a self amplifying process of the effects of FGF-2 on new cell survival and differentiation.

Final Conclusions

FGF-2 expression was previously shown to be decreased in the brain of severely depressed individuals (Evans et al., 2004, Gaughran et al., 2006). However, it remained unclear whether this was a consequence of the illness or whether FGF-2 plays a primary role in the control of mood and emotions. I, therefore, undertook a series of studies using animal models to ask whether members of the FGF family can indeed modulate emotionality, and whether they may constitute predisposing factors for individual differences in emotional reactivity. In this dissertation I focused on the role of the FGF-2 in anxiety-like behavior, given that there is significant co-morbidity between anxiety and major depression and that there is mounting evidence that the two disorders may be closely

associated due to a common etiology or risk factors (Gorwood, 2004). In order to test the role of FGF-2 in anxiety, I relied on two animal models; one which relied on genetic selection strategy in Sprague-Dawley rats to enhance basal differences in anxiety-like behavior (Stead et al., 2006) and another which made environmental conditions more complex. These models enabled us to test both the possible genetic role of FGF-2 and its implication as a result of environmental modulation. Furthermore, we administered FGF-2 in order to directly test its role in modulating anxiety-like behavior and its impact on hippocampal cell genesis.

The discussion above recapitulates the findings supporting the novel role of hippocampal FGF-2 in mediating anxiety-like behavior and hippocampal cell genesis. We have found that endogenous FGF-2 reduced vulnerability to anxiety-like behavior by genetic propensity, as seen in HRs, and by impact of experience, as seen in LRs following EC. In summation, EC selectively increased FGF-2 and reduced anxiety-like behavior in LRs. More importantly, we saw that treatment with FGF-2 differentially reduced anxiety in LRs. Furthermore, the functional role of hippocampal FGF-2 in experience-dependent anxiety-like behavior was supported by the fact that an FGF receptor antagonist blocked the anxiolytic effect of EC. Taken together, our results present FGF-2 as a novel mediator of individual differences and experience-dependent anxiety-like behavior.

Moreover, we expanded on the role of hippocampal FGF-2 on mediating anxiety-like behavior by showing the selective impact of FGF-2 on hippocampal cell genesis in LR animals. First, we showed that exposure to EC resulted in higher rates of cell proliferation and cell survival in the hippocampus. Moreover, FGF-2 facilitated a selective enhancement in cell survival in LR animals. Thus, chronic treatment with FGF-2 for a period of three weeks increased cell survival in the hippocampus of LRs. These results followed a similar pattern seen in response to three weeks of EC where we observed increased FGF-2 expression in LRs. The increase in new cell survival following FGF-2 resulted in a preferential increase in newly generated astrocytes in the hippocampus. Therefore, FGF-2 may reduce anxiety-like behavior by increasing the production of new astrocytes in the hippocampus.

In support of this dissertation, recent results from our group support the importance of the FGF system in depression research, as FGF-2 has been demonstrated to act as an antidepressant in the Porsolt Swim Test (Turner et al., 2008b). Furthermore, antidepressant drugs have been shown to increase FGF-2 expression (Mallei et al., 2002) and to enhance neurogenesis (Malberg et al., 2000). Our body of work is consistent with the current hypothesis that a deficiency in growth factors represents a vulnerability factor for mood disorders (Duman and Monteggia, 2006) and that the induction of growth factors is critical to the effectiveness of antidepressant drugs (Warner-Schmidt and Duman, 2007).

Surprisingly, this dissertation also suggests that EC may mimic the effects of FGF-2 and that its behavioral effects may resemble that of antidepressants. EC functioned to rescue LRs low expression of hippocampal FGF-2, and the result was an increase in hippocampal cell genesis and a subsequent reduction in vulnerability to anxiety. These results also support the hypothesis that low expression of FGF-2, as a result of genetic propensity and/or environmental influence, may contribute to the vulnerability of a depression-like phenotype, as increased anxiety is often observed in major depressive disorder (MDD).

In summary, decreased levels of FGF-2 may be involved in the pathophysiology of mood disorders, and antidepressants or EC may exert their effects on hippocampal cell genesis by increasing FGF-2. More importantly, our findings suggest that FGF-2 is not only a mediator of mood disorder pathology but may also influence the vulnerability to anxiety-like behavior in the context of genetic propensity or environmental influence.

Future Directions

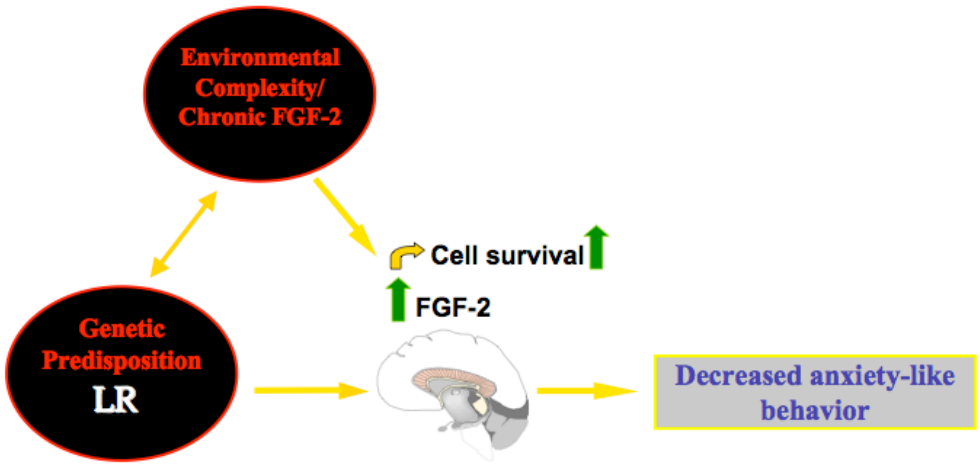
In my discussion above I suggest that hippocampal FGF-2 reduces vulnerability to anxiety via an enhancement of cell genesis in the hippocampus. While I provide evidence to support such a hypothesis it is apparent that most if these data is correlational and a direct role of hippocampal cell genesis on behavior is not directly provided. As a result future studies should be aimed at exploring the direct role of hippocampal cell genesis on anxiety behavior. To this end potential studies should tackle directly the blockade of ongoing hippocampal cell genesis. By blocking ongoing hippocampal cell genesis either with viral vectors at aimed killing dividing cells or chemical agents we could then test the effects of such treatment on anxiety behavior. Such studies could potentially show whether neurogenesis is required for decrease anxiety-like behavior.

Based on the evidence presented above and our conclusions, future translational studies should also be aimed at testing the potential therapeutic value of models reminiscent of EC on individual differences in anxiety. One interesting study could potentially identify individual differences in anxiety behavior using behavioral measures such as those used during clinical trials. One example would be to characterize individual differences in anxiety with questionnaires or similar measures. Once individuals with high anxiety are differentiated from those with low anxiety, a potential next step could be to the test the impact of models similar to EC on their anxiety behavior. More importantly if such studies

do show some promise it would be interesting to follow them with genetic testing to identify potential genetic markers predictive of such treatment response. These would include the potential identification of single nucleotide polymorphims (snps) on the FGF-2 gene to see if they predict differences in high versus low anxiety or treatment response. One other translational study could also test the impact of FGF-2 on anxiety behavior in high anxiety human individuals.

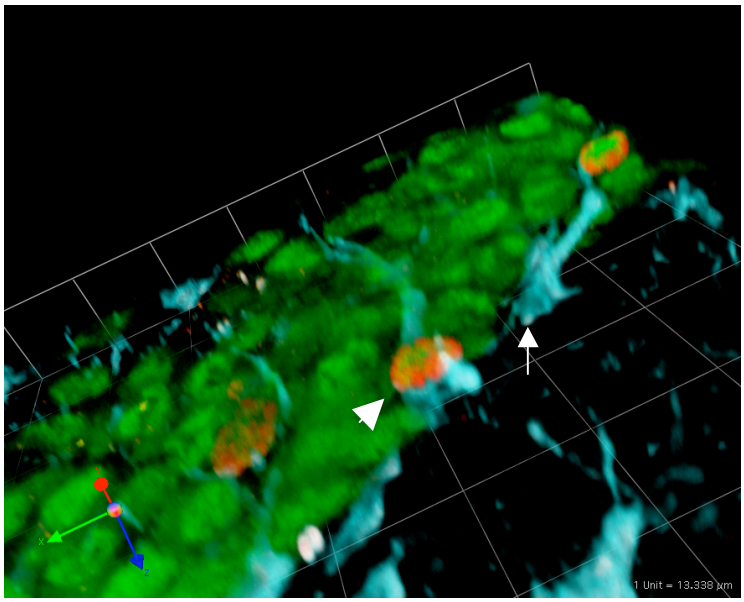
In conclusion future studies should be aimed at testing the potential therapeutic value of EC models in human anxiety patients. These studies could potentially provide a novel treatment strategy for treatment resistant populations suffering form anxiety disorders.

Appendix 5-1: Schematic model: FGF-2 reduces anxiety-like behavior



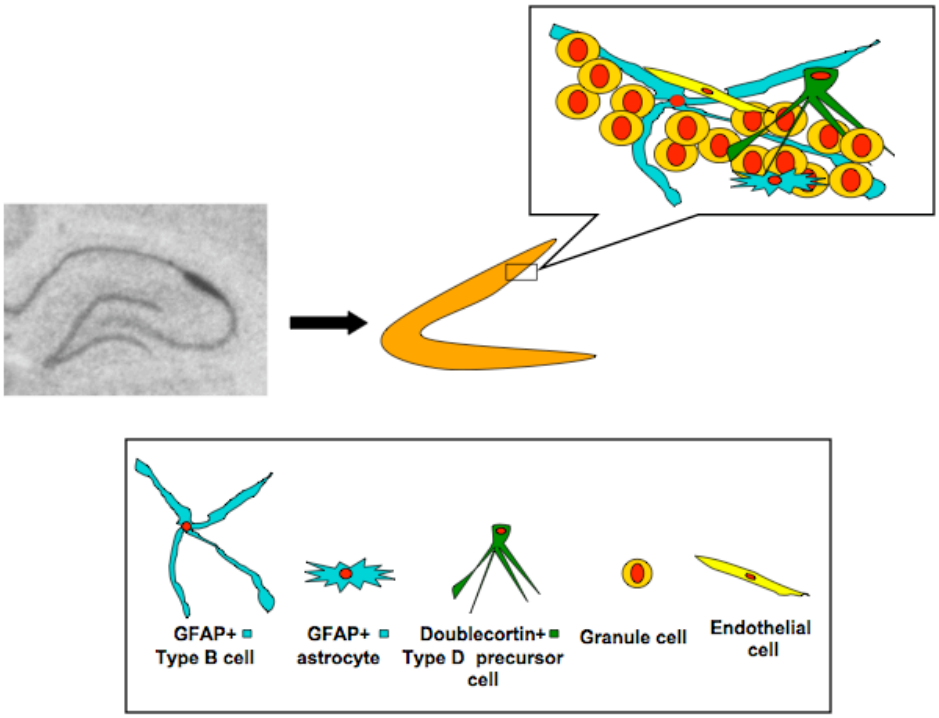
Schematic model of my hypothesis: ***Hippocampal FGF-2 reduces anxiety behavior by promoting glialgenesis and neurogenesis in the hippocampus.*** Low levels of hippocampal FGF-2 brought by genetic endowment contribute to a vulnerable phenotype as seen in LRs. Increasing hippocampal levels of FGF-2 by exogenous treatment or environmental complexity increases the survival of adult born cells which in turn lead to an increase in the generation of new astrocytes and new neurons. This net increase in astrocytes and neurons provide an overall support to the hippocampal circuitry helping reduce anxiety.

Appendix 5-2: Representative images of TypeB GFAP expressing cells



Arrowhead marks a GFAP expressing cell (blue) wrapping around BrdU/NeuN (red/green respectively) positive cell, resembling Type B cells. Arrow marks a GFAP expressing cell (blue) resembling horizontal astrocyte cells

Appendix 5-3: Representative model of the hippocampal stem cell niche.



Cell types found in the subgranular zone of the dentate gyrus as part of the stem cell niche. GFAP expressing cells (blue) wrapping around granule cell layer, resembling Type B cells.

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