

Fibroblast Growth Factor 2 (FGF2) is necessary for the antidepressant effects of fluoxetine in
chronically stressed mice

by

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Abstract

Previous research has shown that fibroblast growth factor 2 protein (FGF2) can act as an anxiolytic and anti-depressive agent in rodents. Levels of hippocampal FGF2 and FGF2 receptors are decreased in post-mortem brains of individuals with mood disorders. No changes in FGF2 were noted in the post-mortem brains of individuals with mood disorders that were successfully treated with antidepressant medication. Mutations in the *FGF2* gene in humans have predicted non-responsiveness to the therapeutic effects of selective serotonin reuptake inhibitors (SSRIs). FGF2 has pleiotropic effects such as promoting neuroplasticity and inflammation response. These findings suggest that FGF2 may be required for the therapeutic effects of antidepressant medications. To test this, we employed a rodent model of depressive behaviour, chronic variable stress (CVS) paired with antidepressant treatment (fluoxetine) in wild-type (WT) and *FGF2* knockout mice (FGF2KO) and examined depressive and anxiety behaviors. We hypothesized that fluoxetine will reverse the effects of CVS on depressive and anxiety behaviours in wild-type mice, but not in FGF2KO, suggesting that the *FGF2* gene is necessary for the therapeutic effects of fluoxetine. Because FGF2 has been previously shown to modify HPA activity through hippocampal glucocorticoid receptors (GR), we also examined levels of GR. We predicted that there would be a decrease in GR in response to CVS, as seen in previous studies, with a further decrease in the FGF2KO mice. Changes in cytokine levels have also been implicated in depression and are generally reversed with fluoxetine therefore we also examined whether these effects were FGF2 dependant. We believed there would be increases in levels of EGF, VEGF, BDNF, and FGF2 in stressed wildtype mice treated with fluoxetine, however we hypothesized that we would not see these changes in FGF2KO mice. Whether astroglia, astroglial functions, or HPA changes are the downstream target of FGF2-mediated changes induced by fluoxetine remains to be

determined, however, the current study reaffirms the potential of FGF2 as a novel therapeutic target in the treatment of depression and anxiety disorders.

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Background on Depression

Etiology

Depression is a debilitating mental illness that affects approximately 300 million individuals worldwide according to the World Health Organization ([WHO], 2018, para. 1). It is one of the leading causes of disability and a major contributor to suicide (“Global, regional, and national incidence, prevalence, and years lived with disability for 310 diseases and injuries, 1990–2015,” 2016). Depression can have adverse effects on the individual’s mind, body, social life, and everyday functioning. The risk factors involved in depression include exposure to stress, genetics, sex and psychological factors such as coping styles (Bierut et al., 1999; Cattaneo et al., 2015; Deverts et al., 2010; Duivis et al., 2011; Jansson et al., 2004; Lohoff, 2010; Rice, 2010; Wankerl et al., 2014). Chronic exposure to stressors, and, uncontrollable and unpredictable stressors have been shown to be highly correlated with an increase in pro-inflammatory markers and increased incidence of depression and anxiety disorders (Cattaneo et al., 2015; Cordner & Tamashiro, 2016; Hill, Hellemans, Verma, Gorzalka, & Weinberg, 2012; Kiecolt-Glaser, Derry, & Fagundes, 2015; Kronfol & Remick, 2000; Menard, Hodes, & Russo, 2016; Raison, Capuron, & Miller, 2006).

Symptoms

The classical Latin word for *depression* is *deprimere*, which literally means “press down” (Kanter, Busch, Weeks, & Landes, 2008). For someone to be diagnosed with depression, they must not only persistently feel sad but also exhibit at least five other symptoms of impairment for at least two weeks. These symptoms may include sleep and appetite changes, guilt and hopelessness, fatigue, anxiety, anhedonia (loss of pleasure), irritability, lethargy, unexplained aches and pains, restlessness, problems concentrating, and thoughts of suicide (American Psychiatric Association,

2013). Because of the complexity of symptoms, depression is very rarely diagnosed independently of other disorders.

Co-Morbidity and Rodent Models of Depression

Depression is often co-morbid with anxiety disorders and inflammatory disease (“Global, regional, and national incidence, prevalence, and years lived with disability for 310 diseases and injuries, 1990–2015,” 2016). Because depression is a unique and complex disorder, it can be difficult to replicate in animal models. For this reason, many animal models use measures such as learned helplessness and sucrose consumption to visualize depressive-like behaviour (Foregard et al., 2011; Krishnan & Nestler, 2011). Chronic exposure to stress has been known to lead to learned helplessness and anhedonia, both of which are symptoms of depression (Foregard et al., 2011; Krishnan & Nestler, 2011).

There are currently several different animal models of depression in place (ex. Maternal separation, social defeat, chronic unpredictable stress, etc..) (Kieseppä & Kansanterveyslaitos, 2005). These models require the rodent to be exposed to stressful situations to induce a depressive-like behavioural phenotype. This study employs the chronic variable stress (CVS) model. CVS is a commonly used paradigm that is designed to introduce recurrent physical, psychological, and social stress that is unavoidable and unpredictable in time and duration (Cordner & Tamashiro, 2016). This paradigm is meant to mimic the perturbations that are implicated in the development of depression in humans. The main strength of the model lies in its ability to induce changes in rodent behaviour which are associated with core symptoms of depression and anxiety including anhedonia, decreased exploratory behaviour and learned helplessness. In addition, changes are observed in peripheral markers such as pro-inflammatory cytokines and in neuroplasticity markers that parallel those findings observed in post-mortem tissue of humans that suffered from mood and

anxiety disorders. Finally, the induction of anxiety and depressive-like behaviours by CVS is generally reversed with chronic antidepressant treatments (Kanter et al., 2008).

Neuroplasticity as a Risk Factor that Mediates Depression

Among the many postulated biological theories concerning the pathophysiology of depression, decreased potential in the brain's neuroplasticity and cellular resilience has been suggested (Serafini, 2012). Cellular resilience is the ability of a cell to cope with environmental changes (Smirnova, Harris, Leist, & Hartung, 2015). The neuroplasticity hypothesis of depression suggests that changes in neuroplasticity and cellular resilience can cause physiological alterations in the brain's structure, thereby impairing neural circuits and their response to stimuli (Serafini, 2012). Neuroplasticity is the ability of the brain's neural circuits, brain nuclei, neurons, and synaptic connections to undergo several lifelong changes due to environmental stimuli through neural plastic mechanisms (Serafini, 2012). Manifestations of neuroplasticity in the adult central nervous system (CNS) include alterations of dendritic function, synaptic remodeling, long-term potentiation (LTP), axonal sprouting, neurite extension, synaptogenesis, and neurogenesis (Charney, DeJesus, & Manji, 2004). Interestingly, impairments in two brain areas are found to be involved in depression: the hippocampus and the prefrontal cortex (PFC). Several risk factors such as genetics or chronic stress from the environment, can affect the morphology of neural cells in the hippocampus and the PFC. Neural cells may react to the exposure of chronic stress by debranching dendrites or with spine loss (Eriksson et al., 1998). Not only are neural cells affected by stress but as are certain molecules, such as neurotrophic factors, which support neurons and further promote neuroplasticity.

A subset of antidepressants (ADTs), known as selective serotonin reuptake inhibitors (SSRIs), increase neurotrophic signaling, which promotes neuronal and synaptic remodelling and

the proliferation of new neurons in the hippocampus and prefrontal cortex (Serafini, 2012). Neurotrophic processes, which are involved in neuroplastic events, have been found to underlie depression. Although precise changes at the synaptic level by ADTs are unclear, it is well known that ADTs may promote neural connections and strengthen the connections through neurotrophins, which may be impaired in depressed individuals.

FGF2 and Neurogenesis

Background on Growth Factors

Previous research has shown evidence that changes in the expression of growth factor signaling molecules are correlated with depression (El Sayed et al., 2012). Growth factors are decreased in post-mortem hippocampal tissue from suicide victims, which suggests that altered trophic support could contribute to the pathophysiology of depression (Schmidt & Duman, 2007). With the administration of antidepressants, growth factors have been shown to exert antidepressant effects on affected brain regions (El Sayed et al., 2012). Research into the neurobiological changes accompanying depression have investigated levels of neurotrophins, such as Fibroblast Growth Factor 2 (FGF2), and the role it seems to play alongside antidepressant administration. Evidence that this system is dysregulated in depressed individuals comes from the findings that their circulation levels seem to be reduced in patients with mood disorders, and that antidepressant treatment reinforces their expression and signaling in the brain (El Sayed et al., 2012).

Growth factors are naturally occurring substances (usually a protein or steroid hormone) capable of stimulating cellular growth, proliferation, and differentiation (Stone & Bhimji, 2018). They act as signaling molecules between cells (ex. Cytokines and hormones that bind to a specific receptor type on the surface of their targets) (Stone & Bhimji, 2018). Different types of growth factors exist and although some functions overlap, growth factors also show specificity between

subtypes (Stone & Bhimji, 2018). For example, insulin-like growth factors (somatomedins) stimulate growth through the mediation of growth hormone secretion from the pituitary gland, or platelet-derived growth factors (PDGF), which stimulate growth of muscle and connective tissue cells (Giustina, Mazziotti, & Canalis, 2008). Some growth factors are small peptides called cytokines. While all cytokines affect signal transduction pathways, only those cytokines affecting cell growth and differentiation are growth factors (Stone & Bhimji, 2018). Examples of protein growth factors are vascular endothelial growth factor (VEGF) and epidermal growth factor (EGF).

VEGF has a role in promoting angiogenesis, neurogenesis, synaptic transmission, and has shown promise as a research target in depression (Strawbridge, Young, & Cleare, 2017). Two recent meta-analyses have shown that increase in expression of VEGF is found in the blood of depressed patients when compared to controls ($p < 0.001$) (Carvalho et al., 2015; Tseng, Cheng, Chen, Wu, & Lin, 2015). Antidepressants such as fluoxetine (a selective-serotonin reuptake inhibitor) have also shown to promote hippocampal expression of neurotrophic factors such as VEGF and brain-derived neurotrophic factor (BDNF) (Warner-Schmidt & Duman, 2007). A study conducted by Warner-Schmidt & Duman (2007) produced results that identified VEGF as a key mediator of antidepressants. An experiment in this 2007 study involved the inhibition of a VEGF receptor, Flk-1, using a complete blockade (SU5416) or a partial blockade (SU1498), to see if VEGF activation affected antidepressant efficacy (Warner-Schmidt & Duman, 2007). SU5416 completely blocked the effects of fluoxetine and desipramine, demonstrating that signaling through Flk-1 is required for the actions of these two antidepressants (Warner-Schmidt & Duman, 2007).

EGF is a potent mitogenic factor that plays an important role in the growth, proliferation, and differentiation of numerous cell types (“EGF epidermal growth factor [Homo sapiens

(human)] - Gene - NCBI," n.d.). Low levels of EGF in the blood plasma has been associated with depression (Tian et al., 2012). A study conducted in 2012 looked at eight single nucleotide polymorphisms (SNPs) within the EGF gene within 463 patients with major depression and 413 control participants among a Chinese population (Tian et al., 2012). The EGF levels of about 200 individuals from each group were determined. The EGF levels in plasma were significantly lower in the patient group than in the control group ($P < 0.0001$) (Tian et al., 2012b). Like VEGF, EGF may also be a useful biomarker for early diagnosis and prognosis of disorders like depression.

BDNF is abundant in the central and peripheral nervous systems of humans and found in both the serum and plasma (Lee & Kim, 2010). BDNF is vital to survival, growth, and maintenance of neurons (Phillips, 2017). There has been strong evidence that neuroplastic mechanisms that involve BDNF are severely altered in individuals with depression (Phillips, 2017). Specifically, clinical evidence has demonstrated that stress-induced depression has resulted in changes in BDNF expression and function, therefore altering neuroplasticity (Phillips, 2017). Untreated patients with depression have shown decreased levels of serum and plasma BDNF and antidepressant treatment promoted increased BDNF activity as well as neuroplastic effects (Lee & Kim, 2010).

Importantly, most growth factors are downregulated at the end of the developmental period. However, a few growth factors remain elevated in the brain throughout adulthood including FGF2 and BDNF (El Sayed et al., 2012). As such, they are thought to be critical for neuroplasticity, which involves similar processes as those seen in development, albeit at a much smaller and restricted scale. It is therefore not surprising that these would be intimately involved in the neuroplasticity hypothesis of mood and anxiety disorders.

Fibroblast Growth Factors (FGFs)

The fibroblast growth factors (FGFs) are a family of cell signaling proteins. They generally act as systemic or locally circulating molecules of extracellular origin that activate cell surface receptors. FGFs bind to heparin (a naturally occurring anti-coagulant produced by basophils and mast cells) and heparan sulfate (linear polysaccharide found in all animal tissue) and have a homologous central core of 140 amino acids (Woodbury & Ikezu, 2014). While many FGFs can be secreted to act on far cellular targets, others can act locally within a tissue. Signaling through heparin and heparan sulfate is what allows FGF2 to induce its pleiotropic effects on different tissues and organs.

FGF2, also known as basic fibroblast growth factor (bFGF) and FGF- β , is encoded by the *FGF2* gene (Dionne et al., 1990) and found in a subset of neurons, glia, the vascular membrane of blood vessels, and in the ependymal cells of the ventricles (Logan, Frautschy, Gonzalez, & Baird, 1992). Brain FGF2 is synthesized by astrocytes and indeed, astrocytic FGF2 has been implicated in enhanced neurogenesis in the subgranular zone (SGZ) of the dentate gyrus following acute stress (Woodbury & Ikezu, 2014). Human FGF2 has high molecular weight (LMW) of 22/21 kDa and low molecular weight (HMW) of 18 kDa isoforms (Woodbury & Ikezu, 2014). LMW FGF2 are cytoplasmic (autocrine) and HMW are nuclear (intracrine) (Woodbury & Ikezu, 2014). In humans, five different polypeptides can be formed from the same *FGF2* gene from five different mRNA translation initiation sites (Woodbury & Ikezu, 2014).

Signaling of FGF2 occurs through four high-affinity tyrosine kinase receptors FGFR1, FGFR2, FGFR3, and FGFR4 (Woodbury & Ikezu, 2014). A closely-related receptor, which lacks the FGF signaling tyrosine kinase domain, FGFR5 (also known as FGFR1L1) was recently discovered (Tiong, Mah, & Leong, 2013). FGFRs are encoded by receptor tyrosine kinases of

around 800 amino acids with several domains including three extracellular immunoglobulin-like domains (I, II, and III), a transmembrane domain (TD), and two intracellular tyrosine kinase domains (TK1 and TK2). FGF2 has a high affinity to IIIc-type FGFR1 and FGFR2, but a low affinity to IIIc-type FGFR3. Binding of FGF2 to FGFRs triggers receptor dimerization and tyrosine receptor kinase activation, resulting in autophosphorylation of the intracellular domain of the receptor and recruitment/assembly of signaling complexes (Woodbury & Ikezu, 2014).

FGF2 Expression Throughout Development of the Central Nervous System

FGF2 is a potent astroglial mitogen that is not only critical during development but, as aforementioned, is one of few growth factors (along with brain derived neurotrophic factor BDNF) that remain upregulated in the adult brain (Zheng, Nowakowski, & Vaccarino, 2004). During development, FGF2 is high in expression from neurulation (the folding process in vertebrate embryos, which includes the transformation of the neural plate into the neural tube) onwards. Expression of FGF2 and its receptors is temporally and spatially regulated during development. For example, expression of FGF2 and FGFR2 in the inferior colliculus and occipital cortex of the postnatal rat brain increased over the first month, with no increase in FGFR1 mRNA. Whereas in the cerebellum, FGF2 and FGFR1 mRNA expression was highest at postnatal day 1, but FGFR2 expression showed no significant change with age (el-Husseini A el-D, Paterson, & Shiu, 1994).

In the adult CNS, FGF2 is also markedly expressed in niches: the subventricular zone of the lateral ventricles – SVZ; and the subgranular zone of the hippocampal dentate gyrus – SGZ. FGF2 has also been implicated in the control of adult neurogenesis based on changes in proliferation and differentiation of adult neural stem and progenitor cells (Woodbury & Ikezu, 2014). Astrocytic FGF2 has been implicated in enhanced neurogenesis in the SGZ following acute

stress (Kirby et al., 2013). FGFR1, which is highly expressed in neural stem cells (NSC) of SVZ and SGZ, is crucial for proliferation of NSC in the hippocampus (Woodbury & Ikezu, 2014).

FGF2 and Proliferation

FGF2 regulates NSC propagation *in vitro* and *in vivo*. *In vitro*, FGF2 has been found to stimulate NSC proliferation and induce proliferation of adult mouse stem cells by keeping progenitor cells in the cell cycle, thereby preventing them from further differentiation (Gritti et al., 1996). *In vivo*, subcutaneous injection of FGF2 produced a 30% increase in proliferating granule cell precursors in the external granule layer of the newborn rat cerebellum, and a significant increase in DNA synthesis in the SVZ and hippocampus (Tao, Black, & DiCicco-Bloom, 1996). In contrast, FGF2 exposure to neutralizing antibodies decreased proliferation of cerebellar and hippocampal precursor cells in postnatal day 1 (P1) rats (Tao, Black, & DiCicco-Bloom, 1997). These studies have shown that pharmacological addition or blockade of FGF2 alters proliferation (Dono, Texido, Dussel, Ehmke, & Zeller, 1998).

FGF2 and Differentiation

In 1998, Dono *et al* found that FGF2-deficient mice viably show normal neuronal progenitor proliferation during development, however, a portion of these progenitors do not colonize to their target layers in the cerebral cortex. This implies that FGF2 is responsible for controlling migration and differentiation more so than proliferation of neural progenitor cells during development. The researchers concluded that even though FGF2 can be seen as an essential proliferative factor *in vitro*, FGF2 functions as a migratory signal for neural progenitors, or its deficiency is compensated for by other FGFs *in vivo* (Dono et al., 1998).

FGF2 and Synaptic Plasticity

FGF2 is also implicated in modulating synaptic plasticity *in vitro* and *in vivo*. FGF2 applied to rat cerebral cortical neurons enhances neurite outgrowth (Morrison, Sharma, de Vellis, & Bradshaw, 1986) and axonal branching in hippocampal neurons (Aoyagi, Nishikawa, Saito, & Abe, 1994). FGF2 application also enhanced long-term potentiation (LTP) in rat hippocampal slices (Terlau & Seifert, 1990). FGF2's exact synaptic effects are unknown but the dual action of FGF2 as a neurotrophic factor and inducer of synaptic plasticity is highly significant for development of therapies for depression since it is capable of incorporating new cells into a strengthened network.

FGF2 and the Depressed Brain

Because of its role in neurogenesis and synaptic enhancement, FGF2 has proven to be highly efficient in regenerating neurons in several animal models, including optic nerve injury and excitotoxic cell death (Sapieha, Peltier, Rendahl, Manning, & Di Polo, 2003). Many researchers have shown the potential therapeutic use of FGF2 for conditions like Alzheimer's disease (AD), Parkinson's disease (PD), and depression. FGF2 levels are shown to be decreased in the prefrontal cortex (PFC) and hippocampus of patients with depression (Evans et al., 2004; Kang et al., 2007). These levels have been shown to increase with antidepressant treatment in both humans and rodent models (Bachis, Mallei, Cruz, Wellstein, & Mocchetti, 2008; Evans et al., 2004).

Previous studies have shown that FGF2 can also act as an anxiolytic and an antidepressant in rodent models (Salmaso et al., 2016). FGF2 has been implicated in depressive behaviours, with FGF2 gene deletion showing an increase in depressive behaviour as well as an increase in hypothalamic-pituitary-adrenal axis activation (Salmaso et al., 2016). It has also been

demonstrated that FGF2-induced changes in HPA Axis activity are necessary for the anxiolytic effects of FGF2 (Salmaso et al., 2016).

Another study with animals conducted in 2012 by El Sayed et al. confirmed the upregulation of FGF2 expression in the rat PFC following antidepressant treatment. The researchers tested the role of FGF2 signaling in behavioural models of depression and anxiety using CVS/sucrose consumption test (SCT), forced swim test (FST) and novelty suppressed feeding test (NSFT). Chronic infusion of FGF2 (intracerebroventricular injection) blocked the deficit in SCT caused by the CVS exposure. Furthermore, after administration of an FGF inhibitor (SU5402), response to antidepressant treatment in CVS/SCT and FST were not effective (El Sayed et al., 2012).

Numerous studies have linked FGF2 to the therapeutic effects of antidepressant treatments such as Selective Serotonin Reuptake Inhibitors (SSRIs). In rodents, FGF2 levels increase in response to SSRI treatment (Bachis et al., 2008; Maragnoli, Fumagalli, Gennarelli, Racagni, & Riva, 2004), and, in humans, FGF2 gene mutations predict responsiveness to SSRI treatments (Kato et al., 2009). Importantly, these studies are mainly correlational in nature, therefore in the current study we will examine whether the *FGF2* gene is necessary for the antidepressant effects of fluoxetine in the presence and absence of perturbations.

Fluoxetine (Prozac) in the new Generation of Antidepressants

The *Diagnostic and Statistical Manual for Mental Disorders 5* (DSM-5) states that the treatment for depression falls into three categories: psychotherapy (i.e., cognitive behavioural therapy and interpersonal therapy), electroconvulsive therapy, and antidepressant medications (Wenthur, Bennett, & Lindsley, 2013). Monoamine oxidase inhibitors (MAOIs), a type of antidepressant, inhibit the effectiveness of monoamine oxidase, which is a chemical in the brain

that removes neurotransmitters from the brain. Individuals who suffer from depression do not have enough neurotransmitters present to keep their neurons communicating with each other. This produces low mood. When monoamine oxidase does not remove neurotransmitters, mood can be lifted because neurons are better able to communicate. MAOIs are more potent than selective serotonin reuptake inhibitors (SSRIs), but because they affect more neurotransmitters, they can cause more side-effects. SSRIs are considered safe and very effective. SSRIs only affect serotonin, which is one of the most important neurotransmitters involved in regulating mood. Because SSRIs influence only one neurotransmitter, they are more predictable and have fewer side effects.

One of the more well-known SSRIs approved to treat depression is fluoxetine (trade name: Prozac). Fluoxetine, (*R, S*)-*N*-methyl-3-phenyl-3-(4-(trifluoromethyl) phenoxy)propan-1-amine (CAS No: [54910-89-3]), is a racemic (composed of both dextrorotatory and levorotatory forms) phenoxyphenylpropylamine, with a molecular weight of 309.3 and an excellent ability to penetrate the central nervous system (CNS). It is FDA approved for major depressive disorder (for ages 8 and up), obsessive-compulsive disorder (ages 7 and up), panic disorder, bulimia nervosa, and premenstrual dysphoric disorder (Sohel & Molla, 2018).

Serotonin and Norepinephrine have been proven to play a large role in depression. Researchers have shown low concentrations of serotonin in the cerebrospinal fluid of patients with depression and lower levels of serotonin uptake sites have been found in platelets of patients with depression (Sohel & Molla, 2018). Pre-synaptic serotonin (5HT1A) receptors in the dorsal raphe nucleus of the brain stem project to the prefrontal cortex. Fluoxetine exerts its effects by blocking the reuptake transporter protein located in the presynaptic terminal (Sohel & Molla, 2018), making a selective serotonin reuptake inhibitor (SSRI). Because of its long half-life of 2 to 4 days, antidepressant effects are seen within 2 to 4 weeks. Upon administration, fluoxetine is subjected

to significant hepatic metabolism by cytochrome P450 enzymes (CYP2D6) forming several metabolites. Fluoxetine is primarily excreted (80%) as either norfluoxetine (*N*-desmethylfluoxetine), or as glucuronides (any substance produced by linking glucuronic acid to another substance via a glycosidic bond) of fluoxetine (Wenthur et al., 2013). Several studies have shown fluoxetine's antidepressant effectiveness in animal and human models.

A meta-analysis in 2012, using individual patient level-data of fluoxetine for the treatment of depression, showed statistically and clinically significant benefits (Gibbons, Hur, Brown, Davis, & Mann, 2012). The researchers wished to determine the short-term efficacy of antidepressants for treating major depression in youth, adults, and geriatric populations. Patients in all age groups had significantly greater improvement relative to placebo controls. The results were irrespective of baseline depression severity.

El Sayed et al. (2012) also demonstrated that the decreased levels of the high affinity receptor, FGFR1, following CVS is then restored to control levels after fluoxetine treatment. This study also determined the influence of the FGFR inhibitor, SU5402, on the FST response to fluoxetine. Based on previous reports of efficacy in this model, 10mg/kg dosage was used. A two-way ANOVA analysis showed a significant interaction between fluoxetine and SU5402 on time spent immobile ($F_{(1,36)}=3.96$; $p= 0.054$), which was then significantly reduced by fluoxetine ($p=0.018$) and abolished by SU5402 ($p=0.029$).

Fluoxetine is effective in treating all degrees of depression and has a distinctly more benign side-effect profile than other antidepressants. It is also safer in cases of overdose than older antidepressant drugs. It also seems that inhibition of the FGFR by SU5402 seems to prevent fluoxetine from exerting its antidepressant effects on the subject after CVS exposure (El Sayed et

al., 2012). This suggests that the FGF family and fluoxetine must work together to exert optimal antidepressant results.

Inflammation as a Mediator for Depression

Cytokines are proteins that are made by the immune system, which act as chemical messengers (Zhang & An, 2007). They are produced by several different cell types, including macrophages, B lymphocytes, T lymphocytes, mast cells, somatic cells, endothelial cells, and fibroblasts (Kronfol & Remick, 2000). Cytokines released from one cell can signal to another cell by binding to the surface receptor (Zhang & An, 2007). The different types of cytokines (chemokines, interferons, interleukins, lymphokines, and tumor necrosis factor) can work alone, together, or even against each other to help in the immune response (Zhang & An, 2007).

Research has shown that immune activation and cytokine production are involved in depressive disorders (Cattaneo et al., 2015; Dantzer, O'Connor, Lawson, & Kelley, 2011; Han & Yu, 2014; Howren, Lamkin, & Suls, 2009; Kiecolt-Glaser et al., 2015; Miller & Raison, 2016; Raison et al., 2006). Inflammation plays a key role in depression's pathogenesis for a subset of depressed individuals (Kiecolt-Glaser et al., 2015). Chronic stress and inflammation have been known to work together in the implication of depression. Chronic stress can cause prolonged inflammation and can trigger the development of depression via changes in neuroplasticity (Cattaneo et al., 2015; Dantzer et al., 2011; Kiecolt-Glaser et al., 2015). The resulting behaviours and health problems (e.g., pain, sleep alterations, or changes in diet) can lead to further inflammation and depression (Han & Yu, 2014). From chronic inflammatory stress, the immune system is activated, which then produces cytokines (small cell-signaling proteins that mediate and regulate immune responses and inflammation) that may be involved in depression (Zhang & An, 2007). Research into the pathology of mood disorders has found a bi-directional relationship

between depression and inflammation, in which depression facilitates inflammatory responses and inflammation promotes depression (Kiecolt-Glaser et al., 2015).

Background on Cytokines

Cytokines can be divided into two categories: (1) pro-inflammatory cytokines interleukin (IL)-1 β , IL-6, IL-12, and tumor necrosis factor (TNF)- α , and (2) anti-inflammatory cytokines IL-4, IL-10, interferon (IFN)- α , and transforming growth factor (TGF)- β 1 (these are only a few examples of pro- and anti-inflammatory cytokines) (Wankerl et al., 2014). As their names suggest, the pro-inflammatory cytokines promote inflammatory responses for diseases, and the anti-inflammatory cytokines antagonize the inflammation to promote healing. Interestingly, a subset of cytokines are capable of both promoting and reducing inflammation responses (Kronfol & Remick, 2000).

Recent meta-analyses have shown pro-inflammatory cytokine differences between patients with depression and control subjects. This includes differences in levels of IL-6, TNF- α , IL-1 β , the soluble IL-2 receptor, the IL-1 receptor antagonist (IL-1ra), and C-reactive protein (CRP) (Dowlati et al., 2010; Howren et al., 2009; Liu, Ho, & Mak, 2012). For example, higher levels of IL-6 and CRP have shown to predict the subsequent development of depressive symptoms, supporting a causal pathway (Valkanova, Ebmeier, & Allan, 2013). Conversely, prospective studies have also shown that depression predicted higher levels of IL-6 and CRP (Copeland, Shanahan, Worthman, Angold, & Costello, 2012; Deverts et al., 2010; Duivis et al., 2011; Matthews et al., 2010).

Other studies have shown that the peripheral levels of IL-6, TNF- α , and IL-1 β are increased in individuals with depression (Dowlati et al., 2010; Kahl et al., 2006; Raison et al., 2006), and antidepressant treatments reverse this increase (Tuglu, Kara, Caliyurt, Vardar, & Abay, 2003).

This suggests that many cytokines are promising biomarkers for depression and cytokine assays might provide greater detail on expression changes in patients with depression.

Inflammatory cytokines have been found to influence the pathogenesis of depression through neuroplastic alterations (Han & Yu, 2014). Pro-inflammatory cytokines like IL-1, TNF- α , and IFN- γ are capable of neurotoxicity leading to apoptosis (i.e., cell death), which is implicated in depression (Han & Yu, 2014; Wankerl et al., 2014). As well, neurotoxicity may be a contributing factor to the decreased hippocampal volume that is seen in depression (Han & Yu, 2014). Several brain regions have been shown to alter in the development of depression (e.g, hippocampus, amygdala, caudate nucleus, putamen, and orbital and medial prefrontal cortex).

Cytokines induce depressive symptoms by influencing pathways related to mood processes. Excess inflammatory signaling dysregulates neurotransmitter metabolism, impairs neuronal health, and alters neural activity in mood-related brain regions (Kiecolt-Glaser et al., 2015). Peripherally released cytokines send signals through molecular, cellular, and neural pathways. These signals eventually reach the brain and increase CNS inflammation. Interactions between cytokines and neurotransmitters such as dopamine, glutamate, and serotonin, have been shown to affect mood (Kiecolt-Glaser et al., 2015). For example, cytokines stimulate indoleamine 2, 3-dioxygenase, an enzyme that affects tryptophan metabolism. This pathway promotes depression by slowing down serotonin production (Kiecolt-Glaser et al., 2015). Even cytokine-induced glutamate dysregulation can lead to excitotoxicity, thereby decreasing production of neurotrophic factors (ex. BDNF and FGF2) that are responsible for supporting neuronal health, neuroplasticity, and neurogenesis (Kiecolt-Glaser et al., 2015).

On top of effects on neural processes, cytokines promote functioning of the hypothalamic-pituitary-adrenal (HPA) axis – a key regulator of mood and severely affected in depression (Stetler

& Miller, 2011). Hyperactivity of the HPA axis plays a crucial role in the pathophysiology of depression. Previous research has shown that inflammatory cytokines such as IL-6, IL-1 α , IL-1 β , and TNF- α activate the HPA axis (Han & Yu, 2014). For example, depressed patients generally show an increased concentration of IL-6, which corresponds with dysregulation of the HPA axis (Jehn et al., 2010). During infection and inflammation, TNF- α is released to activate the HPA axis as well. This results in the release of cortisol (hormone involved in regulation of metabolism in the cells that help regulate stress within the body) (Han & Yu, 2014). Over-activation of the HPA axis creates glucocorticoid receptor (GR) insensitivity and excess glucocorticoids (a class of steroid hormones involved in the metabolism of carbohydrates, proteins, fats, that have anti-inflammatory properties) contribute to the loss of neurons in the hippocampus (Han & Yu, 2014). Reduced hippocampal volume may be connected to glucocorticoids, which are usually expressed in higher levels in patients with depression (Han & Yu, 2014). Glucocorticoids typically mitigate inflammation through a negative feedback loop in the HPA axis. These results indicate that the HPA axis may be a crucial target for antidepressants.

Hypotheses

FGF2 is a growth factor as well as an anti-inflammatory cytokine that is heavily involved in neuroplastic events, which are generally decreased in depression (El Sayed et al., 2012; Salmaso et al., 2016). Decreases in neuroplasticity, increases in pro-inflammatory cytokines, and increases in depressive and anxiety like behaviours are reversed with administration of antidepressants, such as fluoxetine. Interestingly, FGF2 is anti-inflammatory and has also been shown to have antidepressant properties (El Sayed et al., 2012; Salmaso et al., 2016). Because of FGF2's pleiotropic functionality in neurobiological systems affected by depression, we believe that FGF2 is mechanistically involved in antidepressant actions and, as such, a functional *FGF2* gene is

necessary for SSRIs, such as fluoxetine, to exert its antidepressant effects (El Sayed et al., 2012; Salmaso et al., 2016). In the current study, we hypothesize that chronic stress will decrease FGF2, increase pro-inflammatory cytokines and increase depressive and anxiety-like behaviours and that fluoxetine will reverse all these effects of chronic stress in wild-type mice (Salmaso et al., 2016). However, mice that lack a functional *FGF2* gene will not show any therapeutic effects of fluoxetine on chronic stress-induced changes in behaviour or pro-inflammatory cytokine expression.

Methods

Experimental Animals

All animal use procedures were approved by the Carleton University Committee for Animal Care, according to the guidelines set by the Canadian Council for the Use and Care of Animals in Research.

For this experiment, sixty-seven male mice (at least 6 weeks old) were used (Black Swiss background, Jackson Laboratory STOCK Fgf2tm1Doe/J). Seventeen of these mice were FGF2 knockout (FGF2KO) and the other fifty were wildtype (WT) control mice.

All mice were singly housed in standard (27cm x 17cm x 13cm) fully transparent polypropylene cages. Twenty of the WT control mice were placed in a shared control room for five weeks, while the other thirty WT and seventeen FGF2KO mice were placed in an isolated stress room for five weeks. All mice were given basic cage environment (nesting material and shelter). They were also maintained on a 12-hour light/dark cycle at 21°C with food and water provided *ad libidum* unless a stressor required otherwise (see Table 4 below).

Table 1. Treatment Groups and Genotypes

		VEHICLE	FLUOXETINE
CONTROL	Wildtype	Beh <i>n</i> =10 qPCR <i>n</i> =10	Beh <i>n</i> =10 qPCR <i>n</i> =10
STRESS	Wildtype	Beh <i>n</i> =16 qPCR <i>n</i> =9	Beh <i>n</i> =14 qPCR <i>n</i> =9
	FGF2KO	Beh <i>n</i> =8 qPCR <i>n</i> =4	Beh <i>n</i> =9 qPCR <i>n</i> =4

Polymerase Chain Reaction (PCR) for Genotype Affirmation

The FGF2KO mice genotype were confirmed by conducting a Polymerase Chain Reaction (PCR) to establish absence of the *FGF2* gene in these mice. The mice were genotyped using the primers and PCR amplification protocol outlined by The Jackson Laboratory:

DNA was extracted from all mice by collecting ear punches and transferring them into fresh PCR tubes, after which the tissue was lysed. The Master Mix for PCR was created using DreamTaq™ Hot Start PCR Master Mix (catalog number: K9011) from Thermo Fischer Scientific. This mix contained 4 x 1.25 mL DreamTaq Hot Start PCR Master Mix (2X) and 4 x 1.25 mL Nuclease-free water. The Master Mix was stored at -20°C until used. The Mix was gently vortexed and briefly centrifuged after thawing. For each 50 µl reaction, the following components were added into a separate PCR tube:

Table 2. DreamTaq Hot Start PCR Master Mix Kit

DreamTaq Hot Start PCR Master Mix (2X)	25 µl
Forward Primer	0.1-1.0 µM
Reverse Primer	0.1-1.0 µM
Template DNA	10 pg – 1 µg
Nuclease-free water	Fill to 50 µl
Total volume	50 µl

The primers that were added in are custom made primers for WT and FGF2KO (both forward and reverse primers) from Thermo Fischer Scientific. The samples were then be vortexed and briefly centrifuged. The reaction tubes were then placed in a thermocycler and PCR was performed using the recommended thermal cycling conditions outlined below:

Table 3. Cycling Procedure for PCR

Step #	Temperature (°C)	Time	Note
1	94	2 minutes	
2	94	20 seconds	
3	65	15 seconds	-0.5°C per cycle decrease
4	68	10 seconds	
5			Repeat steps 2-4 for 10 cycles
6	94	15 seconds	
7	60	15 seconds	
8	72	10 seconds	
9			Repeat steps 6-8 for 28 cycles
10	72	2 minutes	
11	10		

Once all cycles were completed, a 2% agarose gel was run with the reactions and ethidium bromide to stain and visualize the amplified gene expression of each animal and confirm it's genotype.

Chronic Variable Stress (CVS)

The experiment used a CVS paradigm adapted from the Duman Laboratory (Yale University) to induce a depressive and anxiety-like behavioural phenotype. All animals in the stressor groups were exposed to various mild stressors daily over 35 days (2-3 stressors per 24-hour period) that were uncontrollable in both time and duration to prevent habituation.

Table 4. Chronic Variable Stress Schedule

DAY	STRESSORS	TESTING	OTHER
1	Restraint 1pm, Lights on overnight	Record Weight 1	
2	Cage tilt 11-2pm, Odour 12-4pm		
3	Odour 10-2pm, Empty cage overnight		New bottles
4	Cage tilt 9:30-12:30pm, Restraint 5pm		
5	Odour 9-2pm, Lights on overnight		
6	Restraint 12pm, No bedding overnight		
7	Odour 8-12, Empty cage overnight		
8	Cage tilt 11-3pm, Wet bedding overnight		
9	Restraint 10am, Lights on overnight		
10	Odour 9:30-2pm, Swim 3pm		23-35°C for water temperature
11	Cage tilt 10-12pm, Empty cage overnight		
12	Restraint 11am, Wet bedding overnight		
13	Odour 9-12pm, Cage tilt 1-4pm		
14	Restraint 11am, Lights on overnight	Record Weight 2	
15	Odour 9-12pm, Empty cage overnight		Daily Injections begin today
16	Restraint 10am, Cage tilt 1-4pm		New bottles
17	Swim 10am, Lights on overnight		
18	Restraint 10am, Wet bedding overnight		
19	Cage tilt 9:30-12pm, Empty cage overnight		
20	Odour 9-12pm, Restraint 2pm		
21	Cage tilt 10-1pm, Empty cage overnight		
22	Cage tilt 9-2pm, Lights on overnight		
23	Restraint 9:30am, Odour 1-4pm		New bottles and fresh cages
24	Swim 10am, Wet bedding overnight		
25	Cage tilt 9-12pm, Empty cage overnight		
26	Restraint 11am, Lights on overnight		
27	Cage tilt 9-1pm, Odour 1-4pm		

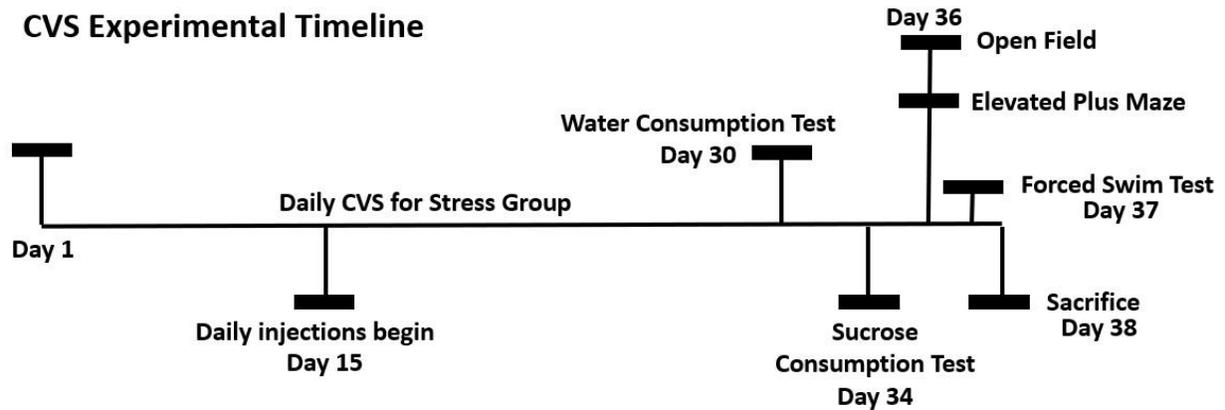
28	Wet bedding 10am, Empty cage overnight		
29	Restraint 1pm, Wet bedding overnight	Take water bottles out at 7:30pm and place water restriction cards on cages.	
30	Odour 1-4pm, Lights on overnight	Water consumption test 9-10am. Replace water with sucrose for habituation.	
31	Cage tilt 9-12pm, Odour 1-4pm		
32	Wet bedding 10am, Restraint 2pm		
33	Odour 9-1pm, Empty cage overnight	Take sucrose out at 7:30pm.	
34	Cage tilt 10-2pm, Wet bedding overnight	Sucrose consumption test 9-10am.	
35	Restraint 11am		
36		Open Field and Elevated Plus Maze	
37		Forced Swim Test	
38	End - All	Sacrifice – live rapid decapitation	Collect Blood, brains, and final Weight

As shown in Table 4., stressors consisted of the following conditions: lights on overnight, cage tilt (30 degrees), exposure to odour (alternating between lemon and mint scents), soiled bedding (10 sprays from a bottle filled with water onto bedding material), empty cage (no bedding or shelter over night), swimming for 10 minutes in large beakers, and restraint (for 15 minutes, in restraint cones that have an opening in the front for ventilation). All animals were monitored both during and immediately after each procedure and stressor.

Antidepressant Treatment and Injection Protocol

Eight of all FGF2KO mice and twenty-six WT control mice received injections of vehicle (sterile saline, VWR Canada) and the other nine of the FGF2KO and twenty-four of the WT mice received injections of the antidepressant treatment fluoxetine (Prozac, Sigma-Aldrich, dose of 10mg/kg, dissolved in sterile saline at a concentration of 2mg/ml). Injections for all mice began on the third week (day 15) of the CVS paradigm and were given intraperitoneally (IP) daily for the final three weeks of the CVS paradigm. Injection sites alternated between the left and right side of the abdomen daily. The injections were given at approximately the same time each day (around 12:00 pm) and mice were again monitored closely afterwards for any signs of discomfort.

Figure 1. Experimental Timeline



Behavioural Analysis

All behavioural tests were conducted as per our previously-published studies.

Sucrose Consumption Test

The sucrose consumption test was conducted as a measure of anhedonia in the mice. Mice were habituated to a 1% sucrose solution for a 48-hour period followed by overnight water deprivation. On day 29 of the CVS paradigm, the water bottles of all mice were taken out of their cages and weighed at approximately 7:30pm. Water restriction cards were placed on the cages. On the next day, the water consumption test was conducted from 9am to 10am. For this test, the water bottles were given back to the mice for an hour and weights of the bottles were recorded afterwards to measure consumption. The water bottles were then replaced with a 1% sucrose solution for a 48-hour period. The sucrose bottles were then removed from the cages on day 33 at approximately 7:30pm. The sucrose consumption test took place on day 34. For the sucrose consumption test, the sucrose bottle was given back for one hour and bottles weighed before and after the test. Data was expressed as sucrose consumption over body weight. Normal *ad libidum* access to food was maintained throughout both habituation and testing periods. Access to water bottles resumed immediately after the test and all solutions were made fresh each day.

Forced Swim Test

To assess the effects of CVS on learned helplessness, a depressive behaviour measure, the forced swim test was used. Mice were individually placed in a glass cylinder (15.5cm diameter) that contained temperature-controlled water (23-25°C) for a period of 5 minutes. Time immobile (i.e., floating while making only necessary movements that require the animal to keep their head above the water), latency to become immobile (time at which animal first shows signs of immobility) and time spend swimming (i.e., active, horizontal movements) was recorded on a video camera and scored by the experimenter. Immediately following the task, animals were dried off using a paper towel and placed back in their home cage and the water in the glass cylinders was replaced before each animal test.

Elevated Plus Maze

The elevated plus maze was used to measure anxiety and exploratory behaviours. The dimensions of the open arms are 30cm x 5cm, with two enclosed with 25cm walls, and elevated 30cm from the floor. The room was brightly lit at 650 lux to ensure the recording software was able to capture all movements of the mouse. The mice were brought into the behavioural testing room one at a time and placed into the centre of the maze, facing an open arm. The mouse was then allowed to explore the maze for five minutes. The mouse was videotaped by an overhead camera while exploring and analyzed using AnyMaze Video Tracking System. Behavioural outputs, including the number of arm entries and time spent on the arms, were analyzed using the AnyMaze Software. The maze was cleaned with Acell Wipes between each animal and wiped dry.

Open Field Test

A second measure of anxiety behaviour was the open field test. Mice were individually placed in a brightly lit (650 lux) box (50cm x 50cm x 35cm) and left to explore for a period of five

minutes. The movement and activity were videotaped with an overhead camera. The footage was analyzed using AnyMaze Video Tracking System. Time spent in the pre-defined zones (periphery and center) were recorded. Anxiety and exploratory behaviours were measured and included the amount of time an animal spent in the center or peripheral zone, as well as general measures of motor activity. The open field box was cleaned using Acell Wipes between each animal and wiped dry.

Emotionality Index

In humans, diagnosis of disorders involves understanding the overall behavioural phenotype instead of individual changes. In order to conceptualize the overall anxiety and depressive phenotype, we included a total “emotionality score” that we have previously employed and was first described by the Sibille laboratory and previously described in Guilloux et al. (2011). Defining depressive- and anxiety- like behaviour in mice (termed “emotionality”) is best characterized using complementary testing (Guilloux, Seney, Edgar, & Sibille, 2011). This, however, can lead to discrepancies between similar paradigms. To address this issue, what Dr. Guilloux hypothesized was that integrating measures along the same behavioural dimensions in different tests would reduce the intrinsic variability of single tests, thereby help us see the underlying “emotionality” of an individual subject or in this case, an individual mouse (Guilloux et al., 2011). For this, we followed Dr. Guilloux’s recommendations and applied z-normalizations across complimentary measures of emotionality in the different behavioural measures we used (equations are listed below). This in turn lowered the variance of emotionality measurement, enhanced the reliability of behavioural phenotyping, and increased the analytical opportunities (Guilloux et al., 2011).

Z score OF=

$$\frac{\left(\frac{X - \mu}{\sigma}\right) TimeCenter + \left(\frac{X - \mu}{\sigma}\right) Proportion Peripheral Distance}{2}$$

Z score EPM=

$$\frac{\left(\frac{X - \mu}{\sigma}\right) TimeOpen + \left(\frac{X - \mu}{\sigma}\right) Proportion Closed Distance}{2}$$

Z score FST=

$$\left(\frac{X - \mu}{\sigma}\right) Time Immobile$$

Emotionality score=

$$\frac{ZscoreOF + ZscoreEPM + ZscoreFST}{3}$$

Animal Sacrifice and Quantitative Real Time Polymerase Chain Reaction (q-rt-PCR)

One day following the completion of the behavioural testing, all mice were sacrificed by rapid decapitation. All brains were collected, wrapped in aluminum foil individually, flash frozen, and stored in a -80°C until use. At the time of decapitation, blood of each animal was also collected and placed in an Eppendorf tube that contains 10µl of Ethylenediaminetetraacetic acid (EDTA) to prevent blood clotting, which was centrifuged to only collect the serum. The serum was then flash frozen and stored in -80°C until it was used for further analysis. Once ready to use, the brains were placed on ice and one hemisphere's hippocampus was dissected using a blade. The dissected hippocampus was stored in an Eppendorf tube for RNA extraction.

RNA was extracted using the STRATAGENE RNA isolation kit (Agilent). Reverse transcription was carried out using Superscript III (Invitrogen) and GR and FGF2 levels were assessed using TAQMAN assays (Life Technologies; Assay ID: Mm00433832_m1 Gene Symbol: NR3C1; Assay ID: Mm01285715_m1 Gene Symbol: Fgf2) as per the manufacturer's instructions. All assays were conducted and analyzed using the Applied Biosystems 7500 machine and software set to detect TAQMAN probes/assays.

RNA Isolation Protocol

The following steps were taken to extract RNA from the hippocampi (as per the Absolutely RNA Nanoprep Kit by Agilent Technologies): 0.7 μ l of β -ME were added to 100 μ l of Lysis Buffer for each sample. 100 μ l of the Lysis Buffer - β -ME mixture was added to each cell sample and vortexed until homogenized. An equal volume (usually 100 μ l) of 80% sulfolane (stored at room temperature) was added to the cell lysate and mixed thoroughly by vortexing for 5 seconds. This mixture was transferred to an RNA-binding nano-spin cup that had been seated within a 2 ml collection tube and the cap was snapped onto the top of the spin cup. The sample was spun in a microcentrifuge at $\geq 12,000 \times g$ for 60 seconds. The spin cup was retained, and the filtrate was discarded. The spin cup was then re-seated in the same 2ml collection tube. 300 μ l of 1 \times High-Salt Wash Buffer was added to the spin cup, capped, and the sample was spun again in a microcentrifuge at $\geq 12,000 \times g$ for 60 seconds. The spin cup was again retained, filtrate discarded, and re-seated in the collection tube. 300 μ l of 1 \times Low-Salt Wash Buffer was added next. The spin cup was capped, and the sample was spun in a microcentrifuge at $\geq 12,000 \times g$ for 60 seconds. Then a low-salt wash was performed once again (removed and retained the spin cup, discarded the filtrate, and re-seated the spin cup in the collection tube. The sample was spun in a microcentrifuge at $\geq 12,000 \times g$ for 60 seconds). Spin cup was retained, filtrate discarded, and re-seated in the

collection tube. The spin cup was capped without any solutions added and spun in a microcentrifuge at $\geq 12,000 \times g$ for 3 minutes to dry the fiber matrix. Spin cup was added to a fresh 2ml collection tube at this time. 10 μ l of Elution Buffer heated to 60°C was added directly onto the fiber matrix inside the spin cup. The spin cup was capped, and the sample was incubated at room temperature for 2 minutes. Sample was spun in a microcentrifuge at $\geq 12,000 \times g$ for 5 minutes. The purified RNA eluate was transferred to a capped microcentrifuge tube to store the RNA. The RNA was stored at -20°C.

Reverse Transcription Protocol

The following steps were taken for reverse transcription (instructions from the SuperScript III First-Strand Synthesis SuperMix for q-rt-PCR Product Information Sheet by Invitrogen). The following kit components were combined in a tube on ice:

Table 5. Master Mix for q-rt-PCR

Component	Amount
2X RT Reaction Mix	10 μ l
RT Enzyme Mix	2 μ l
RNA (up to 1 μ g)	x μ l
DEPC-treated water	to 20 μ l

The RNA previously stored was placed on ice and pipetted into the tubes. Tube contents were gently mixed and incubated at 25°C for 10 minutes. Tube was incubated at 50°C for 30 minutes. Reaction was terminated at 85°C at 5 minutes, then chilled on ice. 1 μ l (2 U) of *E. coli* RNase H was added and incubated at 37°C for 20 minutes and this cDNA was stored at -20°C until use.

Q-RT-PCR using TAQMAN Assays Protocol

The following was thawed on ice, completely resuspended by gently vortexing, then briefly centrifuged to ring liquid to the bottom of the tube:

- a. TaqMan Gene Expression Assays (20X)
- b. cDNA Samples

For each sample (to be run in quadruplicate), the following was pipetted into a nuclease-free 1.5 ml microcentrifuge tube:

Table 6. Plate Preparation for q-rt-PCR

PCR Reaction Mix Component	Volume per 20 μ l reaction (μ L)	
	Single Reaction	Four Replicates
20X TaqMan Gene Expression Assay	1.0	5.0
2X TaqMan Gene Expression Master Mix	10.0	50.0
cDNA template (1 to 100 ng)	4.0	20.0
RNase-free water	5.0	25.0

The previously stored cDNA was thawed on ice and pipetted into the tubes for each sample. The tube was capped and inverted several times to mix the reaction components and centrifuged briefly. 20 μ l of PCR reaction mix was transferred into each well of a 96-well reaction plate in triplicate. The plate was sealed with the appropriate cover. The plate was then centrifuged briefly. Plate was loaded into the reading instrument. To run the q-rt-PCR reaction, the Applied Biosystems 7500 Real-Time PCR System was used using the appropriate parameters outlined in the instructions.

Luminex Assay for Analysis of Cytokine Expression

A custom Cytokine Assay was conducted. This was done using the R&D Systems Mouse Magnetic Luminex Assay Kit (catalog # LXSAMSM). The protocol was per the manufacturer's instruction:

All previously frozen serum samples were centrifuged at 16,000 x g for 4 minutes immediately prior to use or dilution (the Mouse Premixed Multi-Analyte Kit recommended at least a 2-fold dilution such as 75 μ l of sample and 75 μ l of Calibrator Diluent RD6-52 Mix thoroughly).

All reagents provided in the kit were brought to room temperature before use. The standards provided in the kit were reconstituted using the Calibrator Diluent RD6-52. The standard was left to sit for a minimum of 15 minutes with gentle agitation prior to making dilutions. At this point, each standard cocktail was a 10X concentrate. To create Standard 1, 100 μ l of each standard cocktail was combined with Calibrator Diluent RD6-52 into a fresh Standard 1 polypropylene tube. The final volume in the standard 1 tube was 1000 μ l. The number of standard cocktails being combined determined the amount of Calibrator Diluent being used. 200 μ l of Calibrator Diluent RD6-52 was pipetted into each of 5 test tubes labeled 2-6. Standard 1 was used to produce a 3-fold dilution series. 100 μ l of Standard 1 went into Standard 2, 100 μ l of Standard 2 went into Standard 3, and so on until Standard 6 had been given 100 μ l of Standard 5. Each tube was mixed thoroughly before transferring 100 μ l from it.

The Microparticle Cocktail that is provided in the kit was centrifuged for 30 seconds at 1000 x g prior to removing the cap. The vial was gently vortexed to resuspend the microparticles (vial must not be inverted). The Microparticle Cocktail was diluted using the Assay Diluent RD1W in the mixing bottle provided. For 96 wells being used, there was 500 μ l of the Microparticle Cocktail and 5.00 mL of the Assay Diluent RD1W. Microparticles were always protected from light.

The Biotin-Antibody Cocktail that is provided in the kit was centrifuged for 30 seconds at 1000 x g prior to removing the cap. The vial was gently vortexed (vial must not be inverted). The Biotin-Antibody Cocktail was diluted using the Assay Diluent RD1W and mixed gently. For 96 wells being used, there was 500 μ l of the Biotin-Antibody Cocktail and 5.00 mL of the Assay Diluent RD1W.

The Streptavidin-PE vial that is provided in the kit was centrifuged for 30 seconds at 1000 x g prior to removing the cap. The vial was gently vortexed (vail must not be inverted). The Streptavidin-PE Concentrate was diluted in Wash Buffer. For 96 wells being used, there was 220 μ l of the Streptavidin-PE Concentrate and 5.35 mL of the Wash Buffer. Streptavidin-PE Concentrate was always protected from light.

Next, the probe height was adjusted on the analyzer to avoid puncturing the plate. The Luminex® MAGPIX® instrument was calibrated using the proper reagents outlined in the manual.

Once all reagents, standards, and samples were prepared as directed, they were brought to room temperature before use. 50 μ l of standard or sample was added into each well. The diluted Microparticle Cocktail was resuspended by vortexing. 50 μ l of the microparticle cocktail was added to each well of the microplate. A foil plate sealer was used to cover the plate. The plate was incubated for 2 hours at room temperature on a horizontal orbital microplate shaker (0.12' orbit) set at 800 ± 50 rpm. The plate was then washed using a magnetic device designed to accommodate a microplate. The magnet was applied to the bottom of the plate, allowed 1 minute before removing the liquid, then filling each well with 100 μ l Wash Buffer, waiting another minute and removing the liquid again. This was done two more times. Next, 50 μ l of the diluted Biotin-Antibody cocktail was added into each well. Again, the plate was covered with a foil plate sealer and incubated for 1 hour at room temperature on the shaker set at 800 ± 50 rpm. The plate was again washed three

times as mentioned above. Next, 50 μ l of the Streptavidin-PE Concentrate was added into each well, secured with a foil plate cover, and incubated for 30 minutes at room temperature on the shaker at 800 ± 50 rpm. Once again, the plate was washed three times as mentioned above. The microparticle was then resuspended by adding 100 μ l of Wash Buffer to each well and incubated for 2 minutes on the shaker set at 800 ± 50 rpm. The plate was then read using the Luminex® MAGPIX® reader set to the proper calibrations outlined in the manual.

Statistical Analysis

All data was analyzed using IBM SPSS Statistics Data Editor (Version 20). Analysis of Variance was conducted with genotype (WT vs. FGF2KO), condition (control vs. stress), and treatment (vehicle vs. fluoxetine) as independent variables. Because this is an unbalanced model design, we applied a more conservative alpha level to reduce the likelihood of type 1 errors, therefore probability values were considered significant when $p \leq 0.01$. If the overall model was significant then ANOVAs were followed by post-hoc t-test comparisons with Bonferonni correction for non-orthogonal tests; t-tests were considered significant when $p \leq 0.05$.

Results

To examine the effects of *FGF2* gene deletion on fluoxetine's ability to decrease depressive- and anxiety-like behaviours, WT and FGF2KO mice were exposed to chronic variable stress (CVS) after which they were tested on the forced swim test, sucrose consumption test, open field test, and elevated plus maze. As well, mice were injected with either vehicle or fluoxetine and tested on their depressive- and anxiety-like behaviours without exposure to stress. Chronic fluoxetine treatment did not reverse the effects of CVS on depressive- and anxiety-like behaviours in FGF2KO mice. There were no significant results in the sucrose consumption test nor the weights of the mice (data not shown for either measures).

Forced Swim Test - Results

The results from the forced swim test showed a significant overall model for total immobility time ($F_{(5,21)}=28.083$, $p<0.0001$) (Figure 2). Further comparisons of simple effects showed that all stressed groups had significantly longer immobility time when compared to the control, WT, vehicle-treated mice ($F_{(1,61)}=58.532$, $p<0.01$) (Figure 2). As well, stressed, WT mice treated with fluoxetine showed significant decrease in immobility time compared to the stressed WT vehicle-treated group ($F_{(1,61)}=15.203$, $p<0.01$) reaffirming fluoxetine's antidepressant effectiveness following perturbations (Figure 2). FGF2KO mice showed no response to fluoxetine in immobility (Figure 2).

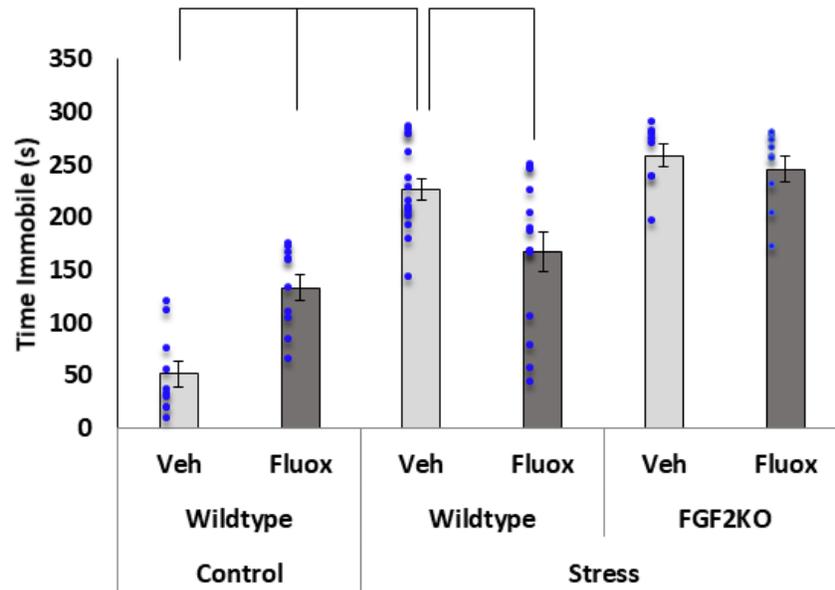


Figure 2. Forced Swim Test – Time Spent Immobile (s). Graphical representation of time spent immobile in seconds, broken down into the different experimental groups. Bars represent group means and error bars represent \pm SEM. Blue dots represent individual sample data points. There was a significant overall model for immobility time ($p < 0.0001$). Comparisons of simple effects revealed that all stress groups had a significant increase in immobility from the control, WT, vehicle-treated mice ($p < 0.01$). Stressed, WT mice treated with fluoxetine showed significantly less immobility when compared their vehicle-treated counterpart ($p < 0.01$). The FGF2KO mice showed no response to fluoxetine ($p > 0.05$).

The results from the forced swim test showed a significant overall model for latency to immobility ($F_{(5,61)}=23.894$, $p < 0.0001$) (Figure 3). Comparisons of simple effects showed that all stressed groups also had a significant decrease in latency to immobility when compared to the non-stressed group ($F_{(1,61)}=108.930$, $p < 0.01$) (Figure 3). The WT stressed mice treated with fluoxetine showed a trend for an increase in the latency to immobility ($p=0.06$) (Figure 3). The FGF2KO mice showed no response to fluoxetine in latency to immobility ($F_{(1,61)}=2.794$, $p > 0.05$) (Figure 3).

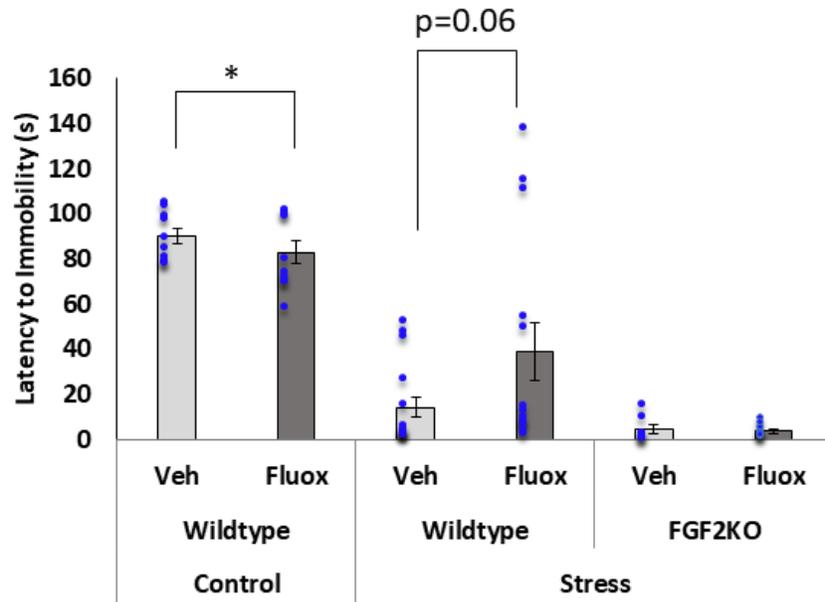


Figure 3. Forced Swim Test – Latency to Immobility (s). Graphical representation of latency to immobility in seconds, broken down into the different experimental groups. Bars represent group means and error bars represent \pm SEM. Blue dots represent individual sample data points. * represents significance where $p \leq 0.05$. There was a significant overall model for latency to immobility ($p < 0.0001$). Comparisons of simple effects showed that all stressed groups had a significantly lower latency to immobility from the non-stressed controls ($p < 0.01$). WT mice treated with fluoxetine showed a trend for an increase in the latency to immobility ($p = 0.06$). FGF2KO mice did not respond to the fluoxetine treatment ($p > 0.05$).

Elevated Plus Maze - Results

Results from the elevated plus maze showed a significant overall model for time spent in the open arms ($F_{(5,61)}=10.337$, $p<0.0001$) (Figure 4). Simple effects showed that the non-stressed mice spent more time in the open arms than the stressed groups ($F_{(1,61)}=46.725$, $p<0.0001$) (Figure 4). No effects were observed in the FGF2KO mice (Figure 4).

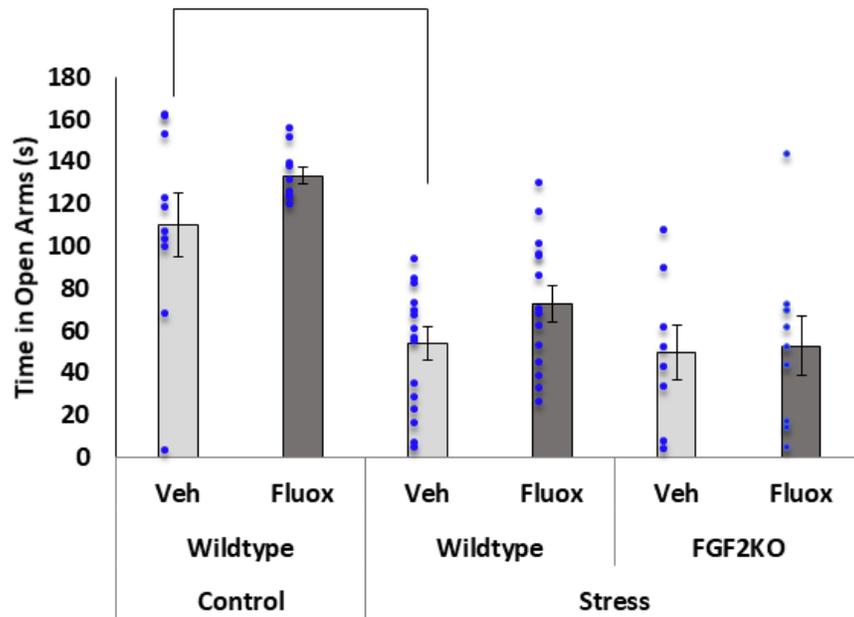


Figure 4. Elevated Plus Maze – Time Spent in Open Arms (s). Graphical representation of time spent in the open arms in seconds, broken down into the different experimental groups. Bars represent group means and error bars represent \pm SEM. Blue dots represent individual sample data points. There was a significant overall model for time spent in the open arms ($p<0.0001$). Simple effects showed that control mice spent more time in the open arms than all the other stressed groups. No effect were observed in FGF2KO mice.

There were no significant results for time spent in the centre ($F_{(3,26)}=1.246, p>0.05$) (Figure

5).

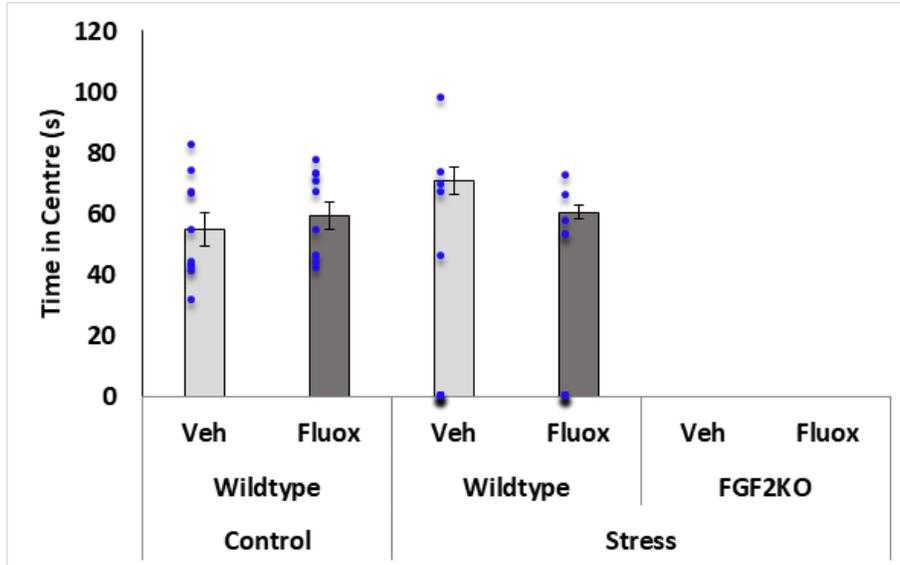


Figure 5. Elevated Plus Maze – Time in Centre (s). Graphical representation of time spent in the centre in seconds, broken down into the different experimental groups. Bars represent group means and error bars represent \pm SEM. Blue dots represent individual sample data points. There were no significant results seen in time spent in the centre.

Results from the elevated plus maze showed a significant overall model for distance travelled in the open arms ($F_{(5,61)}=5.141$, $p=0.001$) (Figure 6). WT stressed mice treated with fluoxetine showed an increase in proportion of distance travelled in the open arms compared to WT stressed mice treated with vehicle (Figure 6). FGF2KO mice showed no effects (Figure 6).

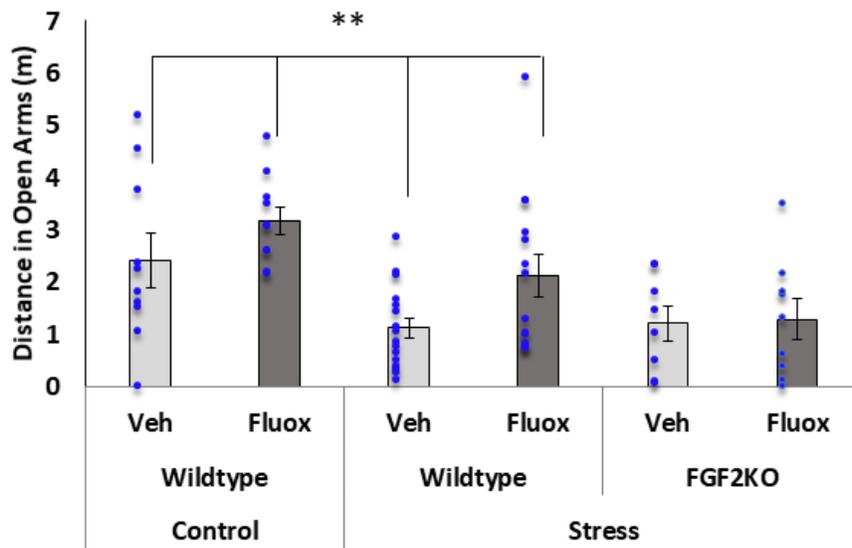


Figure 6. Elevated Plus Maze – Distance Travelled in Open Arms (m). Graphical representation of distance travelled in the open arms in metres, broken down into the different experimental groups. Bars represent group means and error bars represent \pm SEM. Blue dots represent individual sample data points. ** denotes significance where $p \leq 0.01$. There was a significant overall model for distance travelled in the open arms ($p < 0.001$). WT mice treated with fluoxetine showed an increase in the proportion of distance travelled in the open arms compared to vehicle-treated counterparts. No effect observed in FGF2KO mice.

There were no significant results for distance travelled in the center ($F_{(5,61)}=0.876, p>0.05$)

(Figure 7).

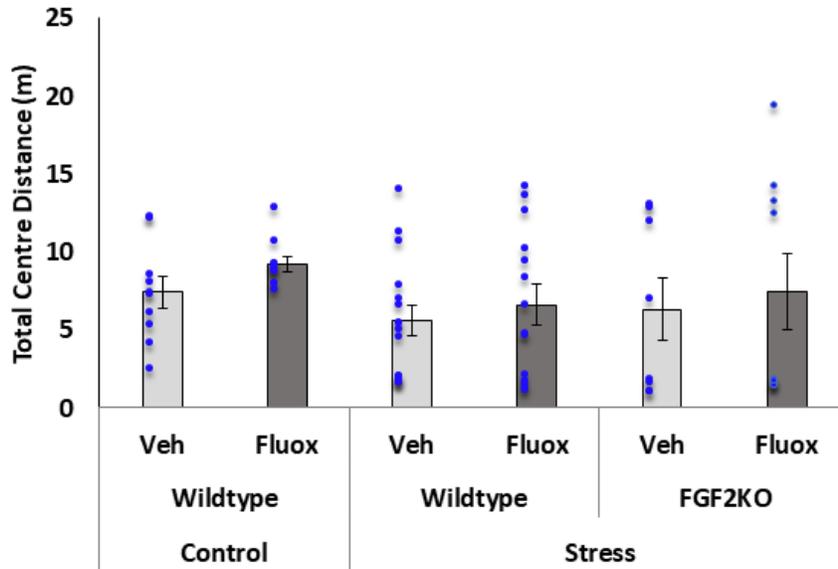


Figure 7. Elevated Plus Maze – Distance Travelled in Centre (m). Graphical representation of distance travelled in the centre in metres, broken down into the different experimental groups. Bars represent group means and error bars represent \pm SEM. Blue dots represent individual sample data points. There were no significant results seen in the total distance travelled in the centre.

Results from the elevated plus maze showed a significant overall model for distance in open arms/total distance travelled ($F_{(5,61)}=4.729$, $p<0.01$) (Figure 8). WT vehicle-treated stressed mice showed a decrease in time spent on the open arms relative to the total distance travelled compared to fluoxetine-treated counterparts (Figure 8). No effect of fluoxetine was observed in FGF2KO animals (Figure 8).

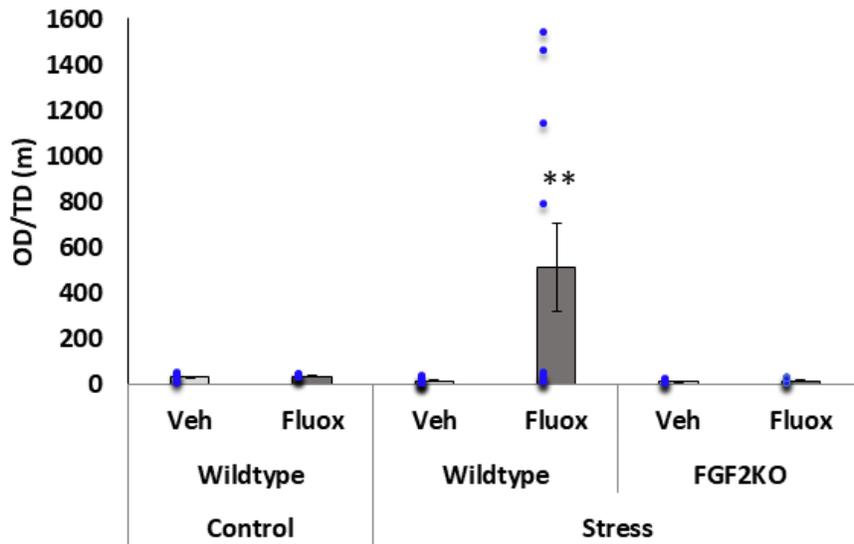


Figure 8. Elevated Plus Maze – Distance in Open Arms/Total Distance Travelled (m). Graphical representation of distance travelled in the open arms/total distance travelled in metres, broken down into the different experimental groups. Bars represent group means and error bars represent \pm SEM. Blue dots represent individual sample data points. ** denotes significance where $p\leq 0.01$. There was a significant overall model for distance in open arms/total distance travelled ($p<0.01$). WT vehicle-treated stressed mice spent less time on the open arms in the total distance compared to their fluoxetine-treated stressed counterparts.

There were no significant results for total distance travelled (Figure 9), suggesting no changes in locomotor activity ($F(5,61)=1.143, p>0.05$) between the groups.

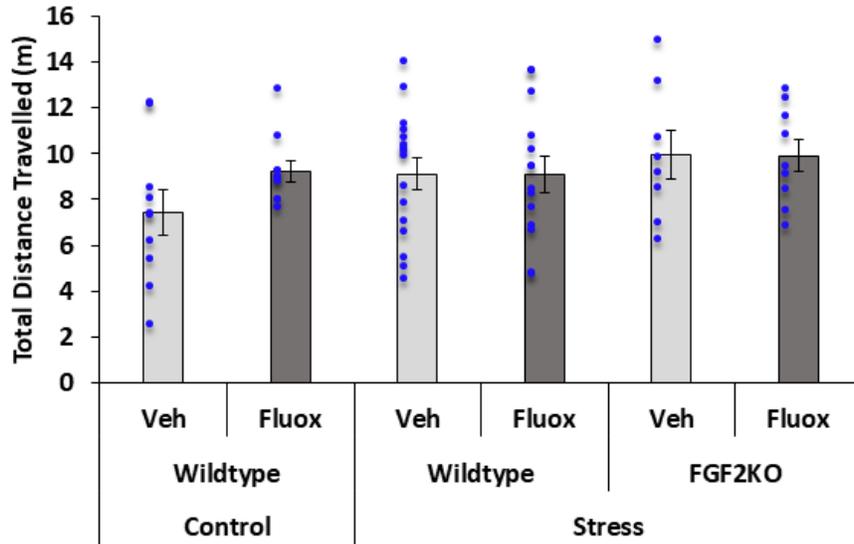


Figure 9. Elevated Plus Maze – Total Distance Travelled (m). Graphical representation of total distance travelled in metres, broken down into the different experimental groups. Bars represent group means and error bars represent \pm SEM. Blue dots represent individual sample data points. There were no significant results seen in the total distance travelled.

There were no significant results for average speed ($F_{(5,61)}=1.1851$, $p>0.05$) (Figure 10).

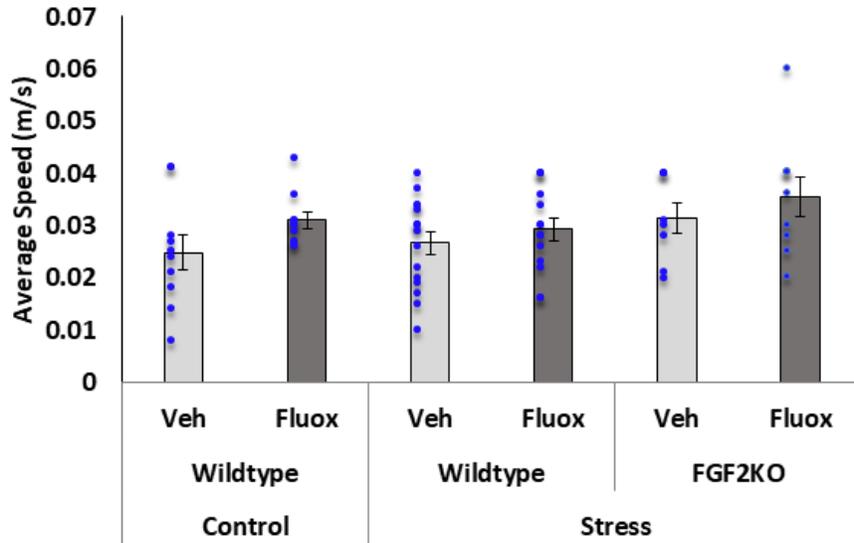


Figure 10. Elevated Plus Maze – Average Speed (m/s). Graphical representation of average speed in metres per second, broken down into the different experimental groups. Bars represent group means and error bars represent \pm SEM. Blue dots represent individual sample data points. There were no significant results seen in the average speed.

Open Field Test - Results

Results from the open field test showed a significant overall model for time spent in the centre zone ($F_{(5,61)}=9.566$, $p<0.0001$) (Figure 11). Post-hoc tests showed that all stressed groups spent less time in the centre ($F_{(1,61)}=43.943$, $p<0.0001$) (Figure 11). FGF2KO mice showed further decrease in time spent in the centre zone ($F_{(1,61)}=20.502$, $p<0.0001$) (Figure 11). No effect of fluoxetine was observed (Figure 11).

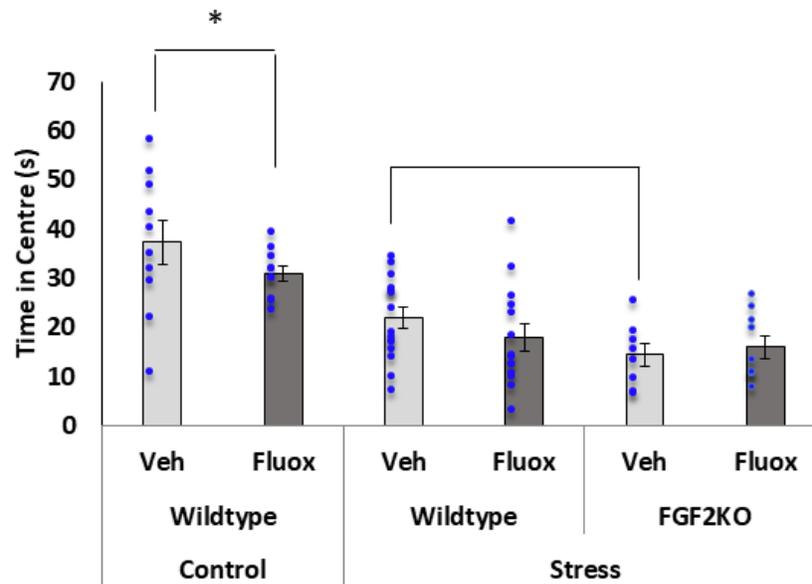


Figure 11. Open Field Test – Time in Centre (s). Graphical representation of time spent in the centre in seconds, broken down into the different experimental groups. Bars represent group means and error bars represent \pm SEM. Blue dots represent individual sample data points. * denotes significance where $p\leq 0.05$. There was a significant model for time in center zone ($p<0.0001$). Post-hoc tests showed that all stressed groups spent less time in the center zone. FGF2KO mice showed a further decrease in time spent in the center suggesting an additive effect of stress and FGF2 gene deletion. No effects of fluoxetine were seen.

Results from the open field test showed a significant overall model for latency to enter the centre zone ($F_{(5,61)}=4.180$, $p<0.003$) (Figure 12). Post hoc tests showed that all stressed groups had a longer latency to enter the centre zone ($F_{(1,61)}=14.990$, $p<0.001$) (Figure 12). FGF2KO mice showed a trend for a longer latency to enter the centre ($p=0.07$) (Figure 12) compared to their WT, stressed counterparts, suggesting an additive effect of stress and FGF2 gene deletion. No effect of fluoxetine was observed here ($F_{(1,61)}=0.246$, $p>0.05$) (Figure 12).

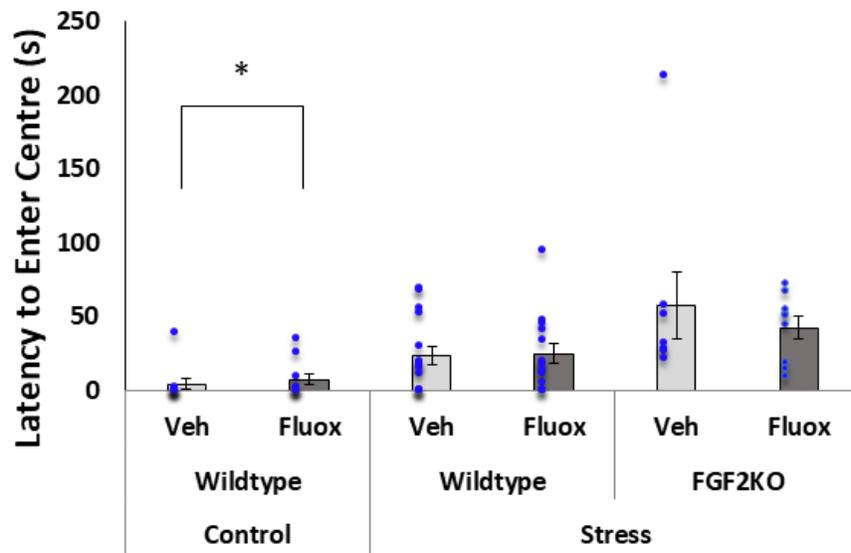


Figure 12. Open Field Test – Latency to Enter Centre Zone (s). Graphical representation of latency to enter the centre in seconds, broken down into the different experimental groups. Bars represent group means and error bars represent \pm SEM. Blue dots represent individual sample data points. * denotes significance where $p \leq 0.05$. There was a significant model for latency to enter center zone ($p < 0.003$). Post-hoc tests showed that all stressed groups had a longer latency to enter the center. FGF2KO mice showed a trend for a longer latency ($p = 0.07$) as compared to their WT, stressed counterparts suggesting an additive effect of stress and FGF2 gene deletion. No effects of fluoxetine were seen.

There were a significant overall effect seen in distance travelled in the centre ($F_{(5,61)}=7.181$, $p<0.0001$) (Figure 13). There was an effect of stress seen on distance travelled in the centre ($F_{(1,61)}=30.613$, $p<0.00001$) (Figure 13). WT stressed mice treated with vehicle travelled more in the centre compared to their FGF2KO counterparts ($F_{(1,61)}=4.139$, $p<0.05$) (Figure 13). As well, WT stressed mice treated with fluoxetine travelled less in the centre than their FGF2KO counterparts ($F_{(1,61)}=22.971$, $p<0.0001$) (Figure 13).

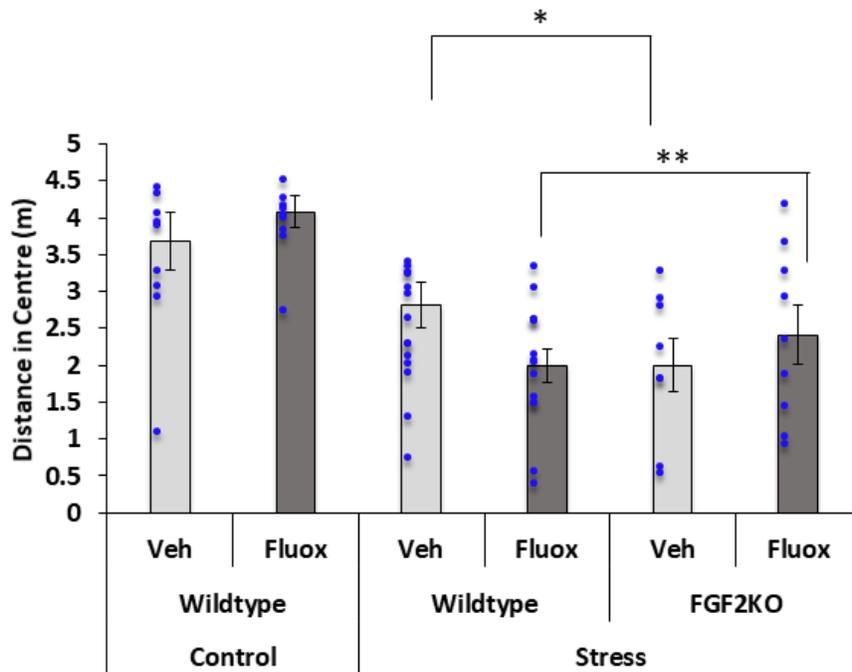


Figure 13. Open Field Test – Distance in Centre (m). Graphical representation of distance in centre in metres, broken down into the different experimental groups. Bars represent group means and error bars represent \pm SEM. Blue dots represent individual sample data points. * denotes significance where $p\leq 0.05$. ** denotes significance where $p\leq 0.01$. There was a significant overall effect seen in distance travelled in the centre ($p<0.0001$). There was also an effect of stress, such that stressed groups travelled less than the non-stressed groups ($p<0.00001$). Stressed, WT, vehicle-treated mice travelled more in the centre than their FGF2KO counterparts ($p<0.05$). Stressed, WT, fluoxetine-treated mice travelled less in the centre than their FGF2KO counterparts ($p<0.0001$).

There was a significant overall effect seen in the total distance travelled ($F_{(5,61)}=2.382$, $p<0.05$) (Figure 14). Stressed, WT, fluoxetine-treated mice travelled less overall compared to their FGF2KO counterparts ($F_{(1,61)}=9.764$, $p<0.01$) (Figure 14).

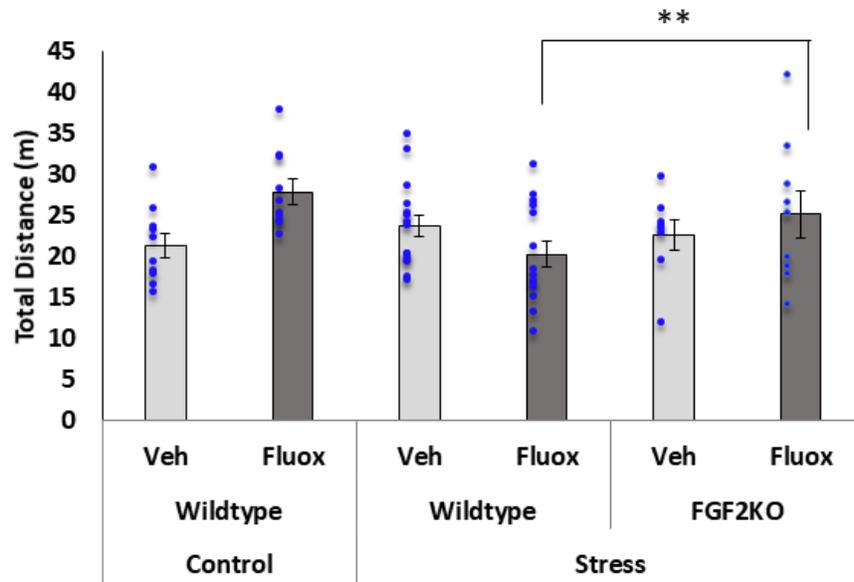


Figure 14. Open Field Test – Total Distance (m). Graphical representation of total distance in metres, broken down into the different experimental groups. Bars represent group means and error bars represent \pm SEM. Blue dots represent individual sample data points. * denotes significance where $p\leq 0.01$. There was a significant overall model for the total distance travelled ($p<0.05$). Stressed, WT, fluoxetine-treated mice travelled less compared to their FGF2KO counterparts ($p<0.01$).

There was a significant overall effect seen in centre distance/total distance ($F_{(5,61)}=9.586$, $p<0.0001$) (Figure 15). Non-stressed, WT, vehicle-treated mice travelled more in the centre relative to total distance, compared to all other groups ($F_{(1,61)}=20.180$, $p<0.0001$) (Figure 15).

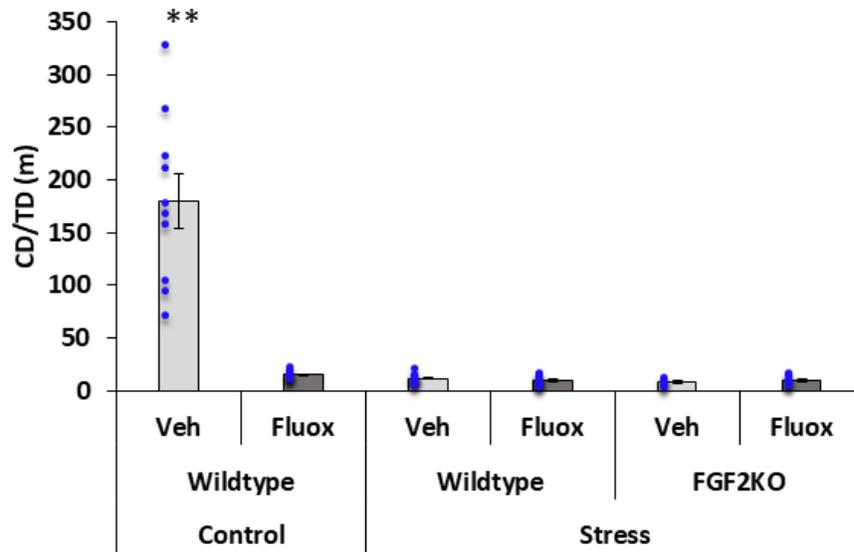


Figure 15. Open Field Test – Centre Distance/Total Distance (m). Graphical representation of centre distance/total distance in metres, broken down into the different experimental groups. Bars represent group means and error bars represent \pm SEM. Blue dots represent individual sample data points. ** denotes significance where $p \leq 0.01$. There was a significant overall model for centre distance/total distance ($p<0.0001$). Non-stressed, WT mice treated with vehicle travelled more in the centre relative to total distance, compared to all other groups ($p<0.0001$).

Glucocorticoid Receptor (GR) Fold Change

The q-RT-PCR results showed a significant overall model ($F_{(5,40)}=7.400$, $p<0.0001$) for glucocorticoid receptor (GR) expression as well as main effects of stress ($F_{(1,40)}=11.784$, $p<0.01$) and genotype ($F_{(1,40)}=8.911$, $p<0.01$) (Figure 16). Stressed mice showed lower GR levels than non-stressed mice and FGF2KO mice showed further decrease in GR compared to WT mice (Figure 16). No effects of fluoxetine administration were observed on GR expression (Figure 16).

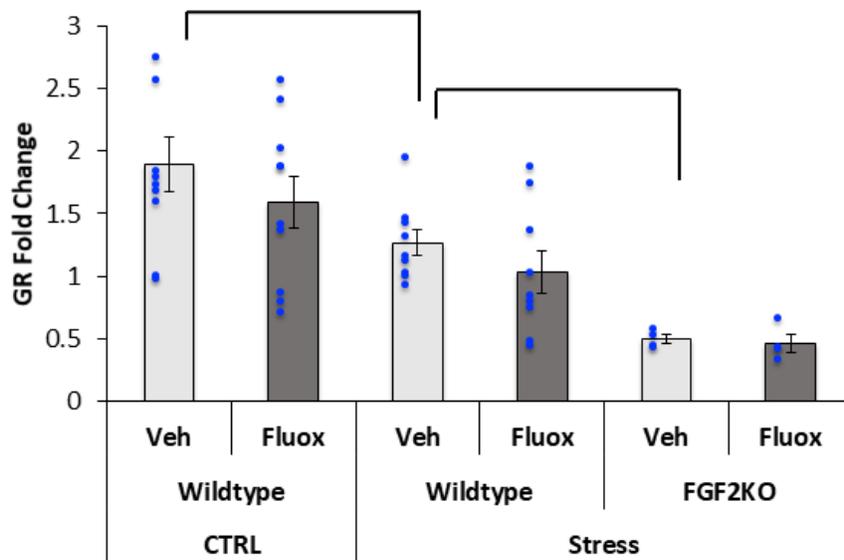


Figure 16. Glucocorticoid Receptor Fold Change. Graphical representation of GR fold change through fluorescence intensity, broken down into the different experimental groups. Bars represent group means and error bars represent \pm SEM. Blue dots represent individual sample data points. There was a significant overall model for *GR* as well as main effects of both stress and genotype ($p<0.01$ for all) such that stressed mice showed lower *GR* levels than non-stressed mice. FGF2KO mice showed a further decrease in *GR*. No effects of fluoxetine were seen on *GR* expression.

Fibroblast Growth Factor 2 (FGF2) Fold Change

FGF2 levels did not change overall ($p=0.04$), however, there were significant main effects of stress ($F_{(1,40)}=7.989$, $p<0.01$) (Figure 17). All stressed groups showed significantly lower levels of FGF2 compared to the non-stressed groups (Figure 17). Because it has been previously shown that acute administration of fluoxetine increases hippocampal FGF2 (Mallei, Shi, & Mocchetti, 2002), we compared FGF2 levels of Vehicle treated, stressed WT mice to their fluoxetine treated counterparts. There was also a trend for an increase in FGF2 levels with fluoxetine treatment ($p=0.06$) (Figure 17).

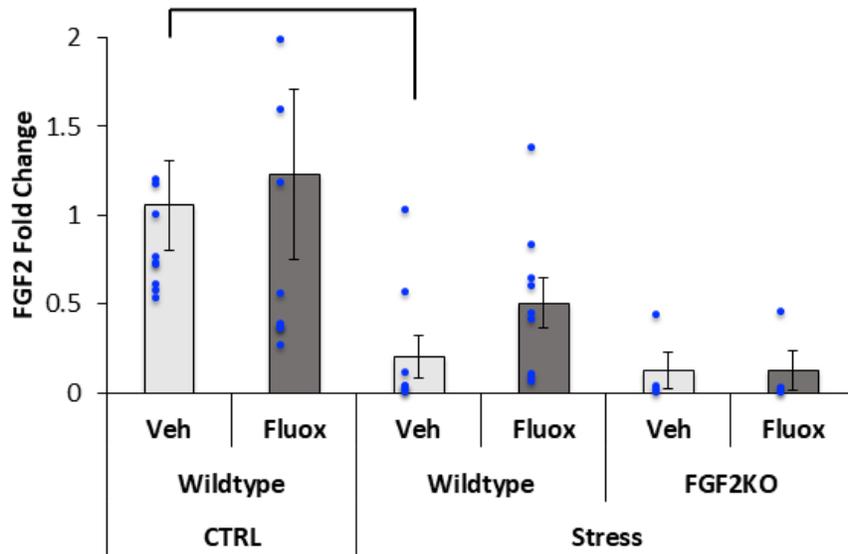


Figure 17. Fibroblast Growth Factor 2 Fold Change. Graphical representation of FGF2 fold change through fluorescent intensity, broken down into the different experimental groups. Bars represent group means and error bars represent \pm SEM. Blue dots represent individual sample data points. *FGF2* levels in the hippocampus did not change overall but there was a significant main effect of stress ($p<0.01$) such that all stressed groups had lower levels of *FGF2* gene expression compared to the non-stressed control mice. There was a trend ($p=0.06$) for an increase in *FGF2* levels with fluoxetine treatment.

Luminex Cytokine Assay - Results

Because FGF2 has been shown to have anti-inflammatory properties and because depression has been associated to a pro-inflammatory state, we examined serum levels of a set of pro-inflammatory and anti-inflammatory cytokines as well as anti-inflammatory growth factors.

Results from the Luminex Cytokine Assay showed detection levels of IFN- γ that were below threshold and so levels were not analyzed further (Figure 18).

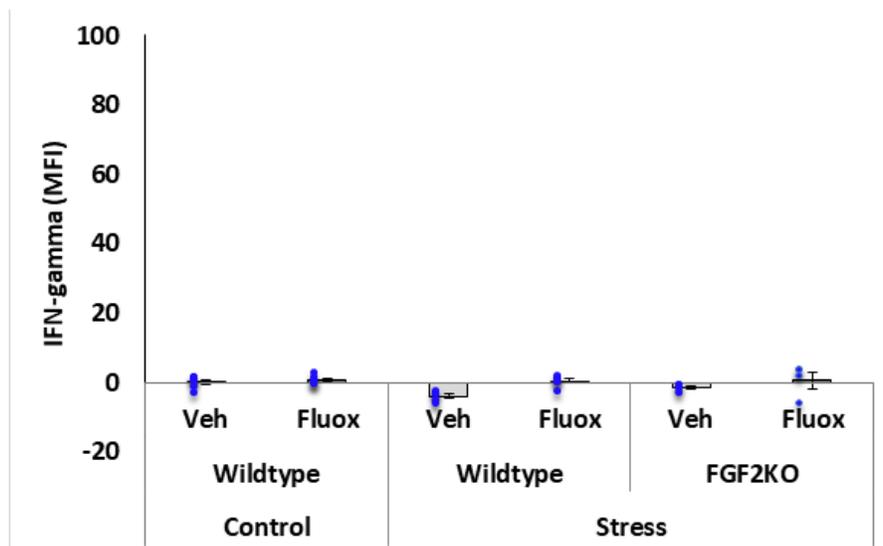


Figure 18. Luminex Data – IFN-gamma. Graphical representation of Median Fluorescent Intensity (MFI) of IFN-gamma expression in the blood serum, broken down into the different experimental groups. Bars represent group means and error bars represent \pm SEM. Blue dots represent individual sample data points. Due to low detection of IFN-gamma, levels were not analyzed further.

Results from the Luminex Cytokine Assay showed detection levels of IL-1 alpha that were below threshold and so levels were not analyzed further (Figure 19).

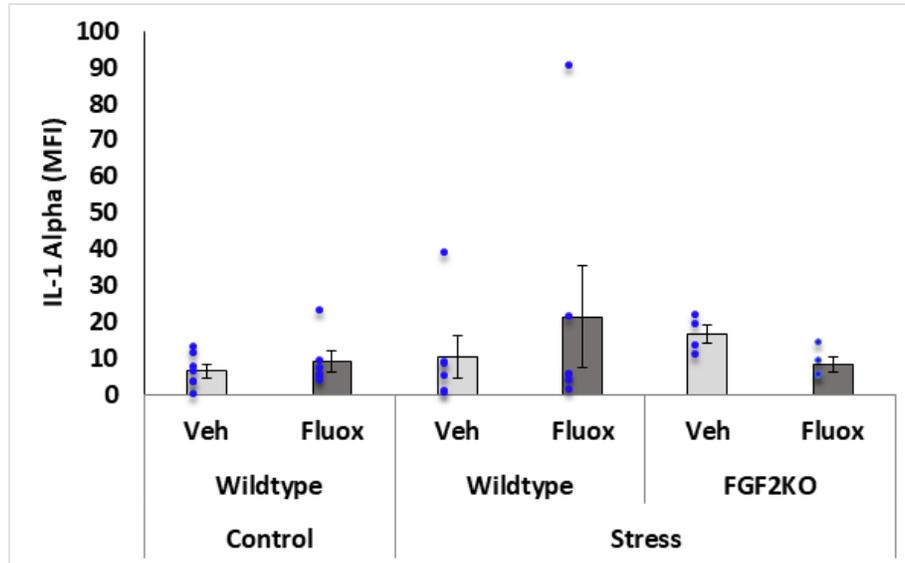


Figure 19. Luminex Data – IL-1 alpha. Graphical representation of Median Fluorescent Intensity (MFI) of IL-1 alpha expression in the blood serum, broken down into the different experimental groups. Bars represent group means and error bars represent \pm SEM. Blue dots represent individual sample data points. Due to low detection of IL-1 alpha, levels were not analyzed further.

Results from the Luminex Cytokine Assay showed detection levels of IL-1 beta that were below threshold and so levels were not analyzed further (Figure 20).

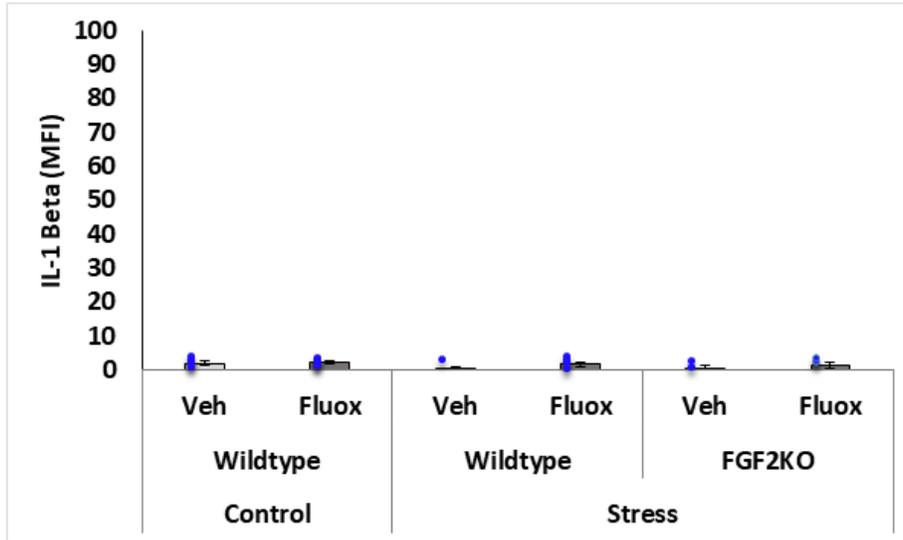


Figure 20. Luminex Data – IL-1 beta/IL-1F2. Graphical representation of Median Fluorescent Intensity (MFI) of IL-1 beta expression in the blood serum, broken down into the different experimental groups. Bars represent group means and error bars represent \pm SEM. Blue dots represent individual sample data points. Due to low detection of IL-1 beta, levels were not analyzed further.

Results from the Luminex Cytokine Assay showed detection levels of IL-4 that were below threshold and so levels were not analyzed further (Figure 21).

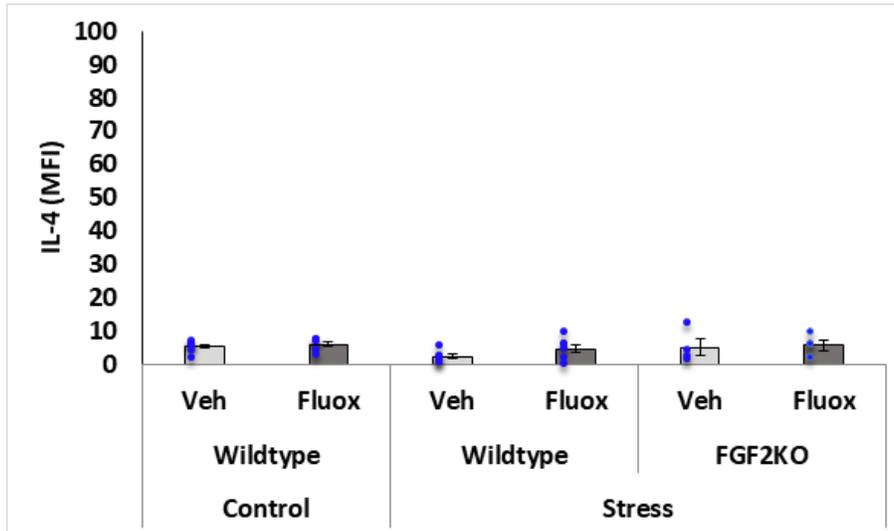


Figure 21. Luminex Data – IL-4. Graphical representation of Median Fluorescent Intensity (MFI) of IL-4 expression in the blood serum, broken down into the different experimental groups. Bars represent group means and error bars represent \pm SEM. Blue dots represent individual sample data points. Due to low detection of IL-4, levels were not analyzed further.

Results from the Luminex Cytokine Assay showed a significant overall model ($F_{(5,27)}=2.970$, $p<0.03$) for IL-6 (Figure 22). Pairwise comparisons show that vehicle-treated FGF2KO mice show significantly higher IL-6 levels than their fluoxetine-treated counterparts ($F_{(1,27)}=6.834$, $p=0.01$) (Figure 22).

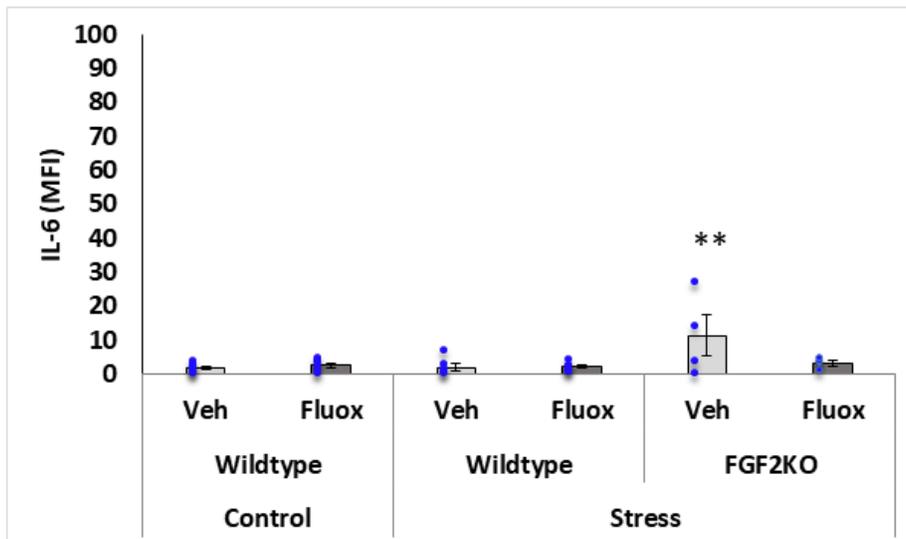


Figure 22. Luminex Data – IL-6. Graphical representation of Median Fluorescent Intensity (MFI) of IL-6 expression in the blood serum, broken down into the different experimental groups. Bars represent group means and error bars represent \pm SEM. Blue dots represent individual sample data points. ** represents significance of group where $p \leq 0.01$. IL-6 levels seem to not be affected in WT mice regardless of stress exposure. However, FGF2KO mice treated with vehicle show increased levels of IL-6, which is then decreased with fluoxetine treatment.

Results from the Luminex Cytokine Assay showed no significant overall model ($F_{(5,27)}=2.141, p=0.091$) for CXCL1 (Figure 23).

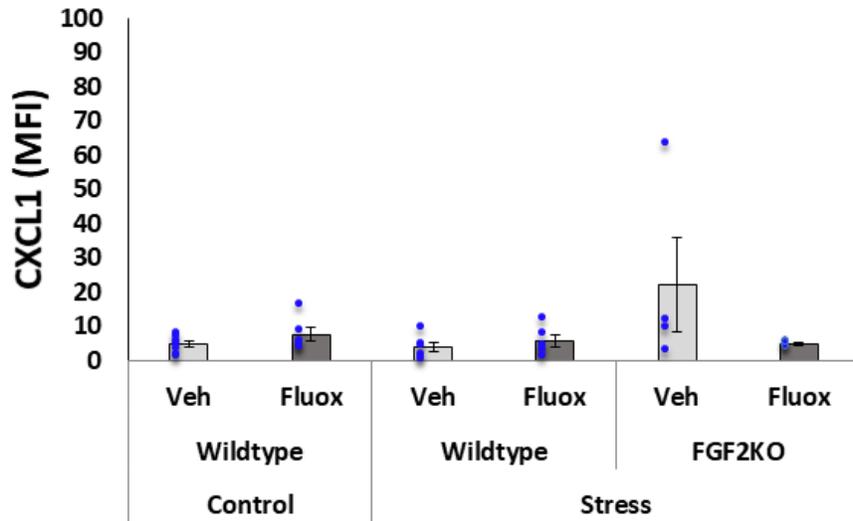


Figure 23. Luminex Data – CXCL1/GRO alpha/KC. Graphical representation of Median Fluorescent Intensity (MFI) of CXCL1 expression in the blood serum, broken down into the different experimental groups. Bars represent group means and error bars represent \pm SEM. Blue dots represent individual sample data points. There was no significant model seen for CXCL1.

Results from the Luminex Cytokine Assay showed a significant overall model for EGF expression ($F_{(5,27)}=3.819$, $p=0.01$) (Figure 24). Stressed, fluoxetine-treated WT mice show significantly lower levels of EGF when compared to their FGF2KO counterparts ($F_{(1,27)}=6.061$, $p<0.03$) (Figure 24).

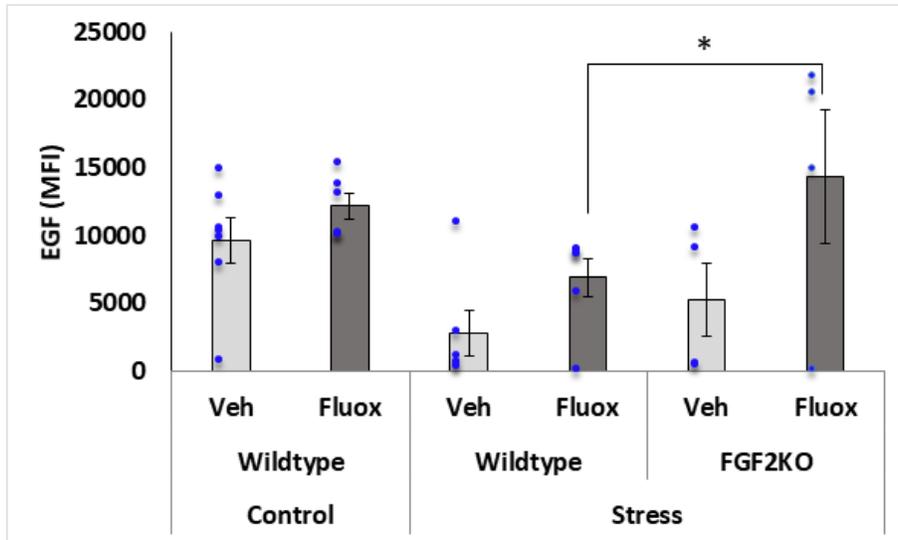


Figure 24. Luminex Data – EGF. Graphical representation of Median Fluorescent Intensity (MFI) of EGF expression in the blood serum, broken down into the different experimental groups. Bars represent group means and error bars represent \pm SEM. Blue dots represent individual sample data points. * represents significant difference where $p \leq 0.05$. Fluoxetine treatment brought EGF levels up in WT stressed mice similar to those seen in the vehicle-treated WT control mice. As well, fluoxetine treatment elevated EGF in FGF2KO mice significantly higher than their WT counterparts.

Results from the Luminex Cytokine Assay showed low detection of FGF2 and so levels were not analyzed further (Figure 25).

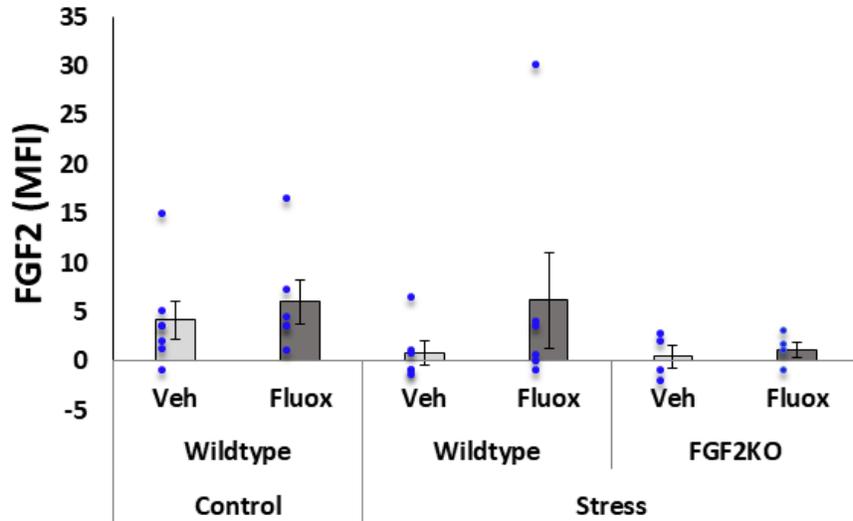


Figure 25. Luminex Data – FGF basic/FGF2. Graphical representation of Median Fluorescent Intensity (MFI) of FGF2 expression in the blood serum, broken down into the different experimental groups. Bars represent group means and error bars represent \pm SEM. Blue dots represent individual sample data points. Due to low detection of FGF2, levels were not further analyzed.

Results from the Luminex Cytokine Assay showed low detection levels of IL-10 and so levels were not analyzed further (Figure 26).

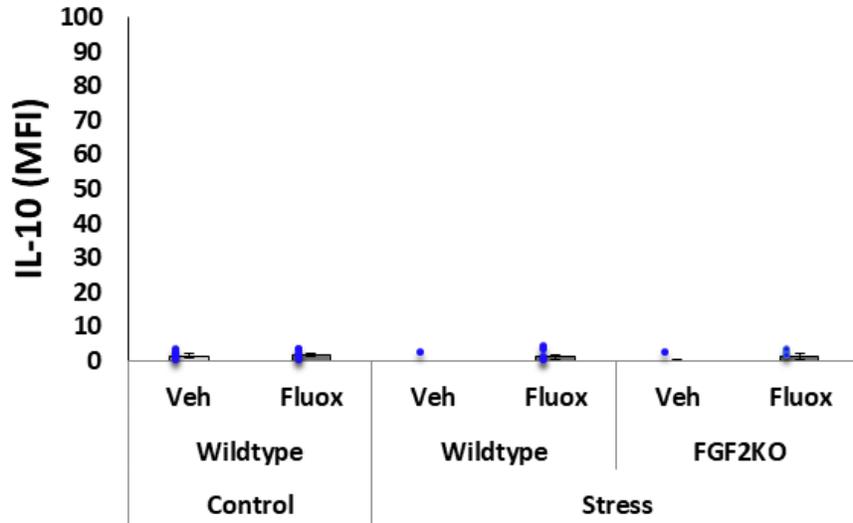


Figure 26. Luminex Data – IL-10. Graphical representation of Median Fluorescent Intensity (MFI) of IL-10 expression in the blood serum, broken down into the different experimental groups. Bars represent group means and error bars represent \pm SEM. Blue dots represent individual sample data points. Due to low detection of IL-10, levels were not analyzed further.

Results from the Luminex Cytokine Assay showed low detection levels of TNF-alpha and so levels were not analyzed further (Figure 27).

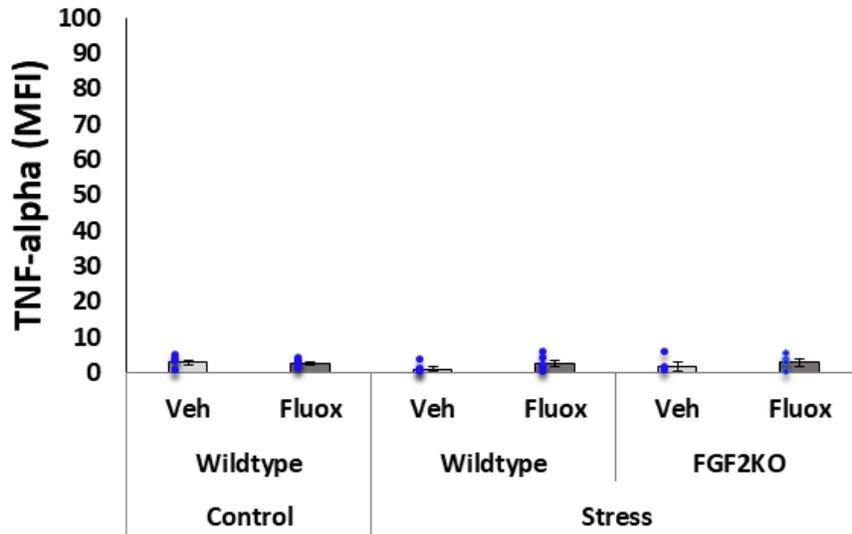


Figure 27. Luminex Data – TNF-alpha. Graphical representation of Median Fluorescent Intensity (MFI) of TNF-alpha expression in the blood serum, broken down into the different experimental groups. Bars represent group means and error bars represent \pm SEM. Blue dots represent individual sample data points. Due to low detection of TNF-alpha, levels were not analyzed further.

Results from the Luminex Cytokine Assay showed no significant overall model for VEGF ($p>0.05$ for all) (Figure 28).

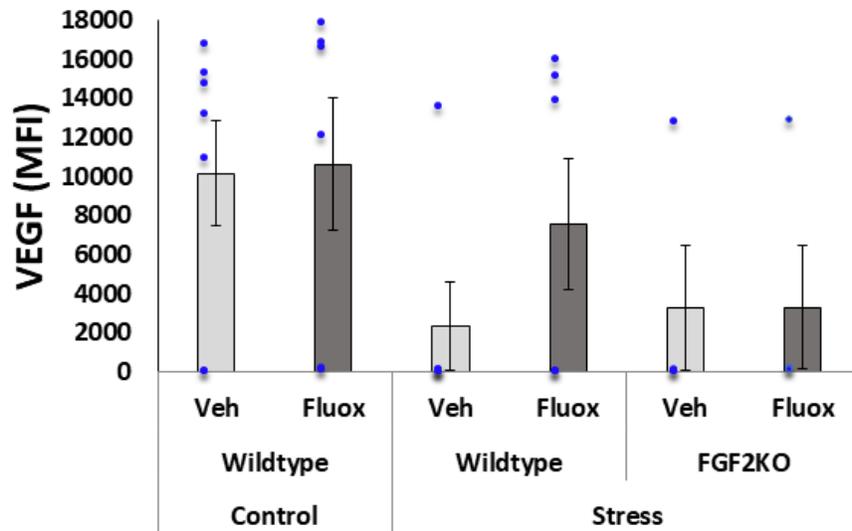


Figure 28. Luminex Data – VEGF. Graphical representation of Median Fluorescent Intensity (MFI) of VEGF expression in the blood serum, broken down into the different experimental groups. Bars represent group means and error bars represent \pm SEM. Blue dots represent individual sample data points. There were no statistically significant findings. No significant effect of stress, genotype or treatment was seen on VEGF levels in any of the groups ($p>0.05$ for all).

In order to conceptualize the overall anxiety and depressive phenotype, we included a total “emotionality score” that we have previously employed and was first described by the Sibille laboratory. The total “emotionality score” showed that a significant increase in emotionality was induced by stress, which was then reduced in the fluoxetine-treated WT mice (Figure 29). No effect of fluoxetine was seen in the FGF2KO mice (Figure 29). GR ($p < 0.01$) and FGF2 ($p < 0.05$) levels significantly correlated to the emotionality score (Figure 30 & 31).

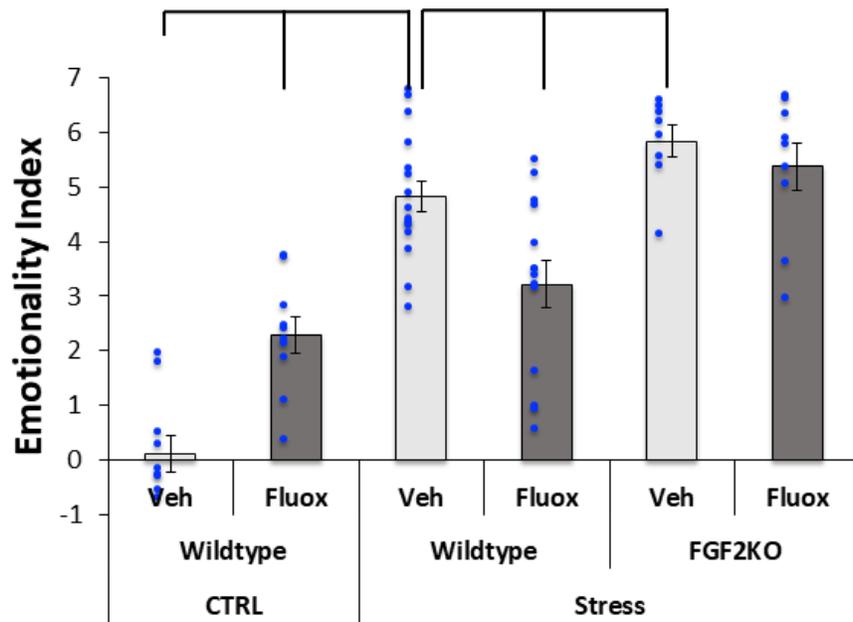


Figure 29. Emotionality Index. Graphical representation of Emotionality index of each subject, broken down into the different experimental groups. Bars represent group means and error bars represent \pm SEM. Blue dots represent individual sample data points. The stressed group had higher emotionality scores than the control counterparts. As well, the stressed FGF2KO mice showed higher emotionality than the stressed WT mice. No effect of fluoxetine was seen in the FGF2KO mice.

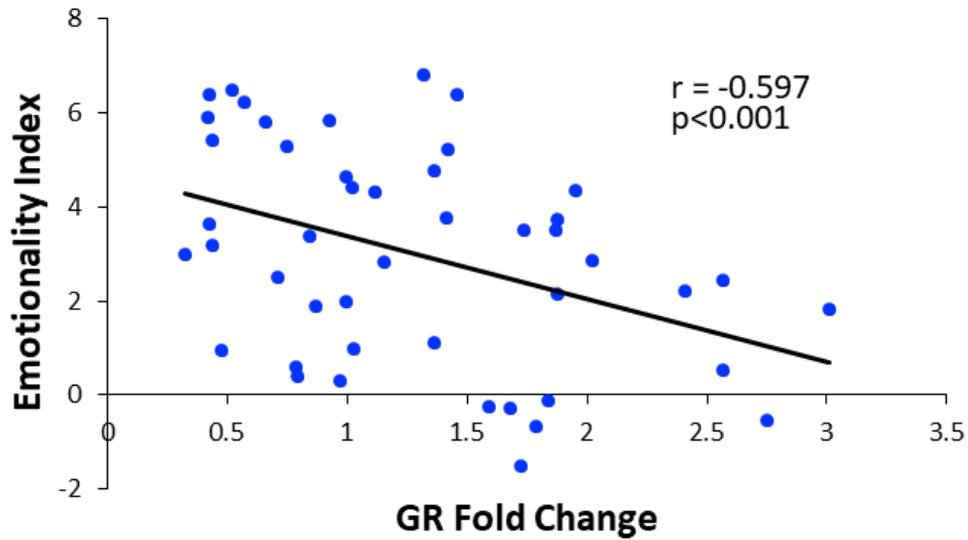


Figure 30. Emotionality Index – GR Fold Change. Scatter plot shows GR Fold change in the hippocampus correlated with emotionality scores of each subject. Blue dots represent independent data points and trendline shows the significant correlation ($r=-0.597$) between GR fold change and emotionality scores ($p<0.001$).

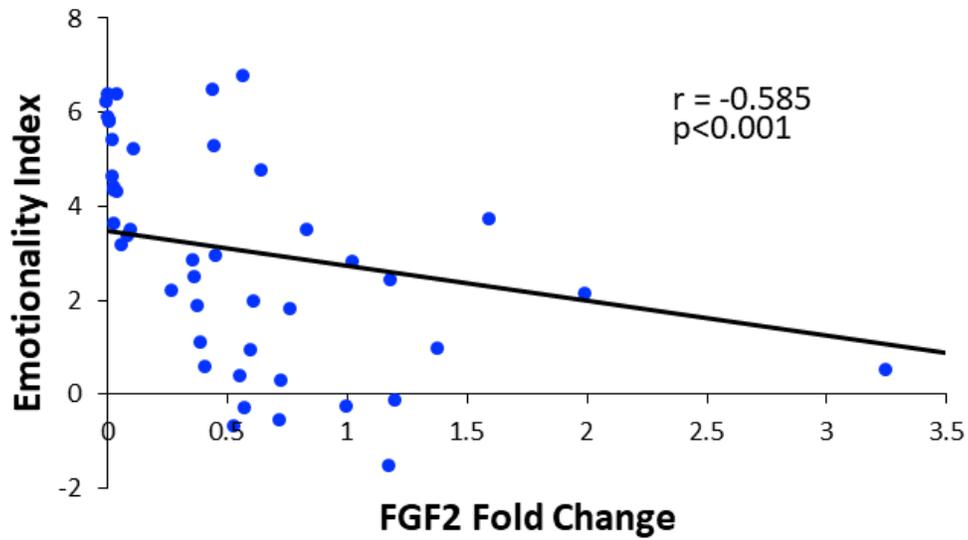


Figure 31. Emotionality Index – FGF2 Fold Change. Scatter plot shows FGF2 Fold change in the hippocampus correlated with emotionality scores of each subject. Blue dots represent independent data points and trendline shows the significant correlation ($r=-0.585$) between FGF2 fold change and emotionality scores ($p<0.001$).

Because behaviour, gene expression levels, and cytokine levels were conducted in the same mice, GR and FGF2 gene expression levels, behavioural outputs, and EGF and VEGF levels were correlated with each other (Table 7). Significant correlations were seen between GR levels and measures of the open field test ($p < 0.01$), elevated plus maze ($p < 0.05$), and forced swim test ($p < 0.01$) (Table 7). FGF2 levels ($p < 0.01$) were significantly correlated with the forced swim test (Table 7). GR levels ($p < 0.01$), FGF2 levels ($p < 0.01$), and VEGF ($p < 0.05$) levels were significantly correlated with the emotionality score (Table 7).

	1	2	3	4	5	6	7	8	9	10
1. Time Centre (OF)										
2. Centre Distance (OF)	0.69 **									
3. Time Open (EPM)	0.45 **	0.46**								
4. Open Distance (EPM)	0.19	0.38**	0.77**							
5. Time Immobile (FST)	-0.54**	-0.30*	-0.43**	-0.28						
6. Latency to Immobility (FST)	0.55**	0.33**	0.59**	0.31*	-0.80**					
7. Emotionality Index	-0.52**	-0.10	-0.32	-0.19	0.86**	-0.79**				
8. GR Fold Change	0.54**	0.33*	0.35*	0.13	-0.47**	0.54**	-0.60**			
9. FGF2 Fold Change	0.20	0.15	0.28	0.03	-0.36*	0.39**	-0.59**	0.36		
10. EGF	0.18	0.25	0.31	0.15	-0.27	0.33	-0.19	-0.05	-0.02	
11. VEGF	0.30	0.04	0.38*	0.33	-0.39*	0.40*	-0.39*	0.14	0.22	0.32

**Correlation is significant at the 0.01 level (2-tailed)

*Correlation is significant at the 0.05 level (2-tailed)

Table 7. Pearson Correlations. Table shows Pearson Correlations between Cytokine Expression, qPCR, and Behavioural Results. ** represents significant correlation where $p < 0.01$. * represents significant correlations where $p < 0.05$. There were significant correlations between GR levels and measures of the open field test ($p < 0.01$), elevated plus maze ($p < 0.05$), and forced swim test ($p < 0.01$). FGF2 levels ($p < 0.01$) significantly correlated with the forced swim test. GR levels ($p < 0.01$), FGF2 levels ($p < 0.01$), and VEGF ($p < 0.05$) levels were significantly correlated with the emotionality score.

Discussion

This study sought to elucidate whether FGF2 is necessary for the antidepressant effects of fluoxetine in mice exposed to chronic variable stress. We hypothesized that CVS exposure would decrease FGF2, increase pro-inflammatory cytokines, and increase depressive- and anxiety-like behaviours, and fluoxetine treatment would reverse these effects in WT mice, but not in FGF2KO mice. As expected, fluoxetine reversed many of the stress-induced depressive- and anxiety-like behaviours in WT mice and these behaviours were not reversed with fluoxetine treatment in FGF2KO mice. This suggests that FGF2 is necessary for the antidepressant effects of fluoxetine. These findings confirm the results seen in studies with humans who have major depressive disorder: in a study conducted by Kato et al. (2009), individuals with a variant of the *FGF2* gene predicted unresponsiveness to SSRI treatments, thereby suggesting an important role for FGF2 in the mechanisms of antidepressant action. Previous studies have also affirmed that FGF2 has inherent anxiolytic and antidepressant properties, however, the results of this current study show that it is also necessary for the therapeutic effects of fluoxetine.

Results from the depressive-like behaviour test, the forced swim test, showed that the effects of chronic variable stress were not reversed in the FGF2KO mice with fluoxetine administration. Additionally, the stressed, WT mice treated with fluoxetine showed individual differences with responses. In humans, approximately half the individuals diagnosed with depression are treatment resistant (Király et al., 2017; Maragnoli et al., 2004) and interestingly we are seeing a difference in response within this group. As well, individual differences were apparent in the latency to immobility in the stressed, WT, fluoxetine-treated group, such that the group was split between high and low latency to immobility. This suggests that there may have been group differences prevalent, which may have influenced performance on the test. Similar individual

differences were seen in the sucrose consumption test, paralleling the typical and atypical development of depression, where some individuals increase food and water consumption, whereas the other half decrease it (Singh & Williams, 2006). One issue with the forced swim test is the potential of a ceiling effect when measuring immobility. Both the stressed WT and stressed FGF2KO mice treated with vehicle produced very similar results, which could be due to this potential ceiling effect.

Results from the anxiety-like behaviour tests showed somewhat discrepant results. Administration of fluoxetine decreased anxiety-like behaviour in the WT mice on the elevated plus maze as expected, however, fluoxetine had no significant effect on anxiety in WT mice in the open field test. There have been previous reports of discrepancies between the findings of the elevated plus maze and the open field test, even though they are both used as measures of anxiety-like behaviour (Carola, D'Olimpio, Brunamonti, Mangia, & Renzi, 2002). This may be because of differences between laboratories in the selection and analysis of behavioural parameters (Carola et al., 2002). As well, mouse strain differences have been previously reported between the two tests that are consistent with the results seen in this study with C57/BL6 mice (Dulawa, Holick, Gundersen, & Hen, 2004). An analysis conducted in 2002 compared the measures of the elevated plus maze and open field test in two strains of mice, which are behaviourally distinct: BALB/c and C57BL/6 (Carola et al., 2002). The researchers observed distinct correlation effects of both strain and test used (Carola et al., 2002). BALB/c mice, an albino laboratory bred strain of the common house mouse, shows greater behavioural response to stress than do C57BL/6 mice (Seibenhener & Wooten, 2015). This may be the reason why the results in this study were discrepant. Regardless, *FGF2 gene* deletion did not show any responses to fluoxetine on the elevated plus maze nor the

open field test. This suggests that regardless of behavioural output, *FGF2* is necessary for fluoxetine's antidepressant effects.

Previous studies have shown that the effects of FGF2 administration on anxiety behaviour were mediated through FGF2's upregulation of hippocampal glucocorticoid receptor (GR) expression (Salmaso et al., 2016). In this study, GR was similarly reduced in expression in the FGF2KO mice. There was also an additive effect seen in GR levels with stress exposure and *FGF2* gene deletion, however, expression did not change with fluoxetine administration in the WT mice. This suggests that changes in expression of hippocampal *GR* cannot be the FGF2-dependant mechanism of action through which fluoxetine reverses the effects of CVS on behaviours. Perhaps analyzing differing time-points will show otherwise. It is important to keep in mind that it is still possible that reduction of *GR* caused by *FGF2* gene deletion may be responsible for the lack of response to fluoxetine in the FGF2KO mice. Whether *GR* is causally related to the effects of chronic variable stress in this study remains to be determined, nevertheless, there was a significant correlation seen with *GR* and all the behavioural tests.

FGF2 levels in response to the CVS model used in this study have not been previously tested. The results show that hippocampal FGF2 levels decreased in WT mice following exposure to chronic variable stress. As well, increases in emotionality were significantly correlated with the decreases in FGF2 ($p < 0.01$). These results further strengthen the relationship between hippocampal FGF2, anxiety- and depressive-like behaviours. In fact, since FGF2 has been previously implicated in several other disorders, such as Alzheimer's Disease and Post Traumatic Stress Disorder, it may be a viable biomarker for many individuals suffering from such disorders (Graham, 2017; Graham, Zagic, & Richardson, 2017; Woodbury & Ikezu, 2014).

The Luminex Assay conducted for cytokine expression looked at a set of pro-inflammatory cytokines (IFN- γ , TNF- α , IL-6, IL-1 α , IL-1 β , and CXCL1), anti-inflammatory cytokines (IL-10 and IL-4), and anti-inflammatory growth factors (FGF2, EGF, and VEGF). The results showed significant interactions in the levels of IL-6 and EGF. Significant effects of *FGF2 gene* deletion were seen in many of the results. As expected, FGF2KO mice treated with vehicle showed higher levels of pro-inflammatory cytokines IL-6 as well as higher levels of the anti-inflammatory growth factor EGF compared to their WT counterparts. This suggests that *FGF2 gene* deletion is producing additive effects on pro-inflammatory cytokine expression levels.

EGF levels in FGF2KO mice were increased with fluoxetine treatment, however, this effect was not seen with VEGF levels. The deletion of *FGF2 gene* and exposure to stress did not promote compensatory actions from VEGF. Interestingly, EGFKO mice display no overt phenotype, which may indicate overlapping or compensatory functions among EGF family members (Luetteke et al., 1999). Studies have also found that VEGF is not altered through compensation and have found varied results of expression changes with exposure to stress (Rubenstein, Hollowell, & Guinan, 2012). FGF2 and VEGF are potent angiogenesis inducers in vivo and in vitro (Seghezzi et al., 1998). Researchers in 1998 showed that FGF2 induces VEGF expression (Seghezzi et al., 1998). Addition of recombinant FGF2 to cultured endothelial cells or upregulation of endogenous FGF2 results in increased VEGF expression (Seghezzi et al., 1998). This may explain the non-compensatory action of VEGF in the FGF2KO mice – *FGF2 gene* deletion may be preventing upregulation of VEGF with fluoxetine treatment as was seen with EGF levels.

In this study, we also sought to determine the emotionality score (previously described in Guilloux, 2011) in which the Z scores were calculated for each test and normalized for locomotor behaviour, as appropriate. As predicted, administration of fluoxetine decreased total emotionality

scores in the CVS-exposed mice. However, a contradictory effect of fluoxetine was also noted in the non-stressed, WT control mice. Here, fluoxetine increased the emotionality score. Since not many studies have used this measure index, therefore unable to show consistency, it is difficult to say whether this is a genuine increase in emotionality. Since locomotor activity is a component in generating the emotionality score, it is possible that fluoxetine-induced locomotor changes contributed to the change in emotionality for this group. Further studies would be needed to determine if this is a consistent effect or a possible limitation in this measure.

The model of stress exposure used in this study is the chronic variable stress (CVS) model, which is often considered a prototypical model (Willner, 2016b). Through this model, rats or mice are exposed to chronic, constant, and unpredictable stressors, resulting in the development of behavioural changes (ex. anhedonia) (Willner, 2016b). One concern in the current study could be the continuous exposure to chronic stressors after antidepressant treatment has begun. Psychologists often recommend avoidance of stressors when depressed patients are prescribed antidepressants or other therapies. The model we used was based on a model used in previous studies in the Duman Laboratory (Bath et al., 2017). Exposure to chronic stress for 4-5 weeks has a robust and chronic effect of stress (Bath et al., 2017). On top of this, at least 3 weeks of antidepressant treatment are needed for effects to be seen (Bath et al., 2017). Since individuals tend to have been diagnosed with depression before being given antidepressants, this model creates face validity by producing depressive-like phenotype before treatment begins.

This does not preclude the possibility that this model may have limitations, particularly in terms of human implications. Depression in humans is generally the result of accumulation of several stressors over a long period of time (ex. Childhood trauma, followed by chronic stress in adulthood) (Brigitta, 2002; Maughan, Collishaw, & Stringaris, 2013; Paykel, 2008). However, the

CVS model requires the subject, in this case a mouse, to be suddenly exposed to chronic stressors to produce depressive- and anxiety-like behaviours, which does not parallel human development of depression. This is critical to keep in mind, especially since mice are highly resilient, and this resiliency varies among humans (Kirkland, Stout, & Sierra, 2016; Sullivan et al., 2017).

The results of the depressive- and anxiety-like behavioural measures produced individual differences. This model has been shown to be difficult to replicate (Willner, 2016a). This could potentially be due to strain differences seen in exposure to stress. For example, C57BL/6 mice, one of the most widely used strains, are more resistant to the commonly used chronic stress protocol, than BALB/c mice (Monteiro et al., 2015). Apart from strain, the type, diversity, and length of stressors are also critical determinants of the impact of chronic stress (Monteiro et al., 2015). A study in 2015 used an extended version of this model and exposed their C57BL/6 mice to varied stressors for 8 weeks, which produced a clear and consistent maladaptive response to chronic stress with behavioural and immunological alterations creating higher face validity (Monteiro et al., 2015). Nonetheless, the high translational potential of the CMS model means that neurobiological mechanisms may be of particular relevance to human depression and mechanisms of clinical antidepressant action. A model that can be translated to human development of depression, with random exposure to stressors over a long period of time, may help better elucidate the generalizability of such models.

In the present study, only male mice were observed. Ideally both male and female mice would be examined especially since depression is highly prevalent among females (Wang et al., 2017). The stress paradigm used in this current study is capable of inducing depressive- and anxiety-like behaviours in both male and female rodents (Willner, 2016b). This will ideally be the next step taken, expanding on this research. Future studies should seek to elucidate the differences

seen in male and female subjects with *FGF2* gene deletion, exposure to stress, and how they respond to antidepressant treatments.

FGF2 signaling occurs through four high-affinity tyrosine kinase receptors FGFR1, FGFR2, FGFR3, and FGFR4 (Woodbury & Ikezu, 2014). A closely-related receptor, which lacks the FGF signaling tyrosine kinase domain, FGFR5 (also known as FGFR1L1) was recently discovered (Tiong et al., 2013). FGF2 has a high affinity to IIIc-type FGFR1 and FGFR2, but a low affinity to IIIc-type FGFR3. Binding of FGF2 to FGFRs triggers receptor dimerization and tyrosine receptor kinase activation, resulting in autophosphorylation of the intracellular domain of the receptor and recruitment/assembly of signaling complexes (Woodbury & Ikezu, 2014). In 2012, El Sayed et al. observed that the decreased levels of the high affinity receptor, FGFR1, following CVS is restored to control levels after fluoxetine treatment (El Sayed et al., 2012). The present study did not look at interactions of FGF2 with its receptors. Interestingly, another study reported that dysregulation of the FGF system in major depressive disorder involves down-regulation of FGFR2 and FGFR3 (Evans et al., 2004). Examining the roles of the FGF family receptors would further elucidate possible biomarkers in depression.

There are several growth factors, like BDNF and VEGF, that remain upregulated in the adult brain (Eriksson et al., 1998). Like FGF2, BDNF and VEGF play important roles in neuroplastic events and have been shown to be implicated in anxiety- and depressive-like behaviours and their treatments (Eriksson et al., 1998). Unlike BDNF and VEGF, however, FGF2 is largely produced by astroglial cells and the effects that FGF2 plays on neuroplasticity are mediated through these astroglial cells (Kirby et al., 2013). Taking this into consideration, it may be important to look to a better understanding of these cells to elucidate the unique role that FGF2 plays in the pathology and treatment of depression. A previous study has shown that injecting a

gliatoxin, not a neurotoxin, into the prefrontal cortex produces behaviours like those seen with exposure to chronic variable stress (Banasr & Duman, 2008), supporting previous implications of astroglial cells in anxiety- and depressive-like behaviours.

Whether astroglial cells, astroglial functions, or HPA changes are the downstream target of FGF2 mediated changes induced by fluoxetine remains to be determined, however, the current study reaffirms the potential of FGF2 as a novel therapeutic target in the treatment of depression and anxiety disorders.

Abbreviations

AD – Alzheimer’s Disease
ADT - Antidepressant
BDNF – Brain-Derived Neurotrophic Factor
CNS – Central Nervous System
CRP – C-Reactive Protein
CVS – Chronic Variable Stress
CXCL – Chemokine Ligand
EGF – Epidermal Growth Factor
EPM – Elevated Plus Maze
FGF2 – Fibroblast Growth Factor 2
FGFR – Fibroblast Growth Factor Receptor
FGF2KO – Fibroblast Growth Factor 2 Knockout
FST – Forced Swim Test
GR – Glucocorticoid Receptor
HMW – High Molecular Weight
HPA – Hypothalamic-Pituitary-Adrenal Axis
IFN - Interferon
IL - Interleukin
LMW – Low Molecular Weight
LTP – Long-term Potentiation
MAOI – Monoamine Oxidase Inhibitor
NSC – Neural Stem Cells
NSFT – Novelty Suppressed Feeding Test
OF – Open Field Test
PCR – Polymerase Chain Reaction
PD – Parkinson’s Disease
PDGF – Platelet-Derived Growth Factor
q-rt-PCR – Quantitative Reverse Transcription Polymerase Chain Reaction
SCT – Sucrose Consumption Test
SGZ – Subgranular Zone
SSRI – Selective Serotonin Reuptake Inhibitor
SVZ – Subventricular Zone
TGF – Tumor Growth Factor
TNF – Tumor Necrosis Factor
VEGF – Vascular Endothelial Growth Factor
WT – Wildtype

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