PRE-CLINICAL ASSESSMENT OF GENETIC AND NEUROBIOCHEMICAL MARKERS FOR DEPRESSIVE BEHAVIOR AND ANTIDEPRESSANT RESPONSE

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ABSTRACT

CRISTINA S. BENTON: Pre-clinical Assessment of Genetic and Neurobiochemical Markers for Depressive Behavior and Antidepressant Response
(Under the direction of Dr. Tim Wiltshire)

Feeling sad and blue,
Is there a marker for you?
Tell me, am I blue?

Identification of biomarkers that can establish diagnosis or treatment response is critical to the advancement of research and management of patients with major depressive disorder. Biomarkers can be used objectively to evaluate clinical progression and response to antidepressant therapy. To identify genetic and neurobiochemical biomarkers of antidepressant response, we compared behavior, gene expression, and levels of thirty-six neurobiochemical analytes proposed to affect anxiety and mood disorders between water and fluoxetine-treated mice in a panel of genetically diverse mouse inbred lines. While responses in the open field (OF) and tail suspension test (TST) contribute to baseline inter-strain differences, chronic fluoxetine treatment predominantly affected behavior in the TST, indicating that the TST is sensitive to the antidepressive effects of fluoxetine. We found that levels of glyoxylase1 (GLO1) and guanine nucleotide binding protein (GNB1) account for most of the covariance in baseline anxiety-like and depressive-like behavior. Overall difference in neurobiochemical levels were observed for positive and negative responders. Biochemical alterations following chronic fluoxetine treatment discriminated positive responders, while baseline neurobiochemical differences differentiated

negative responders. Results show that glial fibrillary acidic protein (GFAP), S100 beta protein (S100β), GLO1, and histone deacytelase 5 (HDAC5) contributed most to fluoxetine response. These proteins are linked within a cellular growth/proliferation pathway, suggesting that positive responses to antidepressants are likely due to increased cellular genesis. In addition, a candidate genetic locus that associates with baseline depressive-like behavior contains a gene that encodes for cellular proliferation/adhesion molecule (*Cadm1),* supporting a genetic basis for the role of neuro/gliogenesis in depression. By using a multi-faceted approach that investigates connections on genetic, neurobiochemical, and behavioral levels, we were able to identify genetic and neurobiochemical markers that can potentially assess risk for despair and poor treatment outcome. Importantly, our research study provides an innovative and powerful platform for pre-clinical assessment of antidepressant drugs in depressive-like susceptible strains and non-responsive lines.

This dissertation is dedicated to Mrs. C and her family.

I still remember!

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LIST OF ABBREVIATIONS

5-HT	Serotonin
ACC	Anterior Cingulate Cortex
B6	C57BL/6J mouse inbred strain
CBF	Cerebral Blood Flow
D2	DBA/2J mouse inbred strain
DPP4	Dipeptidyl Peptidase 4
EMMA	Efficient Mixed Model Association
FST	Forced Swim Test
GWAS	Genome Wide Association Analysis
HAM	Haplotype-Associated Mapping Analysis
HPA	Hypothalamic-Pituitary-Adrenal Axis
LD	Light/Dark Exploration Test
MDD	Major Depressive Disorder
mRNA	Messenger Ribonuecleic Acid
OF	Open Field Test
QTG	Quantitative Trait Gene
QTL	Quantitative Trait Loci
SERT	Serotonin Transporter
SIH	Stress Induced Hyperthermia
SSRI	Selective Serotonin Reuptake Inhibitor
TST	Tail Suspension Test

CHAPTER 1

BIOLOGICAL ALTERATIONS IN DEPRESSION

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1. Introduction

According to the World Health Organization, depression is among the leading cause of disability worldwide with approximately 121 million people affected (http://www.who.int). It is estimated that 5% of men and 9% of women will experience depression in a given year (Kessler et al. 2005). Major Depressive Disorder (MDD) is characterized by persistent depressed mood or loss of interest or pleasure from daily activities. Additionally, patients may experience feelings of guilt or worthlessness, as well as psychomotor, physiological, and cognitive disturbances (DSM IV). Given that the etiology of depression is unclear, current antidepressant treatments are ineffective for most patients. Presently, less than 30% of patients achieve response or remission (Trivedi et al. 2006). Depression is a clinically and genetically heterogeneous disorder, which complicates efforts to identify causative factors of disease and replicate findings. In addition, diagnosis and therapeutic assessment are primarily based on subjective measures, making patient selection and outcome measures amenable to inconsistencies and irreproducibility.

Biomarkers that objectively establish diagnosis, prognosis, and antidepressant response can facilitate research and clinical management of patients with depression. Many analytes, including brain-derived neurotrophic factor (BDNF), serotonin transporter, and monoamines, have been linked with depressive symptoms and response to antidepressant therapy (Manji et al. 2001; Nestler et al. 2002; Thase 2007). Although much progress has been made in identifying neurobiological correlates of depression, it is unclear whether these alterations are causally linked or are due to disease and/or treatment. With the goal of facilitating the search for depression biomarkers, this chapter will discuss several key molecular and neurochemical alterations that have been linked with depressive disorder.

2. Genetic Studies

The role of genetics in the development of MDD is supported by findings from family, twin, and adoption studies. Studies that compared the prevalence of depression in monozygotic versus dizygotic twins indicate a heritability estimate of 35-50% (Bierut et al. 1999; Kendler et al. 1993; Sullivan et al. 2000). There is a two-to-threefold increased risk of developing MDD among first degree relatives of depressed individuals (Kelsoe 2004; Sullivan et al. 2000), indicating that genetic variants can be used as prognostic and diagnostic biomarkers. There are two widely used approaches to determine genetic markers of depression. Candidate gene analysis examines the frequency of genetic alleles between cases and controls. Hypotheses are generated *a priori* based on the likelihood that the gene affects the risk of depression. Alternatively, advances in genotyping capabilities and more recently, gene sequencing, have enabled scientists to look for unbiased genome-wide associations between common single nucleotide polymorphisms (SNPs) and behavior. Genes that confer risk to depression have been primarily identified using candidate gene analysis approach, while recent efforts to uncover genetic markers of antidepressant response include the Sequenced Treatment Alternatives to Relieve Depression (STAR*D) trial and Genome-Based Therapeutic Drugs for Depression (GENDEP) study, which looked at genome-wide associations of common variants with antidepressant response. Genetic studies of depression (Levinson 2006; Lohoff 2010; Shyn and Hamilton 2010) and antidepressant response (Crisafulli et al. 2011; Kato and Serretti 2010; Porcelli et al. 2010) are reviewed in this chapter with a focus on several genes.

2.1 Genetic Predictors of Depression and Antidepressant Response

Antidepressant medications primarily work on altering neurotransmitters in the brain, thus much attention has been given to genes within the monoaminergic pathway (Kato and Serretti 2010). An insertion/deletion polymorphism on the 5' promoter region of the serotonin transporter gene (*5-HTTLPR*) produces a long (*L*) allele or a short (*S*) lower-expressing allele. The *5-HTTLPR* variant alters expression of the serotonin transporter *in vitro* (Lesch et al. 1996) and has been linked with MDD (Caspi et al. 2010; Goldman et al. 2010; Uher and McGuffin 2010), neuroticism (Lesch et al. 1996), affective disorder (Collier et al. 1996; Lasky-Su et al. 2005), suicidality (Anguelova et al. 2003; Lin and Tsai 2004), and anxiety related personality traits (Schinka et al. 2004; Sen et al. 2004). Patients with the low expressing allele exhibited increased amygdala activation in response to sad faces (Hariri et al. 2002), reduced gray matter volume in amygdala and perigenual cingulate cortex (Pezawas et al. 2005), as well as altered functional coupling in both regions (Pezawas et al. 2005), thus supporting the role of the serotonin transporter in the development of the amygdala-cingulate feedback circuitry. Carriers of the *S* allele who experienced stressful life events in the past were more vulnerable to depression and suicidality (Caspi et al. 2003; Kendler et al. 2005). However, several groups did not find an association between depression and *5-HTTLPR* alone (Middeldorp et al. 2010; Munafo and Flint 2009; Risch et al. 2009) or in interaction with stressful life events (Risch et al. 2009). Homozygous carriers of the *L* allele showed higher response and remission rates (Serretti et al. 2005) and more favorable side effect profiles (Kato and Serretti 2010; Kraft et al. 2007; Murphy et al. 2004), which did not replicate in a recent large clinical trial (Kraft et al. 2007). Altogether, these findings indicate that environment must be taken into account when evaluating the potential use of *5-HTTLPR* as a genetic marker of depression.

Other genes in the monoamine pathway have been studied for their link with depressive behavior. The serotonin-1A receptor (*HTR1A*) is located in the serotonergic neurons and on their post-synaptic targets. In the pre-synaptic neuron, 5HT1A auto-inhibits raphe firing and 5-HT synthesis. The -1019C/G variant (rs6295) found in the promoter region of *HTR1A* results in higher expression of serotonin-1A auto-receptor (5-HT1A), which leads to reduction in serotonergic neurotransmission (Stahl 1994). The -1019C/G mutation is correlated with anxiety and depression (Gross et al. 2002; Lemonde et al. 2004; Strobel et al. 2003). In Asians, the G allele is associated with improved treatment outcomes (Hong et al. 2006; Kato et al. 2009). However, this finding was not observed in Caucasians (Lemonde et al. 2004; Serretti et al. 2004), suggesting a confounding effect of race. The relationship between *HTR2A* and antidepressant response is unclear due to conflicting results (reviewed in Kato 2010**)**. A recent meta-analysis did not find any association between *HTR1A* and *HTR2A* and treatment response; however, a polymorphism within *HTR2A* was correlated with tolerability (Kato and Serretti 2010). No association has been established between *HTR2A* and MDD (Anguelova et al. 2003).

The tryptophan hydroxylases 1 and 2 (TPH1 and TPH2) catalyze the rate-limiting step in 5-HT biosynthesis. A functional variant in *TPH2* (Arg441His) results in 80% reduction of 5-HT in the brain (Zhang et al. 2004) and was found to be more frequent in patients with MDD (Zhang et al. 2005). However, other studies failed to replicate this finding (Delorme et al. 2006). Furthermore, the *TPH* 218A allele is associated with poor antidepressant response (Serretti et al. 2001a; Serretti et al. 2001b), a finding that was supported by a meta-analysis study (Kato and Serretti 2010). Patients with the 218 C/C genotype were more likely to respond to antidepressant therapy (Kato and Serretti 2010). Interestingly, the significant pooled odds ratio score (OR) was primarily influenced by the sum of the three studies that looked at the association between

remission rates and the 218 genotype, suggesting that the *TPH* gene may be important in regulating long-term antidepressant response. Of interest is the recent correlation between *TPH2* haplotype markers and suicidality (De Luca et al. 2004; Lopez et al. 2007), suggesting that *TPH2* may mediate a subset of depressive symptoms like suicidal thoughts and feelings of guilt and worthlessness.

Enzymes that mediate clearance of catecholamines, including monoamine oxidase A (MAO-A) and catechol-O-methyl transferase (COMT) have been linked to antidepressant response. Higher transcription efficiency is observed with the variable number tandem repeat (VNTR) sequence located 1.2kb upstream of the *MAO-A* gene (Sabol et al. 1998). Alternatively, the Val to Met substitution at codon 158 for membrane-bound COMT protein (codon 108 for soluble COMT) has been linked to lower enzymatic activity (Mannisto and Kaakkola 1999) and improved response to citalopram (Arias et al. 2006) and mirtazapine (Szegedi et al. 2005) but not paroxetine (Arias et al. 2006; Szegedi et al. 2005).

A locus on Chr. 12 has been linked with MDD (Abkevich et al. 2003; McGuffin et al. 2005) and anxiety (Erhardt et al. 2007). Within this putative region lies the purinergic ATP-binding calcium channel gene (*P2X7).* A non-synonymous coding SNP within *P2X7* (Gln460Arg) is associated with MDD risk (Lucae et al. 2006). P2X7 protein is required for IL-1 (interleukin-1) processing and secretion (Ferrari et al. 2006), highlighting the potential role of immune function in depressive behavior. Moreover, the FK506 binding protein 5 (*FKBP5*) in complex with Hsp90 regulates glucocorticoid receptor sensitivity. A functional variant within *FKBP5* that results in increased intracellular concentration of FKBP5 has been linked with recurrence of depressive episodes (Binder et al. 2004) and antidepressant response (Binder et al.

2004; Lekman et al. 2008b). FKBP5 activates glucocorticoid receptors and the hypothalamic-pituitary-adrenal axis, which regulate response to stress (Binder et al. 2004). Additionally, the corticotropin releasing hormone 1 (*CRH1*) variant is correlated with early onset of depressive symptoms (Papiol et al. 2007). CRH activates the HPA axis, thus supporting the role of the HPA axis in mediating depressive behavior.

Small low-powered studies were combined in a meta-analysis to clarify the associations of several genes with depression, which were unclear due to inconsistent or non-replicated findings. Lopez-Leon et al. found a protective effect for the *APOE 2* allele (combined OR, 0.51; 95% CI, 0.27-0.97) with no evidence of between-study heterogeneity (Lopez-Leon et al. 2008). Alternatively, an increased risk was found for the methylenetetrahydrofolate reductase *MTHFR* C677T polymorphism (pooled OR, 1.36), the guanine nucleotide binding protein 3 *GNB3* C825T variant (pooled OR, 1.38; 95% CI, 1.13-1.69), and the dopamine transporter *SLC6A3* 40 bp VNTR (pooled OR, 2.06; 95% CI, 1.25-3.40) (Lopez-Leon et al. 2008).

Pharmacogenetic studies of antidepressants in the STAR*D trial have identified genes associated with treatment response (Hu et al. 2007; Lekman et al. 2008a; McMahon et al. 2006; Paddock 2008), treatment resistance (Perlis et al. 2008), and treatment-emergent suicidal ideation (Laje et al. 2009; Laje et al. 2007; Perlis et al. 2007). In addition, polymorphisms in genes that encode drug-metabolizing enzymes and transporters have been tested for correlation with treatment response (Peters et al. 2008). Genes that were significantly associated with response to citalopram include *FKBP5* (Lekman et al. 2008a), glutamate receptor, ionotropic kainite 1 (*GRIK1*), N-methyl d-aspartate 2A (*GRIN2A*), 5-hydrxytryptamine receptor 2A (*HTR2A*),

potassium channel, subfamily K, member 2 (KCNK2), phosphodiesterase (PDE), and solute carrier family 6 member 4 (*SLC6A4*) (Lin and Chen 2008).

A link between genes and depression exists, however, putative genes identified to date do not significantly account for the phenotypic variance observed (Mann and Currier 2006). Although these initial results may seem disappointing, they indicate that the genetics of depression is far from simple. It is likely that multiple genes with minor effect sizes interact with environmental factors to affect mood, making identification of genetic biomarkers challenging. Efforts to investigate gene by environmental effects can further delineate the contribution of each gene on disease and treatment outcomes (Lesch 2004; Wermter et al. 2010).

3. Biochemical Alterations

Several mechanisms are altered in depression and these include neurotransmission, neuroendocrine signaling, and neuroimmune functions. It is unclear whether these biochemical alterations are products or causative factors of depression. This section will discuss common biological alterations that have been observed in depression, facilitating identification of candidate biochemical markers for depression and antidepressant response.

3.1 Monoamines

The monoamine theory of depression developed following the observation that iproniazid, a drug that inhibits the metabolism of monoamines by blocking MAO, improved the mood of patients who are taking the drug (Delay et al. 1952). In addition, depletion of monoamines by agents like reserpine was found to induce depression (Goodwin and Bunney 1971). This theory led to the development of antidepressant drugs that elevate monoamine levels

at the synapse by blocking uptake transporters, catabolic enzymes or inhibitory pre-synaptic auto- or hetero-receptors. The monoamines provided a biochemical basis for depression, whereby depression was thought to result from a 'chemical imbalance' of monoamines in the brain (Schildkraut 1965). However, several observations have cast doubt on the major role of monoamines in MDD. In addition to the untimely manner in which elevation of monoamines occur with respect to symptom resolution (Baldessarini 1989), treatments that do not elevate monoamine levels like electroconvulsive therapy (ECT) have been effectively shown to treat depression (Pagnin et al. 2004). The monoamine theory of depression was then modified to indicate that elevation of monoamines is the first step in a cascade of molecular events that ultimately leads to symptom improvement (Pineyro and Blier 1999). Research focus began to shift towards evaluating the long-term adaptive changes that result from increased monoamines in the synapse. It was hypothesized that elevation in monoamines leads to reduction in the sensitivity and/or number of monoamine receptors. Although desensitization and internalization of monoamine receptors have been observed in several animal and post-mortem studies, results were often inconsistent and conflicting (Elhwuegi 2004). Effective antidepressant agents that do not act by inhibiting monoamine reuptake proteins or metabolizing enzymes can still facilitate receptor internalization despite the absence of pre-synaptic input (Fishman and Finberg 1987; Kientsch et al. 2001). More recently, it has been shown that monoamine elevation may lead to cellular genesis. Various antidepressant agents, including specific serotonin reuptake inhibitor (fluoxetine), monoamine oxidase inhibitor (tranylcypromine), specific norepinephrine reuptake inhibitor (reboxetine), and serotonin/norepinephrine uptake inhibitor (tricyclic antidepressants) have been shown to induce cell proliferation and neurogenesis (Santarelli et al. 2003), which

suggests that monoamine elevation leads to other downstream molecular effects that can alter behavior.

Despite decades of research aimed to evaluate the relationship between depression and monoamine alteration, direct evidence supporting the causative role of monoamines in MDD is lacking (Nestler 1998), thus prompting efforts to study other pathways that may underlie depressive behavior.

3.2 Hypothalamic Pituitary Adrenal (HPA) axis

Dysregulation in the HPA axis, which is characterized by elevated plasma cortisol and CRH is a common finding in depressed patients (Holsboer 2000; Raison and Miller 2003). In response to stress, the parvocellular neurons in the hypothalamus secrete CRH, stimulating the release of adenocorticotropin releasing hormone (ACTH) from the anterior pituitary. ACTH, in turn, activates the synthesis and release of glucocorticoids (cortisol from humans and corticosterone in rodents) from the adrenal cortex. Glucocorticoids negatively regulate the HPA axis by inhibiting the synthesis and release of CRH from the hypothalamus.

Activation of the HPA axis mediates physiologic adaptation to stress, however, persistent stimulation can lead to glucocorticoid receptor (GR) desensitization (de Kloet et al. 2005). Patients with depression typically exhibit high levels of cortisol in plasma, saliva, and urine, as well as an increase in the size and activity of the pituitary and adrenal glands (Nemeroff and Vale 2005). Impairment of the HPA axis, which is primarily characterized by the inability to suppress cortisol levels following pharmacologic stimulation of GR by dexamethasone, has been observed in depressed patients (Ising et al. 2005; Kunzel et al. 2003; Sher 2006). HPA alterations

normalize with antidepressant therapy (Holsboer 2000) and this is associated with less relapse (Ising et al. 2007). Glucocorticoids not only exhibit immune and metabolic functions but they also regulate neurogenesis, neuronal survival, hippocampal size and structure, and acquisition of new memories (Herbert et al. 2006). Reduced maternal handling increases CRH signaling (Ladd et al. 1996) and sustains HPA hyperactivity, inducing depressive-like behavior in the pups (Francis et al. 1999; Meaney 2001). In humans, early stressful life event is associated with dysregulated HPA axis (Heim et al. 2002) and development of depressive symptoms (Chapman et al. 2004; McCauley et al. 1997). One of the mechanisms by which antidepressants induce hippocampal neurogenesis is by activating GR (Anacker et al. 2011), thus implicating a direct relationship between HPA axis and neural brain signaling.

3.3 Other Neuroendocrine Markers

It was discovered that hypothyroidism elicits depressive behavior and that these symptoms can be reversed by thyroxine therapy (Asher 1949). Similar symptoms are observed in depression and hypothyroidism, which include dysphoric mood, fatigue, anhedonia, and alteration in weight (Jackson 1998). Low levels of thyroid hormones (T_3 and T_4) stimulate the release of thyrotropin releasing hormone (TRH) from the hypothalamus to the anterior pituitary. The pituitary, in turn, releases thyrotropin-stimulating hormone (TSH), which leads to the release of triiodothyronine (T_3) and thyroxine (T_4) from the thyroid. Thyroid hormones primarily regulate metabolism but may also be involved in neurotransmission (Dratman and Gordon 1996). Although not all depressed patients display abnormalities in thyroid function, alterations have been observed, including elevation in T_4 (Baumgartner et al. 1988; Kirkegaard and Faber 1991), lower TSH levels (Maes et al. 1989), as well as blunted response of TSH to TRH (Hein and

Jackson 1990; Maes et al. 1989). Type-II deiodinase (D-II) catalyzes deiodination of T_4 to T_3. Psychotropic medications like lithium (Baumgartner et al. 1994b), desipramine (Campos-Barros et al. 1994), carbamezapine (Baumgartner et al. 1994a), and fluoxetine (Baumgartner et al. 1994c) stimulate the activity of D-II, indicating that mood regulatory agents indirectly regulate T_3 levels. Another group, however, did not find any effects of antidepressant on thyroid function (Brambilla et al. 1982). Interestingly, one study found that morning and nocturnal changes in TSH may predict antidepressant response (Duval et al. 1996).

There is increasing evidence implicating the involvement of stress-responsive neuropeptide systems in depression and anxiety. The involvement of various neuropeptides has been reviewed (Alldredge 2010; Holmes et al. 2003) and a number of them will be described here. Administration of neuropeptide antagonists/agonists results in altered responses in rodent models of anxiety and depression (Rotzinger et al. 2010). Stress stimulates the release of vasopressin, which in turn enhances the effects of CRH on ACTH (Aguilera et al. 2003; Engelmann et al. 2004; Zhou et al. 2001). Depressed patients display altered levels of vasopressin in the suprachiasmatic nucleus (SCN) (Zhou et al. 2001), paraventricular nucleus (Purba et al. 1996), and supraoptic nucleus (Meynen et al. 2006). A polymorphism in the vasopressin receptor (V_{1B}) may be protective against MDD (Overstreet and Griebel 2005; Salome et al. 2006). Antagonism of the V_{1B} receptor reduced depressive-like behavior (Griebel et al. 2002), which was comparable to treatment with antidepressant agents (Salome et al. 2006). This effect was mainly due to inhibition of the V_{1B} receptors in the lateral septum and amygdala (Stremmelin 2005). Similar to vasopressin, the neuropeptide Y (NPY) is released under stress. NPY is abundantly expressed in the brain and is co-localized with noradrenaline, somatostatin, and GABA (γ-aminobutyric acid) (Kask et al. 2002). Reduction in NPY is associated with

increased sensitivity to depression and stress, indicating that NPY agonists may exhibit antidepressive effects (Redrobe et al. 2002). A variant in the promoter region of *Npy* alters the expression of NPY *in vivo* and is linked with anxiety behavior and neural responses to stress (Zhou et al. 2008). Substance P (SP), a known modulator of pain signaling, has been shown to interact with serotonergic signaling (Schwarz et al. 1999). Substance P binds to neurokinin-1 (NK_1) receptors found in the brain and in the periphery. Genetic ablation or pharmacologic antagonism of NK_1 receptors promotes monoaminergic activity (Froger et al. 2001; Maubach et al. 2002; Santarelli et al. 2001) and reduces anxiety-like behavior (Santarelli et al. 2001). Depressed patients have higher SP levels in the serum (Bondy et al. 2003). Interestingly, NK_1 antagonists activate the serotonergic system similarly to a serotonin reuptake inhibitor (escitalopram) (Guiard et al. 2004), indicating that NK_1 antagonists may have antidepressive effects. Galanin is a 29-30 amino acid peptide that regulates various physiological responses like metabolism and food intake. Galanin binds to several galanin receptors (GALR), which in turn interact with different G proteins, activating various signal transduction pathways (Smith et al. 1998; Wang et al. 1998). Galanin administration in rodents produces a variety of effects, including nerve regeneration, nociception, and alteration in sexual and feeding behavior (Wrenn and Crawley 2001; Yoshitake et al. 2003). Galanin mediates 5-HT and norepinephrine levels (Ogren et al. 2006) and antagonism of GALR can enhance or reduce depressive-like behavior depending on which GALR subtype is being inhibited (Barr et al. 2006; Lu et al. 2005).

Many years of research implicate the role of the neuroendocrine system in depression. Most neuroendocrine regulatory mechanisms occur through the bidirectional communication between the hypothalamus and pituitary. These findings indicate that the neural circuitry,

neuronal signaling, and structural plasticity within this region are likely to be critical in behavioral responses.

4. Metabolic Alterations

Metabolic syndrome is comprised of several features, including central obesity and insulin resistance, which, in concert, increases risk for developing cardiovascular disease and diabetes. Compared to healthy controls, depressed individuals are more likely to develop obesity, diabetes, and hypertension (Lindley et al. 2009), indicating potential overlap between depressive symptoms and metabolic syndrome. Independent of the criteria used to define metabolic syndrome (Raikkonen et al. 2007), a strong bidirectional association between depression and metabolic syndrome exists in women (Gil et al. 2006; Kinder et al. 2004; Raikkonen et al. 2007). The correlation between depressive symptoms and metabolic syndrome is slightly higher in monozygotic twins than dizygotic twins, suggesting that genetics play a critical role in both disorders (McCaffery et al. 2003). Resistance to insulin, which is a risk factor for developing metabolic syndrome, is a common occurrence in depressed patients (Koslow et al. 1982; Okamura et al. 2000; Winokur et al. 1988), which suggests that insulin links depression with metabolic syndrome. Insulin exerts dose-dependent effects on food intake and energy regulation. Ablation of insulin receptors on neuronal cells leads to increased body fat disposition, suggesting that insulin negatively regulates adiposity (Bruning et al. 2000). Additionally, insulin regulates monoamine uptake and metabolism, phosphoinositol turnover, as well as norepinephrine and dopamine transporter mRNA levels (Craft and Watson 2004). It has been shown that insulin can recruit GABA receptors (Wan et al. 1997) and promote internalization of α-amino-3-hydroxy-5-methylisoxazole-4-propionic acid (AMPA) receptors, which suggests that insulin plays a critical

role in neuronal signaling and synaptic plasticity (Huang et al. 1998). Interestingly, brain volume abnormalities and neurocognitive deficits commonly found in MDD patients have been observed in individuals with diabetes mellitus (DM), suggesting overlapping pathophysiology between MDD and DM (McIntyre et al. 2010). Insensitivity to insulin likely develops due to HPA axis hyperactivity (Rizza et al. 1982), impaired immune system (Fernandez-Real et al. 2001; Maes 1995; Moller 2000), and altered central serotonergic signaling (Goodnick et al. 1995; Horacek et al. 1999), all of which are common findings in depressed patients (Belmaker 2008; Krishnan and Nestler 2008).

Association between depression and obesity has been identified in several cross sectional studies (de Wit et al. 2010; Faith et al. 2002; Scott et al. 2008). A recent meta-analysis looked at the association between obesity and depression in a community-based setting and found that obese patients have an 18% increased risk of developing depressive symptoms (overall OR, 1.18) (de Wit et al. 2010). Subsequent sub-group analyses showed that the association with obesity holds true for depressed women but not for men, which suggests that comorbidity is likely to be affected by sex (de Wit et al. 2010). Similarly, a meta-analysis of longitudinal studies showed that baseline obesity increased the risk of depression (pooled OR, 1.57) and that depression increased the odds for developing obesity (pooled OR, 1.40). Prospective analysis of the cause-effect relationship between obesity and depression indicate reciprocal findings, whereby obesity was found to be a predictor of depression in eight out of the ten studies reviewed, while 53% of the studies found that depression predicts obesity (Faith et al. 2011). Interestingly, the positive association between depression and obesity is only detected in studies conducted in the United States but not in other European countries, indicating a strong contributory effect of environment (Atlantis and Baker 2008). It is increasingly recognized that

similar neural circuitry that regulate memory, reward, mood, and emotion also controls appetite, body weight, and energy homeostasis (Dallman 2009; Zheng et al. 2009).

Food induces olfactory and visual sensory inputs, which stimulate the orbitofrontal cortex, where acquisition, storage, and processing of memory and experiences associated with food is thought to occur (Verhagen 2007). Stimulation of the mu-opioid receptor in the nucleus accumbens and ventral pallidum results in further intake of pleasurable foods (Will et al. 2003; Zhang and Kelley 2000). The ventral tegmental area and the nucleus accumbens are part of the mesolimbic dopaminergic system, which regulates behavioral response (motivation) towards favorable stimuli (Berridge 1996; 2007; Pecina et al. 2006), indicating that food intake and motivation are, at least partly, co-regulated by similar circuitry. The hypothalamus regulates homeostatic responses to altered nutrient levels and adiposity levels (Berthoud 2002; Xue and Kahn 2006) through various endocrine hormones, including leptin (Farooqi et al. 2002; Friedman 1999; O'Rahilly 2002) and NPY (Luquet et al. 2005). Although leptin is primarily known for its role in appetite suppression and energy expenditure, leptin also mediates reproduction and cognition (Chehab 2000; Farr et al. 2006). Independent of body mass, depressed patients show lower plasma levels of leptin (Jow et al. 2006; Kraus et al. 2001) although other studies did not find similar results (Antonijevic et al. 1998; Deuschle et al. 1996; Rubin et al. 2002). Rodents exposed to chronic unpredictable stress showed reduction in sucrose preference and higher depressive-like behavior, which was reversed by leptin administration, indicating that leptin exhibits antidepressive effects likely through innervations of the limbic brain circuitry (Lu et al. 2006). In response to stressful events, leptin suppresses CRH, ACTH, and corticosterone secretion, suggesting a direct impact of leptin on the HPA axis (Ahima et al. 1996; Heiman et al. 1997; Huang et al. 1998). In addition, leptin-deficient *ob/ob* mice display altered *Slc6a4*

expression (Collin et al. 2000), decreased neuronal and glial cells, and reduced brain weight and cortical volume (Ahima 1999, Stepan 1999), further supporting the role of leptin in MDD.

A common thread between MDD, DM, and heart disease exists. The co-occurrence and pathophysiologic overlap between metabolic syndrome, obesity, and depression may explain the significant association between depression, diabetes, and cardiovascular disease (Frasure-Smith et al. 1993; Goldney et al. 2004; Paile-Hyvarinen et al. 2007).

5. Neuroimmune

An interaction between behavior and the immune system was first recognized in 200 AD, when Galen observed that melancholic women were more susceptible to cancer (Leonard 1988). Depressed patients exhibit reduced neutrophil phagocytosis, natural killer cell activity, and mitogen stimulated lymphocyte proliferation (Irwin et al. 1990). Furthermore, patients with MDD show increased cytokine secretion from activated macrophages and elevated acute phase proteins in the liver (Sluzewska et al. 1996), indicating dysregulation in immune response. Antidepressants inhibit the ability of lipopolysaccharide (LPS) to induce the synthesis and the release of pro-inflammatory cytokines, likely through elevation of cyclic adenosine monophosphate (cAMP) levels (Xia et al. 1996). It has been hypothesized that abnormal secretion of macrophage monokines leads to depressive behavior (Smith 1991). Macrophages secrete neuroendocrine and immune modulators, including interleukins (IL), tumor necrosis factors (TNF), ACTH, and endorphins (Nathan 1987), thus indicating a regulatory role for macrophages in mediating the neuro-endocrine-immune interface.

A bidirectional relationship between the brain, neuroendocrine, and immune systems exists, particularly in response to stress. Overactivity of the HPA axis, which is a common finding in depressed individuals (Holsboer 2000; Raison and Miller 2003), results in hypercortisolemia and suppression of the immune system. Conversely, persistent stress can result in fewer B cells, T cells, and lymphocytes (Olff 1999), which can confer susceptibility to infections and cancer (Garssen and Goodkin 1999; Kiecolt-Glaser et al. 1995; Reiche et al. 2005). Stressful events like separation or divorce are correlated with increased cancer risk, low proportions of NK and T cells, impairment of DNA repair, and abnormal immune response (Kiecolt-Glaser et al. 1987). The presence of reactive oxygen species has been detected in depressed patients (Irie et al. 2005). Levels of 8-hydroxydeoxyguanosine (8-OH-dG), a biomarker of cancer-related oxidative DNA damage, is positively correlated with depressive symptoms (Irie et al. 2005), which suggests that depression may be associated with cancer.

In 1987, Wagner-Jauregg demonstrated that activation of the immune system can affect various mental states (Raju 1998). Cytokines regulate growth, differentiation, and function of many cells (Turnbull and Rivier 1999). They can be broadly classified as pro-inflammatory or anti-inflammatory cytokines. Pro-inflammatory cytokines like interleukin-1 (IL-1), interleukin-6 (IL-6), and TNF-α stimulate immune cell production, activation, and proliferation. On the other hand, anti-inflammatory cytokines, including interleukin-4 (IL-4), interleukin-10 (IL-10), and interleukin-13 (IL-13) dampen the immune response. The role of cytokines in depression was identified following observation that interferon treatment induces ‘sickness behavior,’ which mimics depressive symptoms such as dysphoric mood, fatigue, anorexia, weight loss, and altered sleep patterns (Papanicolaou et al. 1998; Yirmiya 2000). Depression is characterized by elevation

of pro-inflammatory markers IL-6, c-reactive protein (CRP) (Maes 1995), IL-1, and IL-2 (Dunn et al. 2005; Song et al. 1994). Treatment with LPS stimulated depressive-like behavior and cytokine secretion, which were reversed by antidepressants or cytokine antagonists (Yirmiya 2000). Administration of IL-6 and IL-1 results in elevation of vasopressin, cortisol, CRH, and ACTH (Brebner et al. 2000; Harbuz et al. 1992; Xu et al. 1999), which suggests a pivotal role of cytokines in HPA axis activation (Dentino et al. 1999). In rodents, treatment with IL-1 resulted in increased DA, NE, and 5-HT activity in the brain (Dunn and Swiergiel 1999; Merali et al. 1997; Song et al. 1999). Cytokines acutely stimulate 5-HT neurotransmission and reduce its production by stimulating indoleamine 2,3-dioxygenase (IDO), an enzyme that converts the precursor of 5-HT (tryptophan) into kynurenine (Wichers and Maes 2002). Pro-inflammatory cytokines have been shown to up-regulate serotonin transporter (Morikawa et al. 1998; Mossner and Lesch 1998; Wichers and Maes 2002), while anti-inflammatory cytokines like IL-4 reduces 5-HT uptake (Mossner et al. 2001). Together, these findings suggest that cytokines affect depressive behavior likely through regulation of monoamines and the HPA axis.

The symptom heterogeneity observed in depressed patients suggests that biological abnormalities are likely to be patient-dependent and disease-specific. Collectively, these results indicate that biochemical mechanisms likely interact to mediate a complex behavior like mood and anhedonia. It is therefore unlikely that a single biological marker will characterize a heterogeneous disorder like depression. Significant benefits can be rendered in evaluating the behavioral effects of a panel of biological markers or biochemical signatures, particularly since reciprocal communication between nervous, endocrine, and immune systems have been noted (Cserr and Knopf 1992; Felten 1991; Reichlin 1993). For most cases, when associations between

biochemical alterations and depression are detected, the causal relationship is often poorly understood.

6. Brain and Molecular Correlates

Direct and indirect evidence from neurostructural, neurofunctional, and molecular studies indicate impairments in neural circuitry, structural plasticity, and cellular resilience. These abnormalities reflect the molecular neurobiological underpinnings of depression as discussed below.

6.1 Neurostructural and Neurofunctional Studies

The cortical-limbic circuitry is implicated to mediate emotional processing in depressed patients (Davidson et al. 2002; Dougherty and Rauch 1997; Mayberg 1997). Results from positron emission tomography (PET) studies indicate that unmedicated patients with MDD exhibit increased activity and cerebral blood flow (CBF) to the amygdala, orbital cortex, and medial thalamus, as well as decreased CBF to the pre-frontal cortex (PFC) and anterior cingulate cortex (ACC) (Drevets 2000a; Drevets et al. 1999). Meta-analyses of structural neuroimaging studies indicate that MDD is characterized by reduction of gray matter volumes in the ACC (Koolschijn et al. 2009), subgenual cingulate cortex (Hajek et al. 2008), and hippocampus (McKinnon et al. 2009). Post-mortem neuropathological studies have shown that patients with MDD exhibit reduced cortex volume, decreased number of glial cells, and/or reduced neuron sizes (Ongur et al. 1998; Rajkowska 2000; Rajkowska et al. 1999). Given the functional roles of specific brain regions in emotional processing, neuropathological abnormalities observed in depression suggest that areas that mediate autonomic and neuroendocrine responses (amygdala)

are associated with increased activity and cerebral blood flow, while reduction in activity is observed in brain regions that control emotional processing (cortex) (Manji et al. 2001). Antidepressant treatment reduces CBF and metabolism in the amygadala (Drevets 2000b; Drevets et al. 1999), attenuating hyperresponsiveness to stress (Rosenkranz et al. 2010). Similarly, larger hippocampal volume (Frodl et al. 2008; Kronmuller et al. 2008; MacQueen et al. 2008) and gray matter density in the ACC (Costafreda et al. 2009) were positively correlated with antidepressant response.

Inferences regarding the structural integrity of neural tracts can be made through diffusion tensor imaging (DTI), which measures the diffusion properties of water through brain tissues, *in vivo*. Patients that did not respond to 12 weeks of escitalopram (Alexopoulos et al. 2008) or citalopram (Alexopoulos et al. 2002) treatment showed microstructural abnormalities in white matter pathways connecting the cortex with the limbic and paralimbic areas, which indicates that poor therapeutic outcome is related to impaired cortical-limbic connectivity (Mayberg 2003). Patients with prior exposure to parental verbal abuse (Choi et al. 2009) or have genetic polymorphisms (*5-HTTLPR*) (Alexopoulos et al. 2009) exhibit microstructural white matter abnormalities, suggesting that neural brain structure is subject to genetic and environmental control. Of note, impairment in brain morphology, neural circuitry, and brain function have been linked with monoaminergic and non-monoaminergic genetic variants (Scharinger et al. 2010). In addition to evaluating the structural integrity of neural brain circuits, functional activity within the limbic-cortical circuitry has been investigated. Brain activity can be evaluated by measuring blood oxygen level-dependent (BOLD) signals while patients are resting (intrinsic activity) or when performing a task (task-related activity). BOLD signaling is associated with changes in blood flow and tissue oxygen concentration, which are markers of

brain activity. Depressed individuals have reduced activity in the limbic and cortical regions (Anand et al. 2005), which normalizes as symptoms resolve (Anand et al. 2005). Patients with MDD show hyperactivity in the amygdala (Surguladze et al. 2005) and reduced co-activation of the dorsal ACC (Matthews et al. 2008) when viewing negative facial expressions. These changes in brain activity are ameliorated with chronic antidepressant treatment (Chen et al. 2008; Fu et al. 2004; Sheline et al. 2001).

Similar to the electrocardiogram (ECG), unfiltered electrical activity generated by the brain can be measured by an electroencephalogram (EEG). EEG signals can be converted to show a topographical representation of the distribution of the EEG waveforms across the cortex known as the quantitative electroencephalograph (QEEG) brain map. The QEEG image is used to assess brain activity and metabolism in real-time, providing a global assessment of brain activity. Brain electrical activity can be measured using cordance, low-resolution brain electromagnetic tomography (LORETA), and antidepressant treatment (ATR) index. Cordance, which uses QEEG measurements conducted from a full scalp electrode array, assesses perfusion of cerebral cortex and brain activity on cortical convexities like PFC (Cook et al. 1998; Leuchter et al. 1999). Several groups have demonstrated the usefulness of cordance in characterizing antidepressant response (Bares et al. 2008; Cook et al. 2002). Responders and non-responders differ in QEEG measurements at rest and during task-oriented activities (Bruder et al. 2008). LORETA, which assesses activity of deeper cortical regions like ACC and orbitofrontal cortex (Pizzagalli et al. 2001), identifies cortical alterations in relation to depression and antidepressant response (Anderer et al. 2002; Saletu et al. 2010). Both cordance and LORETA require whole-head electrode montages for data collection, which entails up to 75 minutes of QEEG recording, limiting its clinical utility. On the other hand, the ATR only uses a five-electrode montage on the

frontal brain regions, which limits QEEG recording to 10 minutes (Leuchter et al. 2009a; Leuchter et al. 2009b). The largest study that evaluated the use of ATR in predicting antidepressant response is the Biomarkers for Rapid Identification of Treatment Effectiveness trial in Major Depression (BRITE-MD) trial. In this study, positive ATR predicted response and remission to escitalopram. Patients with negative ATR values were either switched to bupropion or continued to be treated with escitalopram. In comparison to patients who stayed on escitalopram, patients who switched to bupropion were 1.9 times more likely to respond to treatment (Leuchter et al. 2009a; Leuchter et al. 2009b). These findings support the use of ATR as a biomarker for monitoring treatment response and clinical progression.

6.2 Cellular and Molecular Markers

Lower hippocampal volume (Videbech and Ravnkilde 2004), which is commonly found in post-mortem brain tissues of depressed individuals (MacQueen et al. 2003), results in reduced hippocampal plasticity. Reduction in neurogenesis, brain volume, and thickness is likely due to decreased neurotrophins and/or changes in neuroplasticity (Geuze et al. 2005). Neurotrophins, including brain-derived neurotrophic factor (BDNF), have been repeatedly implicated in the pathogenesis and treatment of MDD (Duman and Monteggia 2006). Administration of BDNF induces cell proliferation and neurogenesis (Pencea et al. 2001; Zigova et al. 1998) and leads to decreased depressive-like behavior (Shirayama et al. 2002; Siuciak et al. 1997). Neurogenesis, resulting from either antidepressant treatment or cell implantation, attenuates depressive behavior (Tfilin et al. 2009). Depressed patients show reduced BDNF levels (Sen et al. 2008), which can result in lower number of dendrites in the synapse (Manji et al. 2003; Nestler et al. 2002). Antidepressants stimulate BDNF synthesis (Duman 2004) and normalizes reduced BDNF

levels in depressed patients (Brunoni et al. 2008; Sen et al. 2008). A functional variant located at codon 66 in the BDNF promoter region, resulting in a valanine to methionine change (Val66Met), is reported to correspond with drug response. Carriers of the Met allele were reported to have better treatment outcomes (Gratacos et al. 2008; Kato and Serretti 2010), however, others did not find any correlation between the Val66Met variant and treatment response (Kato and Serretti 2010; Tsai et al. 2003; Wilkie et al. 2007). Furthermore, genetic susceptibility to depression was not associated with the *BDNF* Val166Met variant (Gratacos et al. 2007; Lopez-Leon et al. 2008). The met allele is associated with impaired intra-cellular packaging and activity dependent secretion of BDNF, which disrupts hippocampal function (Egan et al. 2003). Impaired suppression of the HPA axis following dexamethasone treatment was also observed in the *BDNF* Met carriers (Schule et al. 2006). Of note, mouse lines that did not express *Bdnf* during fetal development or post-natal development were hyperactive, hyperaggressive, and showed higher depressive-like behavior compared to transgenic mice that were conditioned to express *Bdnf* during post-natal development (Chan et al. 2006), which suggests that the behavioral effects of BDNF are region and time-dependent. Interestingly, an interaction between the *BDNF* G196A variant, the serotonin transporter gene, and stressful life events have been observed (Aguilera et al. 2009; Pezawas et al. 2008).

BDNF is activated by cyclic-AMP response element-binding protein (CREB). The cAMP-CREB cascade has been extensively studied for its involvement in cell survival and neural plasticity (D'Sa and Duman 2002; Duman et al. 1997). The cAMP-CREB pathway is upregulated following chronic antidepressant treatment (Duman et al. 1999). Activation of the CREB pathway is thought to result in neurogenesis. Activated or phosphorylated CREB is found in actively dividing neural progenitor cells in the hippocampal subgranular zone (SGZ)

(Nakagawa et al. 2002a). Mice lacking *Creb* show markedly reduced cell proliferation (Nakagawa et al. 2002b) and administration of a phosphodiesterase inhibitor, which activates the cAMP cascade, increases neurogenesis and improves depressive behavior (Takahashi et al. 1999). Although CREB plays a critical role in neurogenesis, CREB is not necessary to elicit antidepressant effects. After antidepressant treatment, no difference in depressive-like responses was observed between *Creb* deficient mice and wild-type controls, indicating that the behavioral effects of antidepressant drugs may occur through other CREB-independent mechanisms.

Given that depressed patients exhibit reduced numbers of neuronal and glial cells, molecular mechanisms that stimulate neurogenesis (activation of CREB and BDNF synthesis) are likely to be critical in MDD. Presently, the clinical significance of neuro/gliogenesis in depression is largely unknown. It is likely that cellular proliferative and survival processes interact to facilitate remodeling of synaptic connections that can lead to altered mood. It is noteworthy to consider, however, that in the absence of stress, the neural circuitry underlying depression may be different (Krishnan and Nestler 2008). There is a possibility that reversal of stress-induced neural plasticity changes is not required for antidepressive effects (Nestler et al. 2002).

7. Depression Signatures

7.1 Gene Expression Signatures

Gene expression profiling studies provide an unbiased look at the relationship between gene expression and depressive disorder, which is useful in identifying novel targets for antidepressant therapy (Sequeira and Turecki 2006). Bernard and colleagues collected gene

expression data from the locus coeruleus of healthy, depressed, and bipolar patients. In this study, they found significant alterations in patients with MDD but not bipolar subjects. Gene expression alterations were detected in the glutamate signaling genes (*SLC1A2, SLC1A3* and *GLUL*), growth factor genes (*FGFR3* and *TrkB*), and several astroglial genes (Bernard et al. 2010). Similarly, dysregulation of fibroblast growth factor genes (*FGF1, FGF2, FGFR2,* and, *FGF3)* were detected in cortical regions of depressed patients, irrespective of previous antidepressant treatment (Evans et al. 2004). Consistent with previous findings, expression of genes involved in signal transmission of glutamate and GABA were found to be dysregulated in depressed patients (Choudary et al. 2005) and in suicide victims with and without depression (Sequeira et al. 2009). Alteration in genes regulating oligodendrocyte function (Sequeira and Turecki 2006) and cell-cell communication (Sequeira et al. 2009) were altered in MDD, suggesting impairment in brain circuitry. Notably, reduced oligodendrocyte expression and neuronal changes in amygdala were detected in both depressed individuals and in rodents exposed to unpredictable chronic mild stress (Sibille et al. 2009), indicating a connection between stress response and neural circuitry.

For biomarkers to be clinically useful, putative analytes must be detected in easily accessible samples like plasma or serum. Using LPS-stimulated blood samples, Spijker et al. compared gene expression profiles between healthy and unmedicated patients with MDD. A significant difference in gene expression pattern was observed in a subset of genes, all of which have not been previously associated with depression (Spijker et al. 2010). Transcriptome changes in the leukocyte mRNA is correlated with response to antidepressant agents or lithium therapy (Iga et al. 2008). The authors found that normalization in gene expression pattern correlates with antidepressant response (Iga et al. 2008). In addition to analyzing global changes in the brain or

plasma transcriptome, genetic regulatory elements of depression or antidepressant response can be identified using quantitative trait loci (QTL) mapping analysis. In this approach, DNA variants that regulate gene expression locally or distally (*cis* or *trans*-regulatory elements) are analyzed for correlation with depressive behavior, thereby facilitating analysis for regulatory genes underlying depressive behavior. This approach has been used to detect regulatory genetic elements for several behaviors (Bryant et al. 2009; Radcliffe et al. 2006).

7.2 Protein Signatures

Other efforts to identify depression signatures include protein expression profiling. Plasma samples from control, depressed, and schizophrenic patients were analyzed for 79 plasma biochemical analytes, including cytokines, neurotrophins, and chemokines (Domenici et al. 2010). Interestingly, insulin and matrix metallopeptidase 9 (MMP-9) displayed the biggest difference between control and depressed patients (Domenici et al. 2010). Efforts to expand the panel of protein markers to include peripheral and neuropsychological markers are currently underway (Tadic et al. 2011). The global analysis of protein expression is still in its infancy although several groups have performed proteomic analysis in the cerebrospinal fluid (CSF) (Raedler and Wiedemann 2006) and in discrete brain regions collected post-mortem (Beasley et al. 2006). In order to characterize the cause-effect relationship between biological alterations, treatment, and behavior, protein profiling studies in human samples should be complemented with proteomic studies in animals, which are more amenable for determination of disease and treatment effects.

8. Other Mechanisms

8.1 Epigenetics

Discordance of depression between monozygotic twins suggests other non-genetic factors are involved (Mill and Petronis 2007). Alteration in gene expression can occur without changes in the DNA sequence through epigenetic mechanisms like histone modification and methylation of DNA CpG islands. Deacytelation of histones results in DNA coiling, which prevents binding of transcription factors to the DNA, suppressing gene transcription. Alternatively, methylation alters DNA chemistry, which blocks gene transcription. Epigenetic mechanisms can explain how genetically weak signals of risk combined with environmental factors predispose patients to depression (Caspi and Moffitt 2006).

Adverse childhood experiences confer risk to depressive behavior (Heim and Nemeroff 2001) likely through epigenetic alteration. In animal studies, offspring who received minimal maternal care had higher DNA methylation at the glucocorticoid receptor (GR) promoter region and were more responsive to stress compared to control animals (Liu et al. 1997; Weaver et al. 2004). Methylation in the GR promoter region leads to reduced binding of the nerve growth factor induced protein-A (NGF-1A), affecting GR regulation (Weaver et al. 2004; Weaver et al. 2007). Notably, low levels of maternal care led to epigenetic repression of the estrogen-alpha receptor that resulted in transmission of maternal behavior to offspring (Champagne et al. 2006; Champagne et al. 2003), thus indicating transgenerational phenotypic transfer through epigenetic alterations.

Mice that are deficient in *Hdac5* display enhanced vulnerability to stress, suggesting that stress reduces histone deacytelase activity leading to down-regulation of gene expression (Renthal et al. 2007). The adverse effect of stress on *Hdac5* activity is reversed by chronic antidepressant treatment (Renthal et al. 2007). Antidepressant treatment increases histone acetylation at the *Bdnf* promoter region, activating *Bdnf* expression (Tsankova et al. 2006). BDNF mediates formation and differentiation of new neurons, facilitating long-term potentiation and memory development.

RNA-mediated modifications through non-coding RNAs (ncRNA) and microRNAs (miRNA) can activate or silence gene transcription. The role of miRNA in regulating serotonergic transmission has been reviewed (Millan 2011). MicroRNAs are short RNAs (22-24 nucleotides) that bind to complementary sequences on target mRNAs, typically leading to gene silencing (Bartel 2009; Carthew and Sontheimer 2009; Winter et al. 2009). A recent study by Baudry et al. shows that miR-16 negatively regulates the expression of serotonin transporter (SERT). Fluoxetine treatment stimulates the release of S100 beta protein (S100β) in the raphe, leading to elevation of miR-16 and reduction in SERT (Baudry et al. 2010). MiR-16 also represses the expression of anti-apoptotic protein (B-cell lymphoma 2) Bcl-2 (Cimmino et al. 2005), indicating a critical role of miR-16 in neurotransmission as well as cell proliferation. In addition, genetic studies using seahare (*Aplysia*) identified miR-124 as a translational repressor of CREB, which suggests that microRNAs indirectly regulate secondary messenger pathways by modulating CREB expression (Rajasethupathy et al. 2009). Overexpression of ncRNA was found in Alzheimer's patients (Faghihi et al. 2010), however, an association between ncRNA and depression is yet to be established.

Consistent with the notion that genes are interconnected within a network, it is conceivable that an epigenetic regulatory network exists. Efforts to identify epigenomic signatures are underway (Akbarian and Huang 2009) and this data should be integrated with other data sets like the brain transcriptome and behavior to identify causative pathways in depression. Of great interest is the assessment of epigenetic transgenerational transmission of a trait and genomic imprinting (epigenetic alteration on gene expression is based on whether the gene is inherited from the father or the mother). These epigenetic phenomena facilitate our understanding of how environment and genetics interact to mediate behavior, ultimately providing a comprehensive picture of the molecular mechanisms underlying depression.

8.2 Sleep and Circadian Rhythm

It was previously thought that insomnia is a risk factor for depression (Breslau et al. 1996; Ford and Kamerow 1989; Hohagen et al. 1993) and years of research did not clarify the exact relationship between insomnia and depression (Riemann 2007; Riemann et al. 2001). In an EEG, normal sleep can be partitioned into several stages. The first is progression from light sleep (N1 stage), followed by an "intermediate" level of sleep (stage N2) that leads to the "deep" sleep, which is characterized by slow delta waves on the EEG (stage N3). Stages N1-N3 are part of non-rapid eye movement sleep, which alternates with rapid eye movement (REM) sleep throughout the night (Benca and Peterson 2008). Depression is characterized by abnormal sleep (difficulty falling asleep, nocturnal awakenings, early-morning awakenings), decreased slow-wave sleep, shortened rapid eye movement (REM) latency, and increased REM density (Thase et al. 1997; Tsuno et al. 2005). Interestingly, total sleep deprivation improves symptoms in 40-60% of depressed patients (Giedke and Schwarzler 2002; Wirz-Justice and Van den Hoofdakker

1999), which is thought to be due to activation of the limbic dopaminergic pathways (Ebert et al. 1994; Ebert et al. 1996). Additionally, the slow-wave sleep is marginally affected by antidepressant therapy (Sharpley and Cowen 1995; Tsuno et al. 2005), indicating partial involvement of monoamines in sleep regulation.

In addition to disruption in sleep pattern, depressed patients also exhibit alteration in biological rhythms, including appetite and hormone levels. Patients with seasonal affective disorders (SAD) have depressive symptoms during the winter months when daylight is shorter. The bright light therapy has been effectively used to treat SAD (Lam 2006) and non-seasonal depression (Terman and Terman 2005) and is thought to work by shifting the circadian clock (Wirz-Justice et al. 2005). Similar to 5-HT, melatonin is derived from tryptophan and is a critical regulator of circadian rhythm. Depressed patients display altered melatonin release and abnormal melatonin levels (Rubin et al. 1992; Wetterberg 1999), particularly in the acute phase of depressive illness (Srinivasan et al. 2006). Antidepressant therapy increases melatonin (Srinivasan et al. 2006; Thompson et al. 1985). Of note, a pilot study that looked at the use of melatonin in addition to cortisol as prognostic marker for depression found promising results (Buckley and Schatzberg 2010).

Genetic regulators of the molecular clock (*Clock, Bmal1, Npas2, GSK3β,* and *Timeless*) have been linked with mood disorders (McClung 2007). Mutant mouse models exhibiting point mutations on the *Clock* gene display anxiety-like and depressive-like behavior (Roybal et al. 2007) and increased dopamine transmission in the ventral tegmental area (VTA) (McClung et al. 2005; Nestler and Carlezon 2006), which suggests that the *Clock* gene regulates dopamine signaling. Interestingly, there is circadian rhythm with regards to concentration, release, and

synthesis of 5-HT, norepinephrine, and dopamine (Barassin et al. 2002; Shieh et al. 1997; Weiner et al. 1992), as well as in the expression and activity of monoamine receptors (Kafka et al. 1983; WESEMANN and Weiner 1990; Witte and Lemmer 1991), indicating a link between monoamine signaling and circadian rhythm.

9. Our Approach: Identification of genetic and neurobiochemical markers for depression and fluoxetine response using a panel of genetically diverse mouse inbred strains

Based on these findings, it is unlikely that a single biomarker can describe a multifactorial disorder like depression. Data from the last decades indicate that alterations in MDD are inter-connected (**Figure 1.1**). This figure illustrates that there are neuroanatomical, neurobiochemical, neuroimmune, neuroendocrine, genetic, and metabolic mechanisms underlying MDD. Given the involvement of various biological systems, it is no surprise that depression is characterized by heterogeneous molecular and clinical manifestations, which complicate the search for biomarkers for depressive-like behavior. Therefore, a systems biology approach that investigates connections on genetic, neurobiochemical, and behavioral levels is critical to the identification of prognostic and treatment response biomarkers for depression.

Our laboratory has collected behavioral data for over 30 mouse inbred strains for the tail suspension test, in both naïve mice and mice chronically-treated with the antidepressant fluoxetine. We have also analyzed whole-genome gene expression in the same inbred strains in multiple brain regions believed to play a role in the regulation of mood. In this application, we propose to quantify 40 biochemical biomarkers in the same three brain regions among all 30 inbred strains in both naïve mice and mice that have been chronically-treated with the

antidepressant fluoxetine. The biochemical markers, which were chosen based on literature searches and in consultation with experts in the field of psychiatry and psychiatric genetics, assess multiple mechanisms that have been implicated in human depression, including neuronal modulation, neurogenesis, gliogenesis, and hypothalamic-pituitary mediated immunomodulation. By comparing biochemical and behavioral profiles in both naïve and drug-treated mice, we will identify biomarkers that can predict predisposition to depressive-like behavior and treatment response. Furthermore, comparison of these data with inter-strain gene expression differences will provide information regarding the role of gene regulation on depression. Genetic and biochemical markers that are significantly correlated with differences in behavior in the treatment naïve group can predict predisposition to depressive-like behavior in mice that may influence response to treatment, while genetic and biochemical markers that are significantly different between response groups can provide a biological explanation for differences in treatment response.

10. Figures

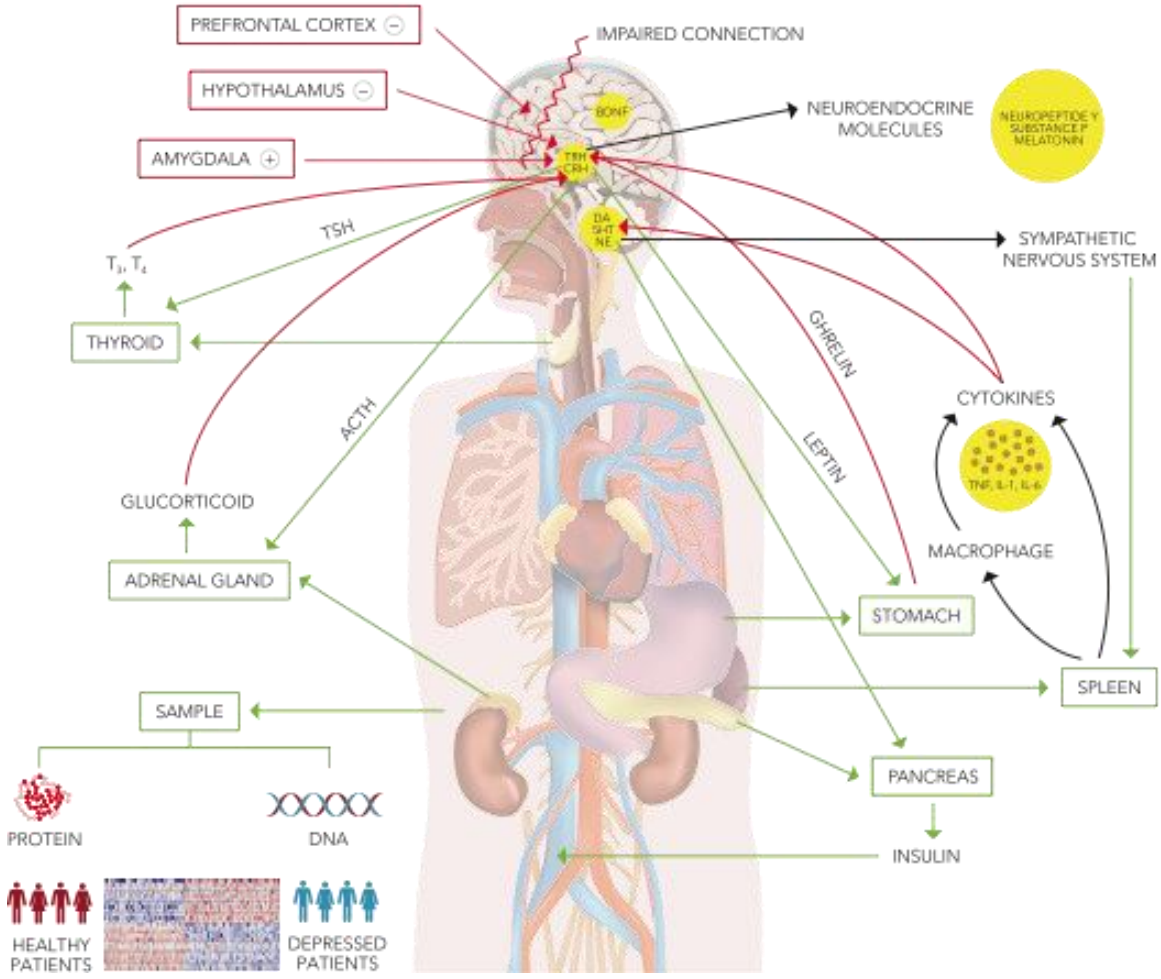

Figure 1.1 Biological alterations in depression

Figure 1.1 Biological alterations in depression. Impairment in the HPA axis, neural circuitry, neuroendocrine, neuroimmune, neuronal signaling, neurogenesis, and metabolic functions have been observed in depressed patients, resulting in symptom heterogeneity. As shown, bidirectional communication among several pathways exists (i.e., crosstalk between sympathetic nervous system and inflammatory markers). Cellular (genetic) and molecular (proteomic) alterations in depression can be identified by performing global gene and protein expression analyses between healthy controls and depressed individuals (bottom left), leading to identification of depression molecular signatures.

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OTHER RESOURCES

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CHAPTER 2

IDENTIFICATION OF QUANTITATIVE TRAIT LOCI FOR DEPRESSION AND ANXIETY IN MULTIPLE MOUSE INBRED STRAINS

1. Introduction

Anxiety and depression have a significant socioeconomic impact. In the United States, the lifetime prevalence rates for anxiety and mood disorder are 31% and 21.4%, respectively (Kessler et al. 2007). A strong comorbidity exists between anxiety and major depressive disorder. Patients with comorbid anxiety and depression are more likely to experience slower recovery, greater psychosocial disability, more chronicity, and increased rates of recurrence and medication use (Hirschfeld 2001). There are minimal studies that have examined treatment efficacy in anxious depressive patients (Hirschfeld 2001). As a result, evidence-based rationale for treatment selection, dose requirement, and duration of therapy in this patient population is lacking.

Evidence from twin studies indicates a strong genetic correlation between anxiety and major depression with minimal to modest shared environmental effects on both disorders (Kendler 1996; Kendler et al. 2007; Roy et al. 1995). It appears that numerous small-effect genes affect both illnesses and that genetic influence on anxiety and depression is independent of age (Demirkan et al. 2011). To elucidate common genetic mechanisms shared between both disorders, we performed genome-wide mapping analysis across multiple mouse inbred strains. The use of mouse inbred strains to investigate genetic variants for behavior offers several advantages, including lower cost, availability of relevant population, ease in sample accessibility, and reduced genomic complexity.

In this study, we have measured baseline anxiety-like and depressive-like responses and performed genome-wide association analysis to identify quantitative trait loci (QTL) or genomic regions associated with anxiety-like and depressive-like behaviors. By conducting a QTL

mapping analysis for both anxiety-like and depressive-like responses, we aim to: 1) identify genetic risk variants for both disorders and 2) propose a molecular pathway that is potentially shared between major depression and anxiety.

2. Materials and Methods

2.1 Animals

Thirty-five mouse inbred strains (129S1/SvImJ, A/J, AKR/J, BALB/cByJ, BTBRT<t>tf/J, BUB/BnJ, C3H/HeJ, C57BL/6J, C57BR/cdJ, C58/J, CBA/J, CE/J, CZECHII/EiJ, DBA/2J, DDY/Jc1SidSeyFrkJ, FVB/NJ, I/LnJ, KK/HIJ, LG/J, LP/J, MA/MyJ, MRL/MpJ, NOD/LtJ, NON/LtJ, NOR/LtJ, NZB/BlNJ, NZO/HILtJ, PERA/EiJ, PL/J, PWD/PhJ, RIIIS/J, SJL/J, SM/J, SWR/J, and WSB/EiJ) aged 8-9 weeks old were obtained from The Jackson Laboratory (Bar Harbor, ME). Male mice were housed four per cage in polycarbonate cages on a 12-hour light/dark cycle (lights on at 0700h) with access to food and water *ad libitum.* The number of animals in each treatment group ranged from 7 to 18 animals per strain. Following one week of habituation, mice were subjected to a behavioral test battery in the order of the least to the most stressful test. Mice were tested every two days for a maximum of three behavioral tests to minimize carryover effects between tests. All procedures were approved by the Institutional Animal Care and Use Committee and followed the guidelines set forth by the National Institutes of Health (NIH) Guide for the Care and Use of Laboratory Animals. Mean behavioral scores are reported in **Table 2.1**.

2.2 Open Field Test (OF)

The OF test was conducted using a 27.3 cm x 27.3 cm open field apparatus (MED-OFA-MS, Med Associates, St. Albans, VT), which was surrounded by infrared detection beams on the x-, y- and z-axes that automatically recorded the animals' position and activity over the course of the experiment. The OF is commonly used to measure exploratory and locomotor behavior in mice. This test exploits the rodent's innate aversion to well-lit open spaces. Based on previous observations that showed increased exploration towards illuminated open areas following administration of anxiolytics (Choleris et al. 2001; Crawley 1985), anxiety-like behavior was measured as percent time spent in the center of the open field. Activity in the open field was recorded for ten minutes.

2.3 Tail Suspension Test (TST)

Mice were tested in a tail-suspension apparatus (PHM-300 TST Cubicle, Med Associates, St. Albans, VT) between 1300h and 1600h. In this test, the mouse is subjected to short-term inescapable stress by being suspended by its tail. Following failed attempts to escape, the mouse becomes immobile, a response generally considered as behavioral despair, a depressive-like behavior that is proposed to model "hopelessness" (Steru et al. 1987; Steru et al. 1985). Immobility was recorded for 6 min in 60-sec blocks. Mice that climbed up their tail during testing were excluded from data analysis.

2.4 Stress-Induced Hyperthermia (SIH)

Mammals, including humans, show an elevation in core body temperature after exposure to stress (stress-induced hyperthermia). Previous studies have shown that mice removed last

from their cage develop hyperthermia compared to mice removed first (Borsini 1989), indicating that SIH response is an index for anticipatory anxiety in mice. Using a modified SIH protocol, rectal temperatures were obtained sequentially, with a 1-min interval between the first and the last mouse in the cage. After 10 min, a second rectal measurement was performed for all mice using the same procedure. For each mouse, the SIH was calculated as the difference in temperature (ΔT) between the first rectal measurement (basal) and the last rectal temperature (after 10 min).

2.5 QTL Mapping

Since mice within the same strain are genetically identical, genetic mechanisms underlying complex diseases like anxiety and depression can be identified by mapping genomic regions associated with inter-strain phenotypic variation. Behavioral measures collected from multiple inbred strains were analyzed on SNPster version 3.3.1 and EMMA *R package* 1.1.2 to infer genomic regions significantly correlated with inter-strain differences in anxiety-like and depressive-like behavior.

Haplotype Associated Mapping (HAM) analysis and Efficient Mixed-Model Association (EMMA) have been well-described elsewhere (Kang et al. 2008; McClurg et al. 2007; Pletcher et al. 2004) and thus will be only briefly summarized here. Experimental SNP genotypes encompassing over seven million polymorphic loci across 49 commonly-used laboratory strains were obtained from the Center for Genome Dynamics at http://cgd.jax.org (Szatkiewicz et al. 2008). Over 190,000 SNPs were informative for the 37 mouse inbred lines, which provided the basis for inferring genotype or haplotype structure at every 3-SNP window. Associations between genotype (1-SNP or 3-SNP window) and phenotype were calculated by an F-statistic

corrected for genome-wide significance and plotted using SpotFire software (TIBCO Palo Alto, CA). Genomic loci that were associated with behavioral phenotypes at an FDR ≤0.05 were considered significant. We also examined if QTLs have been previously linked with behavioral responses related to anxiety and depression. Candidate genes within anxiety-like and depressive-like QTLs are listed in **Tables 2.2 and 2.3.**

2.6 Criteria for depressive-like gene marker characterization

Because our focus is to identify genetic markers for depression, we extensively examined all QTLs significant for depressive-like phenotype as measured by the TST. For candidate gene identification to be effective and efficient, we focused our attention on the top three loci associated with depressive behavior and eliminated any QTLs that were similar to background peaks. This decision was based on previous mapping simulations which showed that the locus most significantly associated with the phenotype is 95% likely to be found within the top peak given that the phenotype has an effect size of 30% (unpublished data).

Genes were prioritized based on the following criteria: a) similar haplotype structure between SNPs within candidate genes and depressive QTLs, b) correlation of candidate gene expression with behavior, c) association between candidate gene expression with levels of neurobiochemical molecules, d) role of genes in behavior based on previous findings, and e) association of genes with major depressive disorder. In addition, we searched for mouse models and experimental assays that can be used for gene validation experiments.

3. Results

3.1 Behavioral QTLs

Baseline behavioral responses were measured in the open field test, stress-induced hyperthermia test, and tail suspension test. Analysis of anxiety-like and depressive-like responses revealed significant effects of strain (percent time spent in the center OF: $F = 37.25$, $p < 0.0001$; change in temperature following stress: $F = 4.86$, $p < 0.0001$; percent time immobile in the TST: $F = 16.37$, $p < 0.0001$). Genetic analysis can be performed using inbred-strain phenotype data to identify genetic loci associated with behavioral phenotypes (Miller et al. 2010; Williams et al. 2009). Loci on Chrs 2 (Chr 2: 161.1 - 162.8; -logP = 5.52, FDR≤ 0.059), 3 (Chr 3: 82.0 - 86.9; -logP = 5.69, FDR≤ 0.059), 6 (Chr 6: 102.7 - 106.7, -logP = 5.52, FDR≤ 0.059), and 17 (Chr 17: 18.7 - 23.7; -logP = 5.70, FDR≤ 0.069) correlated with percent time spent in the center of the OF. The anxiety-like QTL on Chr 17 (**Figure 2.1**) has been previously linked with circadian photosensitivity (Chr 17: 17.9 - 43.3, LOD = 3.7) (Yoshimura et al. 2002), which suggests that similar genetic mechanisms underlie anxiety behavior and circadian rhythm. *Fpr1*, a gene within anxiety-like QTL on Chr 17, has been previously associated with anxiety behavior in rodents (Gao et al. 2011).

We discovered three loci with genome-wide significance for SIH (Chr 1: 64.8 - 65.2, -logP = 5.68, FDR≤ 0.039; Chr 4: 134.8 - 135. 5 -logP = 5.69, FDR≤ 0.039; Chr 5: 108.2 - 110.9, -logP = 6.49, FDR≤ 0.013; Chr 10: 121.3 - 123.6, -logP = 5.88, FDR≤ 0.039). Results from F2 mapping analyses between two phenotypically divergent strains (CBA/J and A/J) reveal two loci on Chr 5 (Chr 5: 107.6 - 123.9, LOD = 16.4 and Chr 5: 130.7 - 142.5, LOD = 30.9) that have been previously linked with preference for entering the open arms, a behavior that indicates less

anxious responses in the elevated plus maze (EPM). The EPM QTLs on Chr 5 account for 30% of the variance in arm preference (Cohen et al. 2001). Additionally, a QTL on Chr 5 has been detected for nurturing ability (Chr 5: 127.7 - 133.5, LOD = 4.5) (Suto et al. 2002). Two out of the thirty-seven genes within the SIH QTL on Chr 5 (*Pde6b* and *Pxmp2*) were previously linked with anxiety or depression (Aston et al. 2005; Cohen et al. 2001; Cook et al. 2001) (**Figure 2.2**).

Independent QTL mapping analysis for depressive-like phenotype identified significant loci on Chrs 2, 4, 9, and 18. Genomic regions on Chrs 2 (Chr 2: 179.5 - 179.6, -logP = 8.52, FDR≤ 0.0006 and Chr 2: 60.1 - 63.3, -logP = 4.92, FDR≤ 0.10), 4 (Chr 4: 129.8 - 132.5, -logP = 4.27, FDR≤ 0.10), 9 (Chr 9: 46.7- 47.6 Mb, -logP = 6.17, FDR≤ 0.059), and 18 (Chr 18: 16.6 - 18.1, -logP = 5.39, FDR≤ 0.074) associated with baseline depressive-like behavior. Although a QTL for depressive-like behavior has not been reported previously at any of the loci we found, a region on Chr 4 has been linked with anxiety-like responses as measured by the EPM (Chr 4: 78.3 - 125.3, LOD = 3.2) (Nakamura et al. 2003) and thermal pain responses in male mice (Chr 4: 115.1 - 139.5, r = 0.34 - 0.42) (Mogil et al. 1997). Interestingly, association with anxiety and/or depression has been reported for two genes (*Hcrtr1* and *Oprd1*) within the despair locus on Chr 4 (Filliol et al. 2000; Scott et al. 2011; van Rijn et al. 2010) (**Figure 2.3**).

3.2 Identifying a candidate genetic marker for depression

Although all candidate genes were found within behavioral loci, only thirty-six out of seventy candidate genes showed similar haplotype structure with the putative depressive QTLs. This information is critical in delineating which genes are likely to drive association between genomic and behavioral differences observed in mice. Convergence of gene expression with behavioral and biochemical data narrowed our list from 36 to 20 priority genes. Using

Spearman's rank correlation coefficient, 20 genes were significantly correlated with behavior and levels of neurobiochemical markers ($p<0.05$). From the list of 20 candidate genes, *Dpp4* is the only gene that has been previously associated with major depression in humans (see Chapter 3 for more details regarding *Dpp4*). A reduction in DPP4 enzymatic activity is common in patients with mood disorder and treatment resistant depression (Maes et al. 1996; Maes et al. 1991; Maes et al. 1997). Animal studies have also found an association between DPP4 and behavioral despair. Mice deficient in CD26/DPP4 exhibit lower immobility scores on both forced swim and tail-suspension tests, which suggests that absence of *Dpp4* produced anti-depressive effects (El Yacoubi et al. 2006). Contradictory results between studies are likely due to biological alterations that occur specifically from activation of DPP4 enzyme, such as change in structural conformations that would otherwise be absent and thus undetected in *Dpp4* knockout studies. Alternative explanations include secondary mechanisms that may arise from genetic ablation of *Dpp4.* Despite ambiguous findings, evidence from human and animal studies suggests that *Dpp4* plays a significant role in depression. This information, in addition to the availability of powerful research tools, led us to investigate the link between DPP4 and depression. To further clarify the connection between *Dpp4* and depressive behavior, we compared DPP4 protein levels as well as enzymatic activity in behaviorally dissimilar strains (Details of the study are found in Chapter 3).

4. Discussion

Given the significant pathophysiologic overlap between anxiety and depression, it is thought that there are common neurogenetic mechanisms underlying these disorders. Findings from genetic twin studies conducted in patients with comorbid anxiety and depression (Kendler

1996; Kendler et al. 2007; Roy et al. 1995) indicate a strong genetic correlation between both disease states. To identify genomic regions shared between these illnesses, we conducted QTL mapping analysis for anxiety-like and depressive-like responses in 35 mouse inbred strains.

Two of the anxiety-like QTLs we identified, Chr 5: 108.2 - 110.9 and Chr 17: 18.7 - 23.7, have been previously associated with anxiety-related responses and circadian photosensitivity, respectively (Cohen et al. 2001; Yoshimura et al. 2002). Three genes within these regions have been previously associated with anxiety or depression. The *Fpr1* gene encodes for *N*-formylpeptide receptor, a G-protein coupled receptor that stimulates the release of pro-inflammatory cytokines by phagocytic cells and negatively mediates the action of glucocorticoids. Mice lacking *Fpr1* exhibit reduced anxiety-like responses, increased exploratory behavior, and impaired fear memory (Gao et al. 2011). Transcription profiling analysis of cortex samples from depressed individuals and healthy control subjects revealed significant differences in the expression of 17 genes involved in oligodendrocyte function; included in that list is *Pxmp2*. The *Pxmp2* gene encodes for a peroxisomal membrane channel, which is critical for myelination. Compared to control subjects, patients with depression show reduced mRNA levels for genes associated with cell communication and neurodevelopment (Aston et al. 2005). This finding indicates that depression may be affected by impairment in synaptic function and cell communication. It is thought that light affects alertness, mood, and anxiety behavior. In mammals, retinal photoreceptors, including rods, cones, and intrinsically photosensitive retinal ganglion cells (ipRGCs), mediate both image-forming and non-image-forming responses to light. The *Pde6b* gene encodes for an enzyme that mediates phototransduction in rods and cones known as the rod phosphodiesterase subunit beta protein. Mutation in *Pde6b* results in visual impairment and age-related degeneration of rods and cones. The SIH locus on Chr 5 where

Pde6b resides has been correlated with anxiety-like behavior (Cohen et al. 2001; Cook et al. 2001), which suggests that similar genetic mechanisms may underlie responses to light and anxiety behavior. Notably, the anxiety-like locus on Chr 17 for time spent in the center OF has been previously linked with circadian photosensitivity. Collectively, our anxiety-like QTL findings indicate that genes affecting circadian rhythm may also influence anxiety responses. Further characterization of candidate anxiety-like genes can help to elucidate molecular pathways linking light responses with anxiety, thus facilitating new avenues for anxiolytic drug research.

We identified five regions that were significantly linked with depressive-like behavior. Of particular interest is the region on Chr 4. This locus has been previously reported to be associated with anxiety behavior and responses to thermal pain (Mogil et al. 1997; Nakamura et al. 2003). Two genes within the despair locus on Chr 4, *Hcrtr1* and *Oprd1*, were previously correlated with depressive and/or anxiety behavior (Filliol et al. 2000; Scott et al. 2011; van Rijn et al. 2010). Orexins are excitatory neuropeptide neurons primarily located in the hypothalamus. Orexin neurons bind to orexin receptors 1 and 2, which are encoded by *Hcrtr1* and *Hcrtr2,* respectively. Reduction in *Hcrtr1* through either pharmacologic intervention or genetic ablation results in decreased depressive-like behavior (Scott et al. 2011). The *Oprd1* gene encodes for the delta1 opioid receptor, which when activated elicits analgesic effects. The *Oprd1* null mice display increased behavioral despair and anxious-like behavior, indicating that delta1 opioid receptor activity is critical for mediating both anxiety and mood disorders (Filliol et al. 2000). Similarly, activation of the OPRD1 receptor reduces depressive-like behavior (Torregrossa et al. 2006) and anxiety-like behavior in ethanol-withdrawn mice (Kraft et al. 2009). Additional studies are needed to identify the molecular pathway underlying opioid effects on anxiety and

depressive behavior. Whether administration of a delta opioid receptor agonist attenuates anxiety and depressive symptoms remain to be seen.

All genes within putative QTLs associated with variable depressive-like behavior were analyzed for its potential role in major depression. Comprehensive analysis of gene expression, neurobiochemical, and behavioral data that were obtained from two independent studies narrowed the list of putative depressive-like genes to 20 promising candidates. Only *Dpp4* has been previously linked with clinical depression and other disorders, including anxiety (Emanuele et al. 2006), depression (Maes et al. 1996; Maes et al. 1991), anorexia (van West et al. 2000), and bulimia (van West et al. 2000). Additionally, availability of research tools, which included commercially-available drugs that target the protein product, ELISA kits, and qPCR primers and probes, made *Dpp4* a good candidate gene to validate. The details of the candidate gene validation study are described in Chapter 3.

Taken together, results from our QTL mapping analyses generated several good candidate genes for anxiety and depression. Importantly, these results indicate that similar genetic mechanisms underlie anxiety and major depression. This study also demonstrates the use of QTL mapping analysis for detection of genetic loci that influence complex phenotypes. Although causative genes for anxiety and MDD are yet to be identified, the road to gene identification is promising. New approaches, such as next-generation sequencing and epigenetic tools (DNA methylation, chromatin, and non-coding RNA analysis) can advance gene mapping analyses, facilitating the identification of genes underlying anxiety and major depression.

5. Tables

Table 2.1 Inter-strain behavioral responses in the open field test, stress-induced hyperthermia, and tail suspension test

Table 2.1. Inter-strain Behavioral Responses in the Open Field Test, Stress-Induced Hyperthermia, and Tail Suspension Test

Strain	Number of Animals (n)	Mean Percent Time Spent in the Center OF	SEM	Mean Change in Temperature (ºC)	SEM	Mean Percent Time Spent Immobile	SEM
129S1/SvImJ	14	2.98	1	1.68	0.18	41.85	2.52
A/J	14	0.91	0.26	1.24	0.11	28.95	2.92
AKR/J	14	10.51	1.22	2.14	0.2	36.41	3.61
BALB/cByJ	14	5.99	1.12	1.79	0.15	33.92	3.08
BTBRT<t>tf/J	16	14.68	1.33	1.49	0.11	35.73	2.85
BUB/BnJ	7	11.91	1.16	2.76	0.16	34.64	5.76
C3H/HeJ	15	12.59	1.18	2.26	0.14	22.58	2.17
C57BL/6J	16	19.72	1.35	1.83	0.14	36.03	7.1
C57BR/cdJ	14	23.88	1.77	2.04	0.1	20.06	2.23
C58/J	16	15.4	1.48	1.63	0.17	22.54	1.58
CBA/J	15	9.95	0.93	2.34	0.14	40.89	2.97
CE/J	8	9.54	1.23	1.84	0.11	27.1	3.32
CZECHII/Ei	9	2.02	0.35	0.99	0.42	59.25	7.17
DBA/2J	14	6.86	1.08	2.56	0.19	21.75	1.74
DDY/Jc1SidSeyFrkJ	8	14.54	2.15	1.8	0.26	73.93	2.25
FVB/NJ	14	14.92	1.12	2.14	0.17	34.94	2.8
I/LnJ	8	10.39	1.33	1.39	0.23	39.11	7.78
KK/HlJ	18	5.43	0.84	1.25	0.12	46.66	4.4
LG/J	12	7.44	1.24	1.93	0.15	25.63	2.53
LP/J	10	4.09	1.25	1.57	0.22	34.45	4.81
MA/MyJ	9	13.24	1.08	1.8	0.26	39.25	5.83
MRL/MpJ	14	9.34	1.38	1.84	0.28	26.98	2.12
NOD/LtJ	17	25.38	2.1	1.88	0.18	41.19	2.54
NON/LtJ	16	13.75	0.95	2.2	0.15	18.87	2.19
NOR/LtJ	10	22.14	1.26	1.78	0.11	35.59	4.1
NZB/BlNJ	14	9.88	0.97	2.14	0.18	48.64	4.15
NZO/HILtJ	11	4.82	0.95	1.61	0.17	33.86	4.49
PERA/Ei	8	15.35	2.21	1.81	0.23	57.99	11.65
PL/J	18	11.72	0.76	2.58	0.18	37.59	2.64
PWD/Ph	10	7.01	1.07	0.79	0.18	Not tested	
RIIIS/J	15	7.54	0.66	2.31	0.21	30.03	1.6
SJL/J	16	10.81	1	2.23	0.25	59.35	2.8
SM/J	14	8.67	0.71	2.1	0.13	41.73	4.79

SWR/J	17	12.71	0.7	2.29	0.17	25.58	1.67
WSB/EiJ	10	14.32	0.74	1.1	0.45	29.61	3.44

Table 2.2 List of candidate genes within anxiety-like QTLs

Table 2.2. List of candidate genes within anxiety-like QTLs	
Gene symbol	**Gene Name**
1010001B22Rik	RIKEN cDNA 1010001B22 gene
1700007G11Rik	RIKEN cDNA 1700007G11 gene
1700013N18Rik	ubiquitin-conjugating enzyme E2D 2 pseudogene
1700029M20Rik	RIKEN cDNA 1700029M20 gene
2610011I18Rik	RIKEN cDNA 2610011I18 gene
2810408I11Rik	RIKEN cDNA 2810408I11 gene
3110052M02Rik	RIKEN cDNA 3110052M02 gene
4930432H08Rik	RIKEN cDNA 4930432H08 gene
4930432O21Rik	RIKEN cDNA 4930428O21 gene
4930515G13Rik	RIKEN cDNA 4930515G13 gene
4930555I21Rik	RIKEN cDNA 4930555I21 gene
4930564K09Rik	RIKEN cDNA 4930564K09 gene
4930565D16Rik	RIKEN cDNA 4930565D16 gene
4933425M03Rik	RIKEN cDNA 4933425M03 gene
5830490A12Rik	RIKEN cDNA 5830490A12 gene
6330415G19Rik	RIKEN cDNA 6330415G19 gene
6820402A03Rik	RIKEN cDNA 6820402A03 gene
9030218A15Rik	RIKEN cDNA 9030218A15 gene
9130219A07Rik	RIKEN cDNA 9130219A07 gene
9330198I05Rik	RIKEN cDNA 9330198I05 gene
9430021M05Rik	RIKEN cDNA 9430021M05 gene
9430099H24Rik	9430099H24Rik Description: RIKEN cDNA 9430099H24 gene
A330049M08Rik	RIKEN cDNA A330049M08 gene
A430028G04Rik	RIKEN cDNA A430028G04 gene
A830010M20Rik	RIKEN cDNA A830010M20 gene
Acadl	acyl-CoA dehydrogenase, long chain
Akr1cl	aldo-keto reductase family 1, member C-like
Ankle2	ankyrin repeat and LEM domain containing 2
Arfip1	ADP-ribosylation factor interacting protein 1
Atp5k	ATP synthase, H+ transporting, mitochondrial F1F0 complex, subunit e
AU019157	expressed sequence AU019157
AU021001	expressed sequence AU021001
Avpr1a	arginine vasopressin receptor 1A
Barhl2	BarH-like 2
Brdt	bromodomain, testis-specific
C1qtnf6	C1q and tumor necrosis factor related protein 6
C630001G18Rik	RIKEN cDNA C630001G18 gene
C77068	expressed sequence C77068
Card10	caspase recruitment domain family, member 10
Ccdc18	coiled-coil domain containing 18
Ccnyl1	cyclin Y-like 1
Cd1d2	CD1d2 antigen
Cdc7	cell division cycle 7 homolog
Chfr	checkpoint with forkhead and ring finger domains

Chl1	cell adhesion molecule with homology to L1CAM
Clic4	chloride intracellular channel 4 (mitochondrial)
Cnr2	cannabinoid receptor 2 (macrophage)
Cntn4	contactin 4
Cntn6	contactin 6
Creb1	cAMP responsive element binding protein 1
Cryga	crystallin, gamma A
Crygb	crystallin, gamma B
Crygd	crystallin, gamma D
Crygf	crystallin, gamma F
Cyth4	cytohesin 4
D4Ertd264e	DNA segment, Chr 4, ERATO Doi 264, expressed
D530037H12Rik	RIKEN cDNA D530037H12 gene
D630023F18Rik	RIKEN cDNA D630023F18 gene
D630033A02Rik	RIKEN cDNA D630033A02 gene
D930015E06Rik	RIKEN cDNA D930015E06 gene
Dclk2	doublecortin-like kinase 2
Dr1	down-regulator of transcription 1
Elfn2	leucine rich repeat and fibronectin type III, extracellular 2
Ephx4	epoxide hydrolase 4
Evi5	ecotropic viral integration site 5
Fam160a1	family with sequence similarity 160, member A1
Fam19a2	family with sequence similarity 19, member A2
Fam69a	family with sequence similarity 69, member A
Fbrsl1	fibrosin-like 1
Fbxw7	F-box and WD-40 domain protein 7
Fga	fibrinogen alpha chain
Fgb	fibrinogen beta chain
Fgg	fibrinogen gamma chain
Fhdc1	FH2 domain containing 1
Fpr1	formyl peptide receptor 1
Fpr2	formyl peptide receptor 2
Fpr3	formyl peptide receptor 3
Fpr-rs3	formyl peptide receptor, related sequence 3
Fpr-rs4	formyl peptide receptor, related sequence 4
Fuca1	fucosidase, alpha-L- 1, tissue
Fzd5	frizzled homolog 5
Gfi1	growth factor independent 1
Glmn	glomulin, FKBP associated protein
Glt28d2	glycosyltransferase 28 domain containing 2
Golga3	golgi autoantigen, golgin subfamily a, 3
Grhl3	grainyhead-like 3
Gtpbp6	GTP binding protein 6 (putative)
Has1	hyaluronan synthase1
Hmgcl	3-hydroxy-3-methylglutaryl-Coenzyme A lyase
Idh1	isocitrate dehydrogenase 1 (NADP+), soluble
Il28ra	interleukin 28 receptor alpha
Il2rb	interleukin 2 receptor, beta chain
Il5ra	interleukin 5 receptor, alpha
Kirrel	kin of IRRE like

Klf7	Kruppel-like factor 7 (ubiquitous)
Lpcat2b	lysophosphatidylcholine acyltransferase 2B
Lrat	lecithin-retinol acyltransferase (phosphatidylcholine-retinol-O-acyltransferase)
Lrba	LPS-responsive beige-like anchor
Mab21l2	mab-21-like 2
Mettl21a	methyltransferase like 21A
Mllt4	myeloid/lymphoid or mixed lineage-leukemia translocation to 4 homolog pseudogene
Mnd1	meiotic nuclear divisions 1 homolog
Mon2	MON2 homolog
Mtap2	microtubule-associated protein 2
Mtap9	microtubule-associated protein 9
Mtf2	metal response element binding transcription factor 2
Myom3	myomesin family, member 3
Ncaph2	non-SMC condensin II complex, subunit H2
Ncrna00085	non-protein coding RNA 85
Nipal3	NIPA-like domain containing 3
Npy2r	neuropeptide Y receptor Y2
P2rx2	purinergic receptor P2X, ligand-gated ion channel, 2
Pde6b	phosphodiesterase 6B, cGMP, rod receptor, beta polypeptide
Pet112l	PET112-like
Pgam5	phosphoglycerate mutase family member 5
Pigg	phosphatidylinositol glycan anchor biosynthesis, class G
Pikfyve	phosphoinositide kinase, FYVE finger containing
Pknox2	Pbx/knotted 1 homeobox 2
Plcxd1	phosphatidylinositol-specific phospholipase C, X domain containing 1
Plekhm3	pleckstrin homology domain containing, family M, member 3
Plrg1	pleiotropic regulator 1, PRL1 homolog
Pnrc2	proline-rich nuclear receptor coactivator 2
Pole	polymerase (DNA directed), epsilon
Ppm1h	protein phosphatase 1H (PP2C domain containing)
Ppp2r1a	protein phosphatase 2 (formerly 2A), regulatory subunit A (PR 65), alpha isoform
Pth2r	parathyroid hormone 2 receptor
Ptpla	protein tyrosine phosphatase-like (proline instead of catalytic arginine), member a
Ptprt	protein tyrosine phosphatase, receptor type, T
Pxmp2	peroxisomal membrane protein 2
Rac2	RAS-related C3 botulinum substrate 2
Rad23b	RAD23b homolog
Rcan3	regulator of calcineurin 3
Rpap2	RNA polymerase II associated protein 2
Rpe	ribulose-5-phosphate-3-epimerase
Rpl5	ribosomal protein L5 pseudogene
Rps3a	ribosomal protein S3A
Sfrp2	secreted frizzled-related protein 2
Sfrs6	serine/arginine-rich splicing factor 6
Sh3d19	SH3 domain protein D19
Srrm1	serine/arginine repetitive matrix 1
Srsf10	serine/arginine-rich splicing factor 10
Sstr3	somatostatin receptor 3
Tgfbr3	transforming growth factor, beta receptor III

Tlr2	toll-like receptor 2
Tmed5	transmembrane emp24 protein transport domain containing 5
Tmem154	transmembrane protein 154
Tmem5	transmembrane protein 5
Trim2	tripartite motif-containing 2
Unc80	unc-80 homolog
Usp15	ubiquitin specific peptidase 15
Vmn2r-ps129	vomeronasal 2, receptor, pseudogene 129
Zfp160	zinc finger protein 160
Zfp213	zinc finger protein 213
Zfp229	zinc finger protein 229
Zfp40	zinc finger protein 40
Zfp51	zinc finger protein 51
Zfp52	zinc finger protein 52
Zfp53	zinc finger protein 53
Zfp54	zinc finger protein 54
Zfp605	zinc finger protein 605
Zfp644	zinc finger protein 644
Zfp677	zinc finger protein 677
Zfp758	zinc finger protein 758
Zfp820	zinc finger protein 820
Zfp944	zinc finger protein 944
Zfp945	zinc finger protein 945

Table 2.3 List of candidate genes within depressive QTLs

Table 2.3. List of candidate genes within depressive QTLs	
Gene symbol	**Gene Name**
2300010F08Rik	RIKEN cDNA 2300010F08 gene
2900052N01Rik	RIKEN cDNA 2900052N01 gene
3100003M19Rik	RIKEN cDNA 3100003M19 gene
4733401A01Rik	RIKEN cDNA 4733401A01 gene
4930451E06Rik	RIKEN cDNA 4930451E06 gene
5830409B07Rik	RIKEN cDNA 5830409B07 gene
9430011C21Rik	RIKEN cDNA 9430011C21 gene
A930004J17Rik	RIKEN cDNA A930004J17 gene
Atpif1	ATPase inhibitory factor 1
C530007A02Rik	RIKEN cDNA C530007A02 gene
Cdh2	neural cadherin
Cdh4	retinal cadherin
Col16a1	collagen, type XVI, alpha 1
Dnajc8	DnaJ (Hsp40) homolog, subfamily C, member 8
Dpp4	dipeptidyl-peptidase 4
Epb4.1	erythrocyte protein band 4.1
Epha7	Ephrin receptor A7
Eya3	eyes absent homolog 3
Fabp3	fatty acid binding protein 3, muscle and heart (mammary-derived growth inhibitor)
Fam76a	family with sequence similarity 76, member A
Fap	fibroblast activation protein, alpha
Gca	grancalcin, EF-hand calcium binding protein
Gcg	Glucagon
Gmeb1	glucocorticoid modulatory element binding protein 1
Hcrtr1	hypocretin (orexin) receptor 1
Ifih1	interferon induced with helicase C domain 1
Itgb6	integrin, alpha 11
Kcnh7	potassium voltage-gated channel, subfamiliy H (eag-related), member 7
Laptm5	lysosomal protein transmembrane 5
Ly75	lymphocyte antigen 75
Matn1	matrilin 1, cartilage matrix protein
Mecr	mitochondrial trans-2-enoyl-CoA reductase
Med18	mediator complex subunit 18
Nkain1	Na+/K+ transporting ATPase interacting 1
Oprd1	opioid receptor, delta 1
Pef1	penta-EF-hand domain containing 1
Phactr4	phosphatase and actin regulator 4
Pla2r1	phospholipase A2 receptor 1, 180kDa

Ppp1r8	protein phosphatase 1, regulatory (inhibitor) subunit 8
Psmd14	proteasome (prosome, macropain) 26S subunit, non-ATPase, 14
Ptafr	platelet-activating factor receptor
Ptpru	protein tyrosine phosphatase, receptor type, U
Pum1	pumilio homolog 1
Rab42	RAB42, member RAS oncogene family
Rbms1	RNA binding motif, single stranded interacting protein 1
Rcc1	regulator of chromosome condensation 1
Rpa2	replication protein A2, 32kDa
Sdc3	syndecan 3
Serinc2	serine incorporator 2
Sesn2	sestrin 2
Sfrs4	serine/arginine-rich splicing factor 4
Slc4a10	solute carrier family 4 sodium bicarbonate transporter, member 10
Smpdl3b	sphingomyelin phosphodiesterase, acid-like 3B
Snhg12	small nucleolar RNA host gene 12
Snhg3	small nucleolar RNA host gene (non-protein coding) 3
Snrnp40	small nuclear ribonucleoprotein 40kDa (U5)
Stx12	syntaxin 12
Taf12	TAF12 RNA polymerase II, TATA box binding protein (TBP)-associated factor, 20kDa
Tank	TRAF family member-associated NF-kappa-B activator
Tbr1	T-box, brain, 1
Tinagl1	tubulointerstitial nephritis antigen-like 1
Trnau1ap	tRNA selenocysteine 1 associated protein 1
Xkr8	XK, Kell blood group complex subunit-related family, member 8
Ythdf2	YTH domain family, member 2
Zcchc17	zinc finger, CCHC domain containing 17

6. Figures

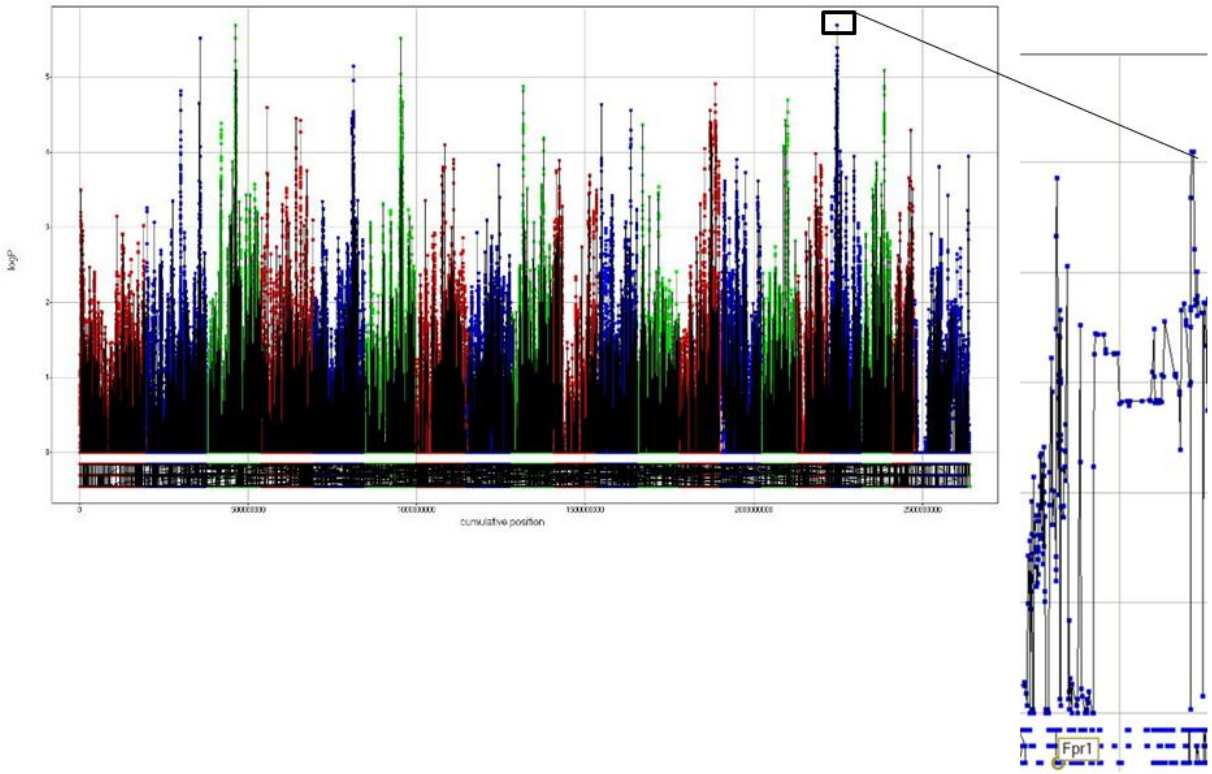

Figure 2.1 Genome-wide association plot for anxiety-like behavior as measured by percent time spent in the center of the open field

Figure 2.1. Genome-wide association plot for anxiety-like behavior as measured by percent time spent in the center of the open field. A genomic region on Chr 17 significantly correlates with amount of time spent in the center of the open field (-logP = 6.17). The figure shows QTL for anxiety-like behavior. To the right, is the putative gene formyl peptide receptor 1 (*Fpr1*) underneath the Chr 17 locus. The y-axis denotes the strength of association between genotype and phenotype (-logP), and the x-axis illustrates the cumulative SNP position in the genome

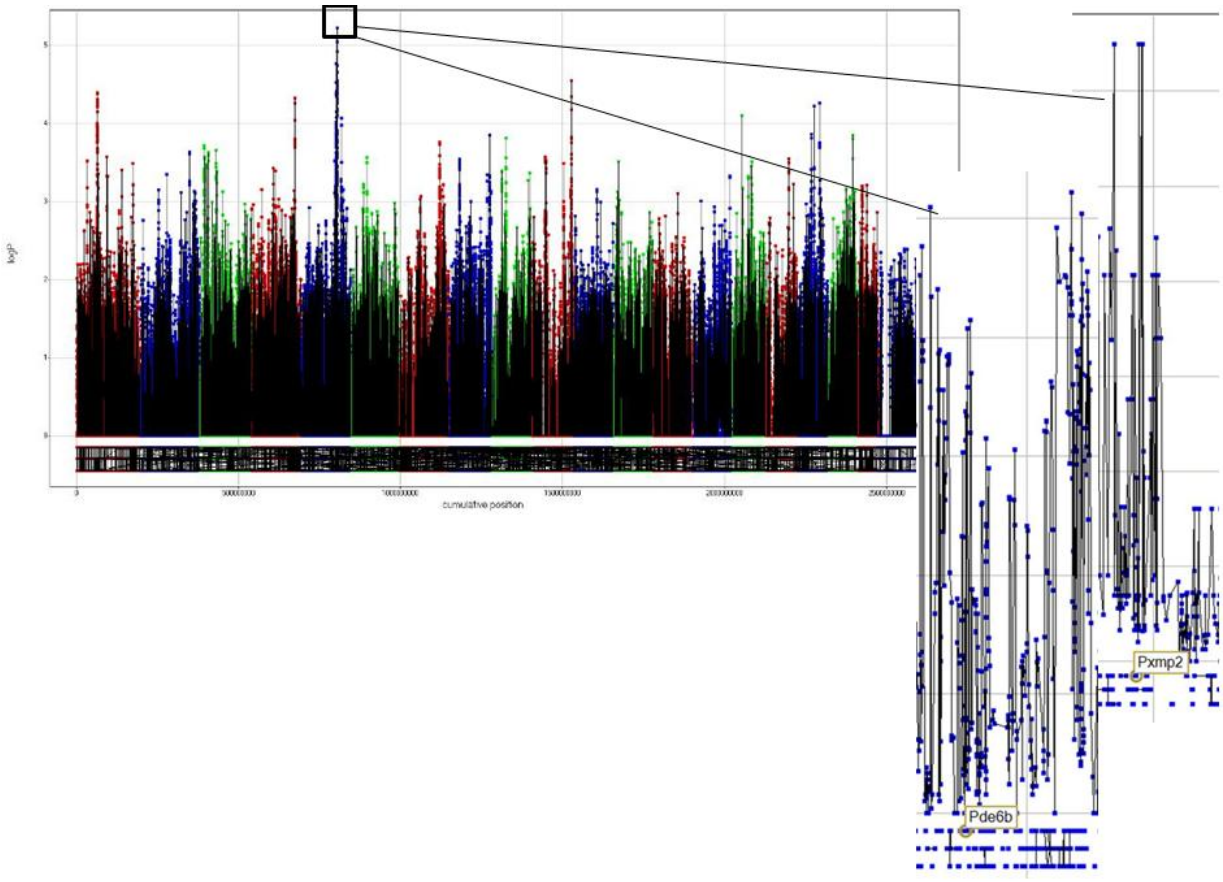

Figure 2.2 Genome-wide association plot for stress-induced hyperthermia

Figure 2.2. Genome-wide association plot for stress-induced hyperthermia. A locus on Chr. 5 significantly associates with changes in temperature following exposure to acute stress (-logP = 6.17). This assay is a measure of anticipatory anxiety. To the right are the putative genes, *Pde6b* (phosphodiesterase 6B) and *Pxmp2* (peroxisomal membrane protein 2) underneath the Chr 5 locus for anxiety-like behavior. The y-axis denotes the strength of association between genotype and phenotype (-logP), and the x-axis illustrates the cumulative SNP position in the genome.

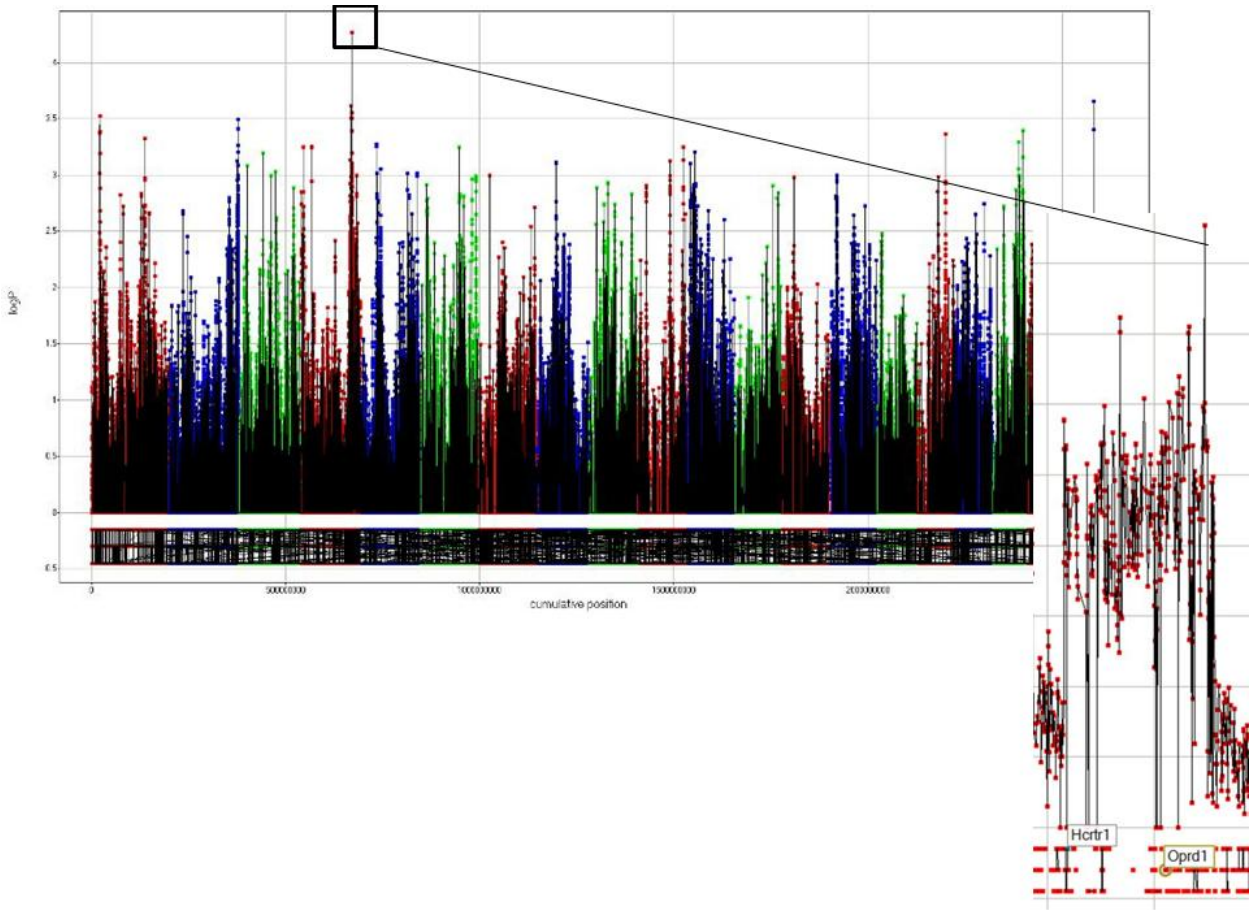

Figure 2.3 Genome-wide association plot for depressive-like behavior

Figure 2.3. Genome-wide association plot for depressive-like behavior. We observed a significant association between a locus on Chr 4 and time spent immobile in the tail suspension test (-logP = 6.17). To the right are the putative genes, hypocretin (orexin) receptor 1 (*Hcrtr1*) and opioid receptor delta 1 (*Oprd1*) underneath the Chr 4 locus for depressive-like behavior. The y-axis denotes the strength of association between genotype and phenotype (-logP), and the x-axis illustrates the cumulative SNP position in the genome.

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CHAPTER 3

CHARACTERIZING THE ROLE OF DIPEPTIDYL PEPTIDASE 4 IN DEPRESSION

1. Introduction

Results from family, twin, and adoption studies indicate that there is a link between genetics and development of major depression. Family and twin studies have estimated the heritability of depression at 35-50% (Kendler et al. 2001; Sullivan et al. 2000), suggesting the potential utility of genetic variants as diagnostic and prognostic markers. An unbiased analysis for genetic regions linked to depression can be performed by investigating genome-wide associations between genetic variants and behavior. Depression is a clinically and genetically heterogeneous disorder, which complicates the identification of causative genes in human populations. Besides ease in accessibility, mice within the same strain are homogeneous at each locus, facilitating gene mapping studies by reducing genetic complexity. Furthermore, the effects of environment and diet can be controlled in mouse populations, thus genetic effects on behavior are more readily discernible compared to human populations.

Using QTL mapping analysis, we identified an association between dipeptidyl peptidase 4 (*Dpp4*) and depressive-like responses (-logP >5.0) (**Figure 3.1**). DPP4 is an ectoenzyme that cleaves polypeptides with proline and alanine, to a lesser extent, at the penultimate position. DPP4 is highly conserved across species and displays considerable sequence homology between the mouse and human DPP4 protein. The crystal structure of this protein contains a catalytic domain found in the internal cavity and a protein binding region located on the external surface (Abbott et al. 1999; Bjelke et al. 2004; Rasmussen et al. 2003). The catalytic domain is involved primarily in cleaving dipeptides with proline residues at the penultimate position, which typically renders molecules less active (**Table 3.1**).

Enzymatic cleavage of peptides that regulate the immune and endocrine processes indicate that DPP4 may mediate the neuroendocrine-immune interface. Although DPP4 is not the only enzyme that can cleave neuropeptide Y, it is the primary peptidase that breaks down plasma NPY levels making DPP4 a major contributory factor in mediating movement of NPY across the blood-brain barrier. Neuropeptide Y (NPY) modulates circadian rhythm (Otori et al. 1993; Weber and Rea 1997), food intake (Levine and Morley 1984; Morley 1987; Morley et al. 1987; Stanley et al. 1985; Stanley and Leibowitz 1985), and learning and memory (Flood et al. 1989; Morley and Flood 1990). N-terminal truncation of NPY by dipeptidyl peptidase IV leads to structural alterations that may influence function and receptor binding (Frerker et al. 2007). Reduction in NPY is associated with depressive behavior and altered response to stress (Redrobe et al. 2002). Additionally, *Dpp4*$^{-/-}$ mice exhibit higher levels of substance P, which corresponds to a decreased in latency in response on the tail pinch and hot plate tests (Guieu et al. 2006). Treatment with DPP4 inhibitor reduced nociceptive response but only on wild type mice suggesting that elevation of substance P is due to lower enzymatic cleavage by DPP4 (Guieu et al. 2006). Substance P (SP) is a potent neuromodulator of pain that can alter behavioral response to stress and serotonergic signaling (Jessop et al. 2000; Malendowicz et al. 1996a; Malendowicz et al. 1996b, Schwarz et al. 1999). Both endocrine mediators, NPY and SP, have been shown to be involved in major depression (see Chapter 1 for details).

DPP4 is a glycoprotein molecule that associates with other immune cell surface markers, including CD3 and CD45 (Ishii et al. 2001; Tanaka et al. 1992). DPP4 functions as a co-stimulatory mediator of T-cell activation, which initiates downstream processes such as interleukin-2 production (Ishii et al. 2001), T-cell proliferation (Ishii et al. 2001), and T-cell

migration (Ikushima et al. 2002). Although its involvement in T-cell activation and proliferation is mainly attributed on its protein binding domain, cleavage of several cytokines by DPP4 may have an indirect effect on immune regulation (**Table 3.1**). Its enzymatic activity may influence its co-stimulatory effects on T-cell activation (Tanaka et al. 1993).

The neuro-endocrine-immune integrated system is critical for many psychiatric disorders including depression, anxiety, and pain as reviewed in (Kopp 2001; Leonard and Song 1996; Miller 1998). The involvement of dipeptidyl peptidase IV in regulating both neuroendocrine and immune processes is suggestive of a DPP4-mediated bidirectional modulation along the neuroendocrine-immune circuitry. Interestingly, DPP4 has been implicated in various mental disorders, including anxiety (Emanuele et al. 2006), depression (Maes et al. 1996; Maes et al. 1991), anorexia (van West et al. 2000), and bulimia (van West et al. 2000). DPP4 enzymatic activity has been associated with depressive symptoms in both clinical and pre-clinical studies (El Yacoubi et al. 2006; Krupina et al. 2009; Maes et al. 2001; Maes et al. 1996; Maes et al. 1991). We hypothesize that *Dpp4* is important for depression due to its role in inflammation and neuropeptide catabolism (**Table 3.1**). The goal of this study is two-fold: first, is to validate our QTL result that showed an association between *Dpp4* haplotype and depressive-like responses and second, we will examine whether behavioral responses to chronic DPP4 inhibition differ between haplotype groups, which will assess functional DPP4 activity between strains.

2. Materials and Methods

2.1 Animals

Twenty-four male mice of the C57BL/6J (B6) and DBA/2J (D2) strains aged 8-9 weeks old were obtained from The Jackson Laboratory (Bar Harbor, ME). Mice were housed four per cage in polycarbonate cages on a 12-hour light/dark cycle (lights on at 0700h) with access to food and water *ad libitum.* Following one week of habituation, mice were randomized to either control or treatment group. All procedures were approved by the Institutional Animal Care and Use Committee and followed the guidelines set forth by the National Institutes of Health (NIH) Guide for the Care and Use of Laboratory Animals.

2.2 Drug Treatment

Sitagliptin is a selective reversible inhibitor of DPP4 that, in addition to diet and exercise, is the currently approved treatment for type 2 diabetes. Sitagliptin was purchased from BioVision (Mountain View, CA) and was administered via oral gavage. Mice were randomized to receive either water or 100 mg/kg of sitagliptin for 14 days (n = 12 mice per strain per treatment group). This dosing regimen was selected based on previous enzymatic and toxicity studies. Treatment with 10 mg/kg of sitagliptin resulted in 90% inhibition after 1 hr and more than 70% inhibition after 8 hr (Davis et al. 2010). Administration of 125 mg/kg of sitagliptin in mice (dose is 12 times higher than the recommended daily dose of 100 mg in humans) did not result in hepatotoxicity and carcinogenicity (Sitagliptin [package insert], 2010). Mice were treated daily with water or sitagliptin throughout the end of the study and were sacrificed by cervical dislocation1 hr after the last dose, which was two days after the last behavioral test.

2.3 Open Field Test (OF)

To minimize potential carryover effects between tests, behavioral tests were conducted every other day, in order from the least to the most stressful test. All behavioral tests were performed between 1300 h and 1600 h. After chronic administration of 0 or 100 mg/kg of sitagliptin for 14 days, mice were tested in the OF. This assay was conducted using a 27.3 cm x 27.3 cm OF apparatus (MED-OFA-MS, Med Associates, St. Albans, VT), which has infrared detection beams that automatically recorded the animals' position and activity on the x-, y- and z-axes over the course of the experiment. The OF is commonly used to measure exploratory and locomotor behavior in rodents. This test exploits the rodent's innate aversion to well-lit, open spaces. Based on previous observations that showed increased exploration in illuminated, open areas following administration of anxiolytics (Choleris et al. 2001; Crawley 1985), we measured anxiety-like behavior as percent time (sec) spent in the center of the OF. Activity in the OF was recorded for 10 min.

2.4 Light-Dark Exploration Test (LD)

The LD test was performed following the OF test. The LD assay is commonly used to detect anxiogenic effects of compounds. Similar to the OF, the LD test is based on the inherent aversion of mice to well-lit open areas and on their spontaneous responses to novel stimuli like light. Half of the LD apparatus was open and brightly illuminated, while the remaining half of the arena was occupied by a small black plexiglass compartment that was dark. Entry between the two compartments was through a small open doorway. Mice were permitted to freely explore the light and dark areas for 10 min. The number of transitions between compartments and the time spent in the light compartment are measures of anti-anxiety-like response.

2.5 Tail Suspension Test (TST)

Following the LD test, mice were tested in a TST apparatus (PHM-300 TST Cubicle, Med Associates, St. Albans, VT). In this test, the mouse is subjected to the short-term, inescapable stress of being suspended by its tail. Following repeated escape attempts, the mouse becomes immobile. This response is generally considered behavioral despair, a depressive-like behavior that is proposed to model "hopelessness" (Steru et al. 1987; Steru et al. 1985). Immobility was recorded for 6 min in 60 sec blocks using the Actimetrics FreezeFrame software version 2.0 (Wilmetter, IL). Since all strains were uniformly active for the first 2 min, percent time spent immobile was calculated for the last 4 min of the test. Mice that climbed up their tail during testing were excluded from analysis.

2.6 Measurement of DPP4 levels

Brain and plasma CD26/DPP4 levels were quantified using Mouse DPP4 ELISA kit (Wuhan EIAab Sciences Co., Wuhan, China) according to the manufacturer's recommendation. In brief, standards ranging from 15.6 ng/mL to 1000 ng/mL were prepared by performing serial two-fold dilutions with sample diluent, which was provided in the kit. Brain and plasma samples were diluted 1:10 and 1:100, respectively. A total of 100 µL of standard, sample or blank were added onto wells that were pre-coated with a specific antibody to DPP4. Optical density of each well was determined using a microplate reader (BioTek Instruments, Winooski, VT) set to 450 nm. Unknown DPP4 sample concentrations were determined from the standard curve, which was generated by plotting the optical densities of the standards against known DPP4 concentrations.

2.7 Assessment of DPP4 activity

DPP4 activity was quantified by measuring the amount of fluorescence generated following enzymatic hydrolysis of the flourogenic substrate H-Gly-Pro-AMC. Recombinant human DPP4 (0.26 mU per well) was added to the appropriate wells, followed by the addition of samples and blanks. DPP4 inhibitor (P32/98; 10 µM per well) was added to the appropriate wells as a negative control. Following a 30-min incubation at 25ºC, 10 µL of H-Gly-Pro-AMC was added to each well. The plate was read at excitation and emission wavelengths of 380 nm and 460 nm, respectively. DPP4 activity was normalized for total protein concentration, as measured by Pierce Coomasie (Bradford) Protein Assay (Thermo Fisher Scientific, Rockford, IL).

2.8 Statistical Analysis

Given the uneven number of strains between haplotypes 1 and 2, behavioral difference between haplotype groups was calculated using a Mann-Whitney U-test. Mean behavioral and biological measurements for C57BL/6J and DBA/2J were calculated and analyzed using Student's t-tests. Statistical analysis was performed using SAS 9.2 (Cary, NC) and Graphpad Prism 5.0 (La Jolla, CA).

3. Results

3.1 Strain distribution pattern within *Dpp4* locus

Our study shows that inter-strain difference in baseline depressive-like responses associate with polymorphic variants within *Dpp4.* As seen in **figure 3.2**, the haplotype structure within *Dpp4* perfectly aligns with the strain distribution pattern across the behavioral locus on

Chromosome 2 (-logP >5.0). Difference in depressive-like behavior was found to be significant between haplotype groups 1 and 2 (U = 6.0, *p<0.007*) (**Figure 3.3**). It is important to note that the region where *Dpp4* resides has been previously associated with high density lipoprotein (HDL) levels, hepatic lipase activity, and insulin levels (Mehrabian et al. 1998), which indicates a potential role of *Dpp4* in diabetes and metabolic syndrome. Both disorders co-occur and exhibit pathophysiologic overlap with major depression (Chapter 1).

3.2 Higher baseline immobility score is linked with higher DPP4 protein levels

A significant difference in plasma (t = 2.96, $p<0.01$) and brain DPP4 (t = 2.44, $p<0.04$) levels was observed between B6 and D2 mice at baseline. Compared to B6, D2 mice have lower DPP4 levels in plasma and brain (**Figures 3.4 and 3.5**). Results from our microarray studies did not show significant difference in *Dpp4* mRNA expression between strains. The expression of one probe set for *Dpp4* (1441342_at) was higher in D2 than B6 mice, while the remaining three probe sets did not show a difference in *Dpp4* mRNA expression between both strains. To determine whether difference in DPP4 protein levels can be attributed to DNA variants that affect amino acid sequence, imputed and experimental SNP genotypes were obtained from the Center for Genome Dynamics (http://cgd.jax.org) for comparison of *Dpp4* sequence across mouse inbred strains (Szatkiewicz et al. 2008). Polymorphic SNPs across 30 strains were all synonymous and were found in the intron, exons, and untranslated regions (UTR). Interestingly, data from Ensembl indicate that six different *Dpp4* transcript variants have been reported for B6. One isoforms leads to an alternative protein product, which may explain the difference in DPP4 protein levels between B6 and D2. Additionally, two *Dpp4* mRNA transcripts with different 3'UTR sequences have been reported in C57BL/10 ScNJ mice, which are closely related to B6

(Bernard et al. 1994). Due to low expression of *Dpp4* in the brain, expression QTL analyses were not performed on *Dpp4,* which limits our ability to predict if *Dpp4* expression is regulated by local or distal QTLs.

No difference in enzymatic activity was observed between strains under control and sitagliptin conditions (**Figures 3.9 and 3.10**), which suggests that inter-strain behavioral differences are likely due to differences in DPP4 protein levels. B6 mice had higher baseline immobility scores ($t = 11.04$, $p<0.0001$) (**Figure 3.8**) and lower DPP4 protein levels than D2 mice (**Figures 3.4 and 3.5**), which suggests that reduced DPP4 protein levels correspond with higher depressive-like behavior. Interestingly, B6 mice displayed lower anxiety-like behavior compared to D2. B6 mice spent more time in the center of the open field ($t = 6.58$, $p<0.0001$) and more time in the light compartment of the LD apparatus ($t = 2.77$, $p<0.008$) (**Figures 3.6 and 3.7**).

3.3 Inter-strain sensitivity to DPP4 inhibition

Plasma DPP4 activity was significantly inhibited for both B6 ($t = 9.03$, $p<0.0008$) and D2 ($t = 15.77$, $p<0.0001$) mice. After chronic treatment with sitagliptin, there was an 88.5% and 84.9% reduction in DPP4 activity for B6 and D2 mice, respectively (**Figure 3.9**). However, treatment with sitagliptin did not alter DPP4 activity in the brain in either strain (**Figure 3.10**). Although no significant strain-specific differences were observed between vehicle and sitagliptin-treated mice in our behavioral tests, B6 mice show a trend of increased sensitivity to DPP4 inhibition (**Figures 3.11 - 3.13**).

4. Discussion

To facilitate the application of pharmacogenetics in drug development and therapeutic selection, research efforts towards gene validation are warranted. Identification of genes underlying depression is challenging to perform in human populations due to limitations of cost, disease heterogeneity, and sample size requirements. However, these limitations can be mitigated through the use of mouse models. The genetic diversity among inbred mouse strains, the availability of murine genetic sequence information, and the development of numerous genetic resources are additional benefits to using mouse models to study the genetic basis of depressive behavior.

This study confirms our initial findings that showed an association between *Dpp4* haplotype and inter-strain baseline depressive-like behavior (Chapter 2). We found that DPP4 protein levels correlate with basal behavioral despair and anxiety-like responses. Higher levels of DPP4 were associated with higher TST immobility scores and lower anxiety-like behavior, as measured by the OF and LD tests. This result is consistent with previous findings that have shown lower depressive-like responses in *Dpp4* null mice. Rodents deficient in *Dpp4* exhibited lower immobility scores for both animal models of behavioral despair (TST and forced swim test), indicating that the absence of *Dpp4* yielded anti-depressive effects (El Yacoubi et al. 2006). No differences in anxiety-like behavior were observed between wild-type and *Dpp4*$^{-/-}$ mice in the light/dark test (El Yacoubi et al. 2006).

We did not observe a significant difference in behavior between vehicle- and sitagliptin-treated B6 and D2 mice. Contradictory to clinical findings that show reduction in plasma DPP4 enzymatic activity is common in patients with mood disorder and treatment-resistant depression

(Maes et al. 1996; Maes et al. 1991; Maes et al. 1997), inhibition of plasma DPP4 activity by sitagliptin did not significantly alter behavior in B6 and D2 mice. This conflicting result may be due to inadequate duration of DPP4-inhibition. It has been shown that 12-week treatment with 20 mg/kg of sitagliptin in mice susceptible to the development of Alzheimer's disease resulted in reduced memory impairment and decreased brain inflammatory markers and amyloid-β protein (D'Amico et al. 2010), suggesting that long-term inhibition may be required to elicit behavioral changes. Additional studies are required to examine whether prolonged DPP4 inhibition alters depressive-like behavior. The results of this study are important, given that sitagliptin is used for long-term management of type 2 diabetes.

The direction of the association between *Dpp4* and depressive behavior remains unclear. Clinical studies indicate that lower DPP4 activity correlates with depressive symptoms, while results from our study and previous *Dpp4* knockout study suggest that increased behavioral despair is linked with higher levels of DPP4 protein and absence of *Dpp4*, respectively. However, because DPP4 protein levels were not quantified in human serum samples, it is unclear whether reduction in DPP4 activity is due to lower DPP4 protein concentration, low or/and altered DPP4 enzymatic activity, or both. Thus, pre-clinical and clinical assessment of DPP4 transcript levels, protein expression, and enzyme activity along with behavior is required to assess the direction of the correlation between *Dpp4* and depression. Furthermore, the relationship between major depression and DPP4 activity in the brain remains unknown. No previous studies have examined DPP4 activity in post-mortem brain tissues of depressed individuals. Additionally, we could not inhibit DPP4 activity in the brain because the ratio of brain to plasma concentration for sitagliptin is fairly low (< 0.06 - 0.1) (Chu et al. 2007). Conseqently, further studies are required to examine if reduction in brain DPP4 activity

influences depressive behavior. It is important to note that the TST is primarily used to evaluate antidepressant activity of novel compounds and does not itself elicit depressive behavior. Behavioral paradigms like chronic social defeat stress and reduced sucrose preference, which assess other aspects of depressive behavior (e.g. anhedonia) (Nestler and Hyman 2010), can be used to further evaluate the role of *Dpp4* in depression.

Given the likelihood of false positive results from genome-wide association studies, it is critical to conduct independent studies for gene validation. Our initial findings indicate that DPP4 mediates baseline depressive-like behavior, which suggests that *Dpp4* may be used as a genetic prognostic or diagnostic marker for depression.

5. Tables

Table 3.1 Potential biological alterations of known DPP4 substrates

Table 3.1. Potential biological alterations of known DPP4 substrates		
Substrate	**Significance**	**Reference**
Neuropeptides		
β-Casomorphin	Inactivation	(Heymann and Mentlein 1986; Nausch et al. 1990)
Endomorphins	Inactivation	(Shane et al. 1999)
Gastric inhibitory peptide	Inactivation	(Ahren and Hughes 2005; Mentlein et al. 1993b; Nausch et al. 1990)
Gastrin-releasing peptide	Inactivation	(Ahren and Hughes 2005; Nausch et al. 1990)
Glucagon-like-peptides (GLP1, GLP2)	Inactivation	(Baggio and Drucker 2007; Drucker et al. 1997)
Growth hormone releasing factor	Inactivation	(Martin et al. 1993)
Neuropeptide Y	Inactivation/ altered receptor specificity	(Frerker et al. 2007; Mentlein et al. 1993a)
Peptide YY	Inactivation/ altered receptor specificity	(Mentlein et al. 1993a; Unniappan et al. 2006)
Pituitary adenylate-cyclase-activating polypeptide	Inactivation	(Lambeir et al. 2001a)
Prolactin	Inactivation	(Nausch et al. 1990)
Tyr-melanostatin	Inactivation	(Shane et al. 1999)
Substance P	Inactivation	(Guieu et al. 2006)
Vasoactive intestinal peptide	Inactivation	(Lambeir et al. 2001a)
Inflammatory mediators		
Eotaxin (CCL11)	Altered receptor specificity	(Forssmann et al. 2008)
IP10 (CXCL10)	Altered receptor specificity	(Ajami et al. 2008; Oravecz et al. 1997)
ITAC(CXCL11)	Altered receptor specificity	(Ajami et al. 2008)
Macrophage derived chemokines (CCL2)	Altered receptor specificity	(Lambeir et al. 2001b; Proost et al. 1999)
Mig (CXCL9)	Reduced activity	(Lambeir et al. 2001b; Proost et al. 2001)
Monocyte chemotactic proteins	Inactivation	(Van Coillie et al. 1998)
RANTES	Altered receptor specificity	(Oravecz et al. 1997)
Stromal-cell-derived-factor (CXCL12)	Altered receptor specificity	(Ajami et al. 2008)
TNF-α	Inactivation	(Bauvois et al. 1992)

6. Figures

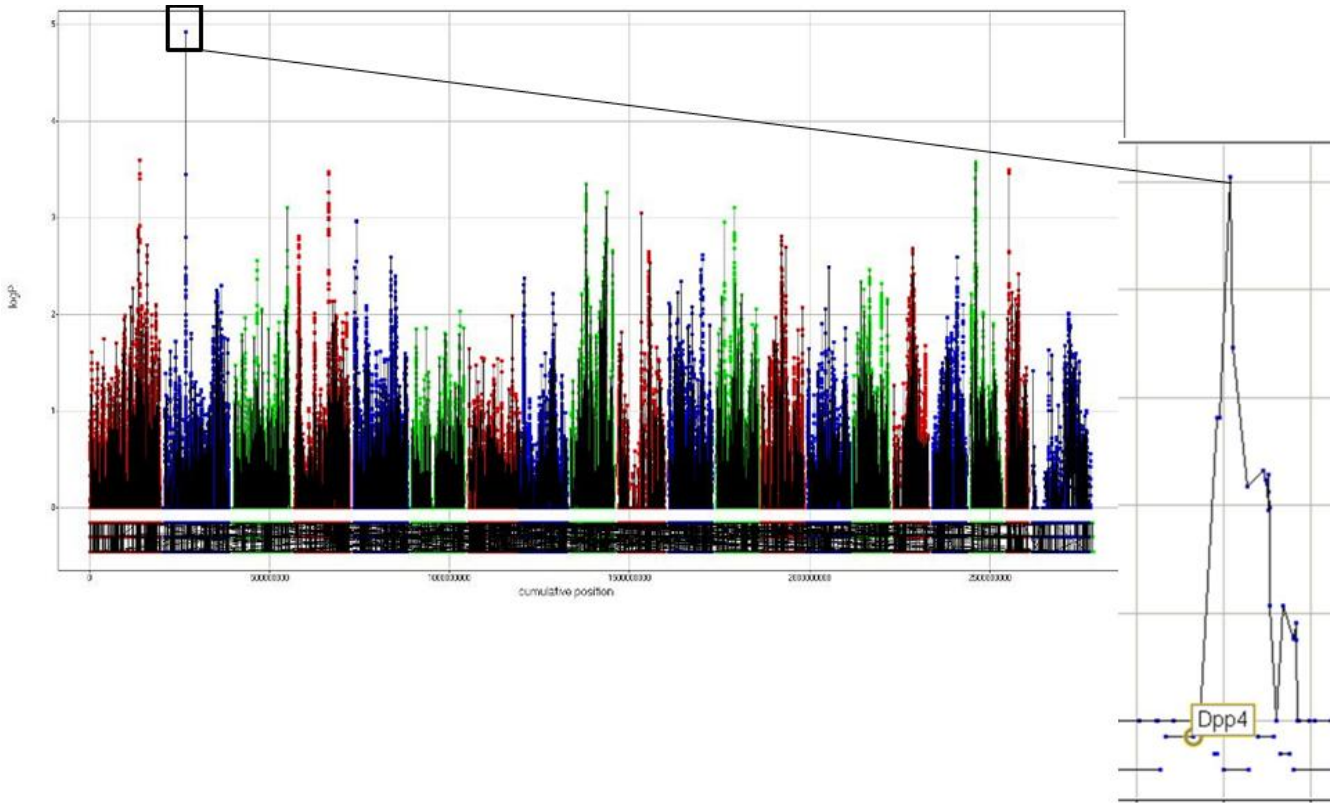

Figure 3.1 Genome-wide association plot for depressive-like behavior

Figure 3.1 Genome-wide association plot for depressive-like behavior. Genomic region on Chr 2 significantly correlates with immobility scores in the tail suspension test (-logP = 5.0). The figure shows the baseline behavioral despair QTL. To the right is the putative gene, dipeptidyl peptidase 4 (*Dpp4*), underneath behavioral locus on Chr 2. The y-axis denotes the strength of association between genotype and phenotype (-logP scores), and the x-axis illustrates the cumulative SNP position in the genome.

3.2

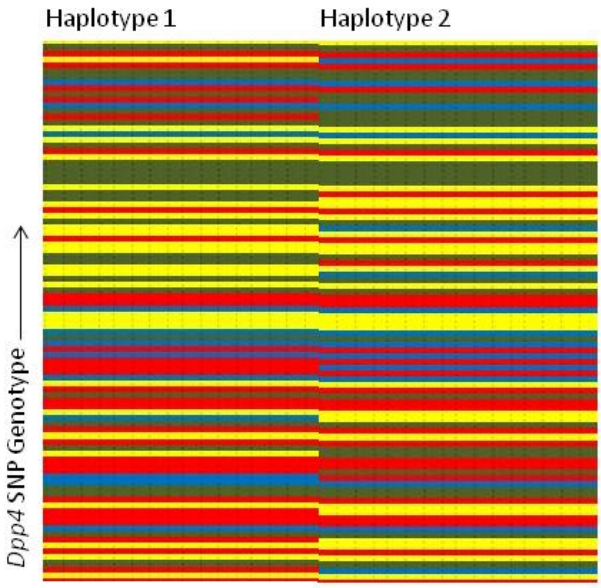

Figure 3.2 Haplotype structure within Dpp4 aligns with the top peak

3.3

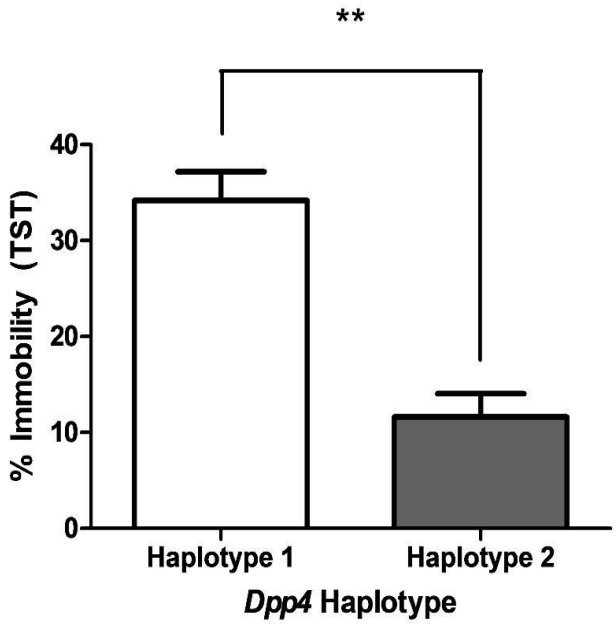

Figure 3.3 Dpp4 haplotype is associated with a difference in baseline immobility score

Figure 3.2. Haplotype structure within Dpp4 aligns with the top peak. The figure illustrates the strain distribution pattern for *Dpp4* (bottom) and for the despair locus on Chr 2 (-logP >5.0, top 3-SNP window). SNP positions are on the y-axis. The x-axis denotes strain. **3.3. Dpp4 haplotype is associated with a difference in baseline immobility score**. Strains in haplotype group 2 (GGG) exhibit lower depressive-like behavior. Percent time spent immobile in the TST is a measure of depressive-like behavior ($p<0.005$).

3.5

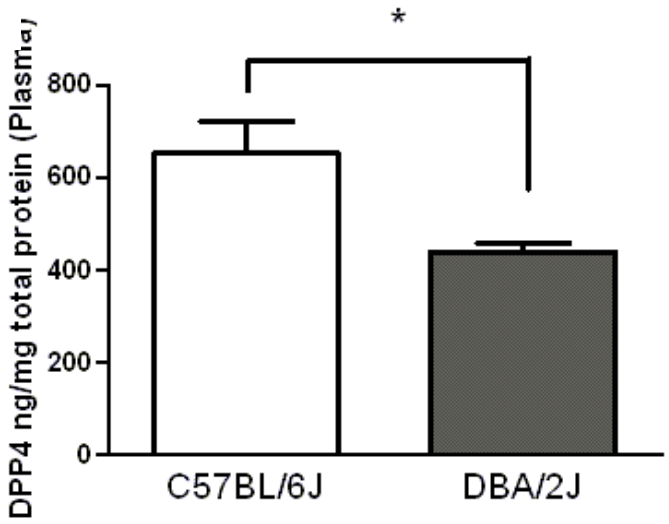

Figure 3.4 Difference in plasma Dpp4 protein levels

3.6

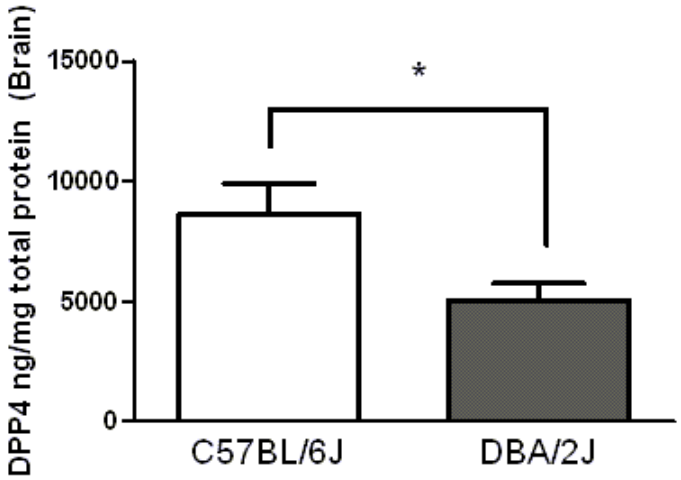

Figure 3.5 Difference in brain Dpp4 protein levels

Figure 3.4. Difference in plasma Dpp4 protein levels. C57BL/6J have higher plasma Dpp4 protein levels in at baseline (*p<0.05).* **3.5. Difference in brain Dpp4 protein levels.** Comparison to DBA/2J, C57BL/6J exhibit higher Dpp4 protein levels in the brain (*p<0.05).*

3.6

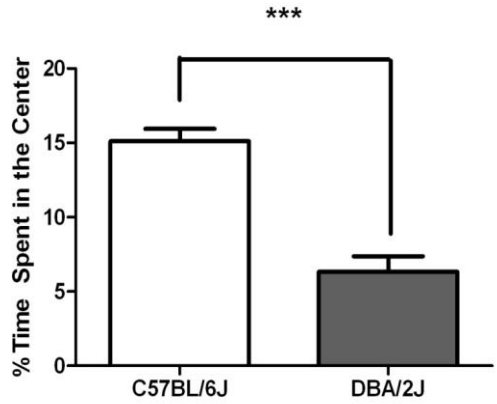

Figure 3.6 Anxiety-like responses in the open field

3.7

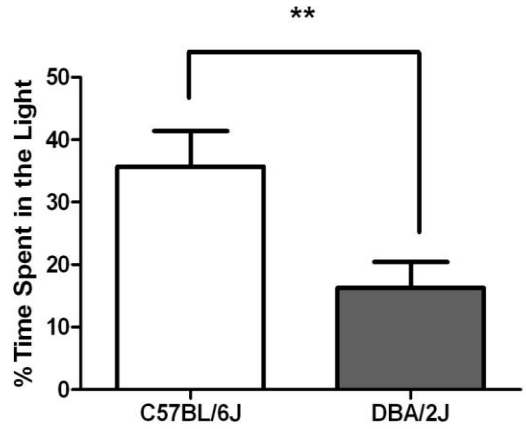

Figure 3.7 Anxiety-like responses in the light-dark exploration test

3.8

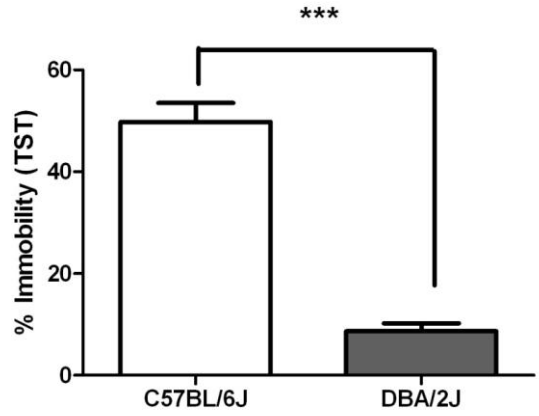

Figure 3.8 Immobility responses in the tail suspension test.

Figure 3.6. Anxiety-like responses in the open field. In comparison to DBA/2J, C57BL/6J mice spent more time in the center of the open field (*p<0.0005)*. **3.7. Anxiety-like responses in the light-dark exploration test.** During the 10 min light-dark test, C57BL/6J mice spent more time in the light compared to DBA/2J (*p<0.005)*. **3.8. Immobility responses in the tail suspension test.** C57BL/6J mice exhibited greater immobilility, indicative of "depressive-like" behavior, compared to DBA/2J mice (*p<0.0005)*.

3.9

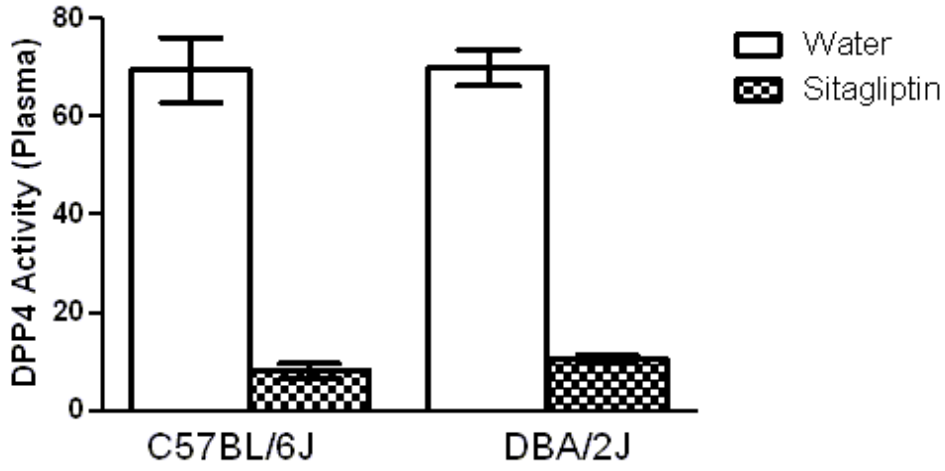

Figure 3.9 Measurement of plasma Dpp4 activity

3.10

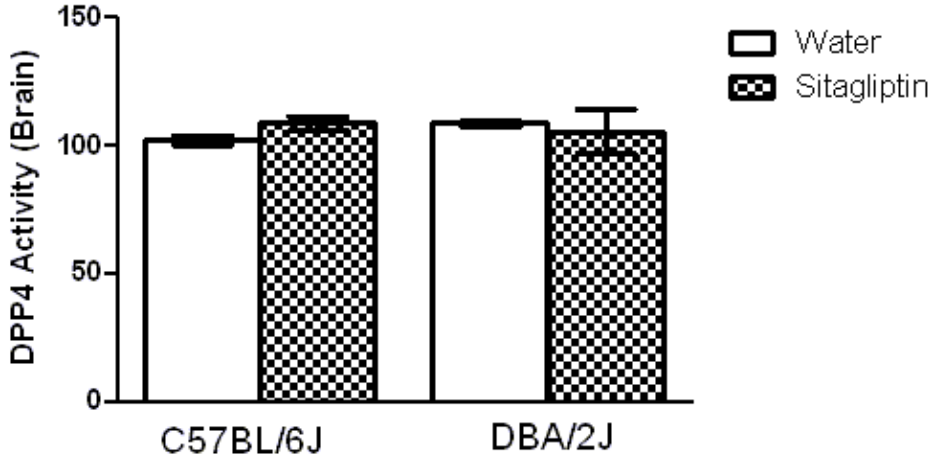

Figure 3.10 Measurement of Dpp4 activity in the brain

Figure 3.9. Measurement of plasma Dpp4 activity. Baseline plasma DPP4 activity did not differ between C57BL/6J and DBA/2J (white bars). Treatment with DPP4 inhibitor (sitagliptin), resulted in greater than 80% reduction of plasma DPP4 activity in both strains (black and white bars) **3.10. Measurement of Dpp4 activity in the brain.** No difference in plasma DPP4 activity was observed between C57BL/6J and DBA/2J (white bars). For both strains, 14-day treatment with sitagliptin did not alter brain DPP4 activity.

3.11

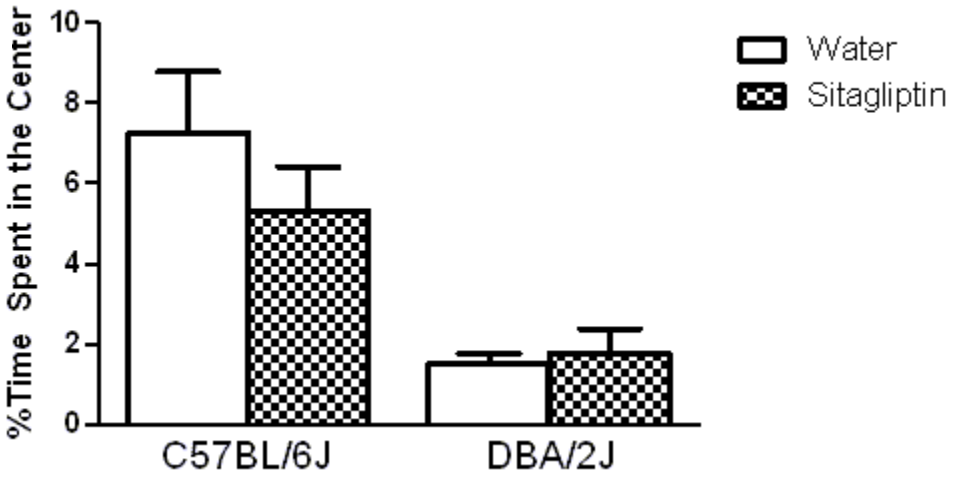

Figure 3.11 Anxiety-like responses in the open field after chronic sitagliptin treatment

3.12

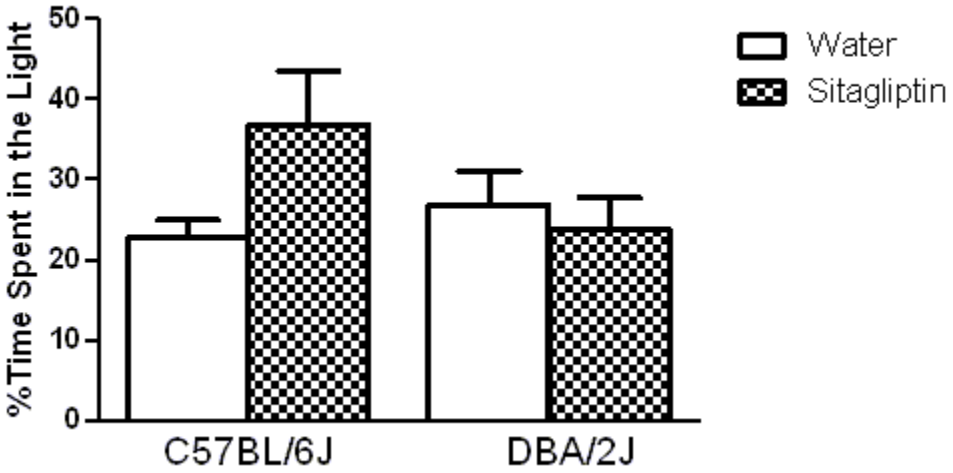

Figure 3.12 Anxiety-like responses in the light-dark assay after chronic sitagliptin treatment

3.13

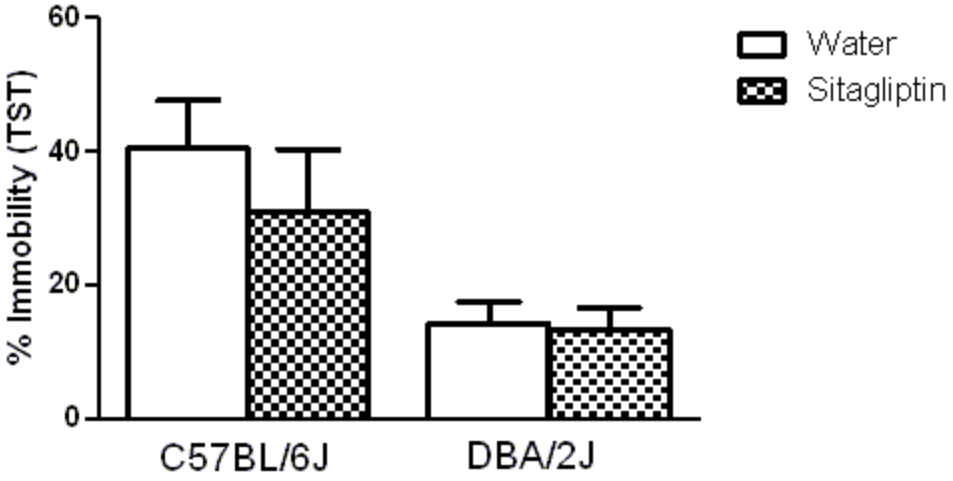

Figure 3.13 Depressive-like responses in the open after chronic sitagliptin treatment

Figure 3.11. Anxiety-like responses in the open field after chronic sitagliptin treatment. For both C57BL/6J and DBA/2J strains, we found no significant difference between treatment groups in the amount of time spent in the center of the open field**. 3.12. Anxiety-like responses in the light-dark assay after chronic sitagliptin treatment.** Fourteen-day treatment with sitagliptin did not alter anxiety-like responses in the light dark test for C57BL/6J and DBA/2J strains. **3.13. Depressive-like responses in the open after chronic sitagliptin treatment.** No difference in immobility was observed between water and sitagliptin-treated mice. White bars denote behavioral scores for control group. Black and white bars indicate mean behavioral responses for the sitagliptin group.

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OTHER RESOURCES

Sitgliptin [package insert], Whitehouse Station, NJ: Merck Sharp & Dohme Corporation; 2010

CHAPTER 4

EVALUATING GENETIC MARKERS AND NEUROBIOCHEMICAL ANALYTES FOR FLUOXETINE RESPONSE USING A PANEL OF MOUSE INBRED STRAINS

This work has been accepted for publication in *Psychopharmacology*

1. Introduction

The social and economic burden of major depressive disorder (MDD) is substantial. Despite the significant cost associated with depression, less than 30% of patients achieve adequate response to a selective serotonin reuptake inhibitor (SSRI) (Trivedi et al. 2006). There are several factors that contribute to poor treatment outcome including gender, disease onset, and presence of comorbid psychiatric disorders (Kornstein and Schneider 2001). Presently, the therapeutic management of patients who relapse or fail to respond is poorly defined. Partial responders or non-remitters typically undergo a series of antidepressant treatment modifications that are largely based on trial and error, leading to delay in symptom resolution and treatment discontinuation (Olfson et al. 2006; Warden et al. 2007). A set of biomarkers that can prospectively identify patients who will respond to a specific medication can provide an evidence-based rationale for selecting an appropriate course of antidepressant therapy.

Several large studies have been undertaken to identify genetic biomarkers of antidepressant response (Garriock et al. 2010; Ising et al. 2009; Uher et al. 2010). Pharmacogenetic studies of antidepressants in the Sequenced Treatment Alternatives for Depression (STAR*D) trial have led to the identification of genes associated with treatment response (Hu et al. 2007; Kraft et al. 2007; Lekman et al. 2008a; McMahon et al. 2006; Paddock 2008), treatment resistance (Perlis et al. 2008), and treatment-emergent suicidal ideation (Laje et al. 2009; Laje et al. 2007; Perlis et al. 2007). In addition, polymorphisms in genes that encode drug-metabolizing enzymes and transporters have been tested for correlation with treatment response (Peters et al. 2008). Although several studies have yielded notable findings, none meet currently accepted standards in human genetics for replication (Chanock et al. 2007).

The search for antidepressant biochemical markers is complicated by multiple neurobiological abnormalities that have been observed in depression, indicating that it is unlikely for a single biological marker to characterize a multifactorial disease like MDD. Many analytes, including brain-derived neurotrophic factor (BDNF), serotonin transporter, and monoamines, have been linked with depressive symptoms and response to antidepressant therapy (Manji et al. 2001; Nestler et al. 2002; Thase 2007). A few studies have looked at biochemical markers that can be used as diagnostic (Domenici et al. 2010; Jehn et al. 2006) or treatment markers (Ising et al. 2007; Schule et al. 2009) for depression. Other physiologic markers including neurostructural and neurofunctional measures have shown promise as potential markers of antidepressant response (Leuchter et al. 2010; Leuchter et al. 2009b). It is yet to be determined if these biomarkers can prospectively predict response prior to treatment.

A major limitation in evaluating the relationship between antidepressant response and biochemical alterations in human subjects is sample accessibility, whereby analysis is typically restricted to human serum and post-mortem brain samples. The assessment of post-mortem brain tissues is limited by the inability to distinguish if neurobiochemical abnormalities are causal or a consequence of disease or treatment, while serum samples are more accessible but provide a relatively myopic view of the neurobehavioral mechanisms that occur in the brain. Importantly, these limitations can be mitigated through the use of inbred mouse strains, which are easily maintained and manipulated for research purposes. Besides ease in accessibility, mice within the same strain are homogeneous at each genomic locus, which makes identification of genetic variants of drug response more feasible due to reduced genomic complexity.

In order to understand how biochemical and genetic differences correlate with treatment response, we measured depressive-like behavior, anxiety-like behavior, gene expression, and the levels

of thirty-six neurobiochemical analytes across a panel of genetically-diverse mouse inbred lines after chronic treatment with vehicle or fluoxetine. Neurobiochemical markers were chosen based on their putative molecular function within pathways proposed to underlie depression and anxiety, which include neuronal transmission, HPA-axis regulation, and neuroimmune processes. To our knowledge, this is the first study that simultaneously measured and examined the role of thirty-six putative neurobiochemical markers across thirty mouse inbred strains. In this study, we examined how baseline and treatment-induced biochemical differences affect depressive-like and anxiety-like responses. Our goal was to establish genetic and biochemical biomarkers that can predict fluoxetine response and to propose a molecular pathway that is critical in mediating response to SSRIs. In addition, we examined baseline neurobiological differences that affect depressive-like and anxiety-like behaviors in an effort to identify biomarkers that contribute to risk for poor treatment outcomes.

2. Materials and Methods

2.1 Animals

Thirty mouse inbred strains (129S1/SvImJ, A/J, AKR/J, BALB/cJ, BTBRT<t>tf/J, BUB/BnJ, C3H/HeJ, C57BL/6J, C57BLKS/J, C57BR/cdJ, C58/J, CBA/J, CE/J, DBA/2J, FVB/NJ, I/LnJ, LG/J, LP/J, MA/MyJ, MRL/MpJ, NOD/LtJ, NOR/LtJ, NZB/BlNJ, NZW/LacJ, P/J, PL/J, RIIIS/J, SJL/J, SM/J, and SWR/J) aged 5-6 weeks old were obtained from The Jackson Laboratory (Bar Harbor, ME). Male mice were housed four per cage in polycarbonate cages on a 12-hour light/dark cycle (lights on at 0700h) with access to food and water *ad libitum.* Following one week of habituation, mice were randomized to either control or treatment group. Depending on cost, availability, and estimated intra-strain response variability, the number of animals treated per treatment group ranged from 9 to 21 animals per strain. All procedures were approved by the Institutional Animal Care and Use Committee

and followed the guidelines set forth by the National Institutes of Health (NIH) Guide for the Care and Use of Laboratory Animals.

2.2 Drug Treatment

Fluoxetine is an SSRI that has been extensively studied for its effect on depressive behavior (Cipriani et al. 2005; Rossi et al. 2004). Fluoxetine HCl was purchased from Spectrum Chemicals (Gardena, CA). Mean water intake for each strain was determined previously by measuring daily water consumption for three weeks. This information, along with average weight measurements for each strain, was used to determine the amount of fluoxetine required to provide a daily oral dose of 0 or 18 mg/kg per mouse. A chronic fluoxetine regimen of 18 mg/kg for 21 days was selected based on results of a previous dose-response study which investigated the behavioral effects of fluoxetine administration at 0, 5, 10, 14, or 18 mg/kg for 1, 6, or 21 days (Miller et al. 2008). In each strain, comparison of mean daily water intake between vehicle and fluoxetine-treated animals indicates minimal effect of fluoxetine on water consumption (**Supplementary Table 4.1**). Mice were treated daily with fluoxetine or water throughout the end of the study and were sacrificed one week after the open field test between 1300 and 1600H.

2.3 Tail Suspension Test (TST)

Strains were randomly tested across day, time of testing, and equipment. At any given time, only four to eight animals per strain were tested. Each animal was tested only once in each procedure. After chronic administration of 0 or 18 mg/kg of fluoxetine for 21 days, mice aged 9-10 weeks were tested in a tail-suspension apparatus (PHM-300 TST Cubicle, Med Associates, St. Albans, VT) between 1300h and 1600h. In this test, the mouse is subjected to short-term inescapable stress by

having its tail suspended. Following failed attempts to escape, the mouse becomes immobile, a response generally considered as behavioral despair, a depressive-like behavior that is proposed to model “hopelessness” (Steru et al. 1987; Steru et al. 1985). Immobility was recorded for 7 min in 60 sec blocks using the following parameters: threshold = 3, gain = 8 and resolution = 200ms. Since all strains were uniformly active for the first min, percent time spent immobile was calculated for the last 6 min of the test. This procedure has been used previously to determine TST responses to antidepressants (Fujishiro et al. 2002; Ukai et al. 1998). Mice that climbed up their tail during testing were excluded from analysis (number of animals excluded = 104 out of 721 mice). Mean depressive-like behavior is reported in **Supplementary Table 4.2**.

2.4 Open Field Test (OF)

To minimize potential carryover effects between tests, behavior in the open field was recorded a week following TST. The OF test was conducted between 1300h and 1600h using a 27.3 cm x 27.3 open field apparatus (MED-OFA-MS, Med Associates, St. Albans, VT), which was surrounded by infrared detection beams on the x-, y- and z-axes that automatically recorded the animals' position and activity over the course of the experiment. Activity in the open field was recorded for ten minutes within an environmental chamber that provided white noise (60-64 dB) and low, indirect lighting (**Supplementary Table 4.2**). The OF is commonly used to measure exploratory and locomotive behavior in mice. This test exploits the rodent’s innate aversion to well-lit open spaces. Based on previous observations that showed increased exploration towards illuminated open areas following administration of anxiolytics (Choleris et al. 2001; Crawley 1985), anxiety-like behavior was measured as percent time spent in the center 25% of the open field. In addition, locomotor activity was recorded

by calculating the total distance traveled in the open field. This behavior was measured to assess if general locomotor activity confounded response in the TST.

2.5 Tissue Collection

Upon completion of the study, mice were sacrificed by cervical dislocation and decapitation between 0900h and 1300h. Trunk blood was quickly collected and allowed to clot on ice. Following centrifugation, serum samples were collected and stored at -20ºC for determination of fluoxetine and norfluoxetine levels using liquid chromatography-mass spectrometry/mass spectrometry (LC-MS/MS). Micro-dissections of individual regions were performed on serial coronal brain sections that were placed on a cold metal block. Cortex was taken from the same section for each animal and immediately snap frozen on dry ice and stored at -80ºC for gene expression and neurobiochemical analysis. Other brain regions were used for RNA isolation and subsequent gene expression analysis. Given that the neurobiochemical markers we examined were primarily expressed in the brain (**Table 4.1**), we measured the levels of these analytes in brain tissue.

2.6 Fluoxetine/norfluoxetine quantification

A solution with 10 µL of serum and 150 µL of acetonitrile was spiked with 5 µg/mL of each internal standard (fluoxetine-$_{D6}$ and norfluoxetine-$_{D6}$). Following sample filtration with 0.45 µm filter plate, 50 µL of the filtrate were diluted with 0.1% formic acid and injected into a Sciex 4000 LC MS/MS (Sciex Inc., Concord ON) equipped with a Waters YMC Cyano HPLC column (2.0 x 23 mm) (Milford, MA). The mobile phase consisted of water/acetonitrile/formic acid (75:25:0.1). A standard curve with concentrations from 20 to 10, 000 ng/mL was used to extrapolate amounts of fluoxetine and norfluoxetine in each sample (n = 8-19 mice per strain per treatment group). Peak areas were detected

for ions with the following mass to charge (*m/z*) ratios: *m/z* 310→44 (fluoxetine), *m/z* 296 →134 (norfluoxetine), *m/z* 316 → 44 (fluoxetine-$_{D6}$), and *m/z* 302 →140 (norfluoxetine-$_{D6}$). This information was used to quantify serum fluoxetine and norfluoxetine levels (**Supplementary Table 4.3**).

2.7 Neurobiochemical analyte quantification

Prior to ELISA experiments, all antibodies were tested for specificity by performing western blot experiments in pooled brain samples acquired from three mouse inbred strains. Abundance of neurobiochemical markers was measured using a parallelized reverse ELISA methodology (Zeptosens – a division of Bayer (Schweiz) AG, Witterswil, Switzerland). From each strain, cortex tissues were taken from three animals in each treatment group and homogenized in CLB1 buffer (Zeptosens) with zirconium oxide beads for 30 sec using QIAgen TissueLyser II (QIAgen, Valencia, CA). Semi-solid brain tissues were mixed gently on a tumbling shaker for 30 min at room temperature. Following centrifugation (2 min at 10,000 x g), supernatants were transferred and stored at -80ºC until further analysis. Total protein concentrations for each sample were determined using a modified Bradford assay (Coomassie Protein Plus Assay, Pierce Biotechnology, Rockford, IL). All samples were adjusted with CLB1 buffer to obtain a uniform concentration of 2 mg/mL. Immediately after dilution, each sample was spotted twice at concentrations of 0.05, 0.10, 0.15, and 0.2 mg/mL of total protein. Reference signals were obtained from simultaneous spotting of assay buffer and labeled antibodies on the array chip. Following overnight incubation with primary antibodies, arrays were washed and incubated with fluorescence-labeled anti-species secondary antibody for 2.5 h in the dark. Arrays were imaged simultaneously using ZeptoREADER (Zeptosens) at excitation/emission wavelengths of 635/670 nm with exposure times of 0.3, 1, 5, and 10 sec.

All images were analyzed using ZeptoVIEW 3.0 (version 3.0.1.17). Background intensities for each individual spot were determined by taking the mean signals of four additional spots equidistant from the sample spot. Net fluorescence intensities (NFI) were calculated by subtracting background signal from each sample spot signaling. Following NFI determination, each NFI value was normalized to the mean intensity of the reference spots. Using least-squares method, eight normalized NFI values obtained from each sample were fitted linearly against tissue lysate protein concentrations. The extrapolated signal intensity that corresponds in the mid-point of the concentration range (0.125 mg/mL) is defined as the reference fluorescence intensity (RFI). Relative abundance for each neurobiochemical marker was obtained by comparing inter-strain RFI values. All neurobiochemical markers measured in this study are listed in **Table 1.** Mean RFI scores for each analyte are listed in **Supplementary Table 4.4**.

Behavioral, neurobiochemical, and drug (fluoxetine and norfluoxetine) concentration data for each animal were deposited at The Jackson Laboratory Phenome Database (http://phenome.jax.org/) under the name Wiltshire 2 and Wiltshire 3**.**

2.8 Statistical analysis

Response to treatment was calculated by taking the ratio of fluoxetine behavioral scores to vehicle behavioral scores. Percent change in immobility was calculated by multiplying the ratio of fluoxetine immobility scores to vehicle immobility scores by 100 and then subtracting the product from 100. We defined positive and negative responders as having at least 20% decreased or increased in immobility scores, respectively. Given that the criterion was defined subjectively, we also investigated difference in response using more stringent criteria, which were 30% and 40% change in immobility scores. Strain and treatment effects on immobility scores and percent time spent in the center of the OF

were obtained using a two-way analysis of variance (ANOVA). Post-hoc pairwise comparisons of behavioral scores between control and fluoxetine groups were performed using a Mann-Whitney U test. False discovery rate is controlled at the 0.10 level using the Benjamini-Hochberg method.

Because both baseline neurobiochemical levels and fluoxetine-induced biochemical alterations can affect SSRI response, we wanted to identify the relationship between behavior and neurobiochemical alterations at baseline and after fluoxetine treatment. Partial least squares (PLS) regression analysis was used to extract latent vectors that account for most of the covariance between biochemical analytes and behavior in each treatment group. Once the vector was extracted, we determined the biochemical analytes that covary most with behavioral response by assessing the PLS loading score for each analyte onto the vector.

To identify biochemical markers that discriminate positive or negative responders, we used a Distance Weighted Discrimination (DWD) analysis. Unlike traditional multivariate analysis, this approach was intended for analysis of high dimension, low sample size data sets (Marron et al. 2007; Qiao et al. 2010). The DWD method was used to identify a hyperplane that best distinguishes the positive or the negative response groups. Following identification of a DWD hyperplane, the DWD loading vector (orthonormal to the hyperplane) was calculated, indicating the contribution of each neurobiochemical marker in discriminating negative or positive responders. The Direction Projection Permutation based hypothesis test (DiProPerm) with DWD was used to test for an overall neurobiochemical mean difference in each response group. In this procedure, the neurobiochemical scores were projected onto the DWD vector and the two sample t-statistic was computed. The t-statistic obtained from the real data set was compared to the t-statistics obtained from 1,000 relabeled group

pairs with recomputed DWD vector to determine if the overall difference in neurobiochemical levels was significant ($p<0.05$).

We performed Pearson's correlation analysis to determine if neurobiochemical levels correspond with fluoxetine, norfluoxetine, or mRNA expression levels. Molecular connections between candidate treatment biomarkers were evaluated using Ingenuity Pathway Analysis (IPA) software (Ingenuity Systems, Inc. Redwood, CA). Statistical analyses were performed using SAS 9.2 (SAS Institute, Cary, NC), R Package version 2.13 (http://www.r-project.org/), and MATLAB 7 (The MathWorks, Natwick, MA).

2.9 Transcriptome gene expression analysis

Brain regions were homogenized in 500 µL of Trizol using a QIAgen TissueLyser at 30 Hz (QIAgen, Valencia, CA). RNA for each animal was isolated by guanidinium thiocyanate-phenol-chloroform extraction and was further purified using the RNAeasy miniprep kit (QIAgen, Valencia, CA) according to the manufacturer's directions. For each strain, RNA samples for three animals from each treatment group were pooled and converted to cDNA and biotinylated cRNA (Enzo kit, Affymetrix, Santa Clara, CA). The resulting cRNA was hybridized to Affymetrix GeneChip Mouse Genome 430 2.0 arrays.

Following normalization with the gcRMA algorithm, expression data were analyzed using ArrayAssist software (Stratagene, La Jolla, CA). To identify genetic alterations associated with fluoxetine administration and treatment response, we analyzed gene expression profiles between control and fluoxetine treated mice as well as profiles between negative and positive responders as defined by the tail-suspension phenotype. Microarray data were preprocessed to identify genes with

expression levels greater than background levels (intensity >50). Gene expression profiles for each treatment group (control vs. fluoxetine) were filtered by calculating fold change difference between probes with minimum and maximum intensity values. In each treatment group, genes that were significantly variable between strains (fold change ≥3 between minimum and maximum values) were analyzed. Alternatively, expression data between response groups (negative vs. positive) were filtered by taking the ratio of fluorescence intensities between fluoxetine and control mice within each strain. Probe sets that were significantly affected by fluoxetine treatment (intensity ratios of fluoxetine to vehicle ≥2 for at least one strain) were selected. Following filtration, genes were prioritized based on their signal-to-noise ratio (SNR), which is the difference in median gene expression values divided by the standard deviation. The *p*-value for each observed SNR was determined from the empirical null distribution of SNRs, which was obtained by permuting the sample labels and recalculating the SNRs 1,000 times (Gould et al. 2006). Genes with a false discovery rate (FDR) of ≤ 0.05 were considered significant.

SNR rank scores were used to identify genes that were differentially expressed in either treatment (control vs. fluoxetine) or response (negative vs. positive responders) groups. These genes were then tested for their ability to assign samples in each response or treatment category using a *k*-nearest-neighbors (*k*-NN) classification with leave-one-out cross-validation test previously described in (Cover T.M. and Hart 1967; Golub et al. 1999). Briefly, the *k*-NN algorithm assigned a treatment or response label on the unknown sample based on the class assignment most frequently represented among its closest neighbors. Class assignments were determined based on the expression profiles of genes deemed to be informative. The validity of the model was tested using a leave-one-out cross-validation test, in which the algorithm withheld a sample, built a class predictor model using the remaining samples, and then predicted the class label of the withheld test sample. Using a distance

function, the model determined which members of the training set were closest to the test sample and then weighted each "vote" according to the distance of the unknown test sample from its neighbors, giving the closest neighbor the biggest "vote." This process was iteratively performed for each sample, and the cumulative error rate was calculated (Cover T.M. and Hart, 1967; Golub et al., 1999) (Golub et al. 1999). An unsupervised hierarchical clustering analysis was used to evaluate the expression pattern of the informative genes for each treatment and response group. Moreover, we performed eQTL analysis on genes with significant SNR values (FDR≤ 0.05) as described in (Wu et al. 2008) to determine if changes in gene expression following fluoxetine treatment were under *cis*-regulation. Gene expression analyses were performed using GenePattern 2.0 (Reich et al. 2006) and R Package version 2.13. Gene expression data were deposited into NCBI Gene Expression Omnibus (GEO) database (GSE28644).

A logistic regression analysis was used to identify baseline genetic markers that can account for variable fluoxetine response. Probe sets that were variable in the control group (fold change ≥3 between minimum and maximum values) were selected and evaluated for their influence on fluoxetine response. By using these analyses, we can identify baseline genetic markers that may be predictive of SSRI response. Statistical analyses were performed using R Package version 2.13.

2.10 QTL Mapping

Efficient Mixed-Model Association (EMMA) has been well-described elsewhere (Kang et al. 2008) and will be summarized here. Experimental SNP genotypes encompassing over 500,000 polymorphic loci across 72 commonly-used laboratory strains were obtained from the Center for Genome Dynamics at http://cgd.jax.org (Szatkiewicz et al. 2008; Yang et al. 2009). Over 190,000 SNPs were informative for the 30 mouse inbred lines, which provided the basis for inferring genotype.

SNPs used in these analyses were fully genotyped, not imputed, and had been specifically selected to discriminate *Mus musculus* sub-species. This set of SNPs avoids some of the previous biases in genome-wide analyses and enables an accurate ascertainment of population substructure. Associations between genotype (1-SNP) and phenotype were calculated by an F-statistic corrected for population structure and genetic relatedness. Results were plotted using SpotFire software (TIBCO Palo Alto, CA). Genomic loci that were associated with behavioral or biochemical phenotypes at an FDR ≤ 0.05 were considered significant.

3. Results

3.1 Behavioral measurements

Behavioral responses were measured in the open field test and tail suspension test following chronic treatment with vehicle (water) or fluoxetine. Analysis of immobility scores across the 30 mouse inbred strains revealed significant strain ($F = 29.83$, $p < 0.0001$) and treatment ($F = 39.14$, $p < 0.0001$), as well as significant strain by treatment effects ($F = 3.83$, $p < 0.0001$). Similarly, significant strain ($F = 23.16$, $p < 0.0001$), treatment ($F = 20.41$, $p < 0.0001$), and strain by treatment effects ($F = 1.63$, $p < 0.02$) were observed for percent time spent in the center of the OF. Compared to the control group, an overall reduction in mean immobility was observed in mice treated with fluoxetine ($t = 3.253$, $p<0.003$). However, change in immobility scores (fluoxetine response) was variable between strains. Thirteen strains exhibited 20% reduction in immobility (positive responders) while five inbred strains exhibited 20% increased in immobility (negative responders) (**Figure 4.1**). Post-hoc pairwise comparisons of behavioral scores between treatment groups revealed significant difference in immobility scores for BALB/cByJ, BUB/BnJ, C57BL/6J, C57BR/cdJ, CBA/J, MA/MyJ, P/J, PL/J, and

SM/J. Significant difference in anxiety-like responses between vehicle and fluoxetine treated mice were observed for DBA/2J, MA/MyJ, SJL/J, and SM/J (**Supplementary Table 5**).

Mean fluoxetine and norfluoxetine levels were variable across the strains, concentration ranges are (470.3 ng/mL - 1274.8 ng/mL) for fluoxetine and (578.6 ng/mL - 1612.2 ng/mL) for norfluoxetine. No significant correlation was observed between mean depressive-like behavior and mean serum levels of fluoxetine ($r^2 = 0.036$) or its metabolite norfluoxetine ($r^2 = 0.047$). This finding is similar to human studies, which do not find significant association with antidepressant response and serum levels of fluoxetine or norfluoxetine (Amsterdam et al. 1997; Beasley et al. 1990; Norman et al. 1993). With the exception of a few markers, no significant correlation was detected between neurobiochemical levels and drug concentration. Fluoxetine level was minimally correlated with GSK3β ($r^2 = 0.213$), HDAC5 ($r^2 = 0.131$), and VEGF ($r^2 = 0.155$). Minimal correlation between MCH protein levels and norfluoxetine levels ($r^2 =0.135$) was also detected. There was no correlation between mean percent immobility in the TST and total distance traveled in the OF for control ($r^2 = 0.013$) and treated animals ($r^2 = 0.083$), indicating that depressive-like behavior was not affected by locomotor activity. We observed a modest correlation between baseline and fluoxetine-induced immobility scores ($r^2 = 0.628$), which suggests that baseline TST behavior is linked with drug response. Genetic analysis can be performed using inbred-strain phenotype data to identify genetic loci associated with behavioral phenotypes (Miller et al. 2010; Williams et al. 2009). Independent QTL mapping analysis for behavioral phenotypes identified significant loci for anxiety, treatment response (data not shown), and baseline behavioral despair. A locus on Chromosome 9 (9: 46.7- 47.6 Mb) associated with baseline depressive-like behavior (-logP = 6.17, FDR$\leq$ 0.059) and this was of particular interest because of its co-localization with genome-wide association analysis for neurobiochemical markers.

3.2 Levels of S100β, GSK3β, HDAC5, and GFAP contribute most to treatment response

Levels of thirty-six neurobiochemical markers proposed to be involved in depression and anxiety were measured in cortex (**Table 4.1**). Dopamine transporter (DAT), interleukin-6 (IL-6), FK506-binding protein 51 (FKBP51), glucocorticoid receptor (NR3C1), and neuron specific family gene member 2 (NSG2) were excluded from the analysis due to weak intensities, irregular staining, or non-linear dose-response signals. In the control group, correlation between protein levels and mRNA expression levels was detected for c-Fos (r^2 = 0.269, 1423100_at), GFAP (r^2 = 0.222, 1426508_at; r^2 = 0.184, 1426509_at; r^2 = 0.187, 1440142_s_at), GLO1 (r^2 = 0.244, 1424108_at; r^2 = 0.274, 1424109_a_at), and PAQR8 (r^2 = 0.1796, 1431042_at). In the fluoxetine group, correlation between protein levels and mRNA expression levels were detected for GFAP (r^2 = 0.183, 1440142_s_at; r^2 = 0.623, 1426508_at; r^2 = 0.692, 1426509_s_at;), GNB1 (r^2 = 0.144, 1454696_at; r^2 = 0.161, 1417432_at), and NPY (r^2 =0.154, 1419127_at). The lack of strong correlation between neurobiochemical levels and corresponding transcript levels indicates an indirect relationship between mRNA and protein expression. Partial least squares analysis of the data indicated that the first vector accounted for 56% and 59% of the covariance in neurobiochemical and behavioral differences in the fluoxetine and control groups, respectively (**Supplementary Table 4.6**). Behaviors in the OF and TST accounted for baseline behavior (PLS loading score onto vector 1, percent time spent in the center OF: 75.1% and immobility TST: 56.4%). However, behavioral differences observed after chronic fluoxetine treatment were mainly due to inter-strain responses in the TST (PLS loading score onto vector 1, percent time spent in the center OF: 17.2% and immobility TST: 72.6%). Levels of glyoxylase 1 (GLO1) and guanine nucleotide binding protein 1 (GNB1) account for most of the covariance in anxiety-like and depressive-like behavior in the control group. Higher levels of GLO1 and GNB1 associated with higher baseline anxiety-like and depressive-like behavior. Alternatively, fluoxetine-

induced immobility behavior was mostly affected by glial fibrillary acidic protein (GFAP), S100 beta protein (S100β), GLO1, and histone deacytelase 5 (HDAC5) (**Figure 4.2**). Lower immobility scores following fluoxetine treatment were linked with higher levels of GFAP, S100β, GLO1, and HDAC5.

In order to identify biomarkers of fluoxetine response, we examined which biochemical analytes were most effective in discriminating positive or negative responders. An overall neurobiochemical difference was observed in positive and negative responders. Neurobiochemical alterations following fluoxetine treatment discriminate positive responders, while baseline biological differences distinguish negative responders. This finding was observed irrespective of the stringency in defining positive or negative response, defined as 20-40% reduction or increased in immobility, respectively (**Figure 4.3**). DWD analysis show levels of S100β, GSK3β, HDAC5, GFAP, and GNB1 account for most of the variance in treatment response (**Figure 4.2**). Baseline levels of S100β, GSK3β, HDAC5, GFAP, and GNB1 can discriminate negative responders. Chronic fluoxetine treatment affects these analytes and the levels of S100β, GSK3β, HDAC5, GFAP, and GNB1 post-treatment indicate positive response to fluoxetine. The direction of the vectors was the same for GSK3β and HDAC5 but opposite for S100β and GNB1 when discriminating positive or negative responders. Negative responders had lower levels of S100β and higher levels of GNB1, HDAC5, and GSK3β at baseline, while positive responders had lower levels of GNB1 and higher levels of S100β, GSK3β, and HDAC5 after fluoxetine treatment (**Figure 4.2**). GSK3β and HDAC5 can discriminate negative or positive response but have poor specificity in identifying negative and positive response groups from each other, while the opposing direction of the S100β and GNB1 vectors indicates that both analytes can discriminate negative and positive response groups from each other. Interestingly, we observed minimal influence of GFAP on positive response relative to its affect on negative response, which suggests that elevation in GFAP is mainly indicative of negative fluoxetine response.

In both analyses, response to fluoxetine was associated with alteration in candidate neurobiochemical levels (**Figure 4.2)**. Results from different methods of analysis (PLS and DWD) show that levels of GFAP, S100β, HDAC5, and GSK3β contribute to differential TST responses after fluoxetine treatment. In order to address whether fluoxetine mediates levels of candidate treatment markers S100β, GFAP, HDAC5, and GSK3β through a common pathway, we looked for molecular connections between them. Using IPA software, we discovered that all candidate treatment markers are linked either directly or indirectly. S100β, GFAP, HDAC5, and GSK3β connect within a common cellular growth and proliferation network, indicating the importance of cellular genesis in mediating response to chronic fluoxetine treatment (**Supplementary Figure 4.1**).

3.3 Gene expression profiles for treatment and response groups

Gene expression data collected from cortex were preprocessed to eliminate invariable probe sets, reducing the number of probes from 45,101 to 4,818. From genome-wide gene expression analysis, we first identified genes that were strongly correlated with control or fluoxetine treatment by computing the SNR ratio for 4,818 probe sets. We found 203 probe sets that were differentially expressed in either treatment group (FDR ≤ 0.10). Based on the SNR rank scores, twelve genes were significantly up-regulated in control or fluoxetine group, indicating that these genes are likely to be informative for assigning treatment category (**Supplementary Table 4.7**). To evaluate whether the expression profiles of the informative genes can be used to predict treatment classifications, we performed the *k*-NN leave-one-out cross-validation test. The twelve informative genes correctly predicted treatment class for 38 out of the 60 samples (absolute error of 36.6%), indicating that the overall expression patterns of the informative genes do not effectively predict treatment class. Because it is likely that gene expression differences between vehicle and fluoxetine-treated mice may be

confounded by inter-strain difference in response, we then analyzed gene expression profiles in negative and positive response groups. There were over 171 genes with variable expression patterns in each response group (FDR≤ 0.05). Based on SNR rank scores, eight genes were significantly up-regulated in either positive or negative responders, which make them likely to be genetic predictors of fluoxetine response (**Supplementary Table 4.9**). Using the *k*-NN leave-one-out cross-validation algorithm, we tested to see if responders can be correctly assigned (negative or positive) based on the expression patterns of the eight informative genes. Sixteen out of the eighteen samples were correctly assigned to the appropriate response groups, providing an absolute error rate of 11.1%. Results from the regression analysis indicate that several baseline genetic markers affect inter-strain variability in fluoxetine response (**Supplementary Table 4.10**). Using previously described eQTL analysis (Wu et al. 2008), we found that changes in gene expression for the 171 genes were not likely due to *cis*-regulation. Gene expression changes as measured by the log ratios of fluorescence intensities (fluoxetine/vehicle) did not significantly associate with polymorphic variants found within 25-500 kb of the putative genes. A heat map was generated following hierarchical clustering analysis, wherein the expression patterns of the informative genes were used to cluster each treatment and response group (**Figure 4.4**). Apart from a few specific strain divergences the gene sets indicate clusters of treatment and response groups. Our results show that gene expression differences were more apparent between response groups (positive vs. negative) compared to treatment groups (control vs. fluoxetine) with the gene set for response groups being able to reasonably predict SSRI response (**Figure 4.5**).

3.4 Quantitative trait loci associated with baseline depressive-like behavior and variable neurobiochemical levels

We also investigated whether differences in neurobiochemical analytes, which have been previously linked to anxiety and/or depression, are subject to a common genetic regulatory mechanism. Of particular interest, convergence of neurobiochemical and behavioral data revealed significant loci co-localization on Chr 9 (9: 46.7 - 47.6 Mb) (**Figure 4.6**). The Chr 9 QTL associated with baseline depressive-like behavior as well as baseline levels of cyclic AMP response element binding protein (CREB) (-logP = 4.46, FDR≤ 0.05) and vascular endothelial growth factor (VEGF) (-logP = 5.56, FDR≤ 0.05). Genes at this locus include *Cadm1* and 2900052N01Rik. *Cadm1* is involved in neural cell adhesion processes, facilitating cellular and molecular communication between cells and has been linked with social impairments and anxiety-like behavior in mice (Takayanagi et al. 2010; Zhiling et al. 2008).

4. Discussion

Identification of biomarkers that can establish diagnosis, prognosis, and response to treatment is critical to the advancement of research and management of patients with mood disorder. Biomarkers can be used to objectively assess clinical progression and response to antidepressant therapy. Relative to human studies, the use of mouse inbred strains can be an effective method for investigating biomarkers of drug response due to lower cost, greater tissue accessibility, reduced genomic complexity, and subject availability. However, the use of animal models to parallel subsets of behaviors that typify human disorders is challenging, and many of the symptoms used to establish psychiatric diagnoses in humans cannot be replicated in animals (Nestler and Hyman 2010). Here, we assess limited sets of specific behaviors that are relatively robust and have been previously validated as

responsive to antidepressive drug treatment (Cryan et al. 2005; Kulkarni and Dhir 2007) in a panel of genetically-diverse inbred mouse strains (Liu and Gershenfeld 2003; Ripoll et al. 2003; Trullas et al. 1989). The TST is primarily used to evaluate antidepressant activity of novel compounds and does not itself elicit depressive behavior, hence evaluation of treatment responses following exposure to chronic stress, an animal model of clinical depression that has both face and predictive validity (Nestler and Hyman 2010; Willner 1997; 2005), is needed for independent validation of treatment biomarkers.

Early studies from the STAR*D trial found a high co-occurrence of anxiety and depression. Over 49% and 42% of patients in the primary care and secondary care setting, respectively exhibited "anxious depressive" symptoms (Fava et al. 2006). Compared to patients with depression alone, patients with comorbid anxiety and depressive disorders are more likely to have prolonged, severe, and recurrent symptoms (Andreescu et al. 2007; Fava et al. 2004) and are less responsive to treatment (Emmanuel et al. 1998; Fava et al. 2008). In this study, we examined how baseline and treatment-induced biochemical differences affect depressive-like and anxiety-like responses. While anxiety-like and depressive-like behavior both contribute to baseline behavioral differences, anxiety-like behavior as measured by percent time spent in the center of the OF was only minimally affected by fluoxetine treatment. This result supports previous findings, which have demonstrated that responses in the TST is more sensitive to antidepressant drugs as opposed to other psychotropic agents like anxiolytics, antipsychotics, and stimulants (Cryan et al. 2005). Levels of GLO1 and GNB1 mostly account for anxiety-like and depressive-like behavior in the control group, suggesting that alterations in GLO1 and GNB1 may be common in both depression and anxiety disorders. GLO1 is an antioxidant enzyme that catalyzes the detoxification of methylglyoxal and has been linked with various psychiatric disorders including depression (Fujimoto et al. 2008), panic disorder without agoraphobia (Politi et al. 2006),

schizophrenia (Arai et al. 2010), and anxiety-like behavior (Hovatta et al. 2005; Williams et al. 2009). Variable mRNA expression of *Glo1* is likely due to difference in copy number variants. Duplication of a region where *Glo1* resides (Chr 17: 30.12 - 30.65 Mb) was found to be associated with difference in anxiety-like behavior across a panel of mouse inbred strains (Williams et al. 2009). In agreement with a previous study that has found higher *Glo1* mRNA transcripts in more anxious mice (Hovatta et al. 2005), we found that elevation in GLO1 protein corresponds with higher anxiety-like behavior. Elevation in GLO1 protein is also correlated with increased baseline immobility in the TST. This result is discordant with other studies that have found higher expression of GLO1 in less anxious and depressed mice (Ditzen et al. 2006; Kromer et al. 2005). Inconsistent findings may be due to a number of factors including strain, age, and protein quantification methodologies. Therefore, functional and metabolic assessment of GLO1 through measurement of methylglyoxal-mediated glycation is required for clarification (Thornalley 2006). GNB1 belongs to a family of heterotrimeric GTP-binding proteins that integrate signals among receptors and effector proteins. In a study that looked at dominant-subordinate behavior as a rodent model of depression, lower mRNA expression of *Gnb1* was associated with dominant behavior (Kroes et al. 2006). In agreement with this finding, we found that increased behavioral despair is associated with higher GNB1 protein. It is interesting to note that mice lacking *Gnb1* exhibit reduced cortical thickness, neural tube closure defects, and impaired neural progenitor cell proliferation, suggesting the importance of *Gnb1* in neurogenesis (Okae and Iwakura 2010). Treatment with psycho-stimulants like cocaine and amphetamines up-regulates *Gnb1* expression, which indicates that *Gnb1* is involved in drug sensitization (Kitanaka et al. 2002). Interestingly, pre-treatment with fluoxetine attenuates methamphetamine-induced locomotor sensitization (Takamatsu et al. 2006), suggesting potential involvement of *Gnb1* in fluoxetine response. Our results show that more anxious mice exhibit elevated levels of GNB1 protein. A recent study by

Benekareddy et al. found that early stress induced by maternal separation up-regulates *Gnb1* expression, implicating the involvement of *Gnb1* in mediating risk to anxiety and mood disorder (Benekareddy et al. 2010). Taken together, the association of GLO1 and GNB1 with baseline behavioral despair and anxiety-like behavior offers a novel possibility in which these proteins can be targeted to modulate both disorders and improve treatment outcomes in patients who suffer with comorbid anxious depressive symptoms.

To date, analysis of biochemical alterations has only been performed for a handful of markers in a few inbred strains despite inter-strain differences in response to antidepressants (Crowley et al. 2006; Miller et al. 2008; Ripoll et al. 2003; Vaugeois et al. 1997). In this study, we show that response to fluoxetine treatment mirrors responses seen in human fluoxetine treatment with a subset of strains exhibiting an "expected response" of reduced immobility as a phenotype of depressive-like behavior. Additionally, a few inbred strains exhibited no significant response while the remaining strains responded with increased immobility. Similar to previous reports, we observed a strong effect of strain and SSRI treatment on TST responses (Crowley et al. 2006; Ripoll et al. 2003; Trullas et al. 1989). Although the TST immobility scores we measured after chronic fluoxetine treatment were slightly different from what others have reported, our findings are in agreement with a previous study that examined TST responses in multiple inbred strains after fluoxetine treatment (Lucki et al. 2001). Following acute administration of fluoxetine, Lucki and colleagues observed reduced immobility responses for BALB/cJ and DBA/2J but not for C57BL/6J, A/J, and C3H/HeJ (Lucki et al. 2001). With the exception of the data for C57BL/6J, these results are consistent with our findings.

Little correlation was observed between change in immobility scores (treatment response) and serum fluoxetine and norfluoxetine levels, indicating that, at least in the mouse, pharmacokinetics does

not significantly modulate behavior and other factors like pharmacodynamics and pharmacogenetics may contribute to differential responses to fluoxetine treatment. Of note, negative and positive responses to fluoxetine can be distinguished based on neurobiochemical variability. Baseline biological differences discriminate negative responders, while biochemical alterations following fluoxetine treatment delineate positive responders. This result suggests that positive response to fluoxetine is mostly due to treatment-induced biochemical alterations, and negative response to fluoxetine is mainly attributed to baseline differences between mouse strains. Therefore, we propose that patients who fail to respond or remit from antidepressant therapy may have baseline biological abnormalities predisposing them to poor therapeutic outcomes.

Results from both DWD and PLS analyses suggest that levels of S100β, GSK3β, HDAC5, and GFAP influence differential TST responses following SSRI treatment (**Figure 2**). Chronic fluoxetine treatment results in higher levels of S100β, which triggers expression of serotonin transporters (SERT) and activates *de novo* serotonin synthesis (Baudry et al. 2010). Similarly, increased levels of GSK3β have been observed following chronic fluoxetine administration *in vivo* (Fatemi et al. 2009; Li et al. 2004). Inhibition of GSK3β blocked fluoxetine-mediated down-regulation of SERT through over-expression of miR 16 (Baudry et al. 2010), thus linking serotonin regulation with GSK3β phosphorylation (Li et al. 2004). Tsankanova *et al* found that treatment with tricyclic antidepressants results in lower mRNA levels of *Hdac5* and that overexpression of *Hdac5* in the hippocampus blocks the antidepressant effects of imipramine in the chronic social defeat stress paradigm (Tsankova et al. 2006). Consistent with previous studies (Baudry et al. 2010), higher levels of S100β following fluoxetine treatment were observed in positive responders. Interestingly, both negative and positive responders displayed higher levels of GSK3β and HDAC5. GSK3β phosphorylates various molecules typically leading to substrate inhibition, while chromatin remodeling by HDAC5 can prevent

transcription of many downstream genes. Thus, elevation of GSK3β and HDAC5 can affect several different downstream molecular processes, leading to variable treatment outcomes. Additional studies are required to examine the relationship between fluoxetine response and levels of GSK3β and HDAC5. Little information is known about the relationship of GFAP with fluoxetine response. In rodents, chronic treatment with fluoxetine results in elevation of amplifying neural progenitor cells (Encinas et al. 2006), while antidepressant treatment in humans results in increased numbers of quiescent neural progenitor cells, including nestin and GFAP (Boldrini et al. 2009). Rat astrocytic cells enriched with GFAP exhibit Na+ dependent and fluoxetine-sensitive serotonin uptake, suggesting involvement of GFAP in mediating serotonin levels (Dave and Kimelberg 1994). The underlying mechanism mediating the influence of GFAP on negative fluoxetine response warrants closer examination, given the low clinical response rates to SSRI treatment (Trivedi et al. 2006).

Although our findings indicate that fluoxetine administration alters levels of S100β, HDAC5, GSK3β, and GFAP, it is likely that these biochemical changes are not specific to SSRIs. Other psychotropic medications, including clozapine, lithium, valproic acid, and haloperidol, have been shown to alter GFAP (Fatemi et al. 2008) and GSK3β levels (Fatemi et al. 2009) *in vivo*. Altered levels of S100β are observed in patients with schizophrenia (Gattaz et al. 2000; Wiesmann et al. 1999), which can be normalized by treatment with antipsychotic agents (Ling et al. 2007; Steiner et al. 2010). Treatment with HDAC inhibitors is associated with neuroprotective effects and may be beneficial in the treatment of various psychiatric disorders, including bipolar and schizophrenia (Kazantsev and Thompson 2008; Tsankova et al. 2007). Collectively, these findings suggest that S100β, GSK3β, HDAC5, and GFAP are likely to be involved in a behavioral pathway common to multiple psychiatric disorders, although the downstream mechanisms mediated by each analyte may be specific for each psychiatric disorder. An important avenue for future study is the assessment of these biomarkers in

easily accessible samples like plasma or serum, thus facilitating translation of pre-clinical findings to human studies. Current work in the lab aims to evaluate the relationship between behavioral responses and serum levels of neurobiochemical markers across multiple inbred strains. Additionally, sex differences in the metabolism of fluoxetine (Hodes et al. 2010) necessitate further investigation of these biomarkers in females.

Overall, we observed minimal difference (203 probe sets with variable expression patterns) in gene expression profiles between vehicle and fluoxetine-treated mice. This result is consistent with what others have found when performing global transcriptome analyses between control and antidepressant treatment groups (Bohm et al. 2006; Lee et al. 2010; Miller et al. 2008; Takahashi et al. 2006). Although other groups have found more genes that were up- or down-regulated following fluoxetine treatment, the filtering criteria we used to preprocess our microarray data are generally more stringent in comparison. We only analyzed genes with a minimum 3-fold difference between strains and had statistically significant SNR values. In addition, other factors including fluoxetine dosing regimen, microarray methodology, and time of tissue collection can influence gene expression results. When we compared gene expression profiles between negative and positive responders, we found that we can accurately assign samples as either negative or positive responders (absolute error rate 11.1%) based on the expression patterns of *Copg2S2, Prcp, Gpr115, Gxylt2, Plb1, Txndc9, Mum1/1*, and *Gm1642*, making these genes potential predictors of SSRI response. Although none of these genes have been directly linked to antidepressant response or major depressive disorder, several are involved in molecular processes that may be critical in mediating behavior or neuronal function, such as neuropeptide signaling and lysosomal serine carboxypeptidase activity. For six of the genetic markers of fluoxetine response, there are commercially available ELISA kits and antibodies that can be used to evaluate the relationship between protein levels and SSRI response. Additionally, we identified

baseline genetic markers that affect inter-strain despair responses to fluoxetine treatment, suggesting its use as a potential prognostic treatment biomarker. Interestingly, one candidate gene is poly (A) binding protein, cytoplasmic 1 (*Pabpc1*), a gene that was significantly associated with SSRI response in a genome-wide association study, which evaluated over 1,800 patients from the STAR*D trial (Shyn et al. 2011). *Pabpc1* encodes for a poly (A) binding protein that binds to the poly (A) tail of mRNA, facilitating ribosome recruitment and translation initiation. Given that the association between *Pabpc1* and antidepressant response was observed in both pre-clinical and human studies, the mechanism underlying this association warrants further investigation.

To identify genetic markers for depressive disorder, we focused on genomic regions that are associated with both behavioral and neurobiochemical phenotypes. We identified a behavioral despair QTL on Chr 9 that correlated with inter-strain neurobiochemical variability. A QTL for depressive-like behavior has not been reported previously at this locus, which contains only two genes - *Cadm1* and 2900052N01Rik. *Cadm1* encodes for neural cell adhesion molecules that mediate cell-to-cell communication. Further, *Cadm1* is involved in synaptic function and neuronal differentiation (Watabe et al. 2003) and has been linked with anxiety-like and social behavior in mice (Takayanagi et al. 2010; Zhiling et al. 2008). A recent study by Takayanagi *et al* showed that mice lacking *Cadm1* displayed higher anxiety-like phenotype and impairment in motor coordination and social behavior compared to wild-type mice (Takayanagi et al. 2010). However, no significant difference in depressive-like behavior as measured by the TST was observed between groups (Takayanagi et al. 2010). Other behavioral paradigms like chronic social defeat stress and reduced sucrose preference, which assess other features of depressive behavior (e.g. anhedonia) (Nestler and Hyman 2010) can be used to further evaluate the role of *Cadm1* in depression. Since CADM1 act as synaptic adhesive molecule, functional disruption of *Cadm1* may lead to impaired neural plasticity and neurogenesis, both of which have been

linked with major depressive disorder (Campbell and Macqueen 2004; Schmidt and Duman 2007). Links defining interactions between *Cadm1* and the two biochemical markers (CREB and VEGF) that co-localize to the same genetic locus on Chr 9 provide a promising avenue for future study.

Altogether, our results highlight the role of cellular proliferation in mediating SSRI response. Similar to *Cadm1, Gpr115, Plb1, Gxylt2,* and *Prcp* are involved in cellular proliferation and/or cellular adhesion (Bjarnadottir et al. 2004; Ganendren et al. 2006; Mallela et al. 2008; Mallela et al. 2009; Okajima et al. 2008; Sethi et al.). Furthermore, candidate fluoxetine markers S100β, GSK3β, HDAC5, and GFAP are linked through a common cellular growth and proliferation pathway, suggesting that treatment with fluoxetine stimulates cell growth. Our results indicate that response to fluoxetine is affected by cellular genesis, which is consistent with previous studies that have shown a critical role of neurogenesis in mediating SSRI response (Deltheil et al. 2009; Manev et al. 2001). Regulation of neurogenesis, cellular resilience, neuroplasticity, and cellular death/atrophy is important in the pathogenesis and treatment of depression (Duman 2002; Krishnan and Nestler 2008; Lucassen et al. 2010; Manji et al. 2001; Manji et al. 2003). Neuroimaging and post-mortem brain studies show that patients with MDD have impaired neural circuitry and structural abnormalities. Compared to healthy controls, patients with mood disorder display lower number of glial cells, reduced cortical volume and thickness, and decreased neuronal size and density, all of which implicate cellular atrophy/loss (Manji et al. 2001; Manji et al. 2003). Antidepressant therapy increases levels of neurotrophic factors (Duman et al. 2001; Malberg et al. 2000; Sairanen et al. 2005) that promote neurogenesis and inhibit cell death signaling pathways (Riccio et al. 1999). Hippocampal neurogenesis is critical in facilitating adaptive response to stress (Duman et al. 2001; Malberg et al. 2000), memory development (Mirescu et al. 2004; Schloesser et al. 2009), and learning new experiences (Kempermann 2008) thus, impairment of neurogenesis may confer risk to depressive behavior. Elucidating the roles of neurogenesis, cellular

resilience, neural plasticity, and cell death in the development of depressive behavior requires a systems level exploration of these processes. Whether interplay between cellular proliferative and survival processes facilitates synaptic remodeling and leads to altered mood remains to be seen.

In the absence of biological markers, therapeutic outcomes are defined based on reduction of baseline symptoms, which can lead to inconsistencies and irreproducibility. Genetic and neurobiochemical markers can provide an objective means to measure prognosis and treatment response. We identified genetic and biochemical markers of fluoxetine response that are involved in cellular genesis, highlighting the role of neurogenesis and neuroplasticity in major depressive disorder. Since neurogenesis is not specific to antidepressant treatment, it is critical for future studies to identify how psychotropic medications differentially affect neurogenesis and which downstream neurobiological pathways are affected. Such studies may illuminate differences between closely related psychiatric disorders including depression and anxiety.

5. Tables

Table 4.1 List of neurobiochemical analytes simultaneously measured across multiple mouse inbred strains and their known functions

Biomarker	Name	Function	GO category
ACTH	Adrenocorticotropin releasing hormone	Neuroimmune	(GO:0005179), (GO:0007218)
APOD	Apolipoprotein D	Neuroimmune	(GO:0006629), (GO:0005215)
BAG1	BCL2-associated athanogene 1	Apoptosis	(GO:0006916), (GO:0006950)
BDNF	Brain-derived neurotrophic factor	Neurogenesis	(GO:0006916) (GO:0048167)
c-fos	FBJ osteosarcoma oncogene	Transcription	(GO:0007399), (GO:0045941)
CREB	cAMP response element-binding	Signal Transduction	(GO:0007165), (GO:0007202)
PPP1R1B	Protein phosphatase 1 subunit 1B	Modulation of phosphatase1	(GO:0007165) , (GO:0004864)
DAT	Dopamine Transporter	Regulation of dopamine levels	(GO:0005329), (GO:0007268)
FKBP51	FK506-Binding Protein 51	Signal Transduction	(GO:0005528), (GO:0031072)
GAD67	Glutamic Acid Decarboxylase-67	Decarboxylation of glutamate to GABA	(GO:0004351), (GO:0006915)
Galanin	Galanin	Neuronal modulation	GO:0005184), (GO:0006950)
GFAP	Glial fibrillary acidic protein	Maintenance of astrocytes	(GO:0005200), (GO:0005882)
Ghrelin	Ghrelin	Neurotrophy and appetite regulation	(GO:0006916), (GO:0008343)
GLO1	Glyoxylase1	Neuroprotection	(GO:0006916), (GO:0006749)
GNB1	Guanine nucleotide-binding protein subunit beta-1	Signal Transduction	(GO:0006112), (GO:0007200)
GSK3β	Glycogen synthase kinase 3 beta	Phosphorylation	(GO:0035255) , (GO:0006916)
GSR	Glutathione Reductase	Neuroprotection	(GO:0006749), (GO:0016491)
HDAC5	Histone deacetylase 5	Transcription	(GO:0004407), (GO:0000122)
IL-6	Interleukin6	Neuroimmune	(GO:0001781), (GO:0006954)
KCNJ9	Potassium inwardly-rectifying channel, subfamily J, member 9	Regulation of K^+ levels	(GO:0015467), (GO:0006813)
MCH	Melanin Concentrating Hormone	Sleep and circadian rhythm	(GO:0007218), (GO:0046005)
NET	Norepinephrine Transporter	Regulation of norepinephrine	(GO:0006836),

		levels	(GO:0008504)
NGF	Nerve Growth Factor	Neuronal growth and signaling	(GO:0008504), (GO:0006954)
NMDA1	Glutamate Receptor, Subunit 1	Neurotransmission	(GO:0004972), (GO:0005234)
NPY	Neuropeptide Y	Neuropeptide signaling	(GO:0005184), (GO:0001664)
NR3C1	Glucocorticoid Receptor	Neuroendocrine	(GO:0004883), (GO:0006351)
NSG2	Neuron specific family gene member 2	Dopamine receptor signaling pathway	(GO:0007212), (GO:0050780)
P2X7	Purinergic Receptor Ligand-Gated Ion Channel7	Regulation of Ca^{2+}	(GO:0010524), (GO:0000187)
PAQR8	Progestin and AdipoQ Receptor 8	Steroid binding	(GO:0004872), (GO:0005496)
PTH	Parathyroid Hormone	Regulation of Ca^{2+}	(GO:0031856), (GO:0007186)
S100β	S100 beta protein	Glial cell proliferation	(GO:0007417), (GO:0008283)
SERT	Serotonin Transporter	Regulation of norepinephrine levels	(GO:0008504), (GO:0015222)
SGNE	Secretogranin V	Neuroendocrine	(GO:0016486), (GO:0007218)
TNF-α	Tumor Necrosis Factor-alpha	Neuroimmune	(GO:0006955), (GO:0005125)
VEGF	Vascular Endothelial Growth Factor	Growth and angiogenesis	(GO:0001525), (GO:0005125)

6. Figures

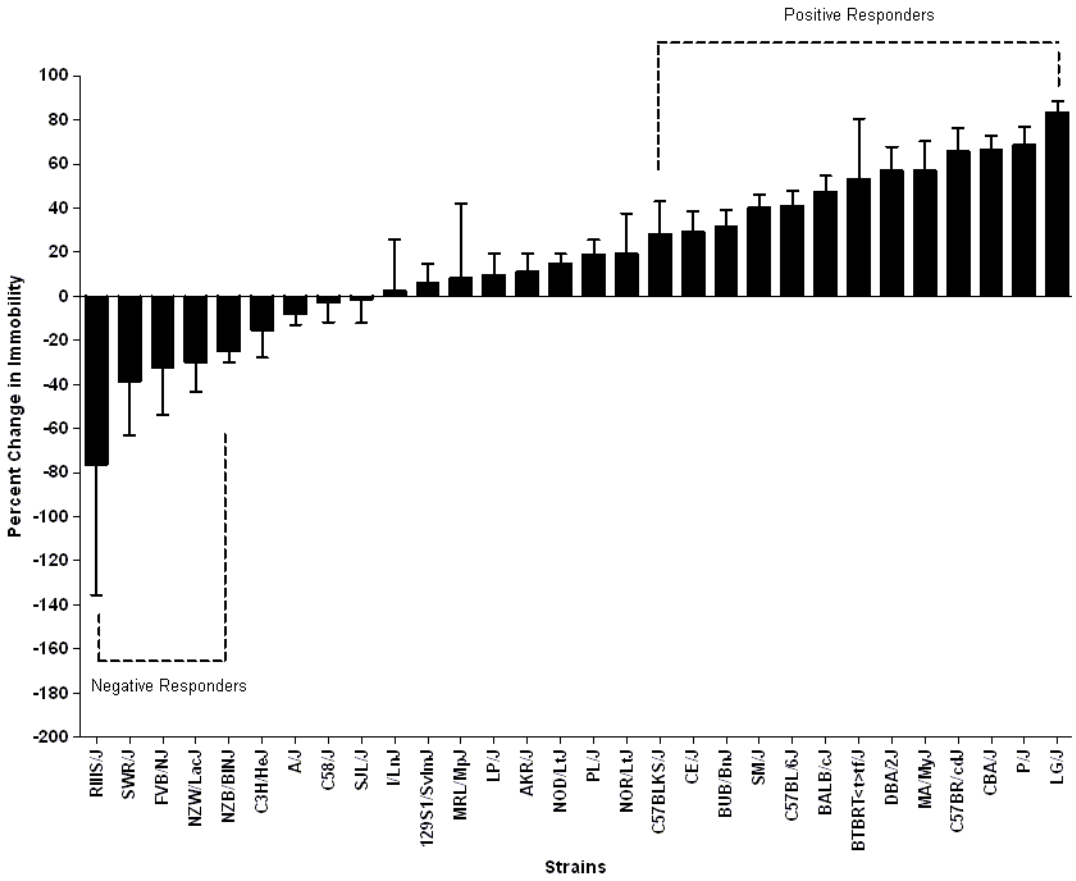


Figure 4.1 Inter-strain difference in fluoxetine response

Figure 4.1. Inter-strain difference in fluoxetine response. Response to treatment was calculated by taking the log ratio of fluoxetine behavioral scores to vehicle behavioral scores. Percent change in immobility was calculated by multiplying the ratio of fluoxetine immobility scores to vehicle immobility scores by 100 and then subtracting the product from 100. We defined positive and negative responders as having *at least* 20% decreased or increased in immobility scores, respectively. Immobility is a measure of "hopelessness" or depressive-like behavior in mice. Strains with positive response to fluoxetine exhibited significant reduction in depressive-like behavior, while negative responders had an increased in immobility

4.2

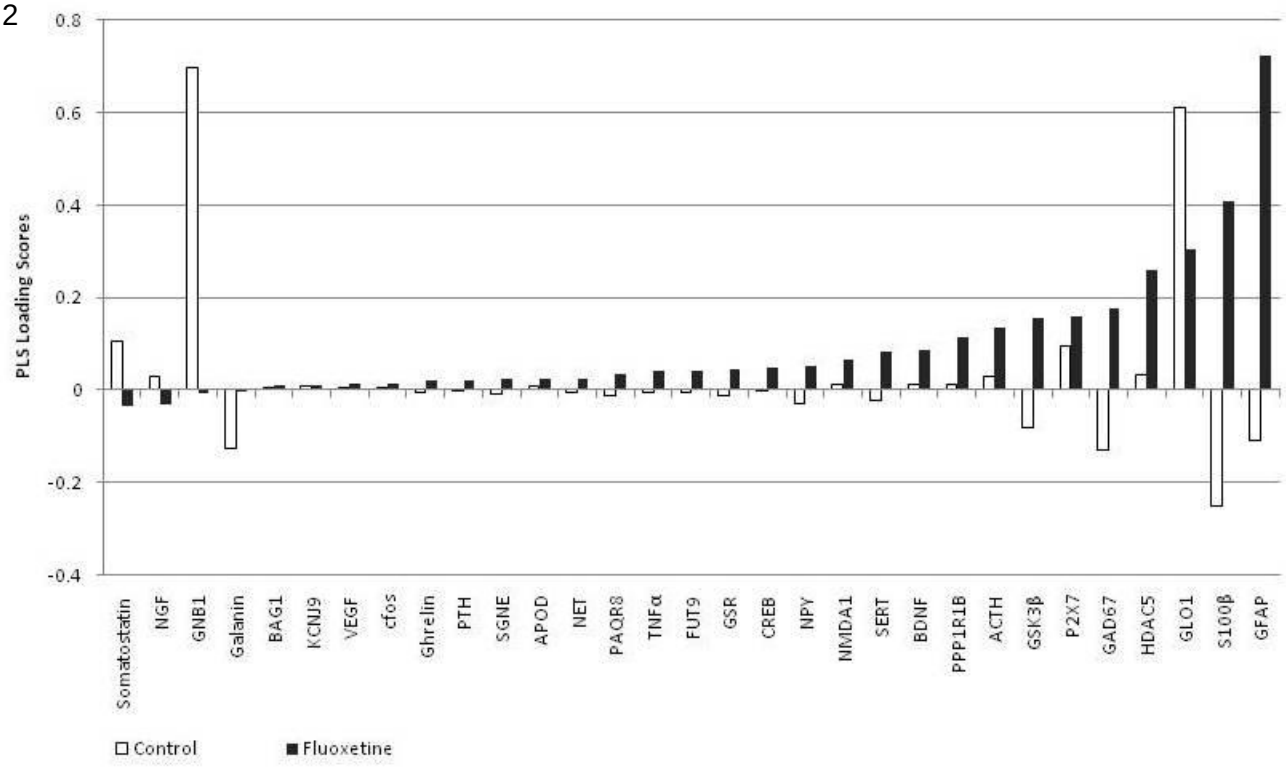


Figure 4.2 Neurobiochemical markers that covary with fluoxetine response

4.3

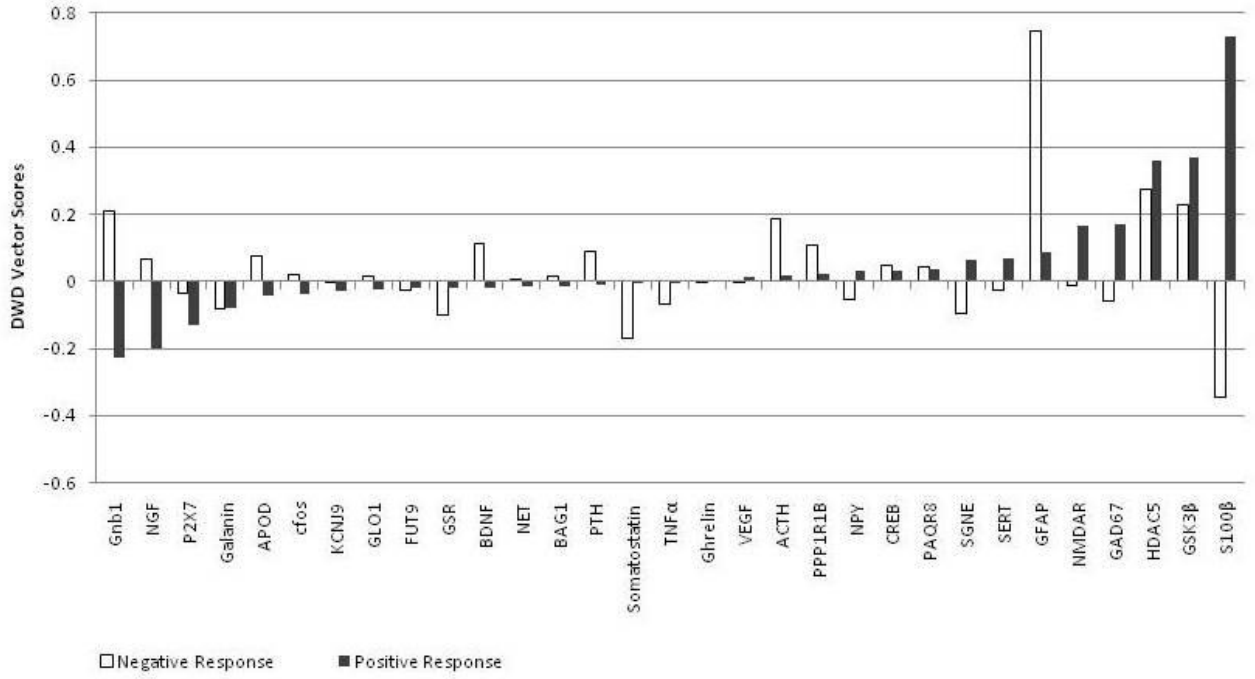


Figure 4.3 Neurobiochemical analytes that contribute most to discriminating positive and negative responders

Figure 4.2. Neurobiochemical markers that covary with fluoxetine response. PLS analysis show levels of GNB1, GLO1, S100β, GAD67, GFAP, and galanin covary most with response to open field and tail suspension tests (white bars). Levels of GFAP, S100β, GLO1, HDAC5, GAD67, P2X7, and GSK3β covary most with depressive-like response following chronic fluoxetine treatment (black bars). **4.3. Neurobiochemical analytes that contribute most to discriminating positive and negative responders**. DWD analysis shows that S100β, GSK3β, HDAC5, and GNB1 discriminate positive responses (black bars) or negative response (white bars). The opposing direction of the S100β and GNB1 vectors indicates that both markers can discriminate negative and positive response groups from each other. Neurobiochemical differences induced by chronic fluoxetine treatment discriminate positive responders while baseline neurobiological differences discriminate negative responders. Overall neurobiochemical difference is observed when we defined positive response as 20% ($p<0.006$), 30% ($p<0.014$) or 40% ($p<0.026$) reduction in immobility or when we defined negative response as 20% ($p<0.006$), 30% ($p<0.036$) or 40% ($p<0.016$) increased in immobility. Data are shown when response is defined as 20% decreased or increased in immobility scores

4.4

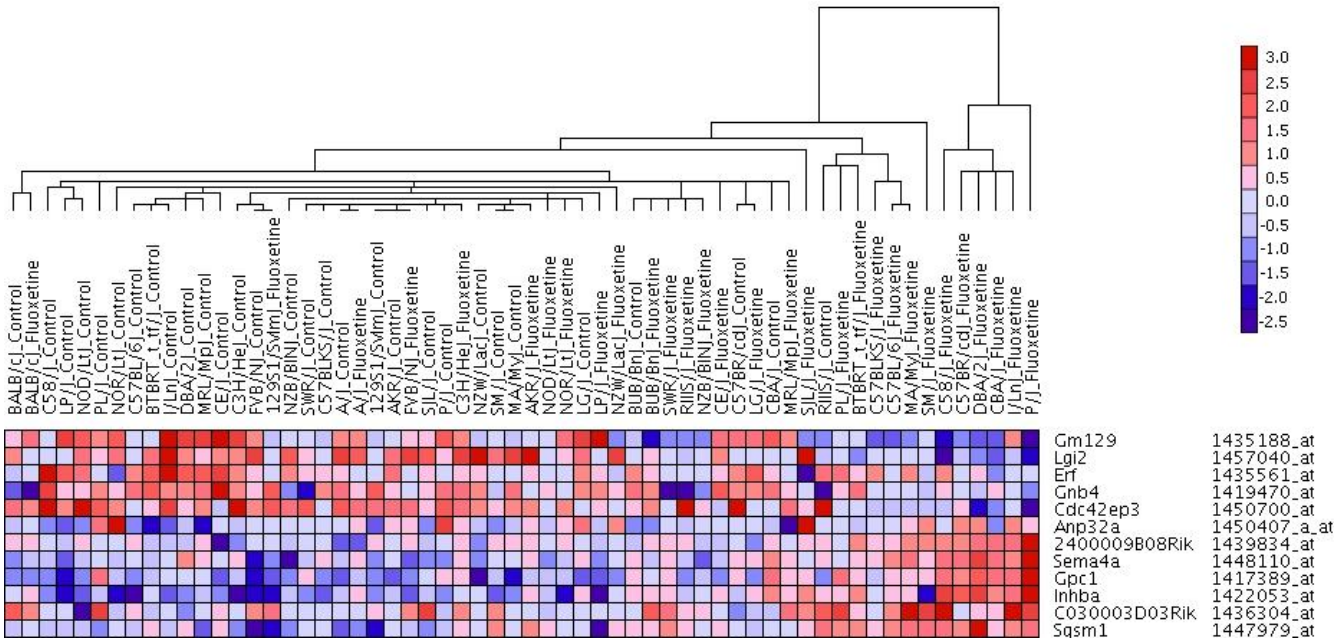


Figure 4.4 Hierarchical clustering of genes discriminating treatment from control

4.5

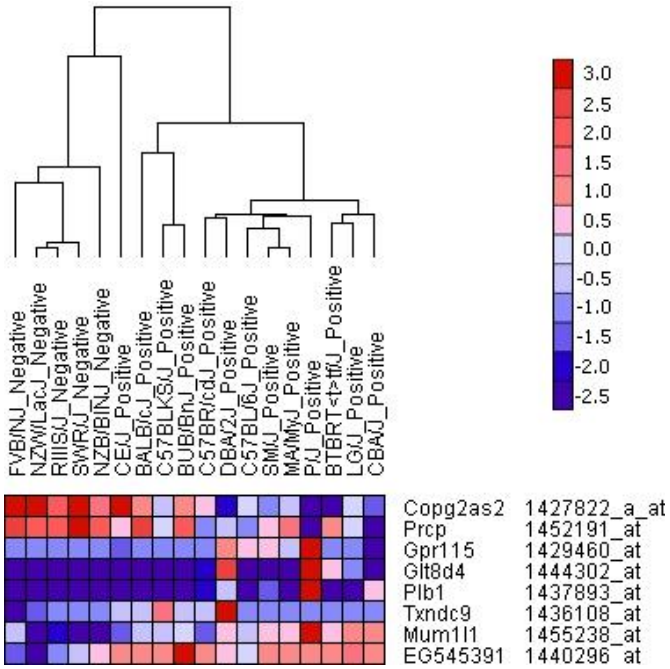


Figure 4.5 Hierarchical clustering of genes discriminating positive and negative response to treatment

Figure 4.4. Hierarchical clustering of genes discriminating treatment from control. Gene expression patterns of the twelve most informative genes on each treatment group. **4.5. Hierarchical**

clustering of genes discriminating positive and negative response to treatment. Gene expression profile of the eight most informative genes on each response group. Only strains deemed to have a response to treatment are shown. In both figures, color denotes direction of gene expression changes (red = up-regulated, blue = down-regulated). Intensity illustrates the magnitude of change in gene expression

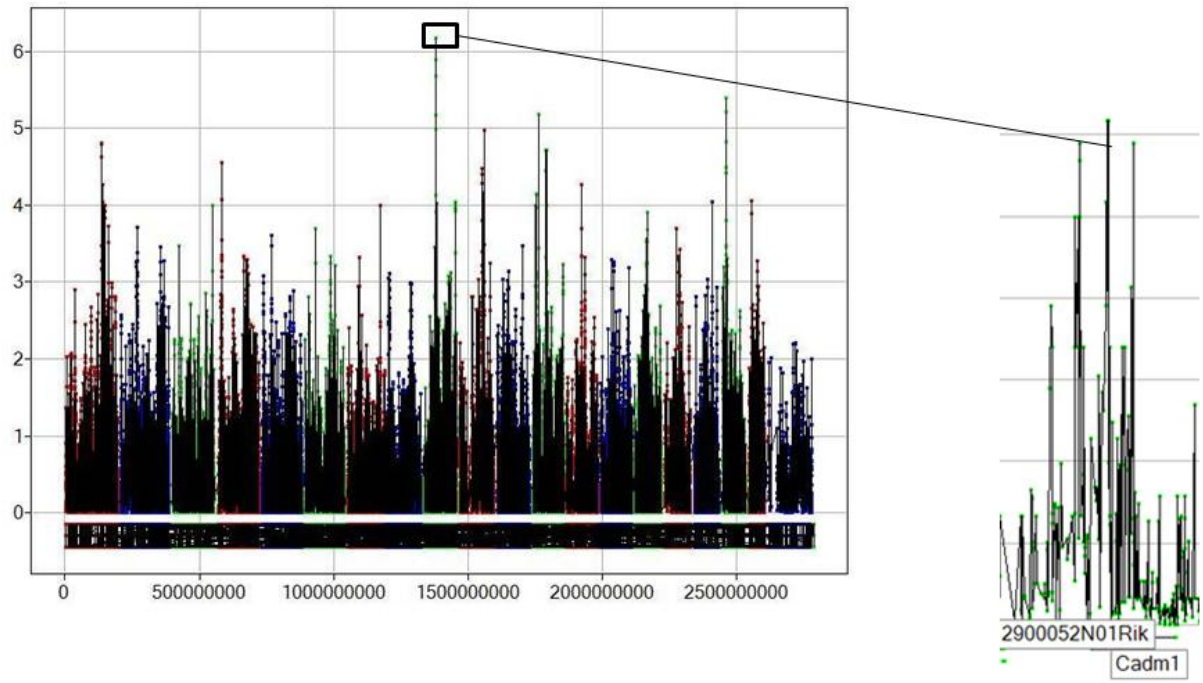


Figure 4.6 Genome-wide association plot for depressive-like behavior

Figure 4.6. Genome-wide association plot for depressive-like behavior. Genomic region on Chr. 9 significantly correlates with depressive-like behavior (-logP = 6.17) and baseline levels of VEGF (-log = 5.56) and CREB (-logP = 4.46). The figure shows baseline behavioral despair QTL. To the right, are the putative genes underneath behavioral locus on Chr 9. The y-axis denotes the strength of association between genotype and phenotype (-logP) and the x-axis illustrates the cumulative SNP position on the genome

7. Supplementary Tables

Supplementary Table 4.1 Mean water intake between water and fluoxetine-treated mice

Supplementary Table 4.1. Mean water intake for vehicle and fluoxetine treated mice.

Strain	Mean Water Intake Per Cage (mL), Control	SEM	Mean Water Intake Per Cage (mL), Fluoxetine	SEM	Difference in Mean Water Intake between Treatment Groups (mL)
129S1/SvImJ	5.2	0.2	3.7	0.3	1.43
A/J	5.3	0.2	4.7	0.2	0.63
AKR/J	7.4	0.2	5.4	0.2	1.98
BALB/cJ	5.7	0.3	5.1	0.3	0.61
BTBRT<t>tf/J	7.1	0.2	6.4	0.2	0.71
BUB/BnJ	7.3	0.4	5.8	0.2	1.53
C3H/HeJ	5	0.2	4.7	0.2	0.27
C57BL/6J	6	0.2	4.5	0.2	1.51
C57BLKS/J	6.1	0.2	4.7	0.2	1.39
C57BR/cdJ	8.9	0.3	8	0.3	0.92
C58/J	5.7	0.2	4.6	0.2	1.08
CBA/J	6.1	0.2	4.9	0.2	1.14
DBA/2J	6.4	0.2	6	0.2	0.37
FVB/NJ	5.4	0.3	5.2	0.2	0.25
I/LnJ	6.4	0.2	4.8	0.2	1.57
LG/J	8.9	0.4	5.8	0.2	3.1
LP/J	3.7	0.2	3.6	0.2	0.14
MA/MyJ	6.7	0.2	5	0.2	1.69
MRL/MpJ	13.2	0.4	8.2	0.2	4.98
NOD/LtJ	7.1	0.2	5.1	0.2	2.04
NOR/LtJ	5.8	0.1	6.6	0.2	0.84
NZB/BlNJ	6	0.2	4.9	0.2	1.16
NZW/LacJ	9.7	0.2	11.2	0.5	1.53
P/J	5.2	0.2	4.9	0.2	0.25
PL/J	5.7	0.2	3.9	0.2	1.82
RIIIS/J	4.2	0.2	4.1	0.2	0.12
SJL/J	7.2	0.3	5.4	0.3	1.76
SM/J	5.4	0.2	5.9	0.3	0.53

SWR/J	**6.9**	**0.3**	**5**	**0.1**	**1.92**
SEM = standard error of the mean					

Supplementary Table 4.2 Behavioral responses in the open field and tail suspension tests

Supplementary Table 4.2. Behavioral responses in the open field and tail suspension tests between control and fluoxetine treatment groups

Strain	Total number of animals tested per strain	Number of animals excluded from the TST analysis	Mean TST Immobility Scores (secs), Control	SEM	Mean TST Immobility Scores (secs), Fluoxetine	SEM
129S1/SvImJ	28	2	122.5	11.87	115.32	10.78
A/J	30	3	106.87	11.66	115.44	5.35
AKR/J	18	2	192.57	10.27	171.21	15.63
BALB/cJ	39	4	107.22	10.64	56.57	7.87
BTBRT<t>tf/J	21	4	15.71	8.83	7.34	4.27
BUB/BnJ	18	1	142.32	10.15	97.28	10.46
C3H/HeJ	30	2	97.34	8.79	112.25	12.32
C57BL/6J	21	5	175.7	18.64	103.5	11.7
C57BLKS/J	24	3	101.31	13.59	72.65	14.84
C57BR/cdJ	27	5	97.51	19.6	33.28	10.39
C58/J	18	0	80.92	13.1	83.22	7.18
CBA/J	30	2	134.98	8.78	44.93	8.53
CE/J	24	3	108.83	16.46	76.9	9.93
DBA/2J	36	9	44.35	9.99	19.06	4.75
FVB/NJ	18	2	53.29	13.63	70.42	11.52
I/LnJ	24	8	79.2	28.57	77.19	18.43
LG/J	21	3	27.25	13.39	4.5	1.42
LP/J	28	9	196.7	13.47	177.73	19.51
MA/MyJ	18	0	79.07	18.01	33.92	10.53
MRL/MpJ	21	4	16.02	5.15	14.65	5.39
NOD/LtJ	24	3	165.4	8.65	141.18	7.44
NOR/LtJ	24	7	104.95	9.85	84.58	18.89
NZB/BlNJ	24	6	161.27	31.01	201.34	8.47
NZW/LacJ	21	4	124.69	19.72	161.92	17.14
P/J	23	3	130.34	17.48	40.71	10.13
PL/J	24	2	169.98	11.91	137.62	11.04
RIIIS/J	21	0	18.58	4.63	32.78	10.98
SJL/J	24	4	143.67	13.13	145.7	15.44
SM/J	18	4	229.53	6.38	137.22	13.29
SWR/J	24	0	50.3	9.91	69.65	12.38

TST = tail suspension test; OF = open field; SEM = standard error of the mean

Supplementary Table 4.2. Behavioral responses in the open field and tail suspension tests between control and fluoxetine treatment groups

Strain	Mean OF Center Time (secs), Control	SEM	Mean OF Center Tme (secs), Fluoxetine	SEM	Mean Total Distance Traveled in the OF, Control (cm)	SEM	Mean Total Distance Traveled in the OF, Fluoxetine (cm)	SEM
129S1/SvImJ	23	17.68	2.33	1.57	450.21	98.51	360.18	87.55
A/J	1.2	1.08	6.6	5.9	257.54	59.18	293.36	76.43
AKR/J	70.89	10.84	71	15.52	1905.12	173.29	1824.08	119.5
BALB/cJ	46.89	23.32	30.82	10.52	968.82	268.46	1018.54	211.55
BTBRT<t>tf/J	56.22	11.15	48.25	9.33	1909.67	192.62	2131.04	237.36
BUB/BnJ	111.88	13.99	147.78	14.61	1606.96	145.31	2392.91	272.73
C3H/HeJ	65	6.84	63.73	5.89	1104.1	60.65	1079.79	42.79
C57BL/6J	77.17	10.82	65.78	9	1128.44	74.57	1174.74	105.63
C57BLKS/J	38.8	6.06	37.25	6.74	775.09	77.77	1004.9	190.83
C57BR/cdJ	193	15.77	156	15.22	2134.34	109.86	2712.47	206.62
C58/J	124.33	15.88	105.56	15.98	1998.07	240	2972.77	583.85
CBA/J	70.47	11.5	56.38	12.35	1051.15	106.42	1506.63	103.85
CE/J	79.22	11.95	89.5	7.62	1546.18	118.34	1518.35	140.48
DBA/2J	33.83	5.84	15.59	3.36	1085.46	84.39	1824.32	255.39
FVB/NJ	136	6.8	112.78	7.29	2209.28	148.26	1869.78	107.35
I/LnJ	60.82	8.54	40.5	12.17	2065.28	124.35	2040.47	284.61
LG/J	85.91	15.99	44.44	21.99	1677.07	97.9	1271.03	63.31
LP/J	56.11	27.35	28.71	8.21	1011	195.36	611.74	90.84
MA/MyJ	131.56	12.53	76.78	14.96	2349.18	182.85	3511.82	395.18
MRL/MpJ	107.91	11.43	85.75	18.27	1471.85	67.62	1395.69	137.08
NOD/LtJ	164.55	18.7	146.64	12.53	2669.31	229.64	2555.08	172.98
NOR/LtJ	145.13	12.13	157.21	14.98	2410.6	180.58	2282.15	94.59
NZB/BlNJ	33.55	6.97	13.55	4.06	1070.27	63.04	942.25	123.13
NZW/LacJ	76.33	7.65	80.56	16.65	1078.48	54.64	1350.67	162.1
P/J	114.67	20.32	59.8	13.12	1807.26	56.44	2445.75	300.53
PL/J	71.33	4.71	57.17	4.46	1840.41	106.79	1770.16	113.83
RIIIS/J	69.22	4.09	79.92	6.57	2050.63	100.38	1824.96	84.83
SJL/J	109.18	37.19	27	17.67	945.1	106.2	853.45	78.73
SM/J	70.63	5.86	38.44	7.68	1999.52	71.84	3180.84	641
SWR/J	91.25	5.4	105.17	11.02	1393.46	83.01	1499.29	67.56

TST = tail suspension test; OF = open field; SEM = standard error of the mean

Supplementary Table 4.3 Serum levels of fluoxetine and its metabolite norfluoxetine following chronic fluoxetine treatment.

Supplementary Table 4.3. Serum Levels of Fluoxetine and its Metabolite Norfluoxetine Following Chronic Fluoxetine Treatment					
Strain	**Number of Animals (N)**	**Fluoxetine (ng/ml)**	**SEM**	**Norfluoxetine (ng/ml)**	**SEM**
129S1/SvImJ	13	1152.92	58.03	1079.77	45.23
A/J	15	937.87	47.72	1222.60	47.96
AKR/J	9	988.11	53.38	1119.22	49.29
BALB/cJ	12	800.17	40.53	930.75	49.93
BTBR T+ tf/J	12	912.08	51.43	1238.50	79.22
BUB/BnJ	9	1396.89	143.39	1391.67	148.09
C3H/HeJ	15	763.07	66.41	949.53	65.12
C57BL/6J	7	1327.57	220.88	1509.00	186.31
C57BLKS/J	12	1243.50	96.14	1195.00	47.55
C57BR/J	10	988.90	151.01	1260.40	153.66
C58/J	9	987.22	116.45	1335.56	105.95
CBA/J	11	1103.91	108.33	1093.27	108.89
CE/J	12	1084.42	73.17	1401.17	74.04
DBA/2J	16	956.50	95.73	1026.69	56.39
FVB/NJ	9	595.19	81.39	798.28	120.00
I/LnJ	8	1606.50	318.33	1438.25	130.94
LG/J	9	1540.00	116.96	1612.22	134.95
LP/J	19	719.63	67.32	783.37	73.67
MA/MyJ	9	1905.56	223.93	1314.33	120.01
MRL/MpJ	9	1066.67	183.54	1047.44	143.05
NOD/LtJ	12	775.50	81.93	912.00	80.58
NOR/LtJ	15	483.33	48.37	746.93	65.22
NZB/B1NJ	12	1311.08	142.63	1156.33	81.24
NZW/LacJ	9	591.44	66.62	762.89	48.00
P/J	11	1885.55	407.27	1267.55	246.70
PL/J	12	757.35	97.23	825.86	111.87
RIIIS/J	12	470.25	36.51	578.58	25.83
SJL/J	11	1274.82	107.88	1124.45	64.77
SM/J	8	1186.95	320.21	1160.23	256.45
SWR/J	12	749.31	110.69	643.79	108.63
SEM = standard error of the mean					

Supplementary Table 4.4 Mean relative fluorescence intensity (RFI) values for thirty-one neurobiochemical analytes measured in the cortex of thirty mouse inbred strains

Supplementary Table 4. 4 Mean relative flourescence intensity (RFI) values for thirty-one neurobiochemical analytes measured in the cortex of thirty mouse inbred strains												
Strains	**ACTH#**	**STD**	**ACTH***	**STD**	**FUT9#**	**STD**	**FUT9***	**STD**	**APOD#**	**STD**	**APOD***	**STD**
129S1/SvImJ	1.80	0.07	1.62	0.11	0.65	0.02	0.50	0.02	0.60	0.01	0.56	0.01
A/J	1.86	0.07	2.04	0.07	0.59	0.02	0.60	0.02	0.52	0.01	0.59	0.04
AKR/J	1.95	0.11	2.05	0.10	0.68	0.01	0.52	0.02	0.56	0.02	0.52	0.04
C57BL/6J	2.49	0.04	2.17	0.08	0.63	0.03	0.61	0.01	0.56	0.05	0.62	0.01
BALB/cByJ	2.05	0.11	2.18	0.07	0.69	0.02	0.61	0.02	0.54	0.03	0.57	0.02
C57BLKS/J	2.10	0.05	2.22	0.07	0.66	0.02	0.66	0.01	0.54	0.02	0.54	0.02
C57BR/cdJ	2.17	0.07	2.47	0.09	0.66	0.02	0.60	0.02	0.57	0.02	0.61	0.03
BTBRT+tf/J	2.25	0.05	2.12	0.05	0.74	0.02	0.60	0.02	0.52	0.01	0.57	0.01
BUB/BnJ	2.28	0.07	2.21	0.10	0.76	0.03	0.71	0.02	0.59	0.01	0.58	0.02
C3H/HeJ	2.26	0.06	2.40	0.05	0.77	0.03	0.55	0.01	0.60	0.02	0.61	0.03
C58/J	2.05	0.05	2.18	0.07	0.73	0.02	0.69	0.02	0.52	0.01	0.53	0.02
CBA/J	2.41	0.07	2.41	0.09	0.78	0.03	0.57	0.02	0.60	0.02	0.66	0.02
CE/J	2.07	0.04	2.08	0.14	0.70	0.01	0.58	0.02	0.56	0.01	0.63	0.01
DBA/2J	1.73	0.04	2.08	0.08	0.57	0.01	0.54	0.01	0.50	0.01	0.56	0.03
FVB/NJ	2.21	0.10	2.34	0.09	0.71	0.03	0.72	0.04	0.63	0.02	0.71	0.03
I/LnJ	2.01	0.07	2.03	0.12	0.75	0.03	0.61	0.02	0.53	0.03	0.57	0.01
LG/J	2.22	0.04	2.50	0.08	0.77	0.02	0.65	0.02	0.57	0.02	0.65	0.02
LP/J	2.45	0.09	1.39	0.07	0.86	0.02	0.45	0.01	0.60	0.01	0.39	0.01
MA/MyJ	1.76	0.06	2.22	0.06	0.72	0.02	0.67	0.02	0.50	0.02	0.64	0.01
MRL/MpJ	2.12	0.06	2.25	0.07	0.70	0.02	0.56	0.02	0.56	0.01	0.67	0.03
NOD/ShiLtJ	2.30	0.05	2.35	0.08	0.83	0.03	0.70	0.01	0.61	0.02	0.71	0.03
NOR/LtJ	1.70	0.06	1.97	0.07	0.65	0.02	0.66	0.02	0.48	0.02	0.62	0.02
NZB/BINJ	2.49	0.06	2.23	0.12	0.84	0.02	0.59	0.01	0.58	0.02	0.65	0.02
NZW/LacJ	2.87	0.11	1.67	0.08	0.87	0.02	0.53	0.01	0.70	0.02	0.47	0.04
P/J	2.22	0.06	2.37	0.07	0.76	0.02	0.80	0.02	0.57	0.02	0.66	0.01
PL/J	2.09	0.06	2.33	0.09	0.71	0.01	0.58	0.02	0.62	0.02	0.70	0.03
RIIIS/J	2.47	0.08	2.30	0.07	0.71	0.01	0.70	0.02	0.65	0.01	0.64	0.02
SJL/J	2.12	0.06	2.08	0.07	0.72	0.02	0.70	0.02	0.62	0.02	0.68	0.02
SM/J	2.11	0.05	2.11	0.06	0.67	0.01	0.69	0.01	0.62	0.01	0.72	0.04
SWR/J	2.26	0.07	2.59	0.08	0.69	0.01	0.75	0.02	0.62	0.03	0.69	0.03

# Denotes RFI values for control mice (water); * Denotes RFI values for fluoxetine-treated mice. STD stands for standard deviation. ACTH = Adrenocorticotropin releasing hormone; APOD= Apolipoprotein D; BAG1= BCL2-associated athanogene 1; BDNF= Brain-derived neurotrophic factor; CREB=cAMP response element-binding; PPP1R1B= Protein phosphatase 1 subunit 1B; DAT= Dopamine transporter; GAD67 = Glutamic acid decarboxylase-67 ; GAL=Galanin; GFAP=Glial fibrillary acidic protein; GLO1=Glyoxylase1; GNB1=Guanine nucleotide-binding protein subunit beta-1; GSK3β=Glycogen synthase kinase 3 beta; GSR=Glutathione reductase; HDAC5= Histone deacetylase 5; KCNJ9=Potassium inwardly-rectifying channel, subfamily J, member 9; MCH=Melanin concentrating hormone; NET=Norepinephrine transporter; NGF=Nerve growth factor; NMDA1=Glutamate receptor; NPY=Neuropeptide Y; P2X7= Purinergic receptor ligand-gated ion channel7; PAQR8=Progestin and adipoQ receptor 8; PTH=Parathyroid hormone; S100β=S100 beta protein; SERT=Serotonin transporter; SGNE=Secretogranin V; TNF-αTumor necrosis factor-alpha; VEGF=Vascular endothelial growth factor

Supplementary Table 4.4 Mean relative flourescence intensity (RFI) values for thirty-one neurobiochemical analytes measured in the cortex of thirty mouse inbred strains

Strains	BAG1#	STD	BAG1*	STD	BDNF#	STD	BDNF*	STD	c-fos#	STD	c-fos*	STD
129S1/SvImJ	0.15	0.01	0.12	0.01	2.08	0.02	1.74	0.06	0.25	0.01	0.28	0.01
A/J	0.17	0.01	0.16	0.01	1.75	0.04	1.65	0.04	0.25	0.01	0.30	0.01
AKR/J	0.17	0.01	0.15	0.01	2.09	0.05	1.73	0.05	0.27	0.01	0.30	0.01
C57BL/6J	0.15	0.01	0.17	0.01	1.74	0.06	2.13	0.06	0.29	0.02	0.31	0.01
BALB/cByJ	0.17	0.01	0.15	0.01	1.80	0.09	1.71	0.08	0.29	0.01	0.35	0.01
C57BLKS/J	0.18	0.01	0.16	0.01	2.08	0.04	2.14	0.06	0.26	0.01	0.28	0.02
C57BR/cdJ	0.16	0.01	0.19	0.01	2.03	0.08	2.07	0.04	0.27	0.01	0.32	0.02
BTBRT+tf/J	0.19	0.01	0.18	0.01	1.94	0.04	2.05	0.08	0.31	0.01	0.33	0.01
BUB/BnJ	0.19	0.01	0.19	0.01	2.15	0.06	1.86	0.05	0.30	0.01	0.31	0.02
C3H/HeJ	0.17	0.01	0.16	0.01	2.02	0.03	1.62	0.06	0.29	0.01	0.32	0.01
C58/J	0.18	0.01	0.17	0.01	1.92	0.04	1.80	0.04	0.29	0.01	0.32	0.02
CBA/J	0.20	0.01	0.18	0.01	2.09	0.03	2.01	0.06	0.32	0.01	0.31	0.01
CE/J	0.15	0.01	0.16	0.01	1.54	0.05	1.71	0.11	0.28	0.01	0.30	0.01
DBA/2J	0.15	0.01	0.17	0.01	1.55	0.07	1.69	0.06	0.27	0.00	0.33	0.01
FVB/NJ	0.18	0.01	0.17	0.01	2.07	0.06	1.91	0.05	0.30	0.01	0.40	0.02
I/LnJ	0.16	0.00	0.16	0.01	1.63	0.05	2.07	0.07	0.28	0.01	0.29	0.01
LG/J	0.18	0.01	0.20	0.01	1.96	0.03	2.14	0.05	0.33	0.01	0.35	0.02
LP/J	0.19	0.01	0.13	0.00	2.11	0.03	1.22	0.06	0.34	0.01	0.23	0.01
MA/MyJ	0.17	0.01	0.18	0.01	1.77	0.05	1.74	0.02	0.24	0.01	0.32	0.01
MRL/MpJ	0.17	0.01	0.20	0.01	1.63	0.03	1.76	0.05	0.31	0.01	0.34	0.01
NOD/ShiLtJ	0.19	0.01	0.19	0.01	1.89	0.06	2.00	0.04	0.30	0.01	0.33	0.02
NOR/LtJ	0.17	0.00	0.17	0.01	1.69	0.08	1.53	0.04	0.25	0.01	0.32	0.01
NZB/BINJ	0.21	0.01	0.19	0.01	1.79	0.04	1.83	0.04	0.36	0.02	0.35	0.01
NZW/LacJ	0.22	0.01	0.14	0.01	2.13	0.06	1.37	0.08	0.40	0.01	0.29	0.01
P/J	0.18	0.01	0.19	0.01	2.13	0.05	1.70	0.02	0.34	0.01	0.32	0.01
PL/J	0.17	0.01	0.21	0.01	1.82	0.03	1.90	0.05	0.33	0.01	0.34	0.02
RIIIS/J	0.19	0.01	0.211	0.013	2.2	0.051	2.041	0.052	0.368	0.017	0.362	0.014
SJL/J	0.174	0.01	0.195	0.011	2.065	0.044	1.887	0.043	0.305	0.011	0.29	0.008
SM/J	0.181	0.01	0.217	0.009	1.939	0.055	2.019	0.034	0.31	0.01	0.311	0.012
SWR/J	0.189	0.01	0.224	0.01	1.931	0.041	2.105	0.069	0.299	0.01	0.351	0.017

Supplementary Table 4.4 Mean relative flourescence intensity (RFI) values for thirty-one neurobiochemical analytes measured in the cortex of thirty mouse inbred strains

Strains	CREB#	STD	CREB*	STD	PPP1R1B#	STD	PPP1R1B*	STD	GAD67#	STD	GAD67*	STD
129S1/SvImJ	0.69	0.03	0.62	0.02	1.08	0.03	0.76	0.03	1.3	0.09	1.13	0.06
A/J	0.73	0.03	0.72	0.01	1.02	0.03	0.88	0.03	1.38	0.05	1.4	0.06
AKR/J	0.75	0.07	0.7	0.02	1.17	0.04	0.87	0.03	1.37	0.07	1.21	0.08
C57BL/6J	0.76	0.06	0.77	0.03	1.07	0.09	0.89	0.02	1.27	0.11	1.7	0.05
BALB/cByJ	0.75	0.04	0.71	0.03	1.17	0.04	0.97	0.02	1.56	0.09	1.57	0.06
C57BLKS/J	0.78	0.03	0.69	0.02	0.94	0.05	0.93	0.04	1.66	0.04	1.87	0.08
C57BR/cdJ	0.81	0.02	0.78	0.03	1.23	0.03	1.07	0.05	1.73	0.02	1.72	0.07
BTBRT+tf/J	0.82	0.04	0.75	0.03	1.38	0.04	1.13	0.05	2.09	0.06	1.41	0.06
BUB/BnJ	0.83	0.02	0.77	0.03	1.17	0.04	1.21	0.09	1.97	0.06	1.87	0.1
C3H/HeJ	0.82	0.03	0.76	0.02	1.23	0.05	0.76	0.01	1.85	0.1	1.21	0.04
C58/J	0.76	0.02	0.8	0.04	0.87	0.04	1.12	0.04	1.72	0.05	2.07	0.09
CBA/J	0.88	0.04	0.82	0.03	1.28	0.05	1.25	0.06	2.08	0.08	2.06	0.06
CE/J	0.78	0.04	0.77	0.02	0.95	0.02	0.82	0.03	1.23	0.03	1.58	0.06
DBA/2J	0.71	0.03	0.76	0.02	0.91	0.02	1.03	0.04	1.66	0.05	1.41	0.07
FVB/NJ	0.82	0.04	0.83	0.04	1.17	0.09	1.21	0.08	1.72	0.14	1.58	0.09
I/LnJ	0.79	0.02	0.7	0.03	1.02	0.03	0.91	0.05	1.57	0.03	1.26	0.06
LG/J	0.85	0.03	0.85	0.05	1.22	0.04	0.87	0.05	1.6	0.06	1.5	0.08
LP/J	0.84	0.03	0.47	0.03	1.28	0.07	0.52	0.02	1.88	0.07	0.72	0.05
MA/MyJ	0.7	0.02	0.83	0.04	1.04	0.03	1.17	0.05	1.67	0.06	1.81	0.07
MRL/MpJ	0.82	0.02	0.85	0.03	0.82	0.05	0.8	0.04	1.85	0.07	1.31	0.05
NOD/ShiLtJ	0.83	0.04	0.78	0.02	1.17	0.05	1.16	0.07	1.84	0.08	1.41	0.06
NOR/LtJ	0.65	0.03	0.73	0.02	1.05	0.03	0.98	0.03	1.5	0.04	1.5	0.06
NZB/BINJ	0.89	0.03	0.8	0.03	1.27	0.06	1	0.05	1.18	0.03	1.23	0.05
NZW/LacJ	0.95	0.04	0.53	0.03	1.29	0.08	0.8	0.02	1.4	0.05	1.11	0.04
P/J	0.82	0.02	0.79	0.03	1.05	0.03	1.3	0.05	1.51	0.08	1.64	0.07
PL/J	0.88	0.03	0.73	0.03	1.37	0.06	0.83	0.04	1.81	0.08	1.28	0.05
RIIIS/J	0.96	0.05	0.7	0.03	1.2	0.08	0.92	0.05	1.82	0.08	1.72	0.05
SJL/J	0.81	0.03	0.7	0.03	1.25	0.04	0.86	0.03	1.73	0.05	1.64	0.07
SM/J	0.84	0.03	0.78	0.03	1.05	0.06	0.84	0.04	1.47	0.09	1.99	0.13
SWR/J	0.82	0.02	0.8	0.02	1.32	0.06	1.08	0.06	1.76	0.05	1.75	0.06

Supplementary Table 4.4 Mean relative flourescence intensity (RFI) values for thirty-one neurobiochemical analytes measured in the cortex of thirty mouse inbred strains

Strains	Galanin[#]	STD	Galanin*	STD	GFAP[#]	STD	GFAP*	STD	Ghrelin[#]	STD	Ghrelin*	STD
129S1/SvImJ	1.41	0.03	0.98	0.02	2.25	0.03	1.51	0.03	0.35	0.01	0.24	0.02
A/J	1.40	0.02	1.19	0.02	2.11	0.08	2.41	0.06	0.38	0.01	0.42	0.03
AKR/J	1.51	0.03	0.99	0.03	3.05	0.05	1.51	0.06	0.39	0.02	0.39	0.03
C57BL/6J	1.14	0.08	1.42	0.03	1.83	0.19	2.06	0.03	0.31	0.04	0.38	0.01
BALB/cByJ	1.37	0.04	1.06	0.04	3.81	0.13	3.32	0.30	0.37	0.02	0.35	0.03
C57BLKS/J	1.40	0.02	1.48	0.04	1.97	0.06	1.97	0.05	0.35	0.02	0.23	0.02
C57BR/cdJ	1.30	0.03	1.01	0.02	2.31	0.12	2.22	0.07	0.35	0.01	0.32	0.02
BTBRT+tf/J	1.54	0.05	1.05	0.02	1.92	0.03	2.18	0.14	0.42	0.02	0.32	0.02
BUB/BnJ	1.52	0.04	1.06	0.03	2.69	0.13	2.23	0.14	0.44	0.01	0.34	0.01
C3H/HeJ	1.49	0.06	1.04	0.03	2.13	0.04	2.22	0.10	0.41	0.02	0.30	0.02
C58/J	1.51	0.03	1.13	0.03	2.11	0.08	7.94	0.15	0.40	0.01	0.35	0.02
CBA/J	1.58	0.04	1.21	0.02	2.63	0.08	5.02	0.11	0.45	0.01	0.34	0.02
CE/J	1.51	0.04	1.06	0.03	1.96	0.03	1.77	0.06	0.38	0.02	0.30	0.02
DBA/2J	1.30	0.04	0.96	0.02	1.74	0.08	6.81	0.18	0.37	0.02	0.33	0.01
FVB/NJ	1.43	0.05	1.20	0.03	2.67	0.10	3.07	0.16	0.44	0.03	0.35	0.02
I/LnJ	1.53	0.04	0.78	0.01	1.70	0.06	1.72	0.11	0.41	0.03	0.30	0.02
LG/J	1.37	0.02	1.01	0.05	2.53	0.11	2.86	0.14	0.46	0.02	0.38	0.01
LP/J	1.30	0.03	0.78	0.03	2.15	0.06	1.21	0.07	0.49	0.03	0.15	0.01
MA/MyJ	1.50	0.03	0.89	0.01	2.47	0.11	3.74	0.13	0.39	0.01	0.35	0.01
MRL/MpJ	2.77	0.12	1.03	0.03	2.60	0.03	3.06	0.20	0.46	0.01	0.32	0.01
NOD/ShiLtJ	2.35	0.09	1.05	0.03	2.87	0.04	2.93	0.20	0.47	0.01	0.33	0.01
NOR/LtJ	1.15	0.02	0.95	0.02	2.20	0.15	2.22	0.12	0.40	0.01	0.31	0.01
NZB/BINJ	1.13	0.03	0.96	0.02	3.08	0.17	2.56	0.13	0.50	0.01	0.31	0.01
NZW/LacJ	1.77	0.07	0.93	0.03	3.48	0.09	1.34	0.10	0.53	0.02	0.21	0.01
P/J	1.27	0.06	0.88	0.02	2.56	0.04	6.50	0.17	0.49	0.02	0.38	0.02
PL/J	1.32	0.02	0.98	0.02	2.09	0.05	2.42	0.09	0.49	0.02	0.33	0.01
RIIIS/J	1.41	0.05	1.08	0.02	5.47	0.14	3.13	0.18	0.45	0.01	0.34	0.01
SJL/J	1.34	0.04	1.05	0.02	3.16	0.04	3.04	0.20	0.47	0.01	0.31	0.01
SM/J	1.48	0.05	1.19	0.03	2.08	0.02	3.17	0.09	0.46	0.02	0.37	0.01
SWR/J	1.47	0.02	1.15	0.02	2.87	0.18	3.50	0.36	0.39	0.01	0.38	0.01

Supplementary Table 4.4 Mean relative flourescence intensity (RFI) values for thirty-one neurobiochemical analytes measured in the cortex of thirty mouse inbred strains

Strains	KCNJ9#	STD	KCNJ9*	STD	GSR#	STD	GSR*	STD	GLO1#	STD	GLO1*	STD
129S1/SvImJ	0.36	0.01	0.33	0.01	0.85	0.03	0.53	0.04	3.49	0.18	1.77	0.26
A/J	0.36	0.01	0.35	0.01	0.72	0.01	0.66	0.02	6.21	0.16	3.61	0.46
AKR/J	0.35	0.01	0.33	0.01	0.87	0.02	0.62	0.03	5.93	0.20	3.83	0.28
C57BL/6J	0.35	0.02	0.34	0.01	0.59	0.02	0.66	0.02	2.96	0.40	2.21	0.15
BALB/cByJ	0.37	0.01	0.33	0.01	0.76	0.03	0.72	0.02	4.04	0.31	2.99	0.27
C57BLKS/J	0.30	0.01	0.33	0.01	0.65	0.02	0.64	0.03	5.02	0.19	3.57	0.14
C57BR/cdJ	0.33	0.01	0.34	0.01	0.76	0.01	0.61	0.02	3.10	0.10	3.35	0.15
BTBRT+tf/J	0.37	0.01	0.36	0.01	0.83	0.02	0.65	0.02	4.17	0.13	3.22	0.12
BUB/BnJ	0.34	0.01	0.36	0.02	0.87	0.01	0.78	0.04	4.34	0.19	3.67	0.12
C3H/HeJ	0.38	0.01	0.37	0.01	0.87	0.03	0.61	0.02	4.38	0.25	3.71	0.16
C58/J	0.37	0.01	0.37	0.01	0.75	0.02	0.67	0.03	3.78	0.14	3.27	0.06
CBA/J	0.37	0.01	0.38	0.01	0.91	0.03	0.81	0.04	5.70	0.15	4.64	0.18
CE/J	0.34	0.01	0.32	0.01	0.78	0.02	0.67	0.02	4.84	0.12	4.43	0.29
DBA/2J	0.28	0.01	0.31	0.01	0.80	0.02	0.62	0.01	4.04	0.21	4.17	0.15
FVB/NJ	0.34	0.02	0.35	0.02	0.74	0.05	0.71	0.04	4.61	0.21	3.57	0.27
I/LnJ	0.35	0.01	0.35	0.01	0.68	0.01	0.63	0.02	4.11	0.16	3.60	0.31
LG/J	0.36	0.01	0.39	0.01	0.76	0.01	0.77	0.03	4.27	0.18	4.11	0.14
LP/J	0.38	0.01	0.26	0.01	0.93	0.02	0.40	0.02	6.51	0.17	2.09	0.21
MA/MyJ	0.36	0.01	0.32	0.02	0.82	0.03	0.76	0.03	2.88	0.11	3.73	0.10
MRL/MpJ	0.36	0.01	0.33	0.01	0.82	0.03	0.72	0.02	4.91	0.18	5.31	0.14
NOD/ShiLtJ	0.35	0.01	0.36	0.01	0.85	0.02	0.77	0.02	4.34	0.21	4.22	0.16
NOR/LtJ	0.32	0.01	0.32	0.01	0.80	0.03	0.76	0.02	2.66	0.16	3.43	0.14
NZB/BINJ	0.40	0.01	0.33	0.01	0.65	0.02	0.64	0.02	4.98	0.18	4.09	0.10
NZW/LacJ	0.41	0.02	0.27	0.01	0.75	0.01	0.45	0.02	5.37	0.16	2.75	0.22
P/J	0.40	0.02	0.35	0.01	0.93	0.02	0.61	0.02	4.01	0.21	4.66	0.12
PL/J	0.36	0.01	0.36	0.01	0.84	0.02	0.71	0.02	4.35	0.10	4.17	0.10
RIIIS/J	0.37	0.01	0.38	0.02	0.83	0.03	0.80	0.03	4.98	0.18	4.07	0.18
SJL/J	0.36	0.01	0.33	0.01	0.93	0.02	0.84	0.03	3.86	0.19	3.41	0.14
SM/J	0.39	0.01	0.33	0.02	0.79	0.02	0.84	0.05	4.39	0.20	3.63	0.14
SWR/J	0.35	0.01	0.38	0.01	0.78	0.02	0.84	0.04	4.26	0.12	4.72	0.27

Supplementary Table 4.4 Mean relative flourescence intensity (RFI) values for thirty-one neurobiochemical analytes measured in the cortex of thirty mouse inbred strains

Strains	GNB1 #	STD	GNB1 *	STD	GSK3β #	STD	GSK3β *	STD	HDAC5#	STD	HDAC5*	STD
129S1/SvImJ	7.79	0.78	8.43	0.65	0.87	0.05	0.67	0.04	2.04	0.09	1.63	0.10
A/J	7.04	1.02	8.57	1.04	0.93	0.04	0.46	0.02	2.52	0.05	1.67	0.07
AKR/J	8.02	0.94	8.39	0.61	0.71	0.04	0.67	0.03	2.01	0.08	2.12	0.10
C57BL/6J	7.98	0.19	8.93	0.46	0.72	0.10	0.69	0.02	2.65	0.14	1.86	0.07
BALB/cByJ	5.48	0.55	7.90	0.87	0.74	0.05	0.65	0.05	2.37	0.07	2.16	0.06
C57BLKS/J	6.58	0.66	6.76	0.78	0.72	0.01	1.15	0.04	2.13	0.10	2.52	0.13
C57BR/cdJ	6.35	0.50	6.03	0.22	1.09	0.06	0.88	0.03	2.36	0.06	2.45	0.11
BTBRT+tf/J	9.31	0.30	5.99	0.41	0.83	0.02	1.57	0.05	2.55	0.08	3.30	0.16
BUB/BnJ	9.14	0.66	11.55	0.94	0.77	0.04	1.13	0.02	2.24	0.05	2.53	0.17
C3H/HeJ	7.24	0.38	9.70	0.86	1.01	0.03	0.55	0.03	2.87	0.18	2.13	0.06
C58/J	5.68	0.44	7.71	0.41	0.92	0.03	0.82	0.02	2.57	0.10	2.42	0.11
CBA/J	10.55	0.48	8.31	0.20	0.89	0.01	1.01	0.03	2.78	0.15	2.68	0.12
CE/J	8.51	0.58	7.64	0.62	0.68	0.01	0.51	0.04	2.21	0.06	1.96	0.08
DBA/2J	7.52	0.71	7.09	0.45	0.68	0.03	0.81	0.03	1.98	0.05	2.17	0.07
FVB/NJ	6.89	0.63	8.60	1.06	0.91	0.03	0.70	0.04	2.64	0.20	2.59	0.17
I/LnJ	10.23	1.26	8.07	1.05	0.53	0.03	0.88	0.10	1.86	0.03	2.32	0.18
LG/J	7.45	0.26	8.15	0.19	1.20	0.02	0.93	0.05	2.76	0.10	2.53	0.10
LP/J	10.81	0.61	7.10	0.40	0.78	0.02	0.26	0.02	2.80	0.11	1.31	0.07
MA/MyJ	6.59	0.73	8.47	0.20	0.86	0.03	1.03	0.02	2.13	0.08	2.47	0.12
MRL/MpJ	9.11	0.69	7.20	0.45	0.72	0.02	0.47	0.03	2.12	0.07	1.80	0.07
NOD/ShiLtJ	9.85	0.88	8.91	0.62	1.59	0.04	1.37	0.04	2.98	0.06	3.05	0.16
NOR/LtJ	6.69	0.75	10.40	0.61	0.83	0.06	0.83	0.04	2.02	0.06	2.57	0.09
NZB/BINJ	14.23	1.11	7.96	0.33	1.45	0.05	0.96	0.03	3.51	0.16	2.65	0.11
NZW/LacJ	11.73	0.66	8.25	0.19	0.58	0.02	0.69	0.05	2.61	0.12	2.14	0.10
P/J	9.02	0.26	11.10	0.37	1.01	0.03	1.68	0.05	2.70	0.10	3.94	0.09
PL/J	10.29	0.56	9.01	0.28	1.11	0.02	0.76	0.03	2.88	0.09	2.35	0.10
RIIIS/J	8.49	0.41	10.41	0.66	1.05	0.02	0.57	0.02	2.62	0.13	2.04	0.11
SJL/J	7.01	0.36	9.86	0.66	1.13	0.02	0.60	0.03	2.63	0.11	2.23	0.11
SM/J	8.31	0.34	7.84	0.39	0.81	0.02	0.60	0.02	2.13	0.07	1.86	0.09
SWR/J	9.77	0.72	12.10	0.34	1.61	0.05	1.15	0.05	3.06	0.11	2.87	0.14

Supplementary Table 4.4 Mean relative flourescence intensity (RFI) values for thirty-one neurobiochemical analytes measured in the cortex of thirty mouse inbred strains

Strains	MCH#	STD	MCH*	STD	NGF#	STD	NGF*	STD	NPY#	STD	NPY*	STD
129S1/SvImJ	0.002	0.008	0.008	0.006	1.78	0.03	1.66	0.04	0.91	0.02	0.47	0.02
A/J	0.012	0.004	0.029	0.008	1.66	0.03	1.94	0.05	0.69	0.02	0.55	0.02
AKR/J	0.015	0.004	0.026	0.006	1.83	0.05	1.88	0.06	0.94	0.03	0.49	0.03
C57BL/6J	-0.004	0.004	0.01	0.005	1.60	0.06	1.62	0.03	0.76	0.05	0.77	0.01
BALB/cByJ	0.009	0.005	0.01	0.004	1.65	0.04	1.82	0.03	0.81	0.03	0.64	0.02
C57BLKS/J	0.014	0.003	0.007	0.005	1.75	0.03	1.83	0.13	0.76	0.02	0.71	0.02
C57BR/cdJ	0.002	0.003	0.009	0.004	1.67	0.03	1.53	0.02	0.97	0.02	0.66	0.03
BTBRT+tf/J	-0.003	0.006	-0.005	0.003	1.52	0.05	1.86	0.02	0.87	0.02	0.63	0.02
BUB/BnJ	0.006	0.005	-0.006	0.005	1.69	0.04	1.65	0.02	0.91	0.03	0.65	0.03
C3H/HeJ	0.01	0.003	0.005	0.004	1.72	0.03	1.54	0.02	0.85	0.03	0.64	0.03
C58/J	0.01	0.003	0.004	0.006	1.65	0.03	1.54	0.04	0.73	0.02	0.67	0.02
CBA/J	0.008	0.004	0	0.002	1.55	0.02	1.87	0.02	0.81	0.02	0.72	0.02
CE/J	0.012	0.002	0.008	0.002	1.62	0.02	1.50	0.04	0.70	0.02	0.57	0.02
DBA/2J	0.012	0.003	0.015	0.004	1.44	0.04	1.54	0.03	0.67	0.03	0.66	0.01
FVB/NJ	0.014	0.004	0.008	0.005	1.75	0.02	1.69	0.13	0.77	0.04	0.64	0.04
I/LnJ	0.02	0.004	0.025	0.003	1.54	0.02	1.48	0.04	0.69	0.03	0.56	0.03
LG/J	0.016	0.004	0.021	0.004	1.69	0.04	1.61	0.02	0.69	0.03	0.60	0.02
LP/J	0.029	0.003	0.001	0.004	1.83	0.02	1.52	0.04	0.70	0.03	0.32	0.02
MA/MyJ	0.03	0.003	0.013	0.004	1.64	0.04	1.39	0.01	0.74	0.02	0.71	0.02
MRL/MpJ	0.011	0.003	-0.005	0.004	1.57	0.02	1.56	0.04	0.91	0.03	0.58	0.01
NOD/ShiLtJ	0.022	0.003	0.006	0.002	1.62	0.02	1.75	0.04	0.98	0.02	0.70	0.02
NOR/LtJ	0.011	0.002	0.003	0.003	1.55	0.04	1.44	0.02	0.69	0.03	0.59	0.02
NZB/BINJ	0.003	0.006	0.001	0.003	1.71	0.02	1.62	0.02	0.66	0.02	0.59	0.01
NZW/LacJ	0.015	0.006	-0.008	0.003	1.71	0.03	1.43	0.04	0.80	0.03	0.43	0.02
P/J	0.001	0.004	0.007	0.004	1.73	0.03	1.41	0.02	0.78	0.02	0.57	0.02
PL/J	0.017	0.004	-0.001	0.003	1.56	0.02	1.63	0.02	0.80	0.02	0.59	0.02
RIIIS/J	0.022	0.003	-0.004	0.005	1.73	0.04	1.68	0.02	0.84	0.03	0.64	0.01
SJL/J	0.021	0.003	-0.003	0.003	1.62	0.02	1.57	0.02	0.80	0.03	0.55	0.02
SM/J	0.019	0.004	0.001	0.002	1.67	0.03	1.57	0.03	0.81	0.03	0.72	0.03
SWR/J	0.006	0.002	0.004	0.003	1.64	0.03	1.81	0.02	0.74	0.01	0.65	0.02

Supplementary Table 4.4 Mean relative flourescence intensity (RFI) values for thirty-one neurobiochemical analytes measured in the cortex of thirty mouse inbred strains

Strains	NMDA1#	STD	NMDA1*	STD	NET#	STD	NET*	STD	P2X7#	STD	P2X7*	STD
129S1/SvImJ	1.04	0.06	0.73	0.05	0.49	0.02	0.40	0.01	2.76	0.06	2.22	0.11
A/J	0.96	0.04	0.92	0.05	0.40	0.01	0.46	0.01	3.55	0.09	2.67	0.08
AKR/J	1.15	0.05	0.89	0.04	0.46	0.01	0.47	0.02	3.20	0.06	2.81	0.07
C57BL/6J	0.80	0.02	1.09	0.03	0.42	0.03	0.53	0.02	3.43	0.16	2.61	0.05
BALB/cByJ	0.88	0.05	0.98	0.03	0.44	0.02	0.47	0.01	3.73	0.13	2.99	0.07
C57BLKS/J	0.98	0.04	0.99	0.04	0.44	0.01	0.49	0.03	3.21	0.06	2.84	0.10
C57BR/cdJ	0.93	0.02	0.87	0.03	0.46	0.02	0.53	0.02	3.07	0.05	3.28	0.10
BTBRT+tf/J	1.19	0.02	0.89	0.03	0.50	0.01	0.48	0.01	3.93	0.09	3.42	0.08
BUB/BnJ	1.03	0.04	1.04	0.05	0.47	0.01	0.52	0.04	3.49	0.12	2.79	0.12
C3H/HeJ	0.93	0.04	0.60	0.03	0.41	0.02	0.48	0.02	3.62	0.14	3.38	0.08
C58/J	0.84	0.02	0.94	0.04	0.43	0.02	0.48	0.02	3.62	0.13	3.10	0.09
CBA/J	1.08	0.04	1.01	0.04	0.45	0.01	0.52	0.03	3.61	0.11	3.07	0.09
CE/J	0.72	0.02	0.80	0.03	0.39	0.01	0.43	0.01	3.42	0.05	2.98	0.07
DBA/2J	0.81	0.03	0.84	0.04	0.40	0.01	0.45	0.01	3.01	0.07	2.65	0.05
FVB/NJ	0.91	0.06	0.96	0.06	0.42	0.03	0.50	0.04	3.65	0.14	3.32	0.15
I/LnJ	0.87	0.03	0.72	0.06	0.39	0.01	0.37	0.01	3.07	0.08	3.00	0.08
LG/J	0.85	0.02	0.98	0.04	0.44	0.02	0.47	0.02	3.63	0.07	3.74	0.13
LP/J	1.10	0.03	0.45	0.02	0.45	0.02	0.29	0.01	4.12	0.10	2.32	0.08
MA/MyJ	0.86	0.03	0.98	0.06	0.40	0.02	0.49	0.01	3.28	0.08	2.97	0.09
MRL/MpJ	1.13	0.03	0.80	0.02	0.51	0.02	0.47	0.02	3.19	0.07	3.15	0.08
NOD/ShiLtJ	1.13	0.06	0.90	0.03	0.53	0.02	0.50	0.02	3.18	0.12	3.31	0.09
NOR/LtJ	0.91	0.03	0.93	0.05	0.38	0.01	0.42	0.02	3.03	0.08	3.05	0.05
NZB/BINJ	0.90	0.04	0.81	0.03	0.49	0.02	0.45	0.02	4.25	0.13	3.26	0.12
NZW/LacJ	1.20	0.05	0.65	0.02	0.54	0.03	0.35	0.01	4.27	0.13	2.35	0.10
P/J	0.94	0.04	1.08	0.03	0.50	0.03	0.44	0.03	4.04	0.14	3.69	0.10
PL/J	1.18	0.05	0.81	0.04	0.48	0.02	0.49	0.02	3.27	0.10	3.53	0.10
RIIIS/J	1.15	0.05	0.91	0.04	0.53	0.03	0.50	0.02	3.54	0.12	3.33	0.13
SJL/J	1.03	0.04	0.97	0.03	0.48	0.03	0.45	0.02	3.45	0.09	3.45	0.10
SM/J	1.04	0.04	1.17	0.05	0.47	0.02	0.52	0.03	3.00	0.08	2.72	0.11
SWR/J	0.99	0.04	0.94	0.02	0.46	0.01	0.50	0.02	2.98	0.07	3.67	0.09

Supplementary Table 4.4 Mean relative flourescence intensity (RFI) values for thirty-one neurobiochemical analytes measured in the cortex of thirty mouse inbred strains

Strains	PTH#	STD	PTH*	STD	PAQR8#	STD	PAQR8*	STD	S100β#	STD	S100β*	STD
129S1/SvImJ	0.72	0.07	0.78	0.08	0.48	0.04	0.32	0.03	3.58	0.31	1.85	0.15
A/J	0.62	0.03	0.61	0.03	0.28	0.02	0.47	0.02	2.91	0.14	3.84	0.33
AKR/J	0.80	0.04	0.72	0.06	0.47	0.03	0.39	0.01	4.30	0.22	2.41	0.29
C57BL/6J	0.79	0.09	0.86	0.09	0.48	0.04	0.55	0.04	1.18	0.20	4.47	0.21
BALB/cByJ	0.65	0.03	0.52	0.02	0.36	0.06	0.47	0.02	3.17	0.39	3.35	0.30
C57BLKS/J	0.77	0.02	0.70	0.05	0.45	0.02	0.42	0.02	3.67	0.12	4.08	0.46
C57BR/cdJ	0.84	0.05	0.90	0.05	0.59	0.03	0.58	0.01	5.06	0.22	3.99	0.21
BTBRT+tf/J	0.73	0.01	0.58	0.01	0.50	0.01	0.51	0.02	3.96	0.16	3.97	0.23
BUB/BnJ	0.81	0.01	0.67	0.06	0.50	0.04	0.49	0.02	4.48	0.48	4.65	0.30
C3H/HeJ	0.90	0.03	0.79	0.05	0.46	0.02	0.49	0.03	2.96	0.10	2.28	0.08
C58/J	0.71	0.02	0.55	0.01	0.41	0.03	0.46	0.02	2.70	0.14	3.69	0.23
CBA/J	0.83	0.01	0.68	0.02	0.46	0.02	0.53	0.03	3.45	0.15	4.21	0.41
CE/J	0.82	0.04	0.70	0.06	0.39	0.02	0.40	0.04	2.50	0.18	3.23	0.37
DBA/2J	0.58	0.02	0.50	0.01	0.31	0.03	0.43	0.04	2.09	0.31	3.18	0.35
FVB/NJ	0.78	0.03	0.80	0.05	0.46	0.02	0.47	0.02	3.08	0.16	3.27	0.11
I/LnJ	0.81	0.05	0.65	0.05	0.41	0.04	0.37	0.03	2.47	0.36	1.66	0.27
LG/J	0.66	0.01	0.58	0.01	0.41	0.02	0.51	0.02	2.54	0.16	3.40	0.20
LP/J	0.83	0.01	0.43	0.01	0.47	0.02	0.23	0.01	2.92	0.11	1.03	0.08
MA/MyJ	0.80	0.04	0.64	0.01	0.41	0.02	0.40	0.01	2.60	0.14	4.12	0.28
MRL/MpJ	0.65	0.03	0.50	0.01	0.39	0.02	0.41	0.05	4.47	0.15	2.67	0.24
NOD/ShiLtJ	0.68	0.06	0.70	0.02	0.46	0.03	0.44	0.02	3.75	0.35	2.47	0.09
NOR/LtJ	0.68	0.04	0.64	0.02	0.45	0.02	0.32	0.03	2.03	0.23	1.03	0.33
NZB/BINJ	0.79	0.02	0.51	0.01	0.51	0.03	0.38	0.02	1.96	0.08	2.70	0.13
NZW/LacJ	0.96	0.02	0.50	0.01	0.66	0.04	0.28	0.01	2.49	0.11	1.86	0.22
P/J	0.89	0.02	0.66	0.01	0.60	0.03	0.38	0.01	2.85	0.10	3.00	0.16
PL/J	0.68	0.01	0.53	0.02	0.52	0.03	0.42	0.02	3.58	0.19	3.07	0.13
RIIIS/J	0.79	0.01	0.68	0.03	0.61	0.05	0.46	0.02	4.05	0.23	3.92	0.28
SJL/J	0.77	0.02	0.66	0.02	0.58	0.03	0.41	0.03	3.91	0.32	2.69	0.18
SM/J	0.69	0.02	0.62	0.02	0.55	0.04	0.53	0.03	3.73	0.22	4.66	0.21
SWR/J	0.84	0.03	0.89	0.04	0.45	0.03	0.49	0.03	2.81	0.28	3.44	0.09

Supplementary Table 4.4 Mean relative flourescence intensity (RFI) values for thirty-one neurobiochemical analytes measured in the cortex of thirty mouse inbred strains

Strains	SGNE#	STD	SGNE*	STD	SERT#	STD	SERT*	STD
129S1/SvImJ	0.50	0.01	0.35	0.01	1.08	0.02	0.81	0.05
A/J	0.40	0.01	0.40	0.01	0.85	0.02	0.84	0.03
AKR/J	0.57	0.02	0.30	0.01	1.08	0.03	0.57	0.02
C57BL/6J	0.46	0.02	0.51	0.01	0.95	0.08	1.01	0.02
BALB/cByJ	0.47	0.01	0.42	0.01	1.08	0.06	0.93	0.03
C57BLKS/J	0.43	0.01	0.38	0.01	0.96	0.03	0.93	0.02
C57BR/cdJ	0.57	0.01	0.47	0.01	1.17	0.03	1.03	0.02
BTBRT+tf/J	0.49	0.01	0.44	0.01	1.10	0.02	1.06	0.02
BUB/BnJ	0.49	0.01	0.38	0.01	1.12	0.03	0.93	0.03
C3H/HeJ	0.49	0.01	0.38	0.01	1.14	0.03	0.84	0.03
C58/J	0.42	0.01	0.46	0.01	0.97	0.02	0.97	0.02
CBA/J	0.48	0.01	0.44	0.01	1.08	0.02	0.97	0.03
CE/J	0.57	0.01	0.38	0.01	0.95	0.02	0.85	0.04
DBA/2J	0.46	0.01	0.43	0.01	0.92	0.06	0.87	0.02
FVB/NJ	0.44	0.02	0.38	0.01	0.97	0.04	0.90	0.02
I/LnJ	0.45	0.01	0.36	0.01	1.02	0.05	0.97	0.03
LG/J	0.48	0.01	0.41	0.01	1.09	0.02	0.87	0.03
LP/J	0.44	0.01	0.26	0.01	1.17	0.02	0.49	0.02
MA/MyJ	0.47	0.01	0.44	0.01	1.00	0.02	1.05	0.02
MRL/MpJ	0.57	0.01	0.41	0.01	1.14	0.03	0.77	0.02
NOD/ShiLtJ	0.50	0.01	0.35	0.01	1.34	0.04	0.88	0.02
NOR/LtJ	0.42	0.01	0.40	0.01	0.92	0.04	0.88	0.02
NZB/BINJ	0.45	0.01	0.44	0.01	1.06	0.03	0.85	0.02
NZW/LacJ	0.40	0.01	0.30	0.01	1.32	0.04	0.62	0.03
P/J	0.46	0.02	0.43	0.01	1.01	0.03	0.91	0.01
PL/J	0.51	0.02	0.40	0.01	1.08	0.03	0.84	0.02
RIIIS/J	0.39	0.01	0.34	0.01	1.16	0.03	0.95	0.02
SJL/J	0.52	0.01	0.37	0.01	1.14	0.03	0.80	0.02
SM/J	0.51	0.01	0.43	0.01	1.07	0.04	0.97	0.02
SWR/J	0.38	0.01	0.34	0.01	1.10	0.04	0.90	0.03

Supplementary Table 4.4 Mean relative flourescence intensity (RFI) values for thirty-one neurobiochemical analytes measured in the cortex of thirty mouse inbred strains								
Strains	**TNF-α#**	**STD**	**TNF-α***	**STD**	**VEGF#**	**STD**	**VEGF***	**STD**
129S1/SvImJ	0.811	0.021	0.393	0.016	0.187	0.010	0.173	0.007
A/J	0.763	0.017	0.531	0.011	0.250	0.008	0.201	0.009
AKR/J	0.821	0.021	0.477	0.015	0.217	0.008	0.183	0.009
C57BL/6J	0.710	0.057	0.555	0.021	0.245	0.010	0.178	0.007
BALB/cByJ	0.729	0.033	0.546	0.018	0.242	0.012	0.220	0.008
C57BLKS/J	0.730	0.016	0.592	0.010	0.202	0.009	0.168	0.010
C57BR/cdJ	0.723	0.018	0.608	0.027	0.202	0.008	0.225	0.014
BTBRT+tf/J	0.815	0.015	0.633	0.012	0.253	0.011	0.228	0.009
BUB/BnJ	0.835	0.030	0.586	0.015	0.221	0.009	0.197	0.012
C3H/HeJ	0.749	0.031	0.609	0.018	0.246	0.015	0.232	0.006
C58/J	0.758	0.023	0.654	0.014	0.249	0.009	0.232	0.010
CBA/J	0.829	0.038	0.610	0.017	0.230	0.008	0.209	0.008
CE/J	0.766	0.019	0.541	0.019	0.285	0.016	0.213	0.006
DBA/2J	0.681	0.035	0.527	0.020	0.232	0.006	0.212	0.007
FVB/NJ	0.718	0.033	0.584	0.029	0.231	0.016	0.209	0.015
I/LnJ	0.754	0.035	0.573	0.024	0.252	0.010	0.227	0.009
LG/J	0.790	0.021	0.687	0.027	0.291	0.011	0.261	0.014
LP/J	0.828	0.021	0.364	0.017	0.261	0.014	0.152	0.003
MA/MyJ	0.750	0.026	0.595	0.018	0.238	0.011	0.254	0.011
MRL/MpJ	0.975	0.014	0.578	0.019	0.276	0.011	0.234	0.008
NOD/ShiLtJ	1.085	0.029	0.597	0.009	0.245	0.009	0.225	0.011
NOR/LtJ	0.716	0.015	0.591	0.011	0.236	0.006	0.240	0.009
NZB/BINJ	0.902	0.021	0.623	0.016	0.332	0.013	0.245	0.015
NZW/LacJ	1.009	0.029	0.439	0.017	0.302	0.014	0.187	0.006
P/J	0.876	0.023	0.633	0.025	0.292	0.012	0.279	0.013
PL/J	0.822	0.016	0.632	0.016	0.289	0.012	0.259	0.013
RIIIS/J	0.877	0.035	0.580	0.018	0.274	0.014	0.206	0.012
SJL/J	0.864	0.019	0.584	0.012	0.273	0.014	0.235	0.016
SM/J	0.803	0.028	0.623	0.018	0.265	0.014	0.219	0.009
SWR/J	0.756	0.022	0.636	0.015	0.236	0.010	0.228	0.010

Supplementary Table 4.5 Pairwise comparisons of behavioral scores between treatment groups

Supplementary Table 4.5 Pairwise comparisons of behavioral scores between treatment groups.				
Strain	**U-Statistic for Percent Time Spent Immobile in the TST**	**Adjusted *p*-values*** **	**U-Statistic for Percent Time Spent in the Center of the OF**	**Adjusted *p*-values*** **
129S1/SvImJ	96	0.537	111.5	0.294
A/J	114	0.264	115	0.917
AKR/J	41	0.345	40	0.965
BALB/cJ	249.5	0.001**	47.5	0.879
BTBRT<t>tf/J	45	0.386	61	0.619
BUB/BnJ	62	0.012*	51.5	0.136
C3H/HeJ	121	0.291	117.5	0.836
C57BL/6J	57	0.007**	63.5	0.5
C57BLKS/J	71	0.26	63	0.843
C57BR/cdJ	95	0.023*	124	0.097
C58/J	42	0.895	53	0.27
CBA/J	190.5	0.00002**	117	0.369
CE/J	69	0.286	70	0.256
DBA/2J	131.5	0.094^	262.5	0.014*
FVB/NJ	43	0.248	64	0.038
I/LnJ	24	0.427	66.5	0.063
LG/J	61	0.070^	77	0.037
LP/J	41.5	0.826	115	0.147
MA/MyJ	63	0.047*	68	0.015*
MRL/MpJ	37	0.923	57.5	0.265
NOD/LtJ	81	0.067^	61	0.974
NOR/LtJ	48	0.205	55.5	0.973
NZB/BlNJ	46	0.594	95.5	0.022
NZW/LacJ	50	0.178	39	0.895
P/J	90	0.001**	79	0.025
PL/J	94	0.025*	102.5	0.078
RIIIS/J	66	0.394	73	0.177
SJL/J	50	0.97	100.5	0.009**
SM/J	49	0.002**	64	0.007**
SWR/J	93	0.225	83	0.525

*** Following Mann-Whitney U test for post-hoc pairwise comparisons of behavioral scores between treatment groups, p-values were adjusted for multiple comparisons using the Benjamini-Hochberg

correction for multiple testing; *** denotes p-values p<0.009; ** denotes p-values p<0.05; ^denotes p-values p<0.10

Supplementary Table 4.6 Covariance of behavior and neurobiochemical levels explained by partial least squares (PLS) latent vectors

Supplementary Table 4.6 Covariance of behavior and neurobiochemical levels explained by partial least squares (PLS) latent vectors		
	Percent covariance explained for behavior and biochemical levels	**Cumulative percent covariance explained for behavior and biochemical levels**
Vehicle		
Vector 1	59	59
Vector 2	29	88
Vector 3	12	100
Fluoxetine		
Vector 1	56	56
Vector 2	33	89
Vector 3	11	100

Supplementary Table 4.7 Informative genes used to predict treatment groups in the k-NN leave-one-out cross-validation test

Supplementary Table 4.7 Informative genes used to predict treatment groups in the kNN leave-one-out cross-validation test	
Genes	**Description**
Gm129	gene model 129
Lgi2	leucine-rich repeat LGI family, member 2
Erf	Ets2 repressor factor
Gnb4	guanine nucleotide binding protein (G protein), beta 4
Cdc42ep3	CDC42 effector protein (Rho GTPase binding) 3
Anp32a	acidic (leucine-rich) nuclear phosphoprotein 32 family, member A
2400009B08Rik	RIKEN cDNA 2400009B08 gene,
Sema4a	sema domain, immunoglobulin domain (Ig), transmembrane domain (TM) and short cytoplasmic domain, (semaphorin) 4A,
Gpc1	glypican 1
Inhba	inhibin beta-A
C030003D03Rik	RIKEN cDNA C030003D03 gene
Sgsm1	small G protein signaling modulator 1

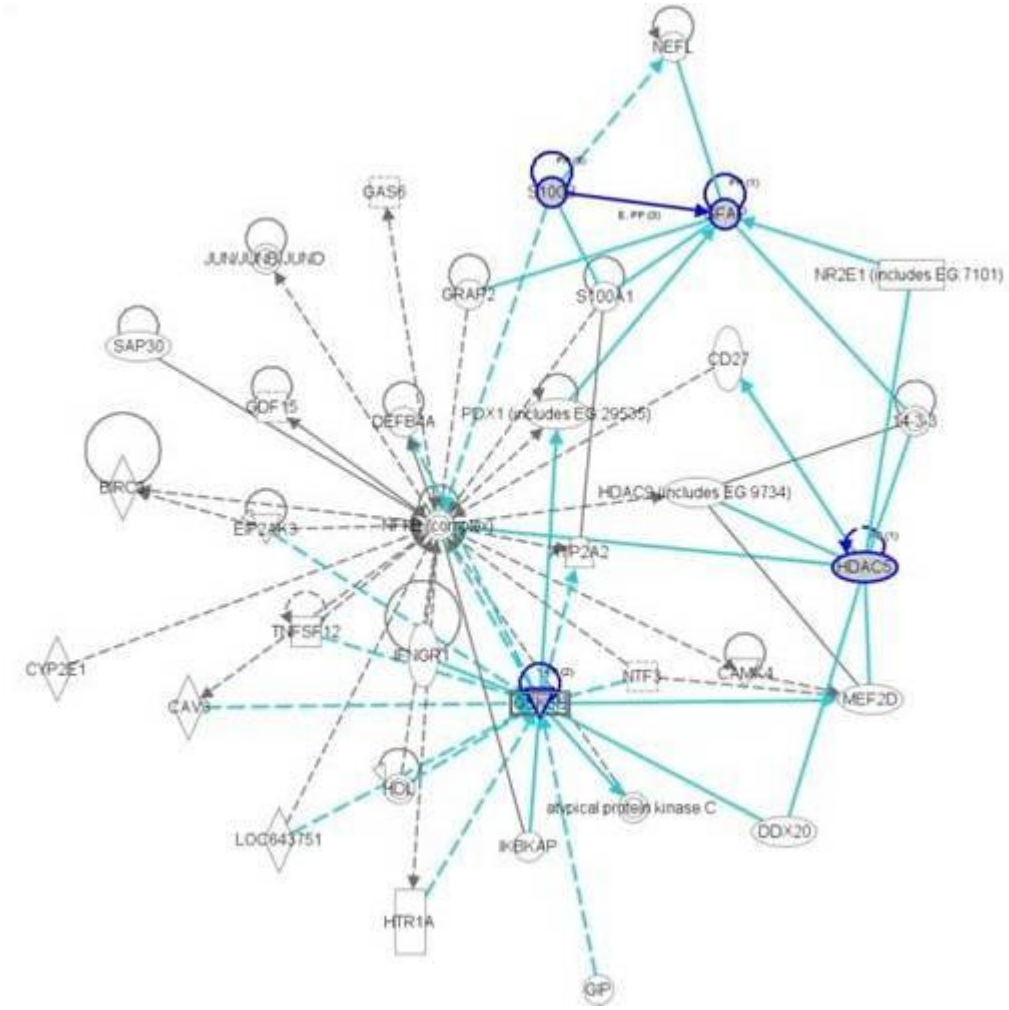
Supplementary Table 4.8 Informative genes used to predict response groups in the k-NN leave-one-out cross-validation test.

Supplementary Table 4.8 Informative genes used to predict response groups in the kNN leave-one-out cross-validation test	
Genes	**Description**
Copg2as2	coatomer protein complex, subunit gamma 2, antisense 2
Prcp	prolylcarboxypeptidase (angiotensinase C)
Gpr115	G protein-coupled receptor 115
Gxylt2	glycosyltransferase 8 domain containing 4
Plb1	phospholipase B1
Txndc9	thioredoxin domain containing 9
Mum1l1	melanoma associated antigen (mutated) 1-like 1
EG545391	predicted gene

Supplementary Table 4.9 Baseline genetic markers that influence variable antidepressant response

Supplementary Table 4.9 List of candidate baseline genetic markers that account for variable fluoxetine response				
Gene	Gene Name	*p*-value	R^2	Adjusted R^2
9230108I15Rik	RIKEN cDNA 9230108I15 gene	0.00014	0.61	0.58
Gm16432	Predicted gene 16432	0.00057	0.53	0.51
C78760	Expressed sequence C78760	0.00069	0.52	0.49
Smurf2	SMAD specific E3 ubiquitin protein ligase 2	0.00086	0.51	0.48
2610005L07Rik	Cadherin 11 pseudogene	0.00113	0.49	0.46
Lmo3	LIM domain only 3	0.00156	0.47	0.44
Tcrb-J	T-cell receptor beta, joining region	0.00206	0.46	0.42
LOC665506	T-cell receptor beta, joining region	0.00278	0.44	0.40
Pabpc1	Poly(A) binding protein, cytoplasmic 1	0.00431	0.41	0.37
Ern1	Endoplasmic reticulum (ER) to nucleus signalling 1	0.00461	0.40	0.37
LOC627901	Similar to zinc finger protein 14	0.00533	0.39	0.36

8. Supplementary Figure



Supplementary Figure 4.1 Cellular adhesion and proliferation pathway links candidate antidepressant biochemical markers S100β GFAP, GSK3β, and HDAC5

Supplementary Figure 4.1. Cellular adhesion and proliferation pathway links candidate antidepressant biochemical markers S100β, GFAP, HDAC5, and GSK3β. Molecular connections between candidate antidepressant biomarkers were evaluated using Ingenuity Pathway Analysis (IPA) software. Results indicate that all candidate antidepressant markers are linked either directly or indirectly. S100β, GFAP, HDAC5, and GSK3β connect within a common cellular growth and proliferation network, indicating the importance of cellular genesis in mediating response to chronic fluoxetine treatment

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CHAPTER 5

TAKING THE YELLOW BRICK ROAD TO BIOMARKER VALIDATION

1. Summary

We collected genome-wide gene expression data and measured the levels of 40 biochemical molecules that have been implicated in anxiety and depression, including neurotransmitters, neurotrophic factors, neuropeptides, and neuroamine regulators . We quantified these biochemical molecules under control, fluoxetine, and treatment-naïve conditions. In addition, we recorded several baseline anxiety and depressive behavioral measures, as well as depressive-like responses, in multiple inbred strains after administration of fluoxetine or water. Our goal is to identify genetic and biochemical markers that can assess SSRI drug response and risk for poor treatment outcome.

2. Translating Genetic Findings from Murine to Humans

Haplotype-behavioral associations were performed, and the locations of the candidate QTLs were examined for previous association with behavioral responses related to anxiety and depression.

We identified two anxiety-like QTLs, Chr 5: 108.2 – 110.9 and Chr 17: 18.7 – 23.7, that have been previously reported for anxiety-related responses and circadian photosensitivity, respectively (Cohen et al. 2001; Yoshimura et al. 2002). Investigation of genes within the anxiety-like behavior loci on Chrs 5 and 7 reveal three genes (*Fpr1*, *Pde6b*, and *Pxmp2*) with previous association with anxiety or depression (Cohen et al. 2001; Yoshimura et al. 2002). Interestingly, we identified a behavioral despair locus on Chr 4 that has been previously linked with anxiety behavior and responses to thermal pain (Mogil et al. 1997; Nakamura et al. 2003). Two genes within the despair locus on Chr 4, *Hcrtr1* and *Oprd1*, were previously correlated with depressive and/or anxiety behavior (Filliol et al. 2000; Scott et al. 2011; van Rijn et al. 2010). Our study demonstrates the power of QTL mapping analysis for the detection of genetic loci that influence complex behavioral phenotypes.

Detecting susceptibility loci for mental health disorders is challenging for human studies given the limited ability to control and account for environmental effects and the need for larger sample sizes to detect genes with moderate effects. Although the use of mouse models can overcome these limitations, depression is challenging to model in animals given that diagnosis and prognosis are based on empirical clinical observations and patients' phenomenological accounts. As a result, rodent behavioral tests for depression often lack face and construct validity and are unable to model certain symptoms, like suicidality. Therefore, an important future direction for mouse QTL behavioral mapping studies is the assessment of candidate genes for depressive disorder. To establish association of murine candidate genes with major depression, we propose a cross-species association analysis approach.

Cross-species association analysis utilizes mouse QTL mapping studies for candidate gene identification and human candidate gene association studies for gene validation. Ease in sample and subject accessibility, reduced genomic complexity, and the ability to control for the effects of environment on behavior makes mouse QTL mapping analysis an effective and efficient method for candidate gene identification, while the use of human samples and data for candidate gene validation provides further information regarding the clinical relevance of genetic biomarkers.

Stage 1: Following identification of loci associated with anxiety-like and depressive-like phenotypes (chapter 2), genes within behavioral QTLs can be prioritized by examining gene expression and biochemical data. Genes with expression levels that correlate with behavioral phenotypes or neurobiochemical levels will be further examined for association with clinical anxiety and depression.

Stage 2: We will perform candidate gene analysis study on the known human homologues of the murine candidate genes identified in Stage 1. Genes that exhibit empirical pointwise p-values of

<0.05 from an allelic association test or haplotype-phenotype association analysis for association with MDD will be selected for further validation. Genotype and clinical information from the Genetic Association Information Network (GAIN) trial are available at http://www.ncbi.nlm.nih.gov/. Details on the methods and study participants have been previously described (Boomsma et al. 2008).

Stage 3: Genotyping experiments will be performed on additional markers within genes that correlate with major depression in stage 2. These markers include haplotype tagging SNPs and SNPs projected to alter gene function (nonsynonymous SNPs and variants for polymorphic microRNA (miRNA) binding sites). Genetic data, clinical information, and biological materials will be obtained from the National Institute of Mental Health Center (NIMH) for Genomic Studies.

It is possible that some genes identified in our mouse QTL mapping analysis may not correlate with major depression. In this case, we will look for similar connections or similar downstream pathways that are shared among candidate behavioral despair genes. We can also compare our pre-clinical findings to human studies that have examined a more homogenous patient population (e.g., patients with recurrent depression or early-onset major depressive disorder).

3. Candidate Gene Validation

3.1 Oprd1 in comorbid anxiety and depression

The locus on Chr 4 for depressive-like behavior has been previously linked with anxiety behavior and responses to thermal pain. Of particular interest is the *Oprd1* gene, which encodes for the opioid receptor, delta1. The *Oprd1* null mice display increased behavioral despair and anxiety-like behavior, indicating that delta1 opioid receptor activity is critical for mediating both anxiety and mood disorder (Filliol et al. 2000). Importantly, this result suggests that similar genetic mechanisms underlie

anxiety and major depression. To further examine the role of *Oprd1* in comorbid anxiety and depression, we can compare the behavioral effects of a delta-selective opioid agonist, selective serotonin reuptake inhibitor (SSRI), and benzodiazepine in attenuating anxiety-like and depressive-like responses. This study can be performed in strains with a) high anxiety-like and minimal depressive-like behavior, b) low anxiety-like and high depressive-like behavior, and c) high anxiety-like and high depressive-like behavior. Strains will be chosen following an exhaustive analysis of our behavioral data and the data from other studies that have assessed several anxiety-like and depressive-like responses between strains.

3.2 Dpp4 in depression

From our QTL mapping analysis, we identified a significant association between *Dpp4* and depressive-like responses. DPP4 is an ectoenzyme that cleaves polypeptides with proline and, to a lesser extent, alanine at the penultimate position. In this study, we found that higher baseline immobility scores are linked with reduced DPP4 protein levels. Compared to D2 mice, B6 mice have higher immobility scores and higher plasma and brain DPP4 protein levels. There are six different *Dpp4* transcript variants that have been reported for B6. One of these isoforms leads to an alternative protein product, which may explain the difference in DPP4 protein levels between B6 and D2 mice. There was no difference in enzymatic activity between B6 and D2, which suggest that baseline inter-strain behavioral differences are likely due to difference in DPP4 protein levels. This result concurs with a mouse knockout study that showed *Dpp4*$^{-/-}$ mice have lower immobility scores compared to wild-type.

Treatment with sitagliptin resulted in greater than 80% inhibition of plasma DPP4 enzymatic activity. However, no significant difference in depressive-like responses was observed between

vehicle- and sitagliptin-treated mice of either strain. It is likely that prolonged DPP4 inhibition is required to mediate depressive-like responses. To assess the direction of the correlation between DPP4 and depressive behavior, we will evaluate the relationship between behavioral responses and DPP4 transcript levels, protein expression, and activity after long-term inhibition of sitagliptin. We will also measure depressive-like responses in mice following intracranial administration of sitagliptin or placebo to ascertain if reduction in brain DPP4 activity is required to affect behavior.

In agreement with this finding, strains that were less immobile or less "depressed-like" after fluoxetine treatment exhibited lower mRNA expression of *Dpp4* after fluoxetine administration (data not reported), indicating a potential role of DPP4 in antidepressant therapy. Reduction in *Dpp4* gene expression after fluoxetine treatment corresponds with lower immobility. This result suggests a potential role of DPP4 in antidepressant therapy. After the direction of the association between *Dpp4* and depressive-like behavior has been established, we will examine the relationship between *Dpp4* and fluoxetine response. In this study, we will assess behavioral responses following administration of water, fluoxetine, and fluoxetine plus sitagliptin.

4. Biochemical Marker Validation

Overall differences in neurobiochemical levels were observed for positive and negative responders. Biochemical alterations following chronic fluoxetine treatment identified positive responders, while baseline neurobiochemical differences differentiated negative responders. Results show that glial fibrillary acidic protein (GFAP), S100 beta protein (S100β), GLO1, and histone deacytelase 5 (HDAC5) contributed the most to fluoxetine response. These proteins are linked within a cellular growth/proliferation pathway, suggesting the involvement of cellular genesis in fluoxetine response.

Our research approach, if validated, provides an unprecedented and feasible means in which we can investigate the effectiveness and clinical utility of new agents in non-responsive lines and strains susceptible to depressive-like phenotypes, ultimately facilitating research efforts towards antidepressant drug development. Unlike other mouse models, our treatment-resistant and depressive-like prone strains were not habituated to exhibit more depressive-like features, nor were their genomes manipulated. Our forward genetic approach uses multiple inbred mouse strains, thereby taking advantage of the naturally-occurring genetic and phenotypic variation extant across inbred mouse strains, which more closely parallels the genetic and phenotypic complexities in the heterogeneous human population. Therefore, our mouse models more closely mimic the conditions of depressed individuals and are powerful pre-clinical models for *in vivo* antidepressant screening and comparative drug studies. For independent validation, we are using a different, but more robust, mouse model of depression, as well as evaluating the antidepressive-like effects of other mood-altering agents.

Study 1: Strains previously shown to have differential depressive-like and anxiety-like behavior at baseline will be selected for this study. We will assess baseline and chronic mild stress (CMS)-induced hedonic responsiveness, behavioral despair, and anxiety-like behavior in the strains that were previously shown to have variable depressive-like and anxiety-like behavior at baseline. The chronic mild stress (CMS) behavioral paradigm presents several advantages. First, CMS is sensitive to chronic antidepressant treatment, thereby providing predictive validity. CMS also reduces hedonic responsiveness to pleasurable items, modeling anhedonia observed in depressed patients. Finally, CMS elicits many of the depressive symptoms commonly found in humans, including reduction in sexual behavior, increased HPA axis activity, and abnormal EEG measures, thereby providing good face validity (Willner 1997; Willner et al. 1992) and making this test a robust animal model of depression

(Nestler and Hyman 2010). *The first study will examine baseline responses to chronic mild stress (CMS) to identify depressive-like prone strains, thus providing an independent validation of our previous findings.*

Study 2: Upon completion of the first study, the mice will be randomized to receive vehicle, citalopram (20 mg/kg), or novel antidepressant agent UNC1 for 28 days. Mice will be exposed to various stressors for 5 days during the second and third week of the treatment study to maintain stress-induced hedonic state in the mice (Strekalova et al. 2006). After chronic treatment, the mice will be tested in the open field, light/dark test, tail suspension test, and forced swim test. Preference and consumption of sucrose-flavored water will be measured weekly using previously published protocols (Strekalova et al. 2006). *The goal of study 2 is to investigate the ability of citalopram and UNC1 to reverse stress-induced depressive-like and anxiety-like response in susceptible and non-susceptible strains, previously identified in study1*. In this study, we will investigate if the same genetic and biochemical markers of fluoxetine response can delineate treatment responders to other antidepressant agents (citalopram and novel agent UNC1), providing an independent validation of predictive biomarkers, which has not been done before in pre-clinical models of depression. Furthermore, we plan to collectively assess molecular, biochemical, and behavioral parameters in order to establish a composite measure of drug response.

Another important avenue for future study is the assessment of these biomarkers in easily accessible samples, like plasma or serum, thus facilitating translation of pre-clinical findings in mice to human studies. Current work in the lab aims to evaluate the relationship between behavioral responses and serum levels of neurobiochemical markers across multiple inbred strains.

5. Conclusion

We conducted a comprehensive analysis of behavioral, neurobiochemical, and transcriptome data across thirty mouse inbred strains, which has not been accomplished previously. We identified biomarkers that influence fluoxetine response, which altogether, implicate the importance of cellular genesis in fluoxetine treatment. More broadly, this approach can be used to assess a wide range of drug response phenotypes that remain challenging to address using human samples.

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