

**The intersection of early life experiences and serotonergic gene expression on  
behavior and physiology**

by

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## **Dedication**

To my parents, who have supported and provided me with the opportunities to shape my own path in life.

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## Abstract

Adverse early life experiences (aELEs), such as childhood abuse, neglect, or trauma, increase lifetime vulnerability for mental illness. Interactions between aELEs and polymorphisms in serotonergic (5-HT) genes can further increase risk. However, understanding of how aELEs shape the serotonergic system remains limited, despite extensive clinical evidence implicating 5-HT abnormalities in mental illness.

This thesis work investigates the long-term consequence of maternal separation (MS), a rat model of aELE, in c57bl/6 mice. Specifically, changes in the expression and DNA methylation of tryptophan hydroxylase 2 (TPH2), the rate-limiting enzyme in neuronal 5-HT synthesis, as well as associated behavioral and molecular correlates, are evaluated. In parallel, a transgenic TPH2 knockdown mouse line is characterized to evaluate the effect of decreased TPH2 expression on behavior.

Brief (MS15) and prolonged (MS180) maternal separation affects long-term decreases in midline dorsal raphe TPH2 mRNA without altering DNA methylation. These changes are concurrent with shared decreases in raphe serotonin transporter mRNA, increases in dorsal hippocampal glucocorticoid receptor mRNA, and decreases in anxiety-like behavior. The expression and behavioral patterns are in contrast to reported changes in MS rats, indicating a unique response to MS that affects a convergence

between MS15 and MS180 c57bl/6 mice that resembles patterns reported in early-life induced ‘resilient’ rats. Underlying convergence, maternal behavior during the MS paradigm is transiently increased as a function of separation duration. This implicates enhanced maternal care as a moderating influence on early life stress, promoting later-life ‘resiliency’ in c57bl/6 mice. In parallel, transgenic knockdown of TPH2 affects a significant decrease in anxiety- and depression- like behavior, supporting a functional role of decreased TPH2 mRNA in shaping long-term behavioral changes during early development.

In summary, this thesis work demonstrates that low TPH2 mRNA during early development alters long-term behavior, affecting a phenotype of decreased anxiety- and depression- like behavior. Moreover, it suggests a unique response to MS in c57bl/6 mice, in which maternal mediation not only mitigates aELEs, but can also promote lifetime ‘resiliency.’

# Chapter 1 – Introduction

## ***I. Central themes***

### **Adverse Early Life Experiences & Vulnerability**

Adverse early life experiences (aELEs), such as childhood abuse, neglect, or trauma, increase lifetime vulnerability for mental illness (6). These include substance abuse, mood and anxiety disorders, schizophrenia, eating disorders, attention-deficit-hyperactivity disorder (ADHD), personality disorders, and increased suicide attempts (7). Limited clinical evidence suggests long-term molecular changes in the brain are associated with a prior history of aELEs. These includes abnormalities in brain morphology (8,9), electrical signaling (10), and stress response (11), suggesting that molecular abnormalities as a result of aELEs may underlie lifetime vulnerability. However, because of the sensitive nature of this population, investigations into long-term consequences of childhood trauma remain limited.

These findings are replicated and elaborated using animal models of aELEs. In particular, studies in rats provide extensive evidence of aELEs shaping development and function of the hypothalamic-pituitary-adrenal (HPA) axis. These include long-term changes in the expression of corticosteroid receptors, altered levels of stress hormones and their regulation, and increases in anxiety- and depression- like behaviors (12). Conversely, specific factors in early life, such as quality or amount of maternal care, can mitigate aELEs and promote resiliency in later life. Early life research remains largely

restricted to investigations in rats, and translation of early life paradigms to a mouse model remains limited. Yet, adaptation of these paradigms into mice is important not just for validating core findings across species, but also because of a more robust genetic toolkit available to probe gene – environment interactions in mice.

In contrast to extensive work delineating the effects of aELEs on HPA axis development and function, understanding of how aELEs shape the serotonergic system remains limited. This is despite overwhelming evidence implicating serotonin (5-HT) in mental illness, for which a substantial risk factor is a prior history of aELEs. A history of aELEs in concert with genetic vulnerabilities in serotonergic genes can predict probability for depression (13), indicating an interaction between aELEs and serotonergic function. However, understanding of how aELEs shape the serotonergic system remains fragmented, due to a limited number and inconsistent findings across studies. Thus, it remains to be articulated how aELEs shape serotonergic development to increase vulnerability.

### **Tryptophan Hydroxylase 2 & Vulnerability**

Altered 5-HT neurotransmission is implicated as a central mechanism underlying a range of psychiatric disorders, including schizophrenia, obsessive-compulsive-disorder (OCD), ADHD, anxiety, depression, and addiction. Underlying altered 5-HT neurotransmission are changes in the function or expression of serotonergic genes. Of particular interest is neuronal tryptophan hydroxylase 2 (TPH2), a paralog of tryptophan hydroxylase (14) abundant in brainstem raphe nuclei (15), where it is the rate-limiting enzyme in brain serotonin biosynthesis. TPH2 polymorphisms, some of which may have



functional significance (16,17), have been associated with mood disorders, attention deficit hyperactivity disorder, executive processing, suicide behavior, personality traits, obsessive-compulsive disorder, Tourette's disorder, aggression, autism and addiction, though as with most genetic studies, negative findings are also reported (18).

Often associated with development of mental illness is severe trauma or chronic stress. These events result in prolonged elevation of corticosteroid concentrations which can adversely effect neuronal signaling and behavior (19,20). The primary source of neuronal 5-HT, the raphe, expresses corticotropin releasing hormone (CRH) receptors, which are important in raphe cell survival and are implicated in modulation of TPH2 expression (21). Chronic infusion of corticotropin releasing hormone (CRH) or urocortin II, a CRF2 receptor agonist, result in decreases in the ratio of ventromedial / caudal-dorsomedial TPH2 expression in the dorsal raphe (22). Similarly, chronic infusions of urocortin 1, a CRF1 receptor agonist, into the basolateral amygdaloid complex increases TPH2 mRNA in the dorsal raphe (23). These studies imply that environmentally induced elevation in CRH also elevates TPH2 expression. This is supported by limited evidence of elevated TPH2 mRNA following aELEs in rodents (24), chronic variable stress (21), and in brains of depressed suicide victims (25).

In summary, converging lines of evidence suggest TPH2 expression is sensitive to stress and may underlie vulnerability for mental illness. Because neuronal 5-HT availability depends on TPH2 levels (26), alterations in its expression has consequences on 5-HT activity and behavioral systems under its influence. However, understanding how environmental experience shapes TPH2 expression and the mechanisms underlying its regulation remains limited.

## **Early life Experience & Epigenetic Regulation**

Emerging evidence suggests epigenetic mechanisms may underlie early life experience-dependent changes in gene expression. Epigenetic mechanisms regulate gene expression through posttranslational modification of histones and DNA methylation, acting to alter chromatin structure to regulate RNA polymerase access to regulatory sites. The relative stability of epigenetic marks and their persistence beyond the presence of factors that induce them, make epigenetic regulation a highly attractive mechanism through which early life experience establishes long-term patterns of expression. In a few studies modeling early life experience in rodents, changes in epigenetic marks are associated with altered expression of hippocampal glucocorticoid receptor (GR) and hypothalamic arginine vasopressin (AVP), which are involved in stress axis function, behavior, and stress response (27,28). However, studies remain limited to the regulation of HPA axis genes, with no studies yet reporting on epigenetic changes in serotonergic genes following aLEEs. This is despite evidence that selective serotonin reuptake inhibitors (SSRIs), used in the treatment of anxiety and mood disorders, affect chromatin remodeling and activate central mediators of epigenetic regulation such as methyl CpG binding protein 2 (MeCP2) and methyl CpG binding domain protein (MBD1) (29,30). The time course required for SSRIs to reach therapeutic effect is consistent with the time course necessary for chromatin remodeling (31), supporting a mechanism of action by which SSRIs achieve therapeutic effect by altering epigenetic marks. In addition, drugs which directly modulate epigenetic changes, such as sodium butyrate, a histone deacetylase inhibitor, have antidepressant-like effects on behavior following chronic

social defeat in rats (32), suggesting that long-term epigenetic changes underlie vulnerability.

The TPH2 gene contains a neural restrictive silencing element (NRSE) in its promoter / 5' untranslated region, which is regulated and targeted by an epigenetic regulator of neuronal genes, the Re-1 Silencing Transcription Factor (REST). REST expression is sensitive to early developmental experiences and is implicated in underlying changes in the expression of CRH receptors (33). This suggests it may play a role in modulating molecular response to early life experience in the brain. Because of evidence suggesting TPH2 expression is sensitive to stress, is regulated by an epigenetic regulator of development inducible by aELEs, and polymorphisms in its gene are linked to vulnerability for mental illness, TPH2 is a prime candidate for investigating early life experience-dependent epigenetic changes as a mechanism underlying long-term changes in gene expression and behavior.

## ***II. Hypothesis & Aims***

This thesis work was designed to test the overall hypothesis that **adverse early life experiences (aELEs) increase DNA methylation of tryptophan hydroxylase 2 (TPH2) and produce persistent decreases in its mRNA levels.** Towards investigating this hypothesis, a well-established rat paradigm of aELE, the maternal separation (MS) paradigm, was used to model aELEs in c57l/6 mice. The specific aims of this thesis sought to test the following specific hypotheses:

1. *Prolonged maternal separation (180 minutes/day) decreases TPH2 mRNA in c57bl/6 mice persisting into adult life.*

2. *Prolonged maternal separation (180 minutes/day) increases anxiety- and depression- like behavior persisting into adult life.*
3. *Prolonged maternal separation (180 minutes/day) increases DNA methylation of the TPH2 promoter and 5' untranslated region persisting into adult life.*
4. *Transgenic knockdown of TPH2 expression decreases anxiety- and depression- like behavior persisting into adult life.*

### **III. Literature Review**

The first section of this review, **A. Serotonin system & Development**, is an overview of the functional anatomy of the serotonergic system, its role in behavior, and the importance of maintaining normal 5-HT levels during development in early life. The second section, **B. Hypothalamic-Pituitary-Adrenal (HPA) Axis & Stress**, is an overview of the HPA axis, its signaling cascade, the receptors regulating stress response, and HPA axis dysregulation in mental illness. The third section, **C. Mental Illness**, is an overview of the functional anatomy underlying anxiety and depression, focusing on evidence of abnormalities in the serotonergic system and HPA axis in vulnerability and pathology. The fourth section, **D. Adverse Early Life Experiences & Vulnerability**, is an overview of the pre-clinical studies implicating early life experience in increasing lifetime vulnerability for mental illness. Specifically, it focuses on a discussion of abnormalities observed in the HPA axis and serotonergic system. The fifth section, **E. Rodent Models of Early Life Experience**, is an overview of the rat models of early life experience and core findings on the HPA axis, serotonergic system, and behavior. In addition, this section highlights mixed findings on the influence of aELEs on serotonergic

development efforts into translating these early life paradigms into mice. The sixth section, **F. Epigenetics & Early Life Experience**, is an overview of studies that implicate epigenetic mechanisms as a mediator of early life mediated changes in behavior, gene expression, and stress response. The aim of this section is to underline the rationale for targeting TPH2 as a prime candidate for investigating early life-mediated epigenetic changes.

## **A. Serotonin System & Development**

### **Functional Anatomy**

The serotonergic (5-HT) system is implicated in the modulation of physiological states, including cardiovascular regulation, respiration, and thermoregulation, as well as a variety of complex behaviors, including sexual behavior, learning, aggression, emotion, circadian rhythm, sleep-wake cycle, appetite, and pain sensitivity (34). The majority of 5-HT containing neurons are localized along the midline of the brainstem in distinct sub-nuclei of the raphe, and send collateralized projections to innervate a wide distribution of spinal, subcortical, and cortical structures. The dorsal and median raphe are of particular interest, with the dorsal raphe projecting heavily to frontal cortex and striatum, whereas the median raphe predominantly innervates hippocampus and septum (35). The midline rostral dorsal raphe innervates a range of motor structures, including striatum, motor cortex, basal ganglia, and putamen, whereas the caudal dorsal raphe is implicated in structures involved in stress and anxiety, including hippocampus, hypothalamus, and amygdala (36). The raphe also receives afferent projections from many of the same regions, as well as internally from the various sub-nuclei of the raphe. The wide

distribution of projections suggests that 5-HT neurons act to influence several regions simultaneously, acting to integrate and fine tune behavioral output. Although implicated in a range of behaviors, 5-HT depletion does not result in physiological failure or cessation of behavior, suggesting that 5-HT is not directly responsible for any one behavior, but functions as a modulator.

### **Early life & 5-HT: Evidence from TPH2 knockouts**

Transgenic knockout of TPH2 illuminates the role of endogenous brain 5-HT in fetal, postnatal, and adult life. TPH2 mouse knockouts (KOs) are viable with normal serotonergic neuron formation, despite near complete depletion of brain 5-HT (26). More extensive characterization suggests 5-HT<sub>1a</sub>, 5-HT<sub>1b</sub>, 5-HT<sub>2a</sub>, 5-HT<sub>2c</sub>, SERT, and TPH1 mRNA and SERT, 5-HT<sub>1a</sub>, 5-HT<sub>2a</sub>, 5-HT<sub>1b</sub>, 5-HT<sub>2c</sub>, and MAO-A protein levels across various cortical, subcortical, and brainstem structures are unchanged (37,38). This suggests either that serotonergic development is independent of 5-HT availability, or else that maternal or placental sources of 5-HT are sufficient in establishing the serotonergic network.

Similarly, knockout of TPH2 has no effect on the activity of other neurotransmitter systems. Whole brain levels of norepinephrine, glutamate, GABA, dopamine, and DOPAC are unchanged (39). Regional analysis of norepinephrine, dopamine, and associated metabolites also indicate unchanged levels in cortex, thalamus, olfactory bulb, cerebellum, hippocampus, brainstem, and striatum (40).

Behavioral characterization of TPH2 KOs suggests lower 5-HT activity decreases anxiety-like and depression-like behaviors. Moreover, TPH2 KOs also exhibit increased

compulsiveness, motor impulsivity, aggressiveness, and increased sexual behavior (38-41). These findings are complemented by evidence of increased anxiety-like and depression-like behaviors from elevated 5-HT in SERT (42) and 5-HT<sub>1a</sub> (43) knockouts. The overall pattern of behavior suggests a wide range of disinhibition across various behaviors from TPH2 knockout, suggesting 5-HT activity normally inhibits the structures modulating these behaviors.

### **Early life & 5-HT: Pre- and postnatal exposure to SSRIs**

The effects of low 5-HT in TPH2 KOs are complemented by reciprocal evidence of SSRI exposure during prenatal and postnatal life. In humans, prenatal exposure to SSRIs is associated with low birth weight, risk for preterm birth (44), internalization behavior during early childhood (45), blunted somatosensory response (46), poor psychomotor development (47). Similarly, prenatal exposure to SSRIs in rodents are associated with reduced weight (48), neonatal mortality (49), and increased behavioral despair (50).

During adolescence, SSRI treatment can increase agitation, depression, anxiety, and suicidal ideation (51-56). Similarly, early postnatal SSRI exposure in rodents increases anxiety-like and depression-like behavior (57,58), decreases sexual behavior (59), reduces aggression (60), anhedonia (61), and reduces active sleep (62). Paradoxically, several of the long-term adverse effects arising for adolescent exposure to SSRIs can be reversed by SSRI treatment as adults (63). Molecular changes are also reported, with down-regulation of SERT and TPH2 mRNA in the brainstem (59) and morphological changes in serotonergic neurons (64).

The abnormalities in behavior and physiology following early life SSRI exposure are in contrast to effectiveness in treating psychopathology in adult life (65). Moreover, several of the behaviors affected by pre- or post-natal exposure to SSRI have an inverse effect compared to TPH2 KOs, including aggression, anxiety- and depression- like behaviors, sleep, and sexual behavior (refer to section entitled Early life & 5-HT: Evidence for TPH2 knockdowns). The pre-clinical and rodent evidence suggests that exposure to excessive 5-HT during pre- and post-natal development has long-term adverse consequences which may contribute to vulnerability.

The studies discussed above indicate a critical period during early life in which normal serotonergic activity is necessary for appropriate brain development. Although experimental manipulation of serotonergic activity highlights the impact of abnormalities in serotonergic activity on early postnatal development, understanding remains limited on the impact of aELEs on serotonergic development and the regulatory mechanisms underlying these long-term changes. Delineating these factors will be critical to developing interventions or treatments to prevent lifetime vulnerability for mental illness.

## **B. Hypothalamic Pituitary Adrenal (HPA) Axis & Stress**

### **HPA Axis & Stress**

A core risk factor in mental illness is stressful life events. In response to psychological or physical stress, the hypothalamic-pituitary-adrenal (HPA) axis is activated, resulting in a cascade of signals preparing the body to effectively deal with the stressor. The stress response is adaptive and designed to deal with acute threats,



facilitating diversion of resources for immediate use, increasing inflammatory response to injuries, and supplying cells with amino acids and fatty acids for energy.

However, prolonged activation of the HPA axis from chronic or traumatic stress has adverse long-term consequences on homeostasis. Rat models of chronic stress, in which animals are subject to prolonged stress paradigms or stress hormone exposure, results in impaired negative feedback response, abnormalities in glucocorticoid receptor expression, and dysregulation of baseline plasma corticosterone (66,67). Persistent activation and hyper-responsiveness of CRH circuits underlies vulnerability for mood and anxiety disorders (68). Abnormal HPA axis function is implicated in systemic illness (69) and affective disorders (70,71). This includes, but is not limited to, hypertension, depression, posttraumatic stress disorder, and anxiety. In parallel, disorders in which the HPA axis is dysfunctional mirrors several symptoms of depression (72), strongly implicating abnormal exposure to stress hormones in the pathology of mental illness. In summary, the evidence suggests environmental stress and dysregulation of the HPA axis underlies vulnerability and / or pathology of mental illnesses.

The HPA axis regulates the stress response through a series of neuroendocrine signaling cascades. The paraventricular nucleus (PVN) of the hypothalamus releases corticotropin releasing hormone (CRH), which stimulates the anterior pituitary to release adrenocorticotrophic hormone (ACTH), which in turn stimulates synthesis and secretion of corticosteroids from the adrenal cortex (20). The initiation of the stress response is governed by activation of the PVN, principally influenced by brainstem catecholaminergic signaling via  $\alpha$ -adrenergic receptors (73). Additional activation originates from the amygdala (74,75), noradrenergic stimulation from the locus coeruleus

(76), and serotonergic stimulation from the raphe (77). In response to immediate physiological threats, signals to the PVN are likely activated directly via brainstem catecholaminergic projections, whereas stressors requiring interpretation may be relayed through limbic structures for processing before PVN activation (66). Ultimately, the end effect is to increase circulating corticosteroids, which exert a range of effects on metabolic and immune function.

A core element of HPA axis regulation is signal termination. Extended exposure to corticosteroids can result in adverse effects, including reduced neurogenesis, altered gene expression, decreased hippocampal volume, and memory impairment (78,79), underscoring the essential nature of a well-regulated stress response. The termination of stress response is primarily mediated via negative feedback, occurring at all levels of the HPA axis, including the adrenal cortex, the pituitary, the PVN, and at limbic structures, including hippocampus, prefrontal cortex (PFC) and lateral septum (LS) (80,81). Evidence for PVN-mediated termination derives from glucocorticoid injections into the PVN, decreasing CRH and ACTH (82), which suggest direct stress receptor-mediated inhibition of PVN activity. Furthermore, non-PVN hypothalamic structures inhibit PVN activity via GABAergic signaling, decreasing CRH and ACTH release (83,84). Evidence for hippocampal involvement in negative feedback is suggested by high expression of corticosteroid receptors (85) and stimulation induced decrease in HPA activity (85). Conversely, hippocampal lesions increase CRH expression and potentiate corticosteroid secretion (86-88).

Negative feedback in the brain is mediated by the mineralocorticoid (MR) and glucocorticoid (GR) receptors. MR is characterized as “high-affinity, low capacity,”

whereas GR is “low-affinity, high-capacity.” The MR is operative at low corticosterone concentrations during tonic release, whereas GR comes into play following MR saturation. Whereas MR is implicated in maintaining the normal circadian corticosteroid rhythm, GR is implicated in negative feedback following acute elevations. GR and MR coordinate regulation of corticosteroid levels, and altered balance in their expression is implicated in depression and PTSD (67,89). In rodent models of early life resiliency, increased hippocampal GR expression is postulated to accelerate negative feedback, quickly reducing corticosterone levels following stress (90).

Although the physiological stress response is a generalized event, the extent and duration of activation varies depending on type and duration of the stressor. The HPA axis is adaptive for responses to acute physical events (evading a predator, for example), whereas extended or extreme elevations in corticosteroid secretion may have adverse consequences.

### **HPA Axis & 5-HT system**

The serotonergic system shares several functional and anatomical connections with the hypothalamic-pituitary-adrenal (HPA) stress axis. The raphe sends direct projections to the paraventricular nucleus (PVN) of the hypothalamus, as well as indirect projections via limbic structures implicated in regulation of the stress axis, including amygdala and hippocampus. In reciprocation, hypothalamic CRH neurons innervate the raphe (91), where CRH primarily acts to inhibit serotonergic neurons (92). Abnormalities of the HPA axis, including increased levels of stress hormones, blunted or elevated corticosterone circadian rhythm, impaired negative feedback response, and altered

corticosteroid receptor expression, concurrent with abnormalities in the serotonergic system, are reported in a subset of the clinically depressed population (93).

Deficits in 5-HT signaling are shown to affect HPA axis regulation of circadian corticosterone rhythm. In early postnatal life, 5,7-dihydroxytryptamine (5,7-DHT) lesion of 5-HT nerve terminals in the suprachiasmatic nucleus (SCN) prevents development of a circadian corticosterone rhythm, resulting in basal corticosterone levels remaining at trough levels throughout the day (94). In adult life, 5,7-DHT lesions to the SCN result in a free-running corticosterone and ACTH rhythm intermediate between peak and trough levels with no clear circadian variation (95,96). These findings suggest that normal 5-HT activity is necessary for development and maintenance of circadian corticosterone rhythm.

The expression of corticosteroid receptors is also modulated by 5-HT activity. Injections of 5-HT up-regulates GR expression in the hippocampus, whereas destruction of 5-HT projections decreases GR and MR mRNA in the hippocampus (97). Changes in stress receptor expression from altered 5-HT activity can affect stress response. 5,7-DHT lesions in the PVN or raphe can blunt ACTH response to acute-restraint and ether stress. This is suggested to be in part modulated by 5-HT<sub>1a</sub>, 5-HT<sub>2a</sub>, and 5-HT<sub>2c</sub> receptor activity, as antagonists to these receptors replicate the effects from 5,7-DHT lesions (98). In summary, 5-HT activity is shown to affect HPA axis function at baseline, in response to stress, and gene expression.

Conversely, abnormalities in the HPA axis also affect the 5-HT system. Elevated levels of stress hormones from environmental stress as well as direct injections of stress hormones are implicated in abnormal 5-HT activity. For example, chronic unpredictable

stress down-regulates 5-HT<sub>1a</sub> binding and mRNA levels in the hippocampus. These changes are rescued by adrenalectomy + low levels of glucocorticoid replacement, implying that elevated plasma corticosteroid levels from environmental stress and GR occupancy underlie changes in serotonergic function (67). This is supported by evidence of chronic social stress or prolonged administration of ACTH up-regulating cortical 5-HT<sub>2a</sub> receptor binding (99,100). Serotonergic activity is also upregulated by environmental stressors, with increased c-fos levels in the dorsal raphe following inescapable stress (101), exposure to novel open field (102), social defeat stress (103), and forced swim stress (104). Similarly, immobilization stress and the Vogel test (drinking behavior punished with an electric shock) increase hypothalamic and hippocampal 5-HT release (105,106). The increases in serotonergic activity are related to levels of stress hormones, with CRH infusions into the raphe inhibiting or exciting serotonergic signaling depending on CRH concentrations (92,107).

In summary, the HPA axis and serotonergic system share extensive anatomical and functional connections. These connections are reciprocal, and affect a dynamic response to environmental challenges. Altered signaling in one system has consequences on functions regulated by the other, emphasizing the high degree of integration between systems.

## **C. Mental Illness**

### **Anxiety**

Anxiety is a complex emotional state influenced by both cognitive and physiological processes, elicited in response to a potential or perceived threat. It is related

to sustained heightened autonomic and behavioral arousal, with increases in avoidance behavior in novel or uncertain environments (36). Anxiety behaviors are related to a degree of risk assessment, in which both rewarding and aversive outcomes are possible. It is suggested a distinction exists between *state anxiety*, reflecting the immediate level of anxiety and *trait anxiety*, reflecting the long-term tendencies of an individual to express anxiety responses (108). *Trait anxiety* is posited to represent inherent baseline levels of anxiety shaped by early life experience and genetic factors.

Non-pathological anxiety is adaptive, protecting an organism from dangerous situations or excessive risk taking. However, in its pathological state, excessive or inappropriate anxiety can severely impact everyday function and quality of life. Pathological anxiety is categorized into six disorders: generalized anxiety disorder, social phobia, simple phobia, panic disorder, post-traumatic stress disorder (PTSD) and obsessive-compulsive-disorder (OCD) (109), which share common themes of excessive rumination, worry, and apprehension about future uncertainties. The prevalence rate for anxiety is 18.1% of the US adult population over a 12-month period (110), which has a significant individual and economic cost.

Trait anxiety of individuals remains fairly consistent throughout life. For example, general behavioral inhibition in early childhood predicts risk for social anxiety in adolescence (111), suggesting baseline levels of anxiety become crystallized by early postnatal development (112). Genetic factors are strongly implicated in establishing trait anxiety. For example, carriers of the (*s*) allele of the serotonin transporter promoter exhibit increased anxiety-like traits (113) and are at increased risk for anxiety disorders (113,114). Morphological abnormalities, arising from genetic or early environmental

factors, are also linked to increased risk for post-traumatic stress disorder (PTSD). Twin studies suggest an inverse correlation between hippocampal volume and development of PTSD, in which smaller volume increases risk for PTSD following trauma (115). Smaller hippocampal volume and increased risk for PTSD are also observed in individuals with a history of early childhood abuse or trauma (116-118), suggesting early environmental influences can mirror genetic vulnerabilities in shaping development and increase risk. In summary, the evidence suggests trait anxiety is established by early postnatal life through interactions between genetic factors and early environmental influences, ultimately contributing to long-term risk for anxiety disorders.

Posited to underlie anxiety disorders is excessive excitatory neurotransmission (119). This originates from the therapeutic success of treating anxiety disorders with GABA-enhancing drugs. More recently, selective serotonin reuptake inhibitors (SSRIs) have been used in treating anxiety disorders, which implicates serotonergic involvement in anxiety. SSRIs have been demonstrated to dampen brain excitability (120,121) and have been shown to increase GABA concentrations (122), consistent with a model of excess excitatory neurotransmission underlying anxiety disorders. The effectiveness of SSRIs in treating anxiety disorders implies that low serotonergic activity underlies vulnerability or pathology of anxiety disorders.

However, studies in rodents suggest decreased serotonergic activity has an anxiolytic-like effect. Lowering neuronal 5-HT content using pharmacological blockades, transgenic methods, and chemical ablations result in decreases in anxiety-like behavior (38,40,123). Conversely, increasing neuronal 5-HT levels via transgenic knockout of the serotonin transporter produces an increase in anxiety-like behaviors (124). The

effectiveness of SSRIs in alleviating anxiety symptoms in humans may be via downstream mechanisms, by which increases in 5-HT levels act indirectly. This is supported by reports of SSRI treatment initially increasing anxiety symptoms before achieving the desired therapeutic effect. This would help to partially reconcile discrepancies between the role of increasing 5-HT on anxiety-like behaviors in clinical vs. animal studies.

The anatomy underlying anxiety is a complex interaction between, cognitive, reward and emotional circuitry. Particularly implicated in this is the septo-hippocampal network, which is suggested to play a role in predicting the outcome of behavioral responses. The core structures of this network include the hippocampus, basolateral amygdala, bed nucleus of the stria terminalis (BNST), and medial prefrontal cortex (mPFC). Afferents and efferents to these structures include the hypothalamus and raphe, by which stress and serotonergic circuits have a modulatory role. The mPFC, amygdala, and hippocampus are implicated in modulating mesolimbic dopaminergic reward circuitry from the ventral tegmental area to the nucleus accumbens (125), and may affect the salience of potential rewards, thus altering expression of approach (exploratory) vs. avoidance (fear) behaviors. Acute stress and specific aversive stimuli, such as diffuse light for rodents, activate the BNST to inhibit emotionally-motivated motor behavior (126).

Structures involved in modulating anxiety send and receive innervations to and from the serotonergic system. Particularly relevant is the dorsal raphe, of which midline vs. lateral dorsal raphe structures are implicated in specific components of anxiety. The dorsal midline structures, in concert with the median raphe (MR), are suggested to



modulate general anxiety state, whereas the lateral structures are implicated in panic-related responses. This is related to an anatomical divergence, in which lateral structures send descending innervations to structures implicated in panic responses (e.g. dorsal lateral periaqueductal gray and rostral ventral lateral medulla) whereas midline structures send ascending innervations to cortical and septo-hippocampal structures, although these are not exclusive and there is overlap (127). Lesions of serotonergic innervations of cortical structures impacts choice on delayed reinforcement trials (128), suggesting 5-HT abnormalities alter decision-making processes and control of behavior. Lesions of serotonergic neurons projecting to the basolateral amygdala both increase and decrease anxiety-like behaviors depending on context. This is particularly relevant to modulating amygdala activity and its role in assigning emotional salience to rewarding and aversive stimuli. Escape behavior is also modulated by serotonergic neurons, particularly via lateral dorsal raphe descending innervations to “fight-or-flight” structures, such as the dorsolateral periaqueductal gray and ventrolateral medulla (36). Upper level control of 5-HT neurons originates from the mPFC, inhibiting dorsal raphe activity depending on context. For example, inescapable stress increases dorsal raphe activity, whereas escapable stress activates the mPFC to inhibit dorsal raphe activity (129). The hippocampus receives particularly dense innervation from the MR, with moderate innervation from midline DR structures (130,131). Lesions of the MR produce an anxiolytic-like effect, which may be related to altered hippocampal influence on emotion and reward salience, biasing towards approach behaviors (132). In summary, the serotonergic system both modulates and is modulated by structures involved in specific aspects of anxiety. Serotonergic signaling modulates the expression of anxiety-related

behaviors, and its dysfunction is linked to vulnerability and / or pathology of anxiety disorders.

### **Anxiety, Behavioral Disinhibition, and 5-HT**

In reconciling animal and clinical studies of complex behaviors, what is interpreted as anxiolytic behavior following reduced serotonergic activity in rodents may also reflect a disinhibition of behavior, i.e. impulsivity. Mice rated as highly impulsive in a delayed reinforcement task also rate as having low anxiety on anxiety metrics (light/dark box & open-field test), supporting an association between impulsivity and an anxiolytic-like phenotype in expressed behavior (133). Serotonin depletion by raphe lesions or chemical ablation of serotonergic neurons is associated with an intolerance to delayed response to rewarding stimuli (123). Low 5-HT in rodents increases punished responding, active avoidance behavior, impulsive actions, and exploratory behavior in novel environments (128,134-136), consistent with a bias towards active responding when presented with the choice of a passive or active behavior. This is also consistent with evidence of low serotonin associated with suicide (137), ADHD (138), and aggression (139,140), disorders associated with a disinhibition of behavior.

It is not clear how disinhibition or “impulsivity” relates to anxiety and depression pathology. To be sure, impulsivity is a multidimensional construct, and it is not clear which dimension(s) of impulsivity are regulated by serotonin. Because 5-HT is implicated in the prediction of aversive events (141-143), a decrease in 5-HT activity may reduce cognitive appraisal of aversive outcomes, lowering the ability to accurately appraise risk. Along with sensation seeking, lack of planning, lack of perseverance and

negative urgency, and tendencies towards acting rashly in the face of negative affect (144), this may increase the frequency and severity of adverse outcomes, contributing to increased risk for psychopathology.

## **Depression**

A history of aELE significantly increases vulnerability for major depressive disorder (MDD), interacting with genetic polymorphisms to further increase risk (13). Symptoms of MDD include depressed mood, irritability, low self esteem, feelings of hopelessness, decreased concentration, insomnia, anhedonia, lost appetite, low energy, and suicidal tendencies persisting for longer than 2 weeks (109). Most episodes of MDD are preceded by stress (145), consistent with evidence of a dysregulated stress axis commonly observed in a subset of depressed patients. In addition, key symptoms of depression are reported in patients with endocrine disturbances (hyper or hypo - cortisolism & -thyroidism), supporting abnormal HPA axis function in the pathology of depression. This is replicated in animal models, in which chronic stress or exposure to CRH produces behaviors resembling anhedonia, despair, and impaired cognitive function (79,146,147). The overexposure to stress hormones has been linked to reduced neurogenesis and abnormalities in neuronal signaling (148). This is supported by pre-clinical studies, in which morphological changes in neurons and glia of frontal cortical and limbic regions of patients with MDD have been reported (149,150).

The successful treatment of depression using monoamine oxidase inhibitors (MAOI), tricyclic anti-depressants (TCA), and SSRIs implicate abnormalities of neurotransmitter systems in the pathology of depression. Their acute mechanisms of

action include inhibition of serotonin and norepinephrine transporters or inhibition of monoamine oxidase, suggesting therapeutic effect is achieved by enhancing monoamine neurotransmission. However, therapeutic efficacy is achieved only after prolonged administration (weeks to months), suggesting the underlying therapeutic action is attributable to a gradual adaptation to enhanced neurotransmission. A leading theory of SSRI action is the progressive desensitization of auto-inhibitory somato-dendritic 5-HT<sub>1a</sub> receptors over several weeks to increase 5-HT neurotransmission (65). The downstream effects of increased 5-HT neurotransmission may be to increase neurogenesis in specific target regions, such as hippocampus. This is supported by evidence of chronic antidepressant treatment acting to increase hippocampal neurogenesis (148), evidence of decreased hippocampal neurogenesis in response to chronic stress during early or adult life (19), and increased hippocampal neurogenesis and 5-HT activity following early handling or high quality maternal care (151,152).

The site of pathology in depression remains unclear. Recent studies implicate abnormalities in the activity of the amygdala, prefrontal and cingulate cortex, hippocampus, striatum, raphe, and thalamus of patients suffering from major depressive disorder (153,154). Impairment of the hippocampus and cortex may affect cognition and executive function. Deficits in the striatum, hippocampus, and amygdala may affect emotional processing and saliency of rewards, decreasing motivation and pleasure-seeking activities. The raphe, the central site of serotonergic neurons, sends extensive innervations to all of these structures, placing it in a unique position to modulate the wide range of behaviors affected by the pathology of depression. Its dispersed and wide-ranging connections may underlie the effectiveness of treating mental illness with serotonin

augmenting agents, by which SSRIs affect neurotransmission simultaneously via serotonergic innervations across multiple structures to re-establish normal brain activity.

Serotonin is a central focus of investigations into the pathogenesis of depression, of which low serotonergic activity is implicated as a vulnerability factor or proximate cause of depression. Major indices of 5-HT function are found to be lower in patients with depression, including reductions in plasma tryptophan levels (155), cerebrospinal fluid 5-hydroxyindolacetic acid (5-HIAA) levels (156), platelet 5-HT uptake (157), and serotonergic responsiveness to neuroendocrine challenge (158). Experimental depletion of tryptophan, a biosynthetic precursor for 5-HT, in healthy volunteers results in lower mood, memory impairment, and increased aggression (159). Similarly, tryptophan depletion can cause relapse in patients recovering from depression (160). The successful treatment of depression using selective serotonin reuptake inhibitors (SSRIs), which increase extracellular 5-HT content, supports a model in which deficits in 5-HT signaling underlie vulnerability or serve as a proximate factor in the pathophysiology of mental illnesses (161).

Binding and neuroimaging studies in post-mortem suicide victims indicate abnormalities in the serotonergic gene expression. In cortical areas, 5-HT<sub>1a</sub> receptor levels are upregulated in the ventrolateral prefrontal cortex (VLPFC), 5-HT<sub>2a</sub> receptors are upregulated in the dorsolateral prefrontal cortex (DLPFC) (162), and serotonin transporter (SERT) levels are either decreased (163,164) or unchanged (165,166). The DLPFC is involved in working memory, and DLPFC lesions are implicated in deficits of executive function and attention, general apathy, and lack of emotional reactivity (167). The VLPFC is involved in goal-directed behavior and decision-making, and lesions have

also been implicated in deficits in motivation and risk appraisal (168,169). Altered function of these structures, potentially from deficits in serotonergic signaling, is consistent with symptoms associated with depression. Serotonergic function in the dorsal raphe is also affected, with evidence of increased 5-HT<sub>1a</sub> receptor mRNA levels and agonist binding, decreased SERT mRNA but unchanged SERT binding (170,171), and increased TPH2 mRNA levels (25). These studies clearly indicate serotonergic abnormalities in suicide victims, with implications for the pathology of depression.

Although serotonergic dysfunction is implicated in depression, pre-clinical studies are unable to delineate a clear pattern of change in serotonergic activity or gene expression. This is likely because depression is not a unitary construct, complicated by genetic heterogeneity, pleiotropy, epigenetics, and phenocopies. Although the evidence strongly associates abnormal serotonergic function with depression, additional work is needed to clarify whether and how these changes contribute to vulnerability and pathology of depression.

#### **D. Adverse Early Life Experiences**

##### **Adverse Early Life Experiences & Vulnerability**

A limited number of studies have investigated the immediate consequence of child abuse and trauma on HPA axis function. In non-abused children, cortisol typically increases in response to social conflict. However, in abused children, cortisol levels in response to social conflict are blunted (172), which either reflects higher basal stress levels throughout the day or else a de-sensitization of the stress response following abuse in early life. Traumatic events also result in abnormalities in HPA axis feedback to CRH

challenge. In sexually abused girls, CRH challenge effects a blunted ACTH response but increased cortisol response (173). In comparison, abused children with a history of depression exhibit enhanced ACTH but normal corticosterone response relative to non-abused depressed or control children in response to CRH challenge (174). The type of traumatic event also differentiates the form of HPA axis abnormality. Children suffering trauma from the death of a parent exhibit hypo-suppression of corticosterone in response to dexamethasone (175), whereas children suffering trauma from an earthquake exhibit hyper-suppression of corticosterone in response to dexamethasone (176). In summary, these studies indicate aELEs affect immediate abnormalities in HPA axis function to adversely affect development and increase lifetime vulnerability.

A few retrospective studies have also attempted to link aELEs with psychiatric vulnerability and abnormalities in the HPA axis. Women with a history of childhood sexual abuse exhibit hyper-suppression of salivary cortisol in response to dexamethasone challenge (177). Women, with or without depression, who were abused as children also exhibit an exaggerated plasma ACTH response to psychosocial stress relative to depressed women without a history of childhood abuse as well as controls (178). These studies indicate that abnormalities in HPA axis function as a consequence of childhood aELE persist into adult life.

A long-term impact of aELEs on serotonergic activity is suggested by increased efficacy of SSRIs in treating individuals with a prior history of childhood maltreatment. For PTSD patients with a history of child abuse, SSRIs are more effective relative to patients with other underlying causes of PTSD (179). Similarly, SSRIs demonstrate enhanced efficacy in treating early-onset depression in children with a prior history of

aELEs (180). These studies suggest underlying serotonergic abnormalities prevail in this population, reflected by greater efficacy of SSRIs in their treatment. However, pre-clinical studies delineating specific effects of aELEs on serotonergic function remain limited.

Genetic vulnerabilities interact with aELEs to increase risk. Individuals with the (*s*) allele of the serotonin transporter interact with childhood trauma to increase risk for depression, whereas probability for depression in carriers of the (*l*) allele remains relatively unchanged irrespective of childhood trauma (13). In support, studies in non-human primates indicate that rearing conditions, in which either mothers or peers raise the infants, interacts with polymorphisms in the serotonin transporter to determine adult 5-HT activity. Specifically, lower 5-HIAA levels are detected in *s/l* heterozygotes for the serotonin transporter in peer-raised primates, whereas peer-raised *l/l* homozygotes have levels similar to maternally-raised *l/l* and *s/l* counterparts (181). The functional consequence of lower 5-HT activity may increase lifetime vulnerability, which in concert with environmental stressors and other genetic vulnerabilities may precipitate mental illness. In summary, pre-clinical studies suggest an association between aELEs, abnormalities in HPA axis, and vulnerability for mental illness.

Primary findings from pre-clinical studies are mirrored by findings from animal models of aELEs. For example, plasma CRH levels are increased in depressed children with a history of abuse, reproduced in rats subject to prolonged separation (MS180) during early life, a rat model of aELE (90). A contrasting effect is also observed in rats subject to early handling, a rat model of early life experience-induced resiliency (33). Similarly, an exaggerated ACTH response in bereaved children (182) is also reproduced



in rats that had experienced prolonged separation (MS180) in early life (183). The convergence between clinical evidence and rodent models of early life-induced vulnerability or resiliency validates their use in investigating long-term molecular consequences of aELEs and translatability of findings into humans.

## **E. Rodent models of adverse early life experience**

### **Overview**

Historically, core animal models of early life experience were developed using the rat system. Thus, much of the evidence pertaining to the impact of early life on long-term changes in behavior, gene expression, and physiology originates from research in Wistar, Long Evans, Sprague Dawley, or Lister Hooded rats. This section discusses the core findings from these studies, the limited evidence of aELEs shaping serotonergic function, and efforts in translating these early life paradigms across species into mice.

### **Early handling: Model of Resiliency**

Work by Levine established that developmental trajectory could be significantly influenced by differential early life experience. Evidence of early handling as a significant influence on adult rodent behavior emerged from efforts to develop a rodent model of early life trauma. In a seminal experiment, pups were exposed from postnatal day 1-20 to either (i) a 3 minute mild shock, (ii) brief handling without the shock, or (iii) a non-handled control condition. Contrary to expectations, non-handled controls rather than shocked pups exhibited poor learning and ‘emotional disturbance’ as adults (184). Importantly, adult behavior of animals receiving shocks in early life resembled handled

controls, exhibiting better avoidance learning and normal emotionality relative to non-handled controls. These findings indicated that stress during early life does not necessarily increase long-term vulnerability. Instead, a mitigating factor induced by handling pups not only prevented disturbance, but promoted changes suggesting resiliency. From this original study, a number of studies have revealed underlying molecular changes associated with early handling including a resilient HPA axis profile, with increased hippocampal GR concentrations (185), reduced corticotropin releasing hormone (CRH), lower basal corticosterone levels (186), and an enhanced negative feedback response to stress (90). Behavioral changes are also apparent, with decreased anxiety- and depression- like behavior in adult rats which were handled in early life (187).

### **Maternal Mediation**

Maternal mediation is suggested to underlie early handling induced resiliency in offspring. This is supported by evidence of early handling increasing levels of maternal care behaviors, specifically licking/grooming and arched-back nursing (188-190). Tactile stimulation from the dam is essential to dampening HPA activity in pups, protecting against the catabolic effects of corticosteroids during a sensitive period of development (191). The effect on the dam-pup dynamic, rather than the experience of handling itself, is central to the long-term changes in behavior, physiology, and gene expression. This is supported by evidence of handled dams while leaving pups un-manipulated. For example, un-manipulated offspring of handled dams also exhibit adult behavioral and HPA axis responses paralleling their handled counterparts (192), strongly implicating changes to

the dam-pup dynamic in increasing long-term resilience. In summary, maternal mediation is central to pup development, with resiliency from early handling induced by enhancing maternal response.

Postulated to underlie enhanced maternal care from early handling are increased auditory ultrasonic vocalizations (USVs) from pups. Because pup USVs are inherently stressful to the dam, maternal response is stimulated in an effort to reduce the frequency of USVs (193), linking pup distress to maternal behavior. This is supported by evidence from mouse dams treated with the anti-anxiety drug, chlorodiazepoxide, reducing maternal response post-separation, and preventing development of a resilient phenotype in their offspring as adults (194). Additional factors which also may enhance maternal care include pup size and sex. In summary, pup USVs stimulate maternal response, underlying early handling-induced enhanced maternal care and long-term offspring behavior.

### **High vs. Low Quality Dam Care**

A more naturalistic paradigm is selecting for high- versus low- maternal care providers. Rather than handling inducing enhanced maternal care, the selection for natural maternal behavior removes potential confounds from handling experience and provides a direct assessment of the influence of maternal care. Similar to early handling, pups raised by high-care providers exhibit lower anxiety- and depression-like behaviors relative to those raised by low-care providers (195), with corresponding changes reflecting resiliency in the stress axis. This includes increased hippocampal GR expression, lower CRH levels, and enhanced negative feedback response to stressors

(189). Neurogenesis and cellular activity are also affected, including increased brain derived neurotrophic factor (BDNF), cyclic-AMP responsive element binding protein (CREB), acetylcholine esterase, and the synaptic marker synaptophysin, in cortex and hippocampus (151,196). Cross-fostering studies between high vs. low care providers suggest long-term offspring behavior is shaped by a combination of early environmental and genetic factors. Cross-fostering offspring of low-care to high-care providers can lower risk of developing anxiety-related behaviors (196), suggesting maternal mediation confers resilience to non-related pups. However, cross-fostering offspring of high-care to low-care providers has no effect on anxiety-related behaviors, indicating genetic or intrauterine factors can also pre-establish resiliency to adverse postnatal experience.

### **Maternal Separation: A Model of Adverse Early Life Experience & Vulnerability**

The maternal separation (MS) paradigm is used to model adverse early life experiences in rats. Although there is no “standard” for MS, a commonly used version entails separation of pups from dams during postnatal days 2-14, using 3 conditions: (i) a prolonged aELE, in which pups experience 180 minutes of separation per day (MS180); (ii) a short aELE, i.e. handling control, in which pups experience 15 minutes of separation per day (MS15); and (iii) an animal facility reared (AFR) control. Because the paradigm requires handling of pups and dams, development is influenced by contributions from early handling, separation duration, and maternal response. As adults, MS15 animals exhibit a profile of resiliency in behavior, stress physiology, and gene expression resembling early handled animals and offspring of high-care dams, whereas MS180 animals exhibit a profile of vulnerability. As adults, MS180 rats exhibit increased

anxiety- and depression- like behaviors, including reduced sucrose consumption, increased freezing, increased despair-like behavior, reduced exploratory behavior, and increased startle response to acoustic stimuli (197,198) (199,200). MS180 rats also exhibit increased ACTH and corticosteroid response to various stressors, including air-puff startle, novel environments, and acute restraint (90,183).

The MS180 condition is proposed to increase lifetime vulnerability by disturbing pup homeostasis and maternal behavior. MS180 Long Evan rat dams exhibit disorganized maternal behavior following separation (201), in contrast to enhanced maternal care following early handling (189). Adverse effects on pup development are prevented by provision of foster litters during separation (such that only the pups, not the dams, experience separation), resulting in offspring with gene expression intermediate between MS15 and AFR counterparts and a stress response / feedback regulation comparable to MS15 animals (202). This suggests that intact maternal care can mitigate stress to pup homeostasis from prolonged separation. There are also reports of a transient compensatory increase in the maternal care of MS180 Wistar rat dams (190). This may reflect a strain-specific response to the MS paradigm, in which the Wistar strain is insensitive to MS180. This is consistent with evidence of no change or improved learning following MS180 in Wistar rats (203). In contrast, MS180 in Long Evans rats more consistently report changes in HPA axis and behavior associated with vulnerability (183,200,201,204). The evidence from MS suggests MS180 affects maternal behavior and pup homeostasis to affect vulnerability in later life, but which may be mitigated by genetic resiliencies.

## **Maternal Separation: Translation to the Mouse System**

In contrast to the extensive early life studies performed in rats, research in mice is limited and often inconsistent. The mouse system offers a richer set of genetic tools for studying gene - environment interactions, but mixed success in mice raises questions regarding translational validity of the paradigm as a model of aELEs across species. Yet, species-specific responses to maternal separation also offers an opportunity to determine factors underlying differential mouse vs. rat responses to aELEs, helping to more clearly define the early life factors governing lifetime resiliency vs. vulnerability.

Reports on depression- and anxiety- related behaviors in adult c57bl/6 mice following MS are mixed. In the Porsolt forced swim test (FST), despair-like behavior is increased (205) or unchanged (206,207) in adult mice subjected to MS180. On anxiety-related measures, some studies report increased anxiety-like behaviors following MS180, suggesting that the MS paradigm translates across species. These studies report less open arm time in the elevated plus maze (EPM), less center time in the open-field test (OFT), and less exploration time and visits in the novel object (NO) test in MS180 mice (208,209). However, another set of studies report unchanged open arm time or entries in the EPM, center time or exploratory activity in the OFT, and transitions in the light/dark (L/D) box (206,208,210,211), suggesting mice are insensitive to the MS180 paradigm. This is challenged by a third set of studies reporting increased open arm time and entries in the EPM, more transitions in the L/D box, and increased center time and vertical exploratory rears in the OFT in MS15 and MS180 adult c5b7l/6 mice (207,212). The third set of findings suggest a unique response to MS, in which both MS15 and MS180 promotes resiliency in mice. However, behavioral results are complicated by evidence for diurnal variation. Specifically, Parfitt et al. showed that anxiety-like behavior in c57bl/6

mice is decreased if tested during the dark phase but increased if tested during the light phase (212).

Understanding of corticosteroid levels at baseline or in response to aELE stress in mice remains limited. In one study, adult MS180 c57bl/6 mice (PND1-10 separation) exhibited elevated corticosterone levels at baseline and in response to stress (28). In another study, adult MS15 and MS180 c57bl/6 mice (PND2-14 separation) exhibited an attenuated or accentuated stress response, respectively, following a 30-minute 100 dB acoustic stress (213), consistent with patterns reported in rats (183). However, a follow-up study from the same lab contradicts earlier reports. Adult MS15 and MS180 c57bl/6 mice exhibit an unchanged or attenuated stress response to a 30-minute 100 dB acoustic stress with the response contingent on whether MS was experienced during the first or last 3 hours of the light phase (212). These findings imply that HPA axis function is altered irrespective of separation duration. The decreased stress response in the adult MS180 c57bl/6 mice would be consistent with an attenuated stress response to mild handling in adolescent MS180 Balb/cJ mice, and leads to the interpretation that HPA response may be altered similarly in MS15 and MS180 mice (211). In conclusion, limited reports exist regarding HPA stress response following aELEs in mice, and these inconsistencies deserve further investigation.

Evidence for long-term changes in gene expression following MS are limited to the HPA axis in mice. Adult MS180 c57bl/6 mice exhibit increased arginine vasopressin (AVP) mRNA levels in the paraventricular nucleus of the hypothalamus, associated with increased inter-male aggression, memory deficits in an inhibitory avoidance task, and increased despair-like behavior (28,208,214). Intriguingly, these changes are associated

with hypo-methylation of a critical regulatory region of AVP, suggesting epigenetic changes as a result of early life experience exists in mice. Though lacking a clear behavior correlate, there is also evidence of a biphasic response of cortical glucocorticoid receptor (GR) expression to MS180 in balb/c and c57bl/6 mice, with decreased GR expression prior to PND21 but increased GR expression after PND21. Hippocampal GR is also decreased in juvenile MS180 balb/c mice, but normalized by adult life (209). The over-expression of GR in the cortex is associated with increased anxiety- and depression-like behavior (215), which may inform the functional significance of increased GR in the cortex of MS180 mice.

In summary, the validity of the MS paradigm in mice is limited by a small number of inconsistent studies. In part, the omission of either the MS15 or AFR condition in comparisons against the MS180 condition complicates cross-study comparisons, making it difficult to discern whether discrepancies between studies are due to handling, separation, strain, or protocol differences. Whereas some are in agreement with the extant model in rats, others report insensitivity or even promotion of resiliency in later life. Understanding the influence of MS in mice is important for validating and strengthening core findings of MS across species. Alternatively, if there is a divergent effect of MS in mice, it offers the opportunity to more clearly delineate the factors that shape early life development in this species.

### **Models of Early Life: Shaping the Serotonergic System**

Although substantial progress has been made in delineating the consequence of early life experience on the HPA axis, its long-term impact on serotonergic development



remains unclear. This section discusses findings from studies investigating the impact of early handling or maternal separation on basal serotonergic activity, serotonergic response to various stimuli, and serotonin-related gene expression in rats.

Early handling can transiently increase 5-HT activity during early postnatal life. In Long-Evans rats, 5-HT turnover in the hippocampus and frontal cortex, but not the hypothalamus, is increased at PND7, but returns to control levels by adult age. Similarly, 5-HT turnover in the olfactory bulb is also reportedly increased in early handled Wistar rats at PND8 (216). The increase in hippocampal 5-HT activity is concurrent with the induction of hippocampal GR expression (217). There is some evidence to suggest that that long-term changes from early handling may act through increased 5-HT activity to effect HPA axis resiliency in later life, although this remains controversial. As adults, early handled rats exhibit lower 5-HT levels and 5-HT<sub>2a</sub> receptor binding, suggesting 5-HT activity in later life is decreased (218). This is supported by decreased expression of the 5-HT<sub>2A</sub>, 5-HT<sub>2C</sub> and 5-HT<sub>3</sub> receptors in the brainstem of MS15 Wistar rats (219), suggesting lower serotonergic activity as a consequence of early handling. Although these reports indicate sensitivity of the serotonergic system to early handling, its functional role remains to be clarified.

The influence of aELEs on development of the serotonergic system is not well understood. In one report, basal 5-HT and 5-HIAA levels in the frontal cortex, hippocampus, and hypothalamus remain unchanged in adult MS180 Long-Evans rats (198), suggesting serotonergic activity is insensitive to aELEs. However, another study reports hippocampal 5-HT, but not 5-HIAA, are decreased following MS180 in adult Sprague-Dawley rats (197). This is consistent with a third report of MS180 decreasing 5-

HT levels in the hippocampus and medial prefrontal cortex of adult Listar Hooded rats (220). These studies imply that 5-HT activity in these regions are reduced following aELEs, which in interaction with other factors, may contribute to long-term vulnerability. However, decreases in hippocampal and cortical 5-HT levels are also reported in early handled rats (discussed previously), highlighting limited understanding of how early life experiences shape development and function of the 5-HT system.

Evidence of altered raphe 5-HT activity in adult life following aELEs is mixed. A modified MS protocol, using a 5 (MS5) or 240 (MS240) minutes/day separation from PND2-20, increases 5-HT raphe immunoreactivity in MS5 and MS240 Sprague-Dawley rats (221), suggesting both short and prolonged MS have similar effects on serotonergic activity. However, other studies report decreased 5-HT levels in the raphe of MS180 Sprague-Dawley rats (222) and low 5-HT immunoreactivity in the median raphe of Wistar rats exposed to chronic foot shock in early life (223). Yet, a fourth study reports unchanged 5-HT and 5-HIAA levels in the raphe of MS180 Sprague-Dawley rats, despite decreases in serotonin transporter mRNA levels (197). In summary, there is evidence to support unchanged, increased, and decreased 5-HT activity in the raphe of rats exposed to MS early in life.

Serotonergic response to antidepressants and stimulants is altered by aELEs. Adult MS180 Wistar rats exhibit increased sensitivity to the SSRI citalopram, increasing somato-dendritic inhibition of raphe firing via enhanced 5-HT<sub>1a</sub> receptor activity without altering its expression (224), which may be related to the increased efficacy of SSRIs reported in individuals with a prior history of abuse (179). Sprague-Dawley rats following a modified MS protocol (60 minutes/day from PND1-9) also exhibit lower

ventral striatum 5-HT levels in response to cocaine (225), which has implications for early life experience-mediated changes in reward circuits.

There is some evidence to suggest aELEs prolong stress-induced 5-HT activity. Raphe 5-HT immunoreactivity immediately after a 1-hour immobilization stress is comparable between AFR and MS180 Sprague Dawley rats (222) and 5-HT turnover is comparable in the frontal cortex, hippocampus, and hypothalamus after a 10-minute immobilization stress between AFR and MS180 Sprague Dawley rats. However, 15 minutes after stress cessation, 5-HT levels remain elevated in the frontal cortex and hippocampus (198), suggesting prolonged serotonergic activity in MS180 rats. Additionally, TPH2 mRNA in the raphe is also elevated following social defeat stress in adult MS180 Long-Evans rats (24), where it may underlie increased serotonergic availability and prolong stress-induced serotonergic activity.

In summary, although studies suggest serotonergic sensitivity to early life, consistent patterns of gene expression, activity, and function are yet to be articulated. Because of strong evidence linking serotonergic abnormalities and aELEs with mental illness, clarifying the role aELEs have in shaping serotonergic systems in not just rat models, but also in mouse models, is critical to understanding lifetime vulnerability to mental illness.

## **F. Epigenetics & Early Life Experience**

Seminal work by the Meaney group provided initial evidence linking epigenetic regulation, long-term changes in gene expression, and early life experience (27). In a model of early life experience-dependent resiliency, offspring of “high-care” mothers,

relative to “low-care” mothers, demonstrate long-term increases in hippocampal glucocorticoid receptor (GR) mRNA and binding. Increases in hippocampal GR mRNA are linked to enhanced negative feedback response to stress, effecting quicker re-establishment of basal plasma corticosterone concentrations. These changes are attributed to maternal behavior, in which high quality care results in downstream mechanisms that increase histone acetylation and decrease DNA methylation of the GR promoter in the hippocampus, affecting long-term levels of hippocampal GR mRNA. In contrast, lower quality care results in histone deacetylation and methylation of the hippocampal GR promoter, presumably resulting in lowering levels of hippocampal GR mRNA. Importantly, pharmacological prevention of histone deacetylation during early life in offspring of low care dams prevents these epigenetic changes. Instead, hippocampal GR expression increases and negative feedback response to stress is enhanced despite not receiving high quality care. This work provides an attractive mechanism, epigenetic regulation, through which early life experience establishes semi-permanent changes in gene expression to affect long-term vulnerability or resilience.

The original study is complemented by recent evidence in a mouse MS paradigm. In mice separated from their mothers in early life, long-term arginine vasopressin (AVP) expression, a potentiator of corticotropin releasing hormone activity, is increased and associated with DNA hypo-methylation of an essential regulatory region (28). A more stressful model, in which mice are separated and weaned early, also reports altered DNA methylation of myelin-related genes in the cortex (226). Abnormalities in the HPA axis and white matter have been reported in clinical populations with a history of childhood

maltreatment (227,228), suggesting epigenetic changes may underlie similar abnormalities in humans.

An essential epigenetic regulator of neuronal development is the Re-1 Silencing Transcription Factor (REST), which binds neural restrictive silencing elements (NRSEs) and recruits additional epigenetic factors to silence target genes. REST regulation is implicated in modulating glucocorticoid receptor mediated gene expression, suggesting it interacts with stress response to control gene expression depending on environmental experience. This is supported by rat models of early life experience. In a rat model of early life experience-dependent resiliency, i.e. brief maternal separation, corticotropin releasing hormone mRNA is decreased concurrent with an increase in REST expression and binding of the CRH gene (33). Conversely, a rat model of early life experience-dependent vulnerability, i.e. prolonged maternal separation, results in decreased REST expression and increased REST4 expression (a dominant-negative splice variant of REST) (229). These changes are associated with impaired negative feedback response to stress and increased despair, anhedonic-, and anxiety- like behavior in adult life. Importantly, cortical injection of REST4 into mice during early life replicates the physiological and behavioral consequences of prolonged separation, implicating REST involvement in establishing long-term resiliency or vulnerability.

The junction between the promoter and 5' untranslated region of the TPH2 gene contains a neural restrictive silencing element (NRSE), with binding of the NRSE by REST demonstrated to reduce expression of TPH2 *in vitro* (230). Because evidence indicates TPH2 is sensitive to stress hormones, regulated by REST, and is implicated in vulnerability for mental illness, TPH2 is a prime candidate for investigating the

intersection between early life experience, serotonergic vulnerability, and epigenetic mechanisms in establishing long-term changes in gene expression and behavior.

#### **IV. Research Objectives**

Adverse early life experience and serotonergic abnormalities are heavily implicated in vulnerability for mental illness. However, understanding remains limited on how aELEs shape the serotonergic system and the mechanisms underlying establishment of long-term vulnerability. This thesis work sought to investigate the influence of MS on TPH2 expression and epigenetic mechanisms associated with and underlying these changes in c57bl/6 mice.

To assess aELE-associated epigenetic changes, a technique for accurately dissecting brain nuclei from thin slide-mounted tissue was developed. Available methods were either prohibitively expensive (laser capture micro-dissection) or insufficiently accurate and unreliable (micro-punch). This novel technique, *in situ* hybridization guided freeze matrix assisted punches (IFAP), dissects thin slide-mounted tissue for DNA methylation analysis. The work presented in **chapter 2** provides an overview of the IFAP technique and its accuracy for dissecting raphe tissue for DNA methylation analysis of the TPH2 promoter & 5' UTR. This work was published in the journal *Biotechniques* in September, 2012.

**Chapter 3** presents a series of maternal separation studies investigating long-term molecular changes in c57bl/6 mice following maternal separation. Both MS15 and MS180 decrease TPH2 mRNA in raphe but do not increase methylation of the TPH2 5' UTR and promoter, indicating that decreases in TPH2 are not regulated by elevated DNA methylation of these regions. Because decreases in TPH2 mRNA were shared in both MS

conditions, the focus of this thesis work shifted towards understanding the unexpected parallel changes in MS15 and MS180 –treated mice, investigating whether behaviors and expression of related genes were similarly coincident and explained by compensatory maternal care. Part of this work was published in the journal *Hormones & Behavior*, December, 2012.

Expression analysis of functionally related genes, serotonin transporter (SERT) and glucocorticoid receptor (GR), suggests MS15 and MS180 both decrease raphe SERT mRNA and increase hippocampal GR mRNA. Behavioral assessment, presented in **chapter 4**, also indicates a shared decrease in anxiety-like behaviors corresponding to common patterns of hippocampal GR mRNA. These results suggest that MS15 and MS180 promotes resiliency irrespective of separation duration in c57bl/6 mice. Investigation into whether maternal mediation underlie resiliency revealed an enhanced level of maternal care in MS180, and to a lesser extent, MS15 dams. An increase in post-separation licking / nursing / covering behavior strongly implicates maternal mediation in mitigating the stress from separation and promoting long-term resiliency in c57bl/6 mice.

The increased hippocampal GR mRNA, decreased anxiety-like behavior, and enhanced maternal care suggests that low TPH2 mRNA has a functional role in establishing an anxiolytic-like phenotype. To clarify its role, a TPH2 transgenic knockdown model was developed and characterized. The work in **chapter 5** provides evidence of decreased anxiety- and depression- like behavior from knockdown of TPH2, supporting a role for MS-mediated decreases in raphe TPH2 in establishing long-term decreases in anxiety-like behaviors.

Taken together, the data indicates that (i) MS, irrespective of 15 or 180 minute separation duration, affects a pattern of gene expression and behavior in c57bl/6 mice resembling long-term resiliency; (ii) direct attenuation of TPH2 mRNA in transgenic mice results in long-term decreases in anxiety-like behaviors, supporting a functional role for decreases in TPH2 mRNA on the behavior of MS mice; and (iii) MS enhances maternal care, possibly mitigating the stress from separation and promoting long-term resiliency in c57bl/6 mice.



## **Chapter 2 - ISH-guided freeze-matrix assisted punches (IFAP): Technique for extracting punches from thin slide-mounted tissues for DNA methylation analysis.**

*(a version of this chapter was published in Biotechniques, September, 2012).*

### **I. Abstract**

Methods of dissection of discrete brain regions for molecular analysis are complicated by tradeoffs between accuracy, flexibility, and costs. Because of this limitation, we developed a flexible and cost-effective method, in situ hybridization (ISH) guided freeze-matrix assisted punches (IFAP), for extracting nanogram quantities of DNA from slide-mounted sections as thin as 12  $\mu\text{m}$ . Using ISH to localize regions of interest, tissue is targeted by applying a small bead of M-1 embedding matrix onto cryosections, snap-freezing, and collecting the beads for nucleic acid purification. The method quantitatively recovers RNA and DNA usable for PCR and DNA methylation analysis. The development of this technique was used for DNA methylation analysis of the tryptophan hydroxylase 2 gene in maternal separation studies.

### **II. Materials and Methods**

DNA methylation is one mechanism by which long-term changes in gene expression are effected. Robust changes in the pattern of DNA methylation in homogeneous macroscopic tissues, e.g., carcinomas or cultured cells, are well established (231,232). It has been posited that experience-dependent DNA methylation also occurs in

healthy normal brain tissue, but data has remained limited and controversial (233). Issues pertaining to anatomical specificity, sample recovery, or high-cost have limited progress.

The micro-dissection of tissue sections is tedious, requires thick sections and is limited by punch availability. Recovery of tissue from fresh frozen thin sections, suitable for anatomical studies, is all but impossible. We developed a technique, based on the principle of ISH-guided laser-capture micro-dissection (234), for punching portions of tissue from slide-mounted frozen thin sections. By applying a small bead of liquid onto cryosections and snap-freezing, underlying tissue is lifted when beads are pulled away, and DNA is extracted from bead-tissue complexes using conventional high-salt DNA extraction. This method, ISH-guided freeze-matrix assisted punches (IFAP), facilitates rapid and efficient recovery of discrete regions under conditions suitable for DNA methylation analysis. IFAP utilizes commonly available histological resources to maintain low-cost, and allows for parallel analyses from source tissue.

The IFAP method synergizes well with standard histological workflows. Tissue processing is streamlined, with sections ( $\geq 12 \mu\text{m}$ ) mounted on  $n$  slide sets (sections 1,  $1+n$ ,  $1+2n$ ...on set 1; sections 2,  $2+n$ ,  $2+2n$ ...on set 2; etc., up to sections  $n$ ,  $2n$ ,  $3n$ ...on set  $n$ ). Each set represents a staggered survey through the region of interest. An *ISH* is performed on one set to localize the target area, and is used to guide IFAP dissection on an adjacent set. IFAP in principal works similar to laser capture micro-dissection (LCM), but instead uses low-temperatures to bond tissue with pipette-applied matrix beads. Although not comparable in spatial specificity to LCM, IFAP is low-cost and rapid, useful in millimeter scale dissections that do not require single-cell resolution.

The default approach to isolating nuclei from surrounding tissue is the micro-punch (MP), which utilizes a hollowed sharpened cylinder to punch tissue (235). In comparison, IFAP offers several advantages: (1) flexibility in sample condition and (2) workflow efficiency for parallel analyses. In our experience, MP is typically more effective on fresh tissues and requires thicker sections ( $\geq 60 \mu\text{m}$ ) if frozen and slide-mounted. Additional difficulty occurs if sections are mounted on charged or treated slides, which are commonly used for histochemistry to improve sample retention. A theoretical workaround is to alternate thick and thin slices along with slide coatings, but in practice is cumbersome and complicated. IFAP offers a slice resolution of at least  $12 \mu\text{m}$ , and works with previously stored slide-mounted sections, including charged slides. Thus, it introduces nothing new to the workflow, and can be readily applied in a range of conditions. In this study, we detail the use of IFAP in excision of brain nuclei and evaluate sample integrity for use in assessing DNA methylation. We use Tryptophan Hydroxylase 2 (TPH2), a predominantly raphe-restricted gene, as our test gene.

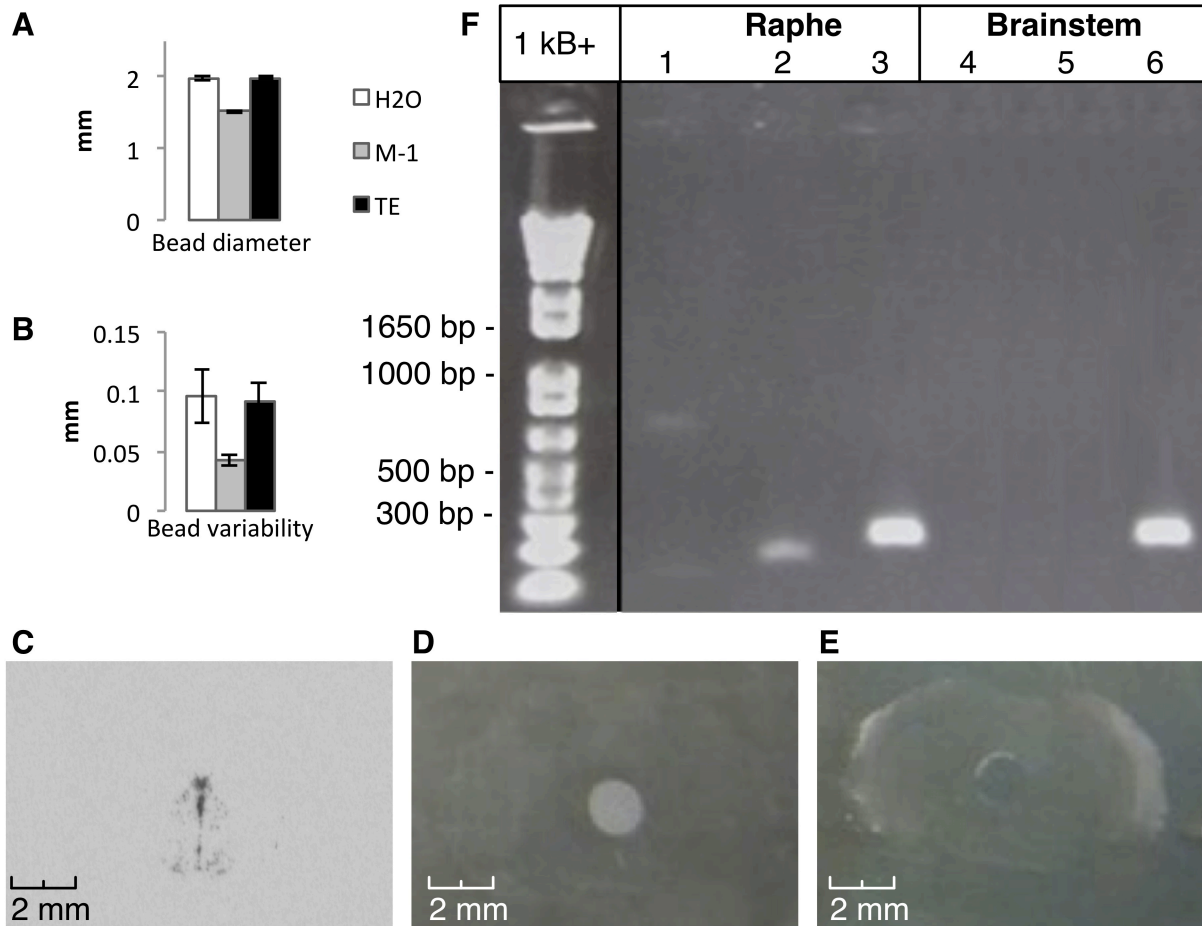
For IFAP, three brains from postnatal day 60 c57/bl6 male mice were sectioned at  $12 \mu\text{m}$  and mounted on Superfrost slides (Fisher Scientific). A fourth mouse brain, for MP comparisons, was sectioned by alternating between  $12 \mu\text{m}$  and  $100 \mu\text{m}$  slices, with the  $12 \mu\text{m}$  sections used for ISH-guidance and  $100 \mu\text{m}$  sections reserved for MP. This alternation of section depth is necessary because MP is impractical on thinly-sliced, frozen slide-mounted sections. Slides were stored at  $-80^\circ\text{C}$ , then re-equilibrated to  $-20^\circ\text{C}$  prior to dissection. All supplies were pre-equilibrated to  $-20^\circ\text{C}$  to prevent beads from re-liquefying. For liquid application, TipOne ultra-low retention pipette tips (USA Scientific) were used to minimize volume variance.

Initially, tests were run to characterize the suitability of different liquids, TE (10 mM Tris-HCl, EDTA 0.1 mM, pH 8.0), H<sub>2</sub>O, or M-1 embedding matrix (Shandon\* Thermo Scientific), for binding and lifting tissue. The rationale for testing M-1, a water-soluble embedding matrix conventionally used to support sectioning of frozen tissue, was that its viscous consistency would minimize variability in bead diameter. Per liquid, beads were tested for (i) dispersal and uniformity and (ii) structural integrity during removal. Diameters were measured (ImageJ) every 30° across each bead. The average bead diameter from 0.5 µl of M-1 was ~1.5 mm, whereas TE and H<sub>2</sub>O were ~2 mm (**Fig. 2-1A**). Moreover, bead diameter variance was less for M-1 compared to TE or H<sub>2</sub>O beads (**Fig. 2-1B**), indicating better uniformity in bead dimensions. Qualitatively, H<sub>2</sub>O and TE beads were more prone to fracture during removal from slides, negatively affecting recovery. For these reasons, M-1 was optimal for IFAP dissections.

To test IFAP specificity, the dorsal raphe, a region rich in serotonergic (5-HT) neurons, was targeted for extraction from surrounding brainstem tissue. *In situ hybridization* histochemistry for TPH2 (**Fig. 2-1C**) guided IFAP dissection on adjacent sections (**Fig. 2-1D**). The ISH was used to identify (i) raphe-containing sections and (ii) localize the tissue areas corresponding to the highest TPH2 optical density within a 1.5 mm diameter circular area. For the full ISH protocol, see Vazquez, 2012 (236). Template for sense and anti-sense probes were generated from cDNA (EST: BG084420). Using ISH as a reference, each slide was briefly brought to room temperature, and a 0.5 µl aliquot of M-1 embedding matrix applied to each section on the slide. After application, slides were quickly placed onto dry ice to snap-freeze the bead (Fig. 1D). For punch removal, slides were transferred to the cryostat, and the blunt edge of a razor blade used

to dislodge each bead from the slide, simultaneously lifting tissue (Fig. 1E). A total of 10 beads per sample was used. After all beads were collected in a chilled 1.5 mL tube, the tube was briefly centrifuged for 5-10 seconds at ~4000g.

To verify IFAP accuracy, RNA was purified (TRIZOL), re-suspended (20  $\mu$ l), reverse-transcribed (10  $\mu$ l) using random 8-mers (Superscript II), and cDNA amplification from raphe-specific genes, TPH2 and LMX1B, compared against RNA from an intact brainstem section from an adjacent slide. For cDNA amplification, the following exon-spanning primers were used: LMX1B: 5'-CTGCTGTGCAAGGGTGACTA / 5'-AAACCAGACCTGGACCACAC, TPH2: 5'-TGTCCTTGGATTCTGCTGTG / 5'-GCCACCAACTTCATTCTTC, and HPRT: 5'-CAGTACAGCCCCAAAATGGT / 5'-GCGCTCATCTTAGGCTTTGT. As expected, due to the higher concentration of raphe tissue extracted using IFAP, IFAP samples successfully amplified TPH2 and LMX1B, whereas RNA from the brainstem section only amplified the ubiquitously expressed HPRT (**Fig. 2-1F**). This confirms specificity for targeting nuclei and regionally-restricted genes, as well as integrity of RNA by this method. Amplicons for TPH2 and LMX1B were TOPO cloned (Invitrogen) and sequence verified.



**Figure 2-1 IFAP extraction of target nuclei and target specificity.**

(A) Mean  $\pm$  SEM of the average bead diameters. (B) Mean  $\pm$  SEM of standard deviations of 6 diameter measurements per bead. (C) TPH2 *ISH* delineates the region of interest. (D) Beads are snap-frozen over the region of interest. (E) Bead removal lifts tissue from the slide. (F) Amplification of target cDNA from reverse-transcribed extracted RNA, run on a 1% TBE agarose gel. Raphe tissue was isolated using IFAP M-1. Brainstem tissue corresponds to an adjacent whole section. Lanes 1 & 4: LMX1b; Lanes 2 & 5: TPH2; Lanes 3 & 6: HPRT.

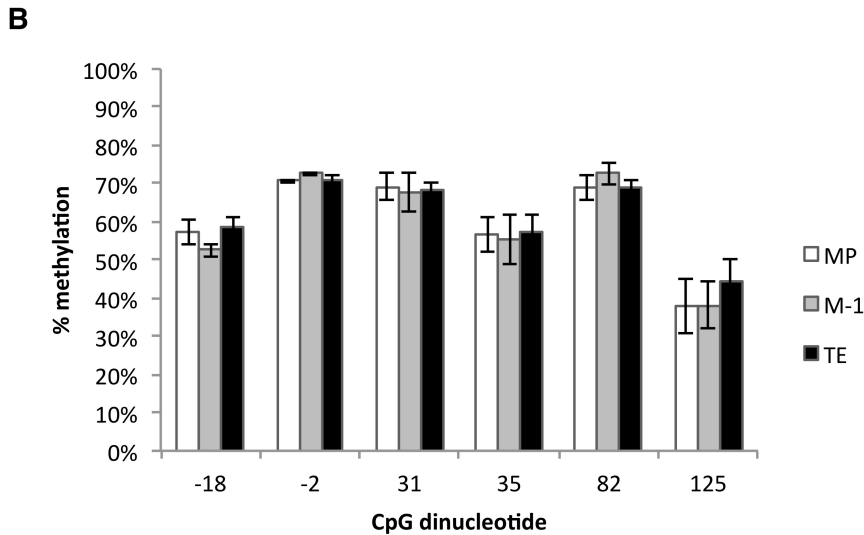
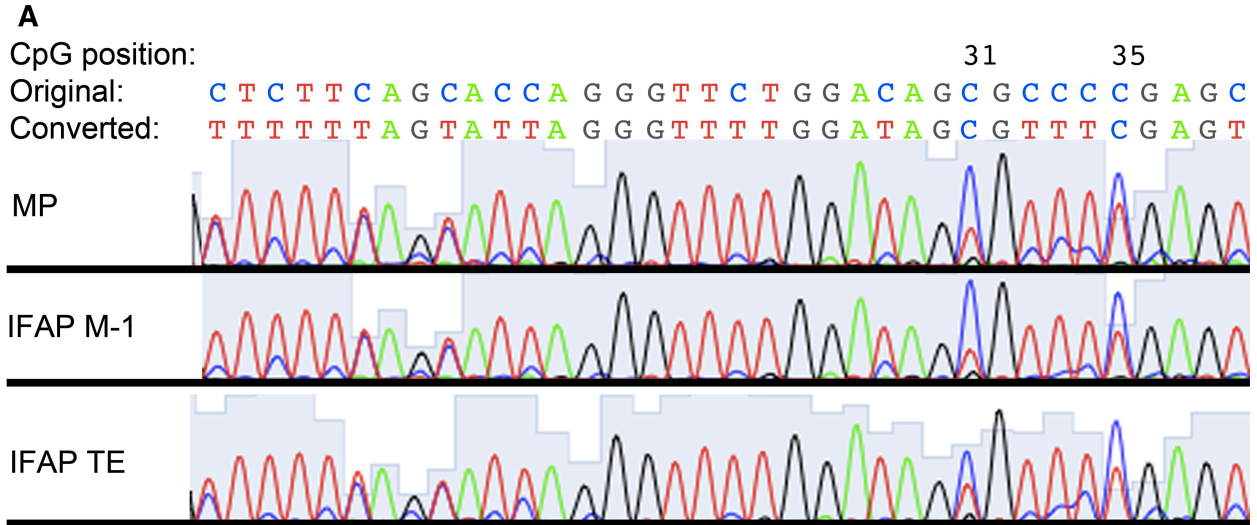
For genomic DNA (gDNA) extraction, 10 beads per sample were treated to a standard high-salt TNES DNA extraction protocol (237) and re-suspended in 25  $\mu$ l TE (10 mM Tris, 0.1 mM EDTA). For TE beads, 1  $\mu$ l of 10 mg/mL glycogen was added as a co-precipitant prior to precipitation, whereas M-1 contained an inherent co-precipitant (likely polyethylene glycol) and did not require glycogen. As a result, DNA pellets were

also more gelatinous and required additional time (5 minutes) and heat (50°C) for re-suspension. Although in our downstream tests, M-1's unknown proprietary ingredients had no adverse effect on bisulfite conversion or sequencing, nanodrop readings revealed elevated peaks at 220-230nm, which complicated accurate gDNA quantitation. This necessitated alternate quantitation by SYBR green fluorescence. Each gDNA sample was diluted 1:100 in TE pH 7.5 (10 mM Tris, 0.1 mM EDTA) and mixed with an equal volume of a 1:1000 dilution of SYBR green I (Invitrogen) in TE pH 7.5, for a final volume of 200 µl. Fluorescence was compared to a DNA standard curve with M-1 added (0.5 µl per bead in sample) to extrapolate yield. Fluorescence quantitation of gDNA from 10 beads returned ~400 ng of gDNA. For comparison, commercial kits typically return ~1-3 µg of gDNA per mg of brain tissue. Based on 0.5 µl IFAP beads generating ~1.5 mm diameter punches of 12 µm thickness, 10 punches would have an estimated mass of ~212 µg (assuming density of 1), returning 212-646 ng of gDNA. Observed yield (400 ng) is well within expectations.

To test usability of IFAP-extracted samples for DNA methylation analysis, we assessed the sequence quality of gDNA obtained using IFAP (M-1 or TE) from slide-mounted thin sections (10 beads) against tissue obtained using a 1.0 mm diameter MP (VWR) on thick sections and replicated across 3 independent samples. Genomic DNA was converted with sodium bisulfite (Qiagen Epitect) and DNA methylation assessed at the TPH2 promoter & 5' untranslated region (UTR) using a nested PCR approach, with inner nested primers fused to an M13 adaptor to improve sequencing. Outer nested primers were: 5'-TAGAYGTGTAATTTGATTGTGGTTATTAGT-3' and 5'-TCCCAACAACRCRCCCAACTAC-3'. Inner nested primers were: 5'-M13fwd-

TGATTGTGGTTATTAGTAATTAGAAATGAGTT-3' and 5'-M13rev-AATCCAAAAACAACCCTCTCC-3'. Amplicons were column-purified (Qiagen Qiaquick) and sequenced bi-directionally (Applied Bio-systems Model 3730 XL). Percent methylation was estimated by comparing cytosine:thymine peak ratios in CpG dinucleotides [% methylation = (C)/(C + T)]. Sequence chromatograms of MP and IFAP M-1 or TE indicated comparable conversion of un-protected cytosines (**Fig. 2-2A**). Furthermore, no difference in CpG dinucleotide methylation was observed, indicating minimal IFAP interference with bisulfite conversion (**Fig. 2-2B**).





**Figure 2-2 Analysis of DNA methylation using MP, IFAP M-1, or IFAP TE extracted samples at the TPH2 promoter and 5' UTR.**

(A) Sequence chromatograms of sodium-bisulfite converted amplicons. Original and expected bisulfite-converted sequences are notated, and CpG positions marked relative to putative transcription start site at the promoter and 5' UTR junction. Bisulfite conversion of cytosine to thymine is comparable between MP and IFAP. (B) Methylation of CpG dinucleotides is indistinguishable between MP and IFAP.

Because there is, to our knowledge, no publication of brain nuclei specific differences in methylation pattern, we're unable to definitively ascertain IFAP's sensitivity for detecting methylation differences. Although this method minimizes

inclusion of non-nuclei tissue by reducing section thickness requirements, it does remain limited by cell heterogeneity, i.e. neuronal vs. glial, in the target nuclei, and is unable to discriminate cell-type restricted changes in DNA methylation. Notwithstanding this limitation, the successful extraction of RNA, conversion, and amplification of TPH2 and LMX1b cDNA suggests a level of sensitivity comparable to MP while offering increased targeting accuracy and streamlined processing when using conventional slide-mounted frozen tissue sections.

In conclusion, IFAP is a flexible, low-cost, and simple method for dissecting nuclei from thin slide-mounted sections. The method synergizes well with conventional histochemical techniques, allowing for parallel investigations with tissue derived from the same animal. The central advantages of IFAP over MP are (i) seamless integration into the histochemical workflow, (ii) quantitative recovery from thin, slide-mounted frozen sections, (iii) no need for specialized equipment and (iv) targeting accuracy using ISH. We verified that gDNA and RNA extracted by this method can be used in DNA methylation analysis and cDNA synthesis, respectively. IFAP uses commonly available equipment, is low-cost, and is capable of extracting nuclei from slide-mounted sections as thin as 12 microns for downstream applications.

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## **Chapter 3 - Maternal separation alters serotonergic and HPA axis gene expression independent of separation duration in c57bl/6 mice**

### ***I. Abstract***

Adverse early life experiences (aELEs), such as child abuse, neglect, or trauma, increase lifetime vulnerability for mental illness. In this study, aELEs were modeled in c57bl/6 mice using the maternal separation (MS) paradigm, in which pups were separated for 180 min/day (MS180), 15 min/day (MS15), or left undisturbed (AFR) from postnatal day 2-14. As adults, pups that experienced MS15 or MS180 demonstrated decreases in tryptophan hydroxylase 2 (TPH2) and serotonin transporter mRNA in the dorsal raphe dorsalis and ventralis, and increases in glucocorticoid receptor mRNA in the dentate gyrus of the hippocampus. To investigate factors underlying shared expression between MS conditions, dam on-nest time and DNA methylation at the TPH2 promoter and 5' UTR were assessed. Post-reunion on-nest time increased as a function of separation duration, potentially serving as a mitigating factor underlying similar expression between MS conditions. TPH2 DNA methylation remained unchanged, suggesting changes in TPH2 mRNA are not mediated by changes in DNA methylation of this region. The shared pattern of expression between MS15 and MS180 conditions suggests a species- or strain- specific response to MS unique to c57bl/6 mice.

## **II. Introduction**

Adverse early life experiences (aELEs), such as child abuse, neglect, and trauma, increase lifetime risk for mental illness. Limited clinical studies suggest immediate and long-term irregularities in stress responses (7). In parallel, abnormalities of the serotonin (5-HT) system, a neurotransmitter network implicated in modulating complex behaviors, is associated with the pathogenesis or vulnerability for mental illness (34). However, much remains unknown regarding the long-term effects of aELEs on serotonergic function.

Polymorphisms in **tryptophan hydroxylase 2 (TPH2)** and **serotonin transporter (SERT)** are implicated in vulnerability for mental illness (13,18). TPH2 is the rate-limiting enzyme in neuronal 5-HT synthesis (238), whereas SERT is the primary transporter responsible for reuptake of 5-HT (239). TPH2 mRNA levels are increased in depressed suicides (25) and polymorphisms are associated with risk for ADHD, anxiety disorders, suicide, and depression (18). Limited reports suggest that aELEs, such as immune system challenge (240) or maternal separation (24) can alter TPH2 expression. Highlighting serotonergic and stress axis sensitivity to early life events, rhesus macaques carriers of a hypomorphic TPH2 allele exhibit increased stress levels if exposed to low-quality peer care as infants (241). Similarly, SERT polymorphisms increase risk for depression and anxiety disorders, and interact with aELEs to increase risk for mental illness (13).

A popular model of aELE is maternal separation (MS), in which rat pups are separated for 15 (MS15) or 180 (MS180) minutes per day from postnatal day (PND) 2-14. MS180 is associated with long-term increases in anxiety-related behaviors and abnormal stress axis function in rats. Conversely, MS15, the handling control, is

associated with a “stress-resilient” profile characterized by lower anxiety-related behaviors and quicker feedback response (242). In part, long-term changes in behavior and stress response are thought to be mediated by changes in expression of the **glucocorticoid receptor (GR)**, a receptor intimately involved in negative feedback and stress response (187). Although the stress axis shares extensive anatomical and functional connections with the serotonergic system (20), reports on serotonergic changes following MS have been inconsistent. For example, adult SERT expression in MS180 rats is reported to increase (243), decrease (197), or remain unchanged (219), highlighting difficulties in demonstrating a consistent effect on serotonergic development.

In parallel, emerging evidence suggests early experience-dependent changes in gene expression are mediated by epigenetic mechanisms (27). Long-term decreases in anxiety behaviors and expression of corticotropin releasing hormone (CRH), a stress peptide, following MS15, are linked to increases in the epigenetic regulator RE-1 Silencing Transcription Factor (REST) (33), a known regulator of TPH2 (230). Conversely, prolonged separations, i.e. MS180, decreases REST mRNA in rats, and injections of a dominant-negative isoform, REST4, during postnatal but not adult life elevates anxiety in c57bl/6 mice (229). This invites the possibility that early experience-dependent changes in expression of other REST-regulated genes, such as TPH2, are epigenetically mediated.

Although predominantly a rat model of aELE, there is interest in adapting the MS paradigm in mice. This is important for the validation and applicability of findings for clinical research, as well as combining a well-validated aELE model with the extensive genetic toolkit readily available in mice. In support, Murgatroyd et. al reports that MS in

c57bl/6 mice induces epigenetic changes and abnormalities in behavior and the HPA axis, suggesting the strain is ideal for investigating aELE-mediated epigenetic changes (28). However, although some studies report expected changes in behavior and stress axis response to MS (208,214,244), others have suggested a resiliency to MS in c57bl/6 mice (206,212). Therefore to clarify and explore the MS paradigm in c57bl/6 mice, we performed MS to investigate (i) long-term changes in the expression of TPH2, SERT, and GR mRNA, (ii) maternal response to MS, and (iii) DNA methylation of the TPH2 promoter and 5' untranslated region.

### **III. Results**

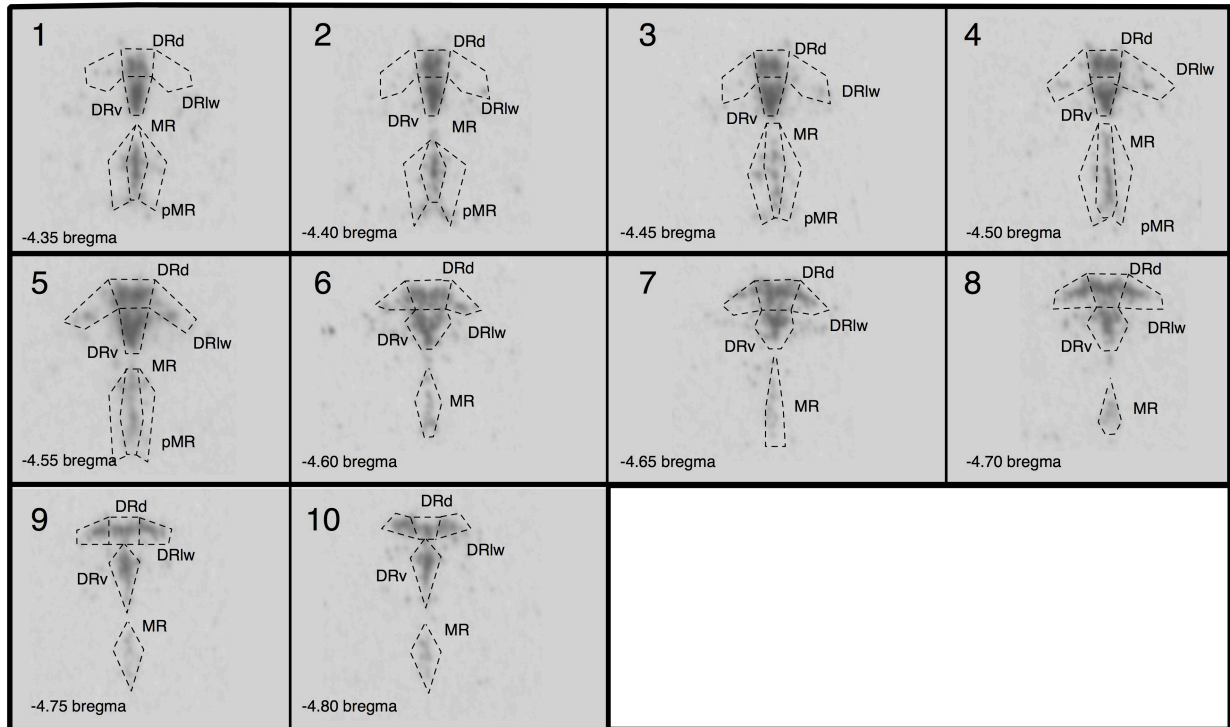
To assess whether maternal separation (MS) affected gross development, mice were weighed on postnatal day (PND) 2, 14, 21, 35, and 60. Weight was not different between groups on all days (**Fig. 3-1**).

	<b>PND2</b>	<b>PND14</b>	<b>PND21</b>	<b>PND35</b>	<b>PND60</b>
MS15	1.70 +/- 0.05	6.75 +/- 0.22	9.49 +/- 0.30	19.94 +/- 0.43	23.91 +/- 0.46
AFR	1.66 +/- 0.08	6.96 +/- 0.30	9.51 +/- 0.27	20.95 +/- 0.35	24.68 +/- 0.38
MS180	1.66 +/- 0.15	6.92 +/- 0.40	9.26 +/- 0.57	20.13 +/- 0.67	23.73 +/- 0.48

**Figure 3-1 Weight across development.**

Weight in grams.

Because analysis incorporates gene expression data from the original and replicate study, optical density from MS15 and MS180 sections were normalized against the AFRs of each study. Raphe expression was assessed across 10 sections from -4.35 to -4.80 bregma (245), covering the dorsal raphe dorsalis (DRd), ventralis (DRv), lateral wings (DRlw), median raphe (MR), and paramedian raphe (pMR) (**Fig. 3-2**).



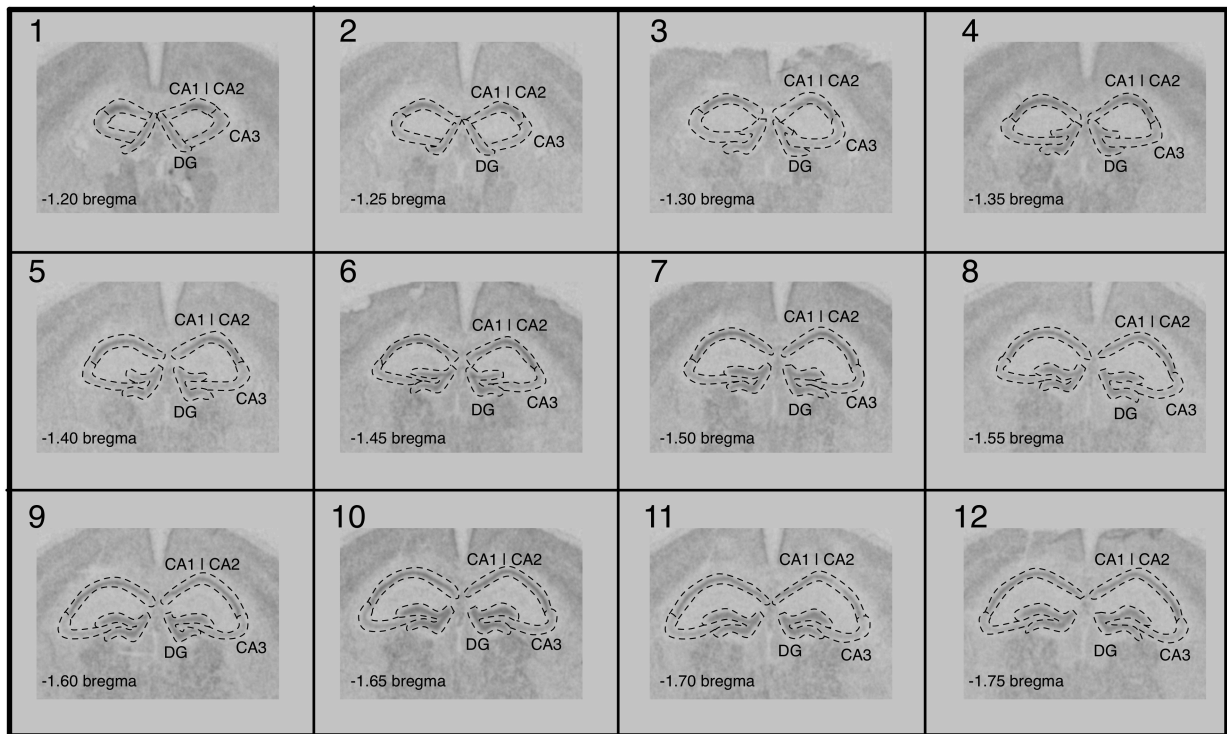
**Figure 3-2 Raphe in situ hybridization for sub-regional analysis.**

Representative ISH for TPH2 and the regional divisions for raphe TPH2 and SERT analyses. Abbreviations: DRd = dorsal raphe (DR) dorsalis; DRv = ventralis; DRlw = lateral wings; MR = median raphe; pMR = paramedian raphe.

Analyses of TPH2 mRNA and SERT mRNA levels across raphe subdivisions revealed significant differences contingent upon having experienced MS, but largely independent of the duration of separation. TPH2 mRNA was decreased in MS15 and MS180 mice in the DRd [ $F(2,82) = 16.676; p < 0.001$ ] and DRv [ $F(2,82) = 22.986; p < 0.001$ ]. However, only MS15 mice had decreased TPH2 mRNA in the MR [ $F(2,82) = 4.545; p = 0.013$ ], with a similar non-significant trend in the dorsal raphe lateral wings (DRlw) [ $F(2,82) = 2.584; p = 0.082$ ] and paramedian raphe (pMR) [ $F(2,82) = 2.739; p = 0.071$ ] (**Fig. 3-4A**). A similar pattern was observed for SERT mRNA in raphe, with decreased levels in the DRd [ $F(2,81) = 12.140; p < 0.001$ ] and the DRv [ $F(2,81) = 19.952; p < 0.001$ ] for MS15 and MS180 mice, but unchanged in the DRlw [ $F(2,81) = 0.407; p = 0.667$ ], MR [ $F(2,81) = 2.318; p = 0.105$ ], and pMR [ $F(2,81) = 1.030; p = 0.362$ ] (**Fig. 3-4B**).



Reports on MS-related changes in TPH2 and SERT mRNA levels are limited. As hippocampal GR has been widely reported to be sensitive to early life experience, we used it as a marker to assess effectiveness of MS180 as an aELE in c57bl/6 mice. Dorsal hippocampal GR mRNA was analyzed in the dentate gyrus (DG), CA3 region, and CA1|CA2 region (**Fig. 3-3**). Hippocampal GR mRNA was increased in MS15 and MS180 conditions in the DG [ $F(2,86) = 5.156$ ;  $p = 0.008$ ] and trended in a similar direction in the CA3 [ $F(2,86) = 2.851$ ;  $p = 0.063$ ] and CA1|CA2 regions [ $F(2,86) = 1.324$ ;  $p = 0.271$ ] (**Fig. 3-4C**).

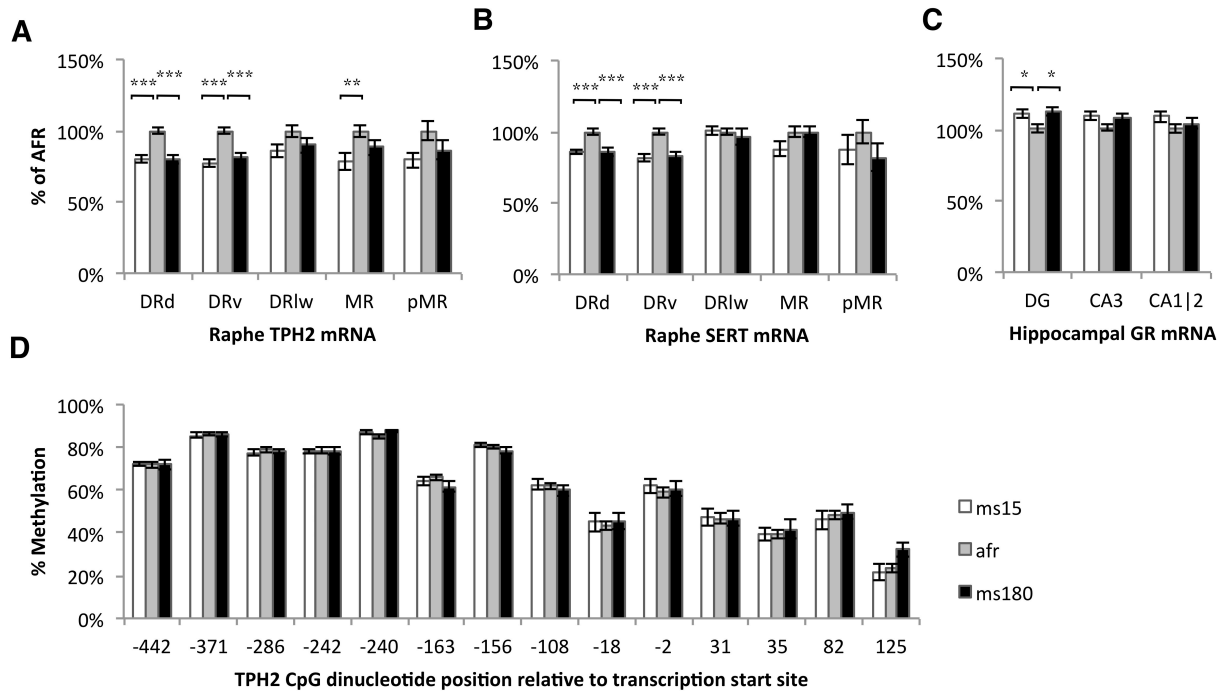


**Figure 3-3 Dorsal hippocampus in situ hybridization for sub-regional analysis**

Representative ISH for GR and regional divisions for dorsal hippocampal analyses. Abbreviations: DG = dentate gyrus. CA = cornu ammonis.

To investigate whether changes in TPH2 mRNA were epigenetically regulated, DNA methylation was assessed at the TPH2 promoter and 5' UTR using sodium bisulfite sequencing. This region was chosen for its high number of CpG dinucleotides, high CG content (50%+), and inclusion of a REST binding site. However, despite differences in

TPH2 mRNA between conditions, DNA methylation in this region remained unchanged (Fig. 3-4D).

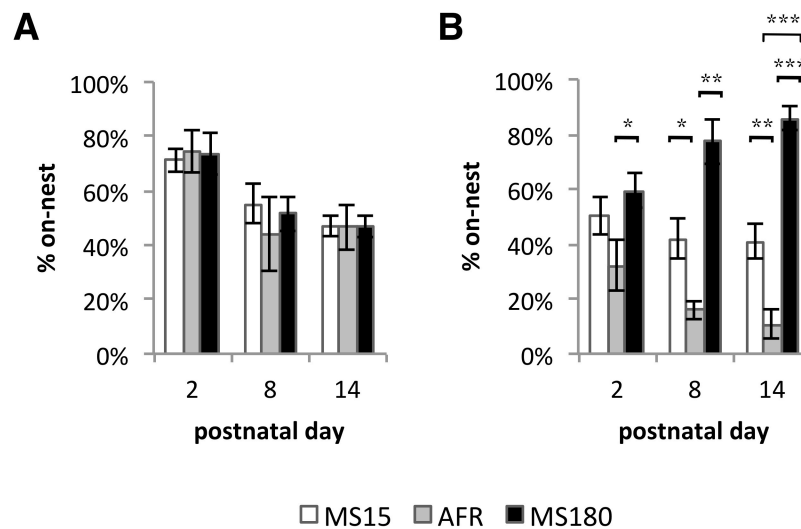


**Figure 3-4 TPH2, SERT, and GR mRNA levels.**

(A) Raphe TPH2 mRNA. (B) Raphe SERT mRNA. (C) Hippocampal GR mRNA. (D) TPH2 promoter and 5'UTR DNA methylation. Nucleotide numbering is in reference to the putative transcription start site. ISH signal was normalized against the AFR condition and expressed as a percentage of AFR expression. DRd = dorsal raphe (DR) dorsalis; DRv = ventralis; DRlw = lateral wings; MR = median raphe; pMR = paramedian raphe; DG = dentate gyrus; CA = cornu ammonis. Statistical significance is denoted by:  $p < .001$  (\*\*\*),  $p < .01$  (\*\*),  $p < .05$  (\*).

We considered the possibility that maternal response may mitigate the stress of separation. A third cohort, independent of animals used for determining expression, was used to determine maternal behavior during the MS paradigm. Pre- and post- separation on-nest time was assessed on PND2, 8, and 14, concurrent with weekly cage cleaning. Thus, pups (handled as a group and remaining with nest) and AFR dams were also briefly removed for 1-2 minutes during this period. Pre-separation on-nest time remained unchanged (Fig. 3-5A), but post-separation on-nest time increased with separation

duration. The effect was marginal on PND2 [ $F(2,20) = 3.01$ ;  $p = 0.07$ ], but significantly differentiated on PND8 [ $F(2,21) = 12.12$ ;  $p < 0.001$ ], and PND14 [ $F(2,21) = 41.53$ ;  $p < 0.001$ ] (**Fig. 3-5B**). MS180 dams spent more time on-nest post-reunion than AFR [ $PND2$ :  $p = 0.06$ ;  $PND8$ :  $p < 0.001$ ;  $PND14$ :  $p < 0.001$ ] and MS15 [ $PND2$ :  $p = 0.25$ ;  $PND8$ :  $p = 0.04$ ;  $PND14$ :  $p < 0.01$ ] dams. Similarly, MS15 dams also exhibited higher time on-nest post-reunion than AFR dams on PND8 [ $p = 0.04$ ] and 14 [ $p < 0.01$ ].



**Figure 3-5 Maternal care behavior.**

(A) Percent time spent on-nest prior to separation by the dam. (B) Percent time spent on-nest immediately post-separation by the dam. Significance is denoted by  $p < .001$  (\*\*\*),  $p < .01$  (\*\*),  $p < .05$  (\*).

#### **IV. Discussion**

##### **Species-specific response to MS**

The results from this study suggest that MS, a common model of aELEs in rats, is not directly transferable to c57bl/6 mice. Instead, both MS15 and MS180 expression patterns for GR, TPH2, and to a lesser extent, SERT mRNA in mice, are similar to

findings from neonatal handled, i.e., MS15 rats. Prior reports in rats suggest MS15 results in a resilient phenotype, characterized by long-term increases in hippocampal GR, blunted stress-induced neuroendocrine response, and decreases in anxiety-like behaviors (246,247). Conversely, MS180 is reported to produce a vulnerable phenotype, with decreases in GR, prolonged neuroendocrine response to stress, and elevated anxiety-related behaviors (183,198). We find GR increases for both MS conditions in c57bl/6 mice, which corresponds with a prior report of blunted MS15 and MS180 stress-induced corticosterone response to acoustic stress (212). These findings would suggest that experiencing MS, irrespective of duration, may result in neuroendocrine resiliency to stress in c57bl/6 mice.

The decrease of TPH2 mRNA in MS15 and MS180 mice is consistent with a stress-resilient pattern of expression. Our findings stand in contrast to reports of TPH2 expression in rat models. In Long-Evans rats, MS15 decreases and MS180 increases TPH2 mRNA, with the magnitude of increase in MS180 rats magnified by social defeat (24). Similarly, chronic immobilization stress increases TPH mRNA and protein levels in rat DR and MR (248,249), and inescapable acoustic stress increases phosphorylation-dependent TPH activity in rat cortex and raphe (250). Our evidence suggests that the stress of MS180 is either insufficient or mitigated in c57bl/6 mice with respect to TPH2 expression. Parallel decreases in raphe TPH2 expression and increases in hippocampal GR mRNA under MS15s and MS180 conditions suggests a stress resilient phenotype in c57bl/6 mice.

The decrease in SERT mRNA for both MS15 and MS180 conditions is consistent with congruent changes in GR and TPH2 mRNA in our study. However, interpreting the

functional effect of a SERT decrease is complicated by mixed reports following MS, which may be due to genetic differences across strains. In the Wistar strain, brainstem SERT mRNA is decreased in MS15, but unchanged in MS180 rats (219). In the Sprague-Dawley strain, raphe SERT mRNA is decreased in MS180 rats (197). In the Long-Evans strains, SERT mRNA in specific sub-regions of the raphe is marginally decreased in MS15 and increased in MS180 rats, with the magnitude of MS180 increase magnified by social defeat (243). Although reports on SERT levels in MS180s are mixed, the few reports regarding MS15 SERT mRNA appear to consistently suggest decreased expression. This corresponds with the decrease we observe in the MS15 condition. The fact that SERT is also decreased in the MS180 condition, suggests this condition also confers stress-resiliency in mice. However, future work will need to clarify the functional implications of lower SERT on behavior and physiology. At minimum, our results suggest a pattern of GR, TPH2, and SERT expression divergent from rats, supporting a species-specific response to MS.

In support of a species-specific response, a number of studies in c57bl/6 mice have reported long-term behavioral and stress axis resiliency to prolonged separation. On behavioral measures, anxiety-related behaviors are reported to be decreased in MS15s (251) and MS180s (207) or unchanged (206), although others have reported an increase (208,244). Neuroendocrine response is also blunted rather than increased, with lower corticosterone levels at baseline in adult mice that experienced a 60 minute MS protocol (252), in MS180s after a 3-4 minute restraint (211), and in MS180s after a 15 minute 100 dB acoustic stressor (212). These limited studies suggest a unique response to MS in

c57bl/6 mice, with changes in GR, TPH2, and SERT mRNA potentially underlying long-term changes in behavior and physiology.

It remains unclear what differentiates the response to MS in c57bl/6 mice. Because the strain is characterized by high licking/grooming and excellent nest construction, it has been suggested that care provided by c57bl/6 dams is analogous to “high maternal care” rat dams (187), which may protect against the stress of aELEs and associated effects on behavior and HPA response (253). The parallel changes in TPH2, SERT, and GR expression in MS15 and MS180 conditions suggest a common unifying experience from maternal separation. The increases in post-reunion on-nest time are suggestive of a compensatory increase in maternal care which may mitigate and promote resiliency. Further, the experience of handling itself may provide supplementary stimulation altering the developmental trajectory. In this case, the inherently high quality of maternal care in this strain protects against separation stress, while handling experience and increases in post-reunion care provide supplementary stimulation to effect long-term changes in gene expression.

A second consideration is species-specific timing of the stress hypo-responsive period (SHRP). During the SHRP, pups exhibit low basal corticosterone secretion and relative insensitivity to experiences that would normally elicit a stress response. In rats, the SHRP lasts from PND4-14 (254), whereas it is shifted to PND1-12 in mice (255). Because MS traditionally occurs between PND2-14, rat pups are exposed to 2 days of separation prior to entering the SHRP, whereas mouse pups are already hypo-responsive before MS begins. Possibly, this may underlie mouse resiliency to MS, as the early postnatal period during MS is likely the most sensitive and traumatic for pups. In future

studies using MS in c57bl/6 mice, it may be necessary to shift the timing of MS, increase separation duration, or supplement MS with additional stressors to overcome resiliency. Indeed, recent studies report increasing PND2-6 separation to 5 hours effectively increases 5-HT levels in PFC, striatum, and hippocampus (256), and combining MS with early weaning increases anxiety and stress response (210).

A final consideration is technical differences across studies. The duration of separation, ambient temperature during separation, maternal care experience (257), litter sex composition (258), whether pups were removed individually or as a litter, pup placement inside or outside of the nest when returned to the cage, and dam proximity during separation, may mitigate or increase the stress of separation. In this study, dams were multiparous (2<sup>nd</sup> litter) with a 1:1 male to female pup ratio, designed to minimize the variability in maternal care for first litters typically observed in c57bl/6 mice. Past studies in rats, specifically those examining TPH2 and SERT mRNA, used primiparous dams and male-exclusive litters (24,243), which may have altered the outcome between studies.

### **Functional consequences and regulation**

Despite the decrease in TPH2 mRNA, DNA methylation of the TPH2 promoter and 5'UTR remained unchanged. This suggests expression is not regulated by methylation in this region. Reports of early-life dependent changes in DNA methylation since the initial report (27) have been infrequent, suggesting that this mechanism may be less common than initially considered.

Presumably, a decrease in TPH2 and SERT would decrease and increase 5-HT signaling, respectively. Transgenic knockout of SERT has been shown to reduce TPH2

levels (259), but transgenic knockout of TPH2 fails to alter SERT expression (37), suggesting TPH2 sensitivity to SERT expression but not vice versa. This indicates changes in TPH2 mRNA may be downstream of changes in SERT, although other factors likely also play a role. Interestingly, increases in hippocampal GR expression following early handling are associated with a transient increase in serotonergic activity during early postnatal life (218), and 5-HT stimulation in hippocampal cells has been previously demonstrated to increase GR expression (260). Potentially, a decrease in SERT levels could underlie a transient increase in extracellular 5-HT in early postnatal life, setting baseline hippocampal GR expression, which is later offset by a decrease in TPH2 levels to re-normalize 5-HT signaling.

Regionally, decreases in SERT and TPH2 mRNA were most robust in the DRd and DRv, with a trend in the DRlw, MR, and PmR for TPH2 but not SERT mRNA. The region-specific expression has functional implications, based on differential projections to and from these sub-regions. The DRv receives dense innervations from the lateral orbital cortex, lateral hypothalamic area, and medial preoptic area, whereas the DRd receives innervations from the ventral orbital cortex, infralimbic cortex, central nucleus of the amygdala, BNST, lateral and posterior hypothalamic area, and preoptic areas, with both sharing innervations from the cingulate cortex and lateral habenula (261). Generally, these areas have been implicated in top-down regulation of anxiety-related behaviors and evaluation of information from limbic systems for integration into an appropriate motor response. The DRlw innervates the dorsolateral periaqueductal gray, ventrolateral medulla, and other structures modulating basic instinctual response to stressors, and exhibits stronger activation in response to stressors compared to DRd or DRv. It has been



suggested that the DRd and DRv are more involved in regulating emotional behavior and motor/cognitive function, whereas the DRlw is involved in the physiological and behavioral response to severe stressors (262). Thus, the primary effect from lower TPH2 and SERT in MS mice may not be on immediate stress response, but instead on higher-order control of emotional and motor behaviors modulated by the DRd and DRv.

Additionally, TPH2 mRNA, but not SERT mRNA, in the MR was decreased in MS15 mice. Although sharing some projections, the MR and DR are also functionally and anatomically distinct. MR efferents project to midline structures, including dorsal hippocampus, medial septum, nucleus accumbens core, and select hypothalamic nuclei, whereas DR efferents branch to lateral structures, including fronto-parietal cortex, amygdala, lateral septum, nucleus accumbens shell, ventral hippocampus, and lateral hypothalamus (263). There is limited evidence suggesting MR sensitivity to early life conditions, with decreases in MR 5-HT immunoreactivity following 3 weeks of early postnatal footshock. This decrease is associated with an anxiolytic effect, reproducible by electrolytic lesion of MR, but not DR (223). A decrease of TPH2 but not SERT mRNA in MS15 mice would potentially also lower 5-HT, but requires future investigation.

In conclusion, we find that MS in the c57bl/6 mouse strain, irrespective of separation duration, decreases raphe SERT and TPH2 mRNA while increasing hippocampal GR mRNA in specific sub-regions. Further, despite changes in TPH2 mRNA levels, methylation of its promoter was unchanged, suggesting other regulatory mechanisms underlie changes in TPH2 expression. To our knowledge, this is the first study to examine TPH2, SERT, and GR expression following MS in c57bl/6 mice. This pattern of expression is consistent with evidence of a minimal effect on behavior and

neuroendocrine response in the c57bl/6 strain (206,212), and suggests more severe stressors are necessary to model aELs in this strain. At the same time, the results suggest MS15 and MS180 expression is distinct from AFR controls, indicating a shared effect from MS. This may be related to the experience of handling and/or increased post-reunion maternal care, which may promote stress-resilience. Future work will be necessary to clarify the functional effect on behavior and 5-HT signaling as a result of subtle but parallel changes in TPH2, SERT, and GR gene expression observed under both MS conditions in c57b/6 mice.

## ***V. Materials & Methods***

Animals: Two independent cohorts of 8-week-old c57/bl6 mice (Jackson Laboratory), were mated 1 male per 2 females at ~10 weeks of age. Each cohort consisted of 10 females mated to 5 males. Males were removed from females ~1 week after mating, females separated into individual cages, and nesting material provided ~1-2 days prior to the day of birth (PND0). MS studies were performed on the second litter. In total, 2 breeding groups were used to obtain 11 MS15, 11 MS180, and 23 AFR males in group 1, and 14 MS15, 13 MS180, and 12 AFR males in group 2. A total of 10 litters were obtained for AFR, 7 for MS15, and 7 for MS180. Expression analyses used both groups 1 and 2 whereas DNA methylation was only assessed in group 1.

Maternal Separation: On PND2, dams were removed from their home cage to an adjacent holding cage. Pups were pooled, sexed, and standardized to foster litters composed of 6-8 pups, at a 1:1 male/female ratio. Each litter was randomly assigned to three rearing conditions: (1) MS180 –180 minute maternal separation during PND2-14 (2) MS15 –15

minute maternal separation during PND2-14, or (3) AFR – animal facility reared – handling of pups only during weighing and cage once per week. Separation of pups occurred between 0800 – 1200 h daily (light cycle from: 0700-1900 hours). Dam and pup holding cages were prepared fresh daily, each containing 400 mL of fresh bedding. During MS, dams were transferred to the dam holding cage, and provided with fresh food and water until the end of separation. Pups were removed individually and transferred to a pup holding cage acclimated to 32 degrees with a heating blanket. On PND2, 8 and 14, for cage cleaning, half of the bedding in the home cage was removed and replaced with fresh bedding during MS for MS15 and MS180 conditions. For AFR cage cleaning, pups were briefly moved as a litter and then returned directly to nest. For MS conditions, pups were placed in a corner opposite the nest at the end of separation prior to returning dams to the home cage. Pups were weaned at PND21, and separated by gender 2-3 per cage until PND60. Mice were weighed on PND2, 14, 21, 35, and 60 prior to a separation or cage change.

Maternal Recording: A total of 7 MS15, 9 AFR, and 8 MS180 dams were used to assess maternal behavior. Pre-separation care was assessed at the onset of the light cycle, 0700-0900 and scored at 30 second intervals for 2 hours on PND2, 8, and 14. Post-separation care was assessed immediately after separation (MS15 & MS180) or cage cleaning (AFR), scored at 10 second intervals for 15 minutes.

Tissue extraction/processing: Mice were anaesthetized with isoflurane and decapitated using a guillotine. Brains were extracted, snap-frozen in -20°C isopentane, and stored at -80°C. Brains were sectioned at 12 micron thickness with a cryostat and slide-mounted (Fisher Superfrost). Slides were fixated using 4% paraformaldehyde, washed 3x in 2X

SSC, incubated in 0.1 M triethanolamine, washed with ddH<sub>2</sub>O, subjected to graded dehydration in EtOH, air-dried, and stored at -80°C.

In Situ Hybridization: A [35-S] labeled ssRNA probe was transcribed from mouse TPH2, SERT, and GR cDNA templates. Template for TPH2 probe originated from a pGEMZ clone containing EST BG084420, generously provided by Dr. Robert Thompson.

Template for SERT originated from a pBS SK(-) clone containing nucleotides 1723-2136 of NM\_01264, generously provided by Dr. Stanley Watson. Template for GR originated from a pBSKII clone containing nucleotides 1-596 of NM\_008173, generously provided by Dr. Audrey Seasholtz (264). For full ISH protocol, see reference (236).

Image Analysis: Films were scanned at 3200 dpi per slide and analyzed using ImageJ (NIH). For assessing optical density (O.D.), sections were set to threshold against a background +/- 3.5 standard deviations. Measurements were normalized against the average AFR O.D. for each cohort, and collapsed across studies.

Sodium Bisulfite Sequencing: Genomic DNA was extracted from slide-mounted tissue punches using in-situ guided freeze matrix assisted punches (IFAP) (265). Purified gDNA was sodium bisulfite treated (Qiagen Epitect) and amplified using a nested-PCR approach. Outer nested primers were: 5'-

TAGAYGTGTAATTTGATTGTGGTTATTAGT-3' and 5'-

TCCCAACAACRCRCCCAACTAC-3'. Inner nested primers were: 5'-M13fwd-

TGATTGTGGTTATTAGTAATTAGAAATGAGTT-3' and 5'-M13rev-

AATCCAAAACAACCCTCTCC-3'. Amplicons were column-purified (Qiagen

Qiaquick) and sequenced bi-directionally (Applied Bio-Systems Model 3730 XL) by the institutional sequencing core. Percent methylation was estimated by comparing ratios of

cytosine:thymine peaks in CpG dinucleotides [ % methylation = (C)/(C + T)] and taking the average across sequence runs.

### **Acknowledgements**

We would like to thank Dr. Robert Thompson, Dr. Delia Vazquez, Dr. Juan Lopez, and Dr. Robert Denver for advisory role in this work and Dr. Audrey Seasholtz for providing the template plasmid for the GR probe. We would also like to thank our undergraduate researchers Shama Patel and Sushma Rao for their assistance in maternal scoring.

## **Chapter 4 - Maternal behavior and offspring resiliency to maternal separation in c57bl/6 mice**

### ***I. Abstract***

Adverse early life experience, such as childhood abuse, neglect, and trauma, increases lifetime risk for mental illness. To investigate underlying mechanisms, the maternal separation (MS) paradigm was developed and validated as an animal model of early adversity in rats, reliably effecting long-term changes to anxiety, gene expression, and stress response. However, across-species validation of core findings in mice has met with limited success. To re-visit parameters governing the effectiveness of MS in mice, this study investigated the effect of MS on maternal care, offspring behavior, and offspring stress-induced corticosterone response in the c57bl/6 mouse strain. The results from this study suggest that: (i) levels of maternal care increase as a function of separation duration immediately after daily MS, but long-term care remains unchanged; and (ii) c57bl/6 mice are resilient to MS, exhibiting only subtle decreases in anxiety and unchanged stress-induced corticosterone response as adults, irrespective of separation duration.

### ***II. Introduction***

Adverse early life experiences, such as child abuse, neglect, or trauma increase lifetime risk to mental illness (6), and are associated with long-term changes in brain development (10,227). A widely-used animal model for the investigation of molecular

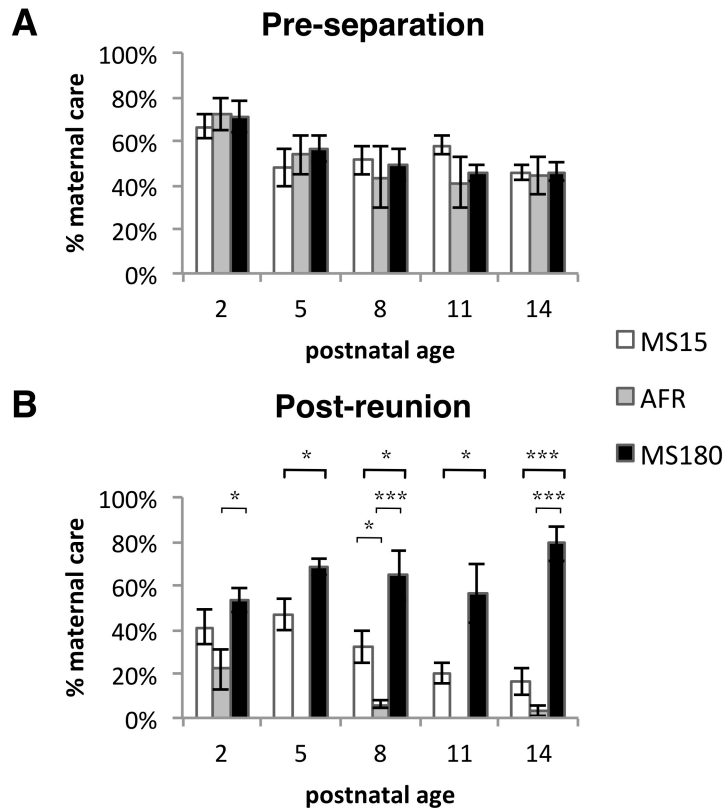
and behavioral responses to early life stress is the maternal separation (MS) paradigm. Developed as a rat model, pups are treated to either a mild (15 minute, i.e. MS15) or prolonged (180 minute, i.e. MS180) daily maternal separation from postnatal day 2-14. As adults, relative to animal facility reared (AFR) animals, MS180 animals exhibit increased anxiety, elevated stress hormone levels (183,198,266), and impaired negative feedback (90,267). Conversely, MS15 animals exhibit decreased anxiety, reduced stress hormone levels (33), and enhanced negative feedback (90).

Efforts have been made to adapt the paradigm to mice, provided advantages in lower husbandry costs and advances in mouse genetics. However, attempts at replication in mice have met with mixed success. Anxiety and stress response in MS180 mice have been reported to be increased (205,208,213,244), decreased (207,212), or unchanged (206,268,269). Further, omissions of the MS15 or AFR condition in comparisons against MS180 (205,207-209,244,270) complicates across-study comparisons, making it difficult to determine whether discrepancies between studies are due to handling, separation, strain, or protocol differences. Of the more comprehensive studies in mice, results suggest either a resistance to the MS paradigm (206) or a marginal “stress-resilient” phenotype in both MS15 and MS180 conditions under certain circumstances (212). Because of inconsistencies, an extensive behavioral and physiological re-evaluation of MS as an early life stress paradigm is necessary to review its effectiveness in mice. By including additional behavioral measures and assessing dam maternal behavior during MS, this study aimed to clarify the role of dam care on offspring behavior and stress response following MS in the c57bl/6 mouse strain.

### III. Results

Pre-maternal separation (Pre-MS) and post-reunion maternal care was recorded and analyzed on PND2, 5, 8, 11, and 14. Dam behavior pre-MS was recorded for 2 hours and post-reunion care recorded for 15 minutes. Recordings of AFR post-reunion care were coincident with the weekly partial cage cleaning, and therefore limited to PND2, 8, and 14, as no manipulations occurred on PND5 and 11 for AFR cages. Pre-MS dam care was not different between groups, suggesting that MS had a minimal effect on long-term (pre-separation) levels of care (**Fig. 4-1A**). Conversely, levels of post-reunion care were significantly different on PND2 [ $F(2,21) = 3.82$ ;  $p = 0.04$ ;  $\eta^2 = 0.28$ ], PND5 [ $F(1,14) = 6.94$ ;  $p = 0.02$ ;  $\eta^2 = 0.31$ ], PND8 [ $F(2,21) = 12.12$ ;  $p < 0.001$ ;  $\eta^2 = 0.54$ ], PND11 [ $F(1,14) = 7.20$ ;  $p = 0.02$ ;  $\eta^2 = 0.30$ ], and PND14 [ $F(2,21) = 41.53$ ;  $p < 0.001$ ;  $\eta^2 = 0.80$ ] (**Fig. 4-1B**). Effect size estimates for ANOVA, using partial eta squared ( $\eta^2$ ), were above 0.16 for all days, suggesting MS manipulations accounted for a significant proportion of the differences in post-reunion care. Similarly, effect size estimates for pair-wise comparisons, using Cohen's d (d), were above 0.77 for all MS comparisons against AFR. Relative to AFR, MS180 post-reunion care was higher on PND2 [ $p = 0.03$ ;  $d = 1.29$ ], PND8 [ $p < 0.001$ ;  $d = 1.55$ ], and PND14 [ $p < 0.001$ ;  $d = 1.79$ ]. Relative to MS15, MS180 post-reunion care was not different on PND2 [ $p = 0.23$ ;  $d = 0.60$ ], but higher on PND5 [ $p = 0.02$ ;  $d = 1.11$ ], PND8 [ $p < 0.05$ ;  $d = 1.04$ ], PND11 [ $p = 0.02$ ;  $d = 1.11$ ], and PND14 [ $p < 0.001$ ;  $d = 1.66$ ]. MS15 post-reunion care was only significantly higher on PND8 relative to AFRs [ $p = 0.05$ ;  $d = 1.55$ ], but was not significantly different on PND2 [ $p = 0.23$ ;  $d = 0.77$ ] and PND14 [ $p = 0.31$ ;  $d = 0.85$ ].



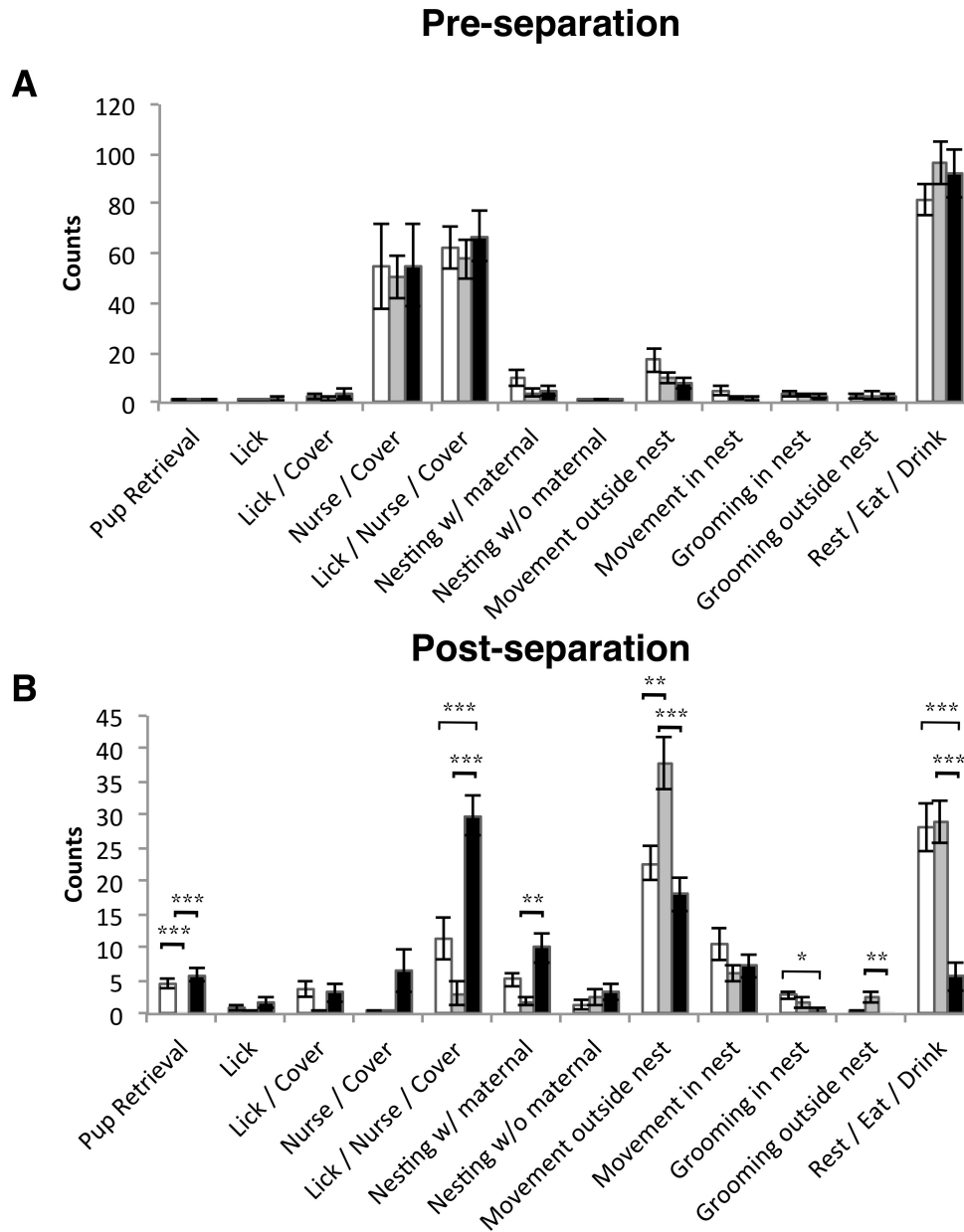


**Figure 4-1 Short- and long-term maternal care during maternal separation.**

(A) Levels of pre-separation maternal care as a measure of changes to long-term care. (B) Levels of post-reunion maternal care immediately following maternal separation and / or partial cage cleaning. Note: no AFR post-reunion data on PND5 & PND11 as there was no cage cleaning on these days. Significance denoted by  $p < .001$  (\*\*\*),  $p < .01$  (\*\*),  $p < .05$  (\*).

In-depth analysis of specific maternal behaviors, averaged across PND2, 8, and 14 observations, revealed significant increases in post-reunion, but not pre-separation, maternal behaviors in MS15 and MS180 dams (**Fig. 4-2A, B**). Significant differences were observed in post-separation pup retrieval [ $F(2,21) = 16.08$ ;  $p < 0.001$ ], combined nursing / covering [ $F(2,21) = 3.63$ ;  $p = 0.04$ ], combined licking / nursing / covering [ $F(2,21) = 22.31$ ;  $p < 0.001$ ], nesting with maternal [ $F(2,21) = 6.86$ ;  $p = 0.005$ ], movement outside nest [ $F(2,21) = 11.22$ ;  $p < 0.001$ ], grooming inside nest [ $F(2,21) = 3.69$ ;  $p = 0.04$ ], grooming outside of nest [ $F(2,21) = 7.00$ ;  $p = 0.005$ ], and

combined resting / eating / drinking [ $F(2,21) = 17.64; p < 0.001$ ] behavior. Compared to AFR, MS180 dams exhibited more pup retrieval [ $p < 0.001$ ], more combined licking / nursing / and covering [ $p < 0.001$ ], more nesting with maternal [ $p = 0.004$ ], less movement outside of nest [ $p < 0.001$ ], less grooming outside of nest [ $p = 0.005$ ], and less combined resting / eating / drinking behavior [ $p < 0.001$ ]. Similarly, MS15 dams exhibited more pup retrieval [ $p = 0.001$ ], less movement outside of the nest [ $p = 0.005$ ], and less grooming outside of nest behavior [ $p = 0.02$ ]. MS15 dams also exhibited less combined licking / nursing / covering [ $p < 0.001$ ], more grooming inside of nest behavior [ $p = 0.03$ ] and combined resting / eating / drinking behavior [ $p < 0.001$ ] relative to MS180 dams.



**Figure 4-2 Maternal behaviors during maternal separation.**

Maternal behavior was averaged and collapsed from PND2, 8, and 14 assessments. (A) Specific pre-separation maternal behaviors by the dam averaged across postnatal day 2, 8, and 14 assessments. (D) Specific post-separation maternal behaviors by the dam averaged across postnatal day 2, 8, and 14 assessments. Abbreviations: w/ = with; w/o = without. Significance is denoted by  $p < .001$  (\*\*\*),  $p < .01$  (\*\*),  $p < .05$  (\*).

Offspring weight was assessed on PND2, 14, 21, 35, and 67 to control for possible gross developmental differences as a result of separation. A marginal difference in weight was observed on PND14 [ $F(2,80) = 3.23$ ;  $p = 0.04$ ;  $\eta^2 = 0.08$ ] and PND67 [ $F(2,80) = 3.48$ ;  $p = 0.04$ ;  $\eta^2 = 0.08$ ], wherein MS15s were marginally lighter than AFRs [PND14:  $p = 0.04$ ; PND67:  $p = 0.04$ ] (Fig. 4-3).

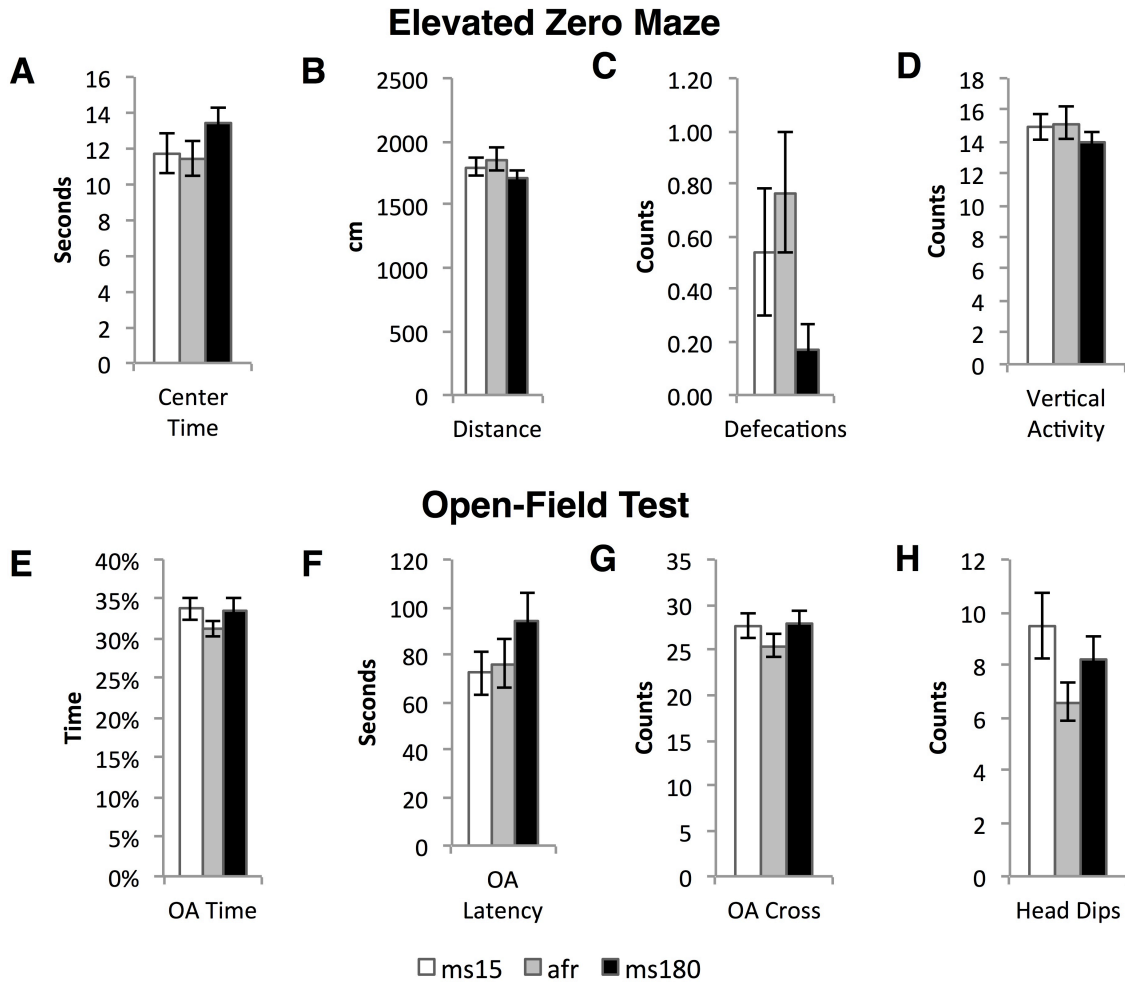
Weight (grams)					
	PND2	PND14	PND21	PND35	PND67
MS15	1.59 +/- 0.02	6.60 +/- 0.11*	9.10 +/- 0.17	19.68 +/- 0.30	23.09 +/- 0.31*
AFR	1.59 +/- 0.02	7.13 +/- 0.18	9.42 +/- 0.25	19.76 +/- 0.25	24.19 +/- 0.30
MS180	1.58 +/- 0.03	6.97 +/- 0.10	9.42 +/- 0.15	20.24 +/- 0.26	23.45 +/- 0.27

**Figure 4-3 Offspring weight across development.**

PND = postnatal day. \* =  $p < 0.05$ .

To characterize the effect of MS on adult behavior, mice underwent a series of behavioral tests from PND67-79 to assess anxiety, behavioral despair, and activity levels. The behavioral battery included the elevated zero maze (EZM), a 5-minute open-field (OFT.5), Light/Dark box (L/D), Porsolt forced swim test (FST), a 20-minute open-field (OFT.20), and novelty suppressed feeding (NSF). The OFT.5 and OFT.20 were also differentiated based on field dimensions (circular vs. square) and area (2920 cm<sup>2</sup> vs. 1600 cm<sup>2</sup>), enclosed space (open vs. covered), and analysis method (video-tracking vs. IR sensors).

On measures of anxiety, no difference in EZM open arm (OA) latency [ $F(2,80) = 1.30$ ;  $p = 0.28$ ;  $\eta^2 = 0.03$ ], OA time [ $F(2,80) = 1.05$ ;  $p = 0.35$ ;  $\eta^2 = 0.03$ ], OA crossings [ $F(2,80) = 1.18$ ;  $p = 0.31$ ;  $\eta^2 = 0.03$ ] or head dips [ $F(2,80) = 2.23$ ;  $p = 0.11$ ;  $\eta^2 = 0.05$ ] was observed (Fig. 4-4 A-D).



**Figure 4-4 Elevated zero maze behavior and open-field 5 minute (OFT.5) behavior.**

Abbreviations: PND = postnatal day; OA = open arm; Vert. = vertical. Significance denoted by  $p < .001$  (\*\*\*),  $p < .01$  (\*\*),  $p < .05$  (\*).

However, testing in the L/D box revealed differences in L/D transitions [ $F(2,80) = 3.58$ ;  $p = .03$ ;

$\eta^2 = 0.08$ ] and vertical activity [ $F(2,80) = 5.15$ ;  $p < 0.01$ ;  $\eta^2 = 0.12$ ]. MS15 mice transitioned more

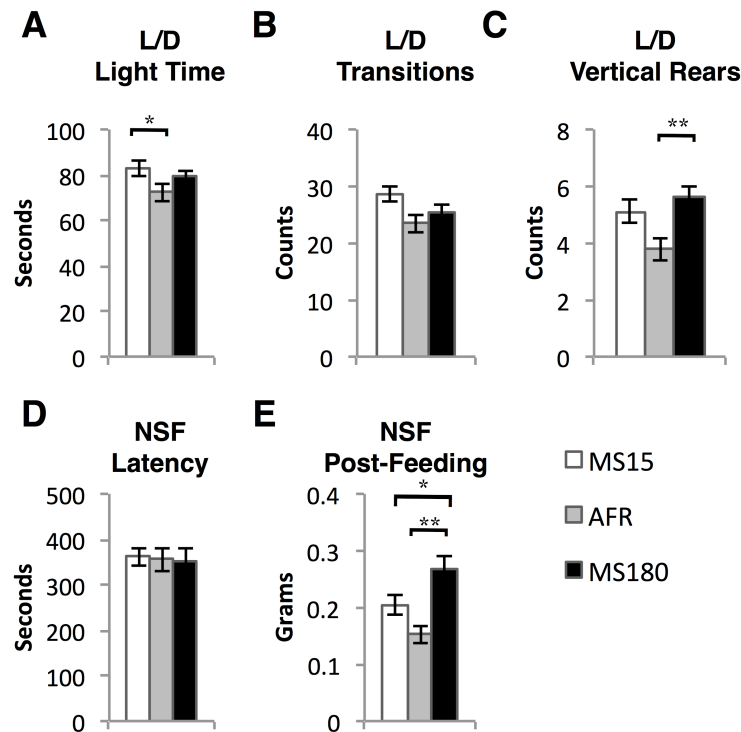
frequently [ $p = 0.02$ ;  $d = 0.68$ ], whereas MS180 mice exhibited more frequent vertical rears on

the light-side [ $p < 0.01$ ;  $d = 0.77$ ] relative to AFR mice (**Fig. 4-5 A-C**). In the NSF, latency to

consume food remained unchanged [ $F(2,80) = 0.03$ ;  $p = 0.97$ ;  $\eta^2 < 0.01$ ] (**Fig. 4-5D**). However, a

difference in post-NSF food consumption was detected [ $F(2,80) = 12.19$ ;  $p < 0.001$ ;  $\eta^2 = 0.23$ ], with

higher consumption in MS180 relative to MS15 [ $p = 0.03$ ;  $d = 0.67$ ] and AFR [ $p < 0.001$ ;  $d = 1.05$ ], but unchanged in MS15 relative to AFR [ $p = 0.09$ ;  $d = 0.59$ ] (**Fig. 4-5E**).

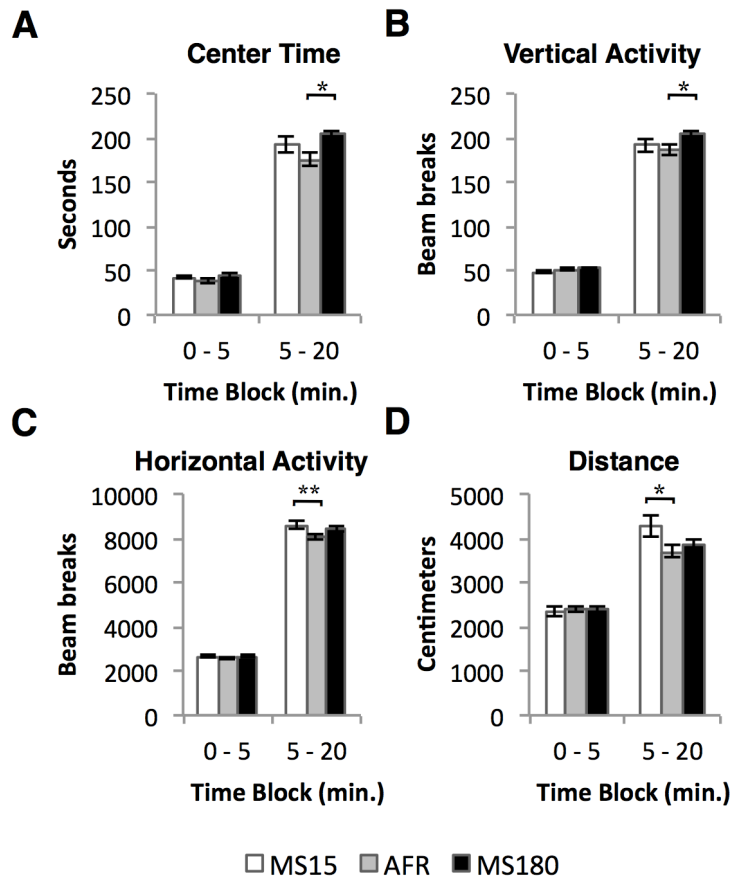


**Figure 4-5 Light/Dark (L/D) box and novelty suppressed feeding.**

(A) Light to dark transitions in L/D. (B) Light time in L/D. (C) Vertical rears in L/D. (D) Latency to consume food in the NSF. (E) Change in pellet weight after 5 minutes of home cage consumption post-NSF. Significance denoted by  $p < .001$  (\*\*\*),  $p < .01$  (\*\*),  $p < .05$  (\*).

In the OFT.5, center time [ $F(2,80) = 0.21$ ;  $p = 0.81$ ;  $\eta^2 < 0.01$ ], distance [ $F(2,80) = 0.86$ ;  $p = 0.43$ ;  $\eta^2 = 0.02$ ], vertical activity [ $F(2,80) = 0.609$ ;  $p = 0.546$ ;  $\eta^2 = 0.015$ ], and defecation [ $F(2,80) = 2.47$ ;  $p = 0.09$ ;  $\eta^2 = 0.06$ ] were unchanged (**Fig. 4-4 E-H**). Similarly, in the OFT.20, center time [ $F(2,80) = 1.89$ ;  $p = 0.16$ ;  $\eta^2 = 0.05$ ], distance traveled [ $F(2,80) = 0.08$ ;  $p = 0.93$ ;  $\eta^2 < 0.01$ ], horizontal activity [ $F(2,80) = 0.21$ ;  $p = 0.81$ ;  $\eta^2 < 0.01$ ], and vertical activity [ $F(2,80) = 1.83$ ;  $p = 0.17$ ;  $\eta^2 = 0.04$ ] were not different in the initial 5 minutes. However, in the last 15 minutes of testing, differences emerged in center time

[ $F(2,80) = 3.23$ ;  $p < 0.05$ ;  $\eta^2 = 0.08$ ], distance traveled [ $F(2,80) = 3.60$ ;  $p = 0.03$ ;  $\eta^2 = 0.08$ ], horizontal activity [ $F(2,80) = 5.14$ ;  $p < 0.01$ ;  $\eta^2 = 0.11$ ], and vertical activity [ $F(2,80) = 3.32$ ;  $p = 0.04$ ;  $\eta^2 = 0.08$ ] (**Fig. 4-5A-D**).



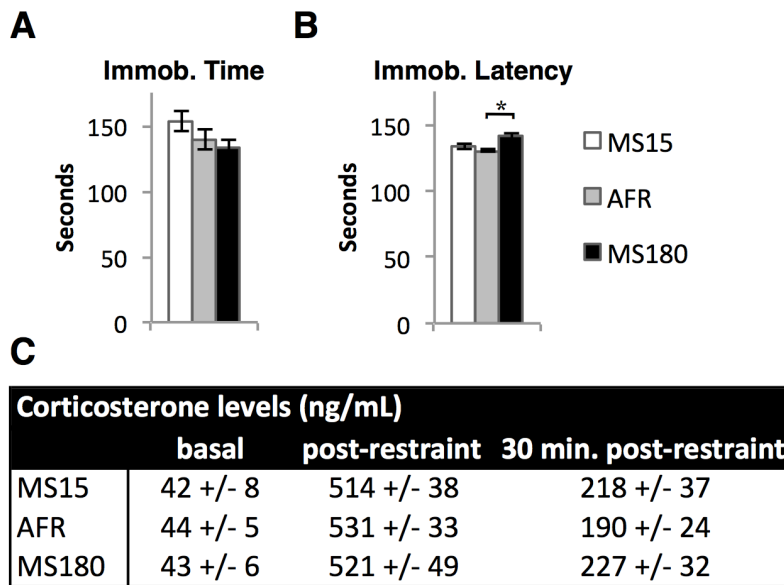
**Figure 4-6 Open-field 20 minutes [activity monitor].**

Open-field behavior in the initial 5 minutes (“0-5”) and last 15 minutes (“5-20”). (A) Center time. (B) Vertical activity. (C) Horizontal activity. (D) Distance traveled. Significance denoted by  $p < .001$  (\*\*\*),  $p < .01$  (\*\*),  $p < .05$  (\*).

MS180s increased in center time [ $p = 0.04$ ;  $d = 0.74$ ] and vertical activity [ $p = 0.03$ ;  $d = 0.70$ ], whereas MS15s increased in distance [ $p = 0.03$ ;  $d = 0.60$ ] and horizontal beam breaks [ $p < 0.01$ ;  $d = 0.74$ ]. On measures of behavior despair, immobility time was not different [ $F(2,80) = 1.83$ ;  $p = 0.17$ ;  $\eta^2 = 0.04$ ] (**Fig. 4-7A**), whereas latency to immobility [ $F(2,80) = 4.98$ ;  $p < 0.01$ ;  $\eta^2 = 0.11$ ] was lower in MS180 relative to AFR mice [ $p < 0.01$ ;  $d = 0.73$ ] in the FST (**Fig. 4-7B**). Across behavioral measures,

medium to large effect size estimates, ranging from  $\eta^2$  values of 0.075 – 0.233, were associated with significant behavioral differences. Conversely, non-significant differences in behavior were associated with smaller effect size estimates, ranging from  $\eta^2$  values of 0.001 – 0.053. This suggests that MS manipulations accounted for a minimal part of any behavioral difference between groups along non-significant measures.

To assess stress-induced corticosterone response, animals underwent a 30 minute restraint stress on PND90, 11 days after concluding behavioral testing. AFR, MS15, and MS180 mice were divided equally into basal, post-restraint, and 30-minutes post-restraint cohorts for terminal trunk blood collection. Trunk serum corticosterone levels were not different between groups at basal [ $F(2,25) = 0.03$ ;  $p = 0.97$ ;  $\eta^2 < 0.01$ ], post-restraint [ $F(2,25) = 0.04$ ;  $p = 0.96$ ;  $\eta^2 < 0.01$ ], and 30-minutes post-restraint [ $F(2,24) = 0.43$ ;  $p = 0.66$ ;  $\eta^2 = 0.04$ ] (**Fig. 4-7C**).



**Figure 4-7 Forced swim test and corticosterone stress response.**

(A) Immobility time in FST. (B) Immobility latency in FST. (C) Corticosterone levels at baseline, after 30 minute acute restraint (post-restraint), and 30 minutes post-restraint. Significance denoted by  $p < .001$  (\*\*\*),  $p < .01$  (\*\*),  $p < .05$  (\*).



#### ***IV. Discussion***

##### **Maternal Behavior**

As a paradigm of early life stress, MS has been successful in modeling adverse early life experiences in rats. However, as demonstrated in our study, its effectiveness in altering behavior and physiology of c57bl/6 mice is limited. Although c57bl/6 dam maternal care is sensitive to MS, offspring are resilient to the stress of prolonged separation. Across multiple measures, the duration of separation had a limited impact on behavior and stress response, suggesting a “stress-resilient” phenotype. This has clinical implications for the extent to which findings from MS are translatable across species, indicating further work is necessary to develop an effective model of early life stress in mice.

As a means of validating implementation of MS protocol, pre-MS and post-reunion levels of maternal care were recorded during PND2-14. Post-reunion care was significantly higher in MS180 relative to MS15 and AFR dams, and higher in MS15 relative to AFR dams, in agreement with reports in Lister-hooded and Wistar rats (190,271). However, long-term care, assessed by a 2 hour pre-separation block, remained unchanged, whereas it has been reported to be unchanged in MS180 but increased in MS15 Wistar rats (190). This is likely a species-divergent effect rather than due to a technical difference, as our findings correspond to reports in c57bl/6 mice of transient increases in MS180 post-reunion care (206), but no long-term change (244). The concordance with prior studies suggests successful implementation of the MS protocol.

A differentiation of specific post-separation maternal behaviors was observed between MS15, MS180, and AFR c57bl/6 dams. Whereas MS15 and / or MS180 dams increased nesting, pup retrieval, and combined licking / nursing / covering behavior, AFR dams increased movement outside of the nest, grooming, and combined resting / eating / drinking behavior. An increase in MS15 and MS180 pup retrieval was expected, as pup displacement post-MS necessitated pup retrieval whereas AFR pups were returned directly to the nest following cage cleaning. More noteworthy was the pronounced increase in MS180 dam licking / nursing / covering behaviors relative to other non-maternal behaviors. These findings are in contrast to reported effects in rats, in which MS180 delays pup retrieval and initiation of licking, grooming, and nursing behaviors relative to MS15 and AFR dams (201). Moreover, vulnerabilities associated with maternal deprivation or separation in rats are mitigated by artificial stimulation (using a wet brush to mimic maternal licking), preventing development of HPA axis abnormalities and decreasing anxiety-like behaviors (272-274). This is further supported by evidence of early handling and high-quality maternal providers conferring resiliency through increased licking / grooming behavior and arched-back nursing (189,190). In this context, the increase in licking / nursing / covering behavior in MS180 c57bl/6 dams strongly implicates enhanced maternal care in mitigating stress from separation and promoting long-term resiliency in MS180 offspring.

Functionally, the transient increase in MS15 and MS180 post-reunion care was likely effected by several factors, including compensatory care, pup displacement, and past experience. Post-reunion care was highest in MS180, followed by MS15, indicating that levels of care increased as a function of separation duration. However, whereas

MS15 post-reunion care progressively decreased as pups aged, MS180 care was unchanged or increased from PND2-14. In comparison, AFR post-manipulation care (after cage cleaning) declined in a pattern similar to MS15, indicating that decreases in post-reunion care naturally occur as pups age. This suggests MS180 dams became progressively sensitized to prolonged separations, maintaining or increasing levels of post-reunion care from PND2-14.

A second factor, pup displacement, may also have effected levels of post-reunion care. For MS conditions, pups were single-handled and returned to a cage position furthest away from the nest, whereas AFR pups were returned directly to the nest following cage cleaning. The rationale for this was to preserve the standard facility rearing experience for AFRs as a comparison against manipulations unique to the MS experience. However, it is possible that displacement catalyzed an increase in post-reunion care, in which returning to find pups displaced away from the nest caused maternal drive to override other behaviors, such as self-maintenance or exploratory behavior. In the future, it will be of interest to determine whether pup displacement is a significant variable in shaping maternal and offspring behavior during MS.

There were a few limitations to the assessment of maternal behavior. First, as a measure of long-term change in care, the 2 hour pre-separation recording (22-hours following the last separation) only represented part of the diurnal rhythm of maternal behavior. We cannot exclude the possibility that maternal behavior changed at other points in time, nor that the pattern of care was unchanged. Secondly, the AFR condition was designed to be a post-reunion assessment and not a non-manipulated assessment of AFR maternal care, which means this study had no measure for basal levels of maternal

care during that period of time. Thus, while the post-reunion assessment demonstrates a difference in response to mother-infant disturbance, the extent and directionality of the change relative to baseline levels of maternal care is undefined.

### **Offspring Behavior & Stress Response**

For behavioral measures reaching statistical significance, MS15 and MS180 performance trended in the same direction. Underlying this may be effects from shared secondary aspects of MS, such as handling, separation experience, or post-reunion maternal care. However, even in rats, it has been difficult to isolate the degree of influence each has on behavioral development. Brief handling elevates post-reunion maternal care and lowers anxiety in adult rats (271). Yet, brief separation without handling still reduces anxiety, despite no change in post-reunion care (275), suggesting MS shapes behavior through individual pup experience. Conversely, although MS180 elevates offspring anxiety in rats, the provision of foster litters to dams during separation decreases offspring anxiety to levels resembling MS15 (202), suggesting MS also shapes offspring behavior through dam response.

Irrespective of mechanism, the directionality of change suggests MS15 and MS180 results in a limited “stress-resilient” behavioral phenotype in c57bl/6 mice, with minor reductions in anxiety, altered coping response, and minor increases in activity. These effects are subtle, and nowhere near as robust as reported effects in rats. Notwithstanding, on anxiety measures, MS15 mice exhibited slight increases in L/D transitions, whereas MS180 mice exhibited increases in L/D vertical activity and a marginal decrease in OFT.5 defecation. Savignac et al. recently reported MS180 decreases open-field defecation and increases L/D transitions in c57bl/6 mice (207),

supporting development of a minor “stress-resilient” phenotype in the c57bl/6 strain following MS180. Yet, NSF feeding latency; EZM OA latency, time, and crosses; and open-field.5 center time and vertical activity, remained unchanged. The discrepancy between parallel measures for anxiety may be attributable to unique test conditions. For example, the L/D box might offer finer discrimination based on its contrast in lighting conditions, providing a more robust conflict between exploratory drive and natural preference for dark safe areas.

Additionally, we observed a time-dependency for detection of anxiety-related behavior. In the OFT.20, MS180 center time and vertical activity were increased in the last 15 minutes, but not the initial 5 minutes of testing. This suggests subtle decreases in anxiety are initially masked, requiring more time for detection. At minimum, a minor increase in exploratory behavior or lack of change suggests c57bl/6 resiliency to the stress of MS180. This resiliency may underlie mixed reports on c57bl/6 performance on the EPM or EZM, as OA time has been reported to be decreased (208,212,244), increased (207,212), or unchanged (206) in MS180 mice.

Similarly, MS had no effect on basal corticosterone levels, nor following a stressor. This corresponds to reports of unchanged corticosterone response to other forms of stress, including novelty and social interactions in the CD-1 strain (269) and acoustic stress in the c57bl/6 strain (212). This is in contrast to the divergent corticosterone response reported between MS15 and MS180 rats (90,247). In part, differences may be attributable to species-specific timing of the stress hypo-responsive period (SHRP), a period in which the stress axis is relatively quiescent and requires major stressors to effect corticosterone response. In rats, the SHRP begins on PND4 and extends to PND14

(276), whereas in mice it begins earlier on PND1 and extends to PND12 (255). Because MS begins on PND2, rat pups are already exposed to MS prior to entering the SHRP, whereas mouse pups are hypo-responsive at the start of MS, potentially conferring resistance to the paradigm. In the future, increasing separation duration, altering timing, or supplementing MS with additional stressors may be necessary to overcome the SHRP in mice to effect a reliable model of early life stress.

Alternatively, we cannot exclude that the lack of change could have been influenced by behavioral testing. The prior 2 weeks of testing could have normalized corticosterone levels through routine exposure to handling and stressful environments. Although short-term effects were minimized by separating the corticosterone assay from testing by 11 days, long-term changes in stress response may have developed and persisted. Notwithstanding, the results are consistent with prior reports of unchanged corticosterone response in c57bl/6 mice, suggesting the lack of effect is attributable to inherent strain resiliency.

In addition to limited effects on anxiety-related measures, minor differences to activity level, behavioral despair, and post-stress food consumption were observed. On measures of activity, distance and horizontal beam breaks in the OFT.20 remained unchanged during the initial 5 minutes, in agreement with results from OFT.5 and a prior report in the c57bl/6 strain (206). However, in the last 15 minutes, MS15 distance and horizontal activity increased. One interpretation is that increased locomotor activity reflects a dis-inhibition of exploratory behavior from lower anxiety. Alternatively, it has been suggested that initial behavior in the open-field is significantly influenced by fear,

anxiety, and exploratory drive, whereas later behavior is a measure for activity. In this case, the results would suggest slightly hyperactivity in the MS15 condition.

On measures of behavioral despair, immobility time remained unchanged, consistent with prior findings in c57bl/6 mice (206,207,268). However, on a secondary measure, MS180 latency to immobility was slightly higher, indicating a marginally more proactive coping response. Regardless, the effect is marginal, and results from the FST suggests that maternal separation has a minimal effect on antidepressant-like behavior in c57bl/6 mice.

Finally, although NSF latency to consume food was unchanged, post-test food consumption increased in MS180s. This corresponds to prior work in rats, where 24-hour food deprivation increased levels of MS180 post-deprivation feeding beyond non-handled controls (277). Unfortunately, this study did not include a pre- or post-assessment of mouse weight, which might have provided evidence for or against a metabolic role in post-NSF food consumption. Alternatively, consumption could be tied to emotionality, in which feeding is a coping response to handling or novelty stress. In the future, including supplementary assessments of food consumption in other tests, such as L/D, EZM, FST, or OFT, would help clarify whether observed consumption was linked to an emotional response to stress.

A final technical consideration is the influence of past maternal experience on offspring outcome. MS studies in c57bl/6 mice have used both multiparous (278) and primiparous (207) dams. While the use of multiparous dams minimizes variable quality in care and litter loss associated with primiparous c57bl/6 dams (279), prior maternal experience may also protect against or compensate for separation stress. In this study, we

used multiparous dams that had 7 days with their 1<sup>st</sup> litter prior to culling and re-breeding 2 weeks later. Although a non-traditional manipulation, the intent was to provide dams with sufficient experience during a critical period for pup survival while limiting the entirety of maternal experience until the 2<sup>nd</sup> litter. Specifically arched-back nursing and anogenital licking is reported to appreciably decrease by PND7 in c57bl/6 dams (280), an age we considered optimal for removing the 1<sup>st</sup> litter from dam care. Further, past evidence indicates that loss of the 1<sup>st</sup> litter does not adversely effect outcome of future litters (279), suggesting that the PND7 cull would not have a significant effect on the 2<sup>nd</sup> litter. However, we acknowledge that early removal could still uniquely alter dam response to MS compared to primiparous dams and / or multiparous dams with a complete 1<sup>st</sup> litter experience, although the effect would extend across all subject groups. Nevertheless, the increase in post-reunion, but not long-term care in MS180 dams is consistent with past reports in nulliparous dams (206), suggesting that the impact from past maternal experience on MS response may be minimal.

## **Conclusion**

Relative to other strains, c57bl/6 mice have been noted for resiliency to early environmental stressors (281,282). This may be tied to higher quality care provided by c57bl/6 dams and an inherent pup resiliency. To elaborate, c57bl/6 foster mothers of balb/c pups lowers adoptee anxiety to levels expected in c57bl/6, whereas balb/c foster mothers, which are “low-quality” providers, of c57bl/6 pups results in no change in adoptee behavior (187). Demonstrating a similar effect, selection for low or high care c57bl/6 mothers also has a minimal impact on offspring behavior and stress response (283). These converging lines of evidence suggest an inherent resiliency in c57bl/6 pups,



suggesting that alternative more severe models of early life stress, beyond MS180, are necessary. Indeed, a recent report suggests combining MS180 with early weaning can reliably increase anxiety and stress response in adults (284). Future avenues of research include pursuing alternative early life stress models and clarification of female-specific or strain-dependent response to MS in mice. In conclusion, we find that MS in the c57bl/6 strain (i) increases post-reunion maternal care; (ii) does not change long-term maternal care; (iii) has a limited impact on offspring behavior; and (iv) does not alter offspring hormonal stress response. The evidence suggests that MS is not effective as a model of early life stress in the c57bl/6 mouse strain, and that c57bl/6 mice are inherently resilient to early life stress.

## ***V. Materials and Methods***

**Animal Husbandry:** A total of 28 female and 14 male c57bl/6 mice, 7 weeks old, were ordered from Jackson Laboratory. Mice were habituated to the breeding facility for 2 weeks prior to mating. Each male was housed with 2 females, maintained in a plexi-glass cage (22 cm x 16 cm x 14 cm) filled with ~400 cm<sup>3</sup> of Corncob bedding, and kept under standard housing conditions (room temperature ~22°C, 55% humidity) in a 12-h light/dark cycle (lights on 0700-1900). Visibly pregnant dams were moved to individual cages and checked daily for litters. If birth occurred < 10 AM, age was designated PND0, and a ¾ inch cotton square added for nesting material. The 1<sup>st</sup> litter was culled after 7 days, dams re-bred 2 weeks after culling, and the 2<sup>nd</sup> litter used for MS studies. All animals were cared for in compliance with national guidelines.

**Maternal separation:** On PND2, pups were selected for an even sex-ratio to minimize the influence of litter sex composition on maternal behaviors (285). To maintain sex

ratios, litter size was targeted to 8, but culled to 6 if necessary to maintain the ratio at the start of the paradigm. Average litter size for dams was: AFR = 7.29 +/- 0.29, MS180 = 7.43 +/- 0.20, MS15 = 7.38 +/- 0.26 at the start of the MS paradigm (PND2). From PND2-14, pups were separated daily for 15 minutes (MS15), 180 minutes (MS180), or left undisturbed (AFR) between 0900-1300 hours, based on prior MS protocols (286). Dams and litters were placed in separate holding cages and rooms. Litter cages were pre-warmed and maintained at 31°C using an adjustable heat mat during separations. Ambient cage temperature measured after separation remained within +/- 0.5°C of starting temperature. MS15 and MS180 pups were handled individually during transfers and when returned, placed into the corner opposite of the nest. MS15 and MS180 pup displacement outside of nest was based on a previously established MS protocol (Dr. Paul Plotsky, personal communication, 2009). To control for time-of-day effects on post-separation maternal behavior, MS15 separation began 15 minutes before the end of MS180 separation. Partial cage cleaning, in which half of the bedding was replaced, occurred on PND2, 8, and 14 during separations. AFR litters were left in the nest during cage cleaning, with bedding cleaned from around the nest to minimize handling effects. On PND21 males were weaned to 3-4/cage and females culled. On PND35, males were further separated to 2-3/cage. Weight was assessed on PND2, 14, 21, 35, and 67.

**Maternal care recording/scoring:** Pre- and post- separation maternal care levels were recorded for MS15 (n = 9), AFR (n = 7), and MS180 (n = 8) dams. Pre-separation care was assessed at the onset of the light cycle, 0600-0800 and scored at 30 second intervals for 2 hours on PND2, 5, 8, 11, and 14. Post-separation care was assessed immediately after separation (MS15 & MS180) or cage cleaning (AFR), scored at 10 second intervals

for 15 minutes. Post-separation recording occurred on PND2, 5, 8, 11, and 14 for MS groups, but only during partial cage changes for AFR on PND2, 8, and 14. For AFR cage cleaning, pups remained undisturbed and bedding around the nest replaced, while MS cage cleaning occurred during separations. Behaviors of pup handling, licking, nursing, covering, and nesting were scored as maternal. Behaviors of movement outside/inside nest, grooming, and eating/drinking were scored as non-maternal. For analysis of specific maternal behaviors, maternal care on PND2, 8, and 14 was collapsed and averaged. For more information on scoring, refer to Stern (287).

**Overview of behavior battery:** On PND60, male AFRs (n = 30), MS15s (n = 24), and MS180s (n = 29) were moved to the behavioral suite and separated to individual cages. On PND66, mice were weighed and cages cleaned. To acclimate mice to manipulations, mice were handled on PND66/67 for 5 minutes. Over the next 2 weeks, mice were tested on the elevated zero maze (PND68), open field test (PND70), light/dark box (PND72), forced swim test (PND74), activity monitor (PND77), and novelty suppressed feeding (PND79). Each test was staggered by a day except following FST, in which 2 days were given. All manipulations were performed between 0900-1300 hours, with a 30 minute pre-acclimation to the behavioral suite prior to testing. On PND78, cages were changed at the start of an 18-hour food deprivation for NSF. The following week, animals were sacrificed (PND90) following an acute-restraint stress.

**Elevated Zero Maze (EZM):** Mice were placed facing the closed arm and tracked for 5 minutes. The room was illuminated with diffuse light at ~50 lux. Trials were captured and analyzed with Limelight video-tracking software (Actimetrics).

**Open-field 5 minutes [video-tracked] (OFT.5):** Mice were placed in the center of a large field (61 cm diameter) and tracked for 5 minutes. Room condition, video-tracking, and analysis were identical to EZM.

**Light/Dark Box (L/D Box):** Mice were placed in the light compartment facing the wall opposite the dark compartment and tracked for 5 minutes. The light compartment was illuminated with a directional 60 watt bulb at 500 lux. Behavior was analyzed using a computer-assisted scoring program, similar to a prior published EPM program (288).

**Forced Swim Test (FST):** Mice were placed in a 19 cm diameter cylinder filled with 20 cm of water acclimated to 23-25°C and tracked for 6 minutes (289). The height and volume of water was sufficient to prevent hind paws or tail from coming into contact with the bottom of the tank. Room illumination was at ~50 lux. Behavior from 2-6 minutes, after an initial 0-2 minute acclimation period (290), was analyzed using a computer-assisted scoring program (288) re-coded for the FST (FSTscore). Behavior was scored in 5 second blocks according to the predominant behavior observed within each block (289).

**Open-field 20 minutes [activity monitor] (OFT.20):** Mice were placed into a 40 x 40 x 35.5 cm square, covered open-field and tracked for 20 minutes using an activity monitor (Accuscan) with IR sensors. Testing illumination was diffuse at ~50 lux.

**Novelty-Suppressed Feeding (NSF):** A 48 x 48 x 72 cm box was layered with ~4 cm of bedding and a pellet of food placed on a 10 cm square piece of filter paper in the center of the box. Testing area was illuminated diffusely at ~50 lux. Mice were food deprived for 24 hours prior to testing. Mice were placed facing a wall and assessed for feeding latency, with a max limit of 10 minutes. Feeding behavior was defined as rearing with

visible food consumption. Upon feeding, testing was terminated and animals returned to their home cage. Post-NSF food consumption was assessed by a 5 minute period of free-consumption in the home cage after testing and pre-/post- assessment of pellet weight.

**Acute-restraint + corticosterone assay:** A restraint tube was used to immobilize mice for a 30 minute duration, following a prior protocol (291). Separate mice were used to assess corticosterone levels at basal (AFR = 10, MS15 = 8, MS180 = 10), post-restraint (AFR = 10, MS15 = 8, MS180 = 10), and 30 minutes post-restraint (AFR = 10, MS15 = 8, MS180 = 9). For trunk blood collection, mice (PND90) were anaesthetized using isofluorane (10 seconds) and rapidly decapitated with a guillotine. All collections were performed between 0700-0930 hours. Trunk blood was mixed with 50  $\mu$ l EDTA (0.5 M) + 2  $\mu$ l aprotinin (1 mg/mL). Serum corticosterone was measured using a DetectX colorimetric immunoassay (Arbor Assays). The sensitivity of the kit was 18.6 pg/mL with a limit of detection of 16.9 pg/mL. The intra-assay variance was 6.9% and inter-assay variance was 7.93%.

**Statistical analyses:** Three-way comparisons for maternal care behavior, offspring weight, offspring behavior, and offspring corticosterone response were determined by analysis of variance (ANOVA) and post-hoc Tukey's honestly significant difference test. Effect size for pair-wise comparisons were calculated using Cohen's d, whereas for ANOVA, comparisons were calculated using partial eta squared ( $\eta^2$ ). Cohen's d estimates were converted to  $\eta^2$  for across measure comparisons of effect size. Cohen suggests  $\eta^2$  estimates of 0.01 as small, 0.06 as medium, and 0.14 as a large effect and Cohen's d estimates of 0.2 as small, 0.5 as a medium, and 0.8 as a large effect (292).

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## **Chapter 5 - Behavioral Disinhibition and Stress Axis Abnormalities in a shRNAi-based Tryptophan Hydroxylase 2 (TPH2) Knockdown Model**

### ***I. Abstract***

Serotonin (5-HT) is implicated in modulating complex behaviors, and its dysfunction is implicated in psychopathology and abnormalities of the stress axis. Using a shRNAi-based transgene, we knocked down tryptophan hydroxylase 2 (TPH2), the rate-limiting enzyme in neuronal 5-HT synthesis, and investigated the functional consequence of decreased TPH2 on behavior and stress axis function. Mouse TPH2 expression was attenuated using a synthetic miR-155 based transgene. Subjects were phenotyped for behavior in the rotorod, open-field test, elevated plus maze, forced swim test, and sucrose preference. Serotonin levels, circadian corticosterone rhythm, stress-induced corticosterone response, and expression of serotonin and stress axis –relevant genes were measured. Transgenic mice with attenuated TPH2 expression demonstrate an anxiolytic, anti-depressant, and hyperactive behavioral phenotype. The mice show reduced cortical 5-HT, a flattened corticosterone rhythm, lower hippocampal mineralocorticoid receptor expression, and decreases in hippocampal mineralocorticoid / glucocorticoid receptor ratios. Our findings support the hypothesis that decreased raphe serotonin lowers the threshold for active responding, reflected in increased exploration, motor facilitation, and proactive coping in the paradigms tested. Further, our findings support serotonergic

involvement in stress axis function, modulating corticosterone rhythm and hippocampal stress receptor gene expression.

## ***II. Introduction***

Neuronal tryptophan hydroxylase (TPH2) is a paralog of tryptophan hydroxylase (14), abundant in brainstem raphe nuclei (15) where it is believed to be the rate-limiting enzyme in brain serotonin biosynthesis. Serotonin is critical to the treatment of psychopathology but its specific role in complex behaviors remains enigmatic. A pivotal role for serotonin is supported by common serotonergic endpoints of diverse classes of antidepressants (293). Specific functions are attributed to individual serotonin receptors (294), but the most effective and widely used psychotropics affect global serotonin signaling through reuptake inhibition. Global limbic 5-HT levels are related to TPH2 levels (26). Polymorphisms in the TPH2 gene, some of which may have functional significance (16,17), have been associated with mood disorder, attention deficit hyperactivity disorder, bipolar disorder, schizophrenia, autism, anxiety and obsessive-compulsive disorder (18). TPH2 levels are increased in the brains of depressed suicides (25) and in rodent models of chronic exposure to corticotropin releasing hormone (22,23), indicating sensitivity to stress axis abnormalities.

Broad implications for brain serotonin in psychopathology contrasts with a less clear understanding of its specific role. Early studies implicated raphe serotonin in the anxiolytic effects of benzodiazepines (295), but inconsistent results and variable effects on locomotion suggested a more general role in threshold for 'active responding' (296). While some studies support this hypothesis, contradictory results preclude a cogent



theory and highlight limitations of the techniques used to interrogate the serotonergic system. Chemical ablation of serotonergic neurons suffers from limited pharmacologic specificity and loss of co-transmitter, while mechanical disruption, electrolytic ablation, and stimulation also affect adjacent and intrinsic structures (296,297). Precursor depletion (298) has been insightful, but the behavioral consequences cannot be separated from the potential effects of reduced melatonin production by TPH1 or other processes dependent on aromatic amino acid transport into brain. Indeed, the discovery of TPH2 highlighted the lack of specificity of pharmacologic approaches to modulate serotonergic function (299). The observation that a natural functional TPH2 polymorphism among mouse strains correlates with behavioral differences is provocative but confounded by the myriad of other genetic differences between inbred lines (300,301).

Transgenic manipulation of TPH2 expression has offered an opportunity to examine the specific effects of brain serotonin biosynthesis on complex behaviors, physiology, and gene expression. Behavioral characterization of TPH2 homologous knockouts (KO) indicate changes in motor impulsivity (38), reproductive behaviors (41), and anxiety- and depression- like behaviors (38,40). Yet, inactivation of TPH2 has been reported to have little effect on levels of other neurotransmitters (39,40), or on the expression of other serotonergic genes at the transcriptional (37) or translational levels (38). Although a knockout model offers the advantage of examining behavior and development in the absence of TPH2, a partial reduction may more accurately model vulnerability and disease states, in which expression is typically only partially increased or decreased.

In this study, we use a short-hairpin RNA interference (shRNAi) based stem-loop structure targeting TPH2 under control of an RNA polymerase II promoter to partially knockdown TPH2 in mice. We show that *tgTPH2i* transgenic mice exhibit decreased cortical serotonin levels and a phenotype consistent with decreased anxiety- and depression- like behavior and increased locomotion. Further, we show changes in the stress axis, including flattened corticosterone rhythm and abnormal corticosteroid receptor gene expression in the hippocampus.

### **III. Results**

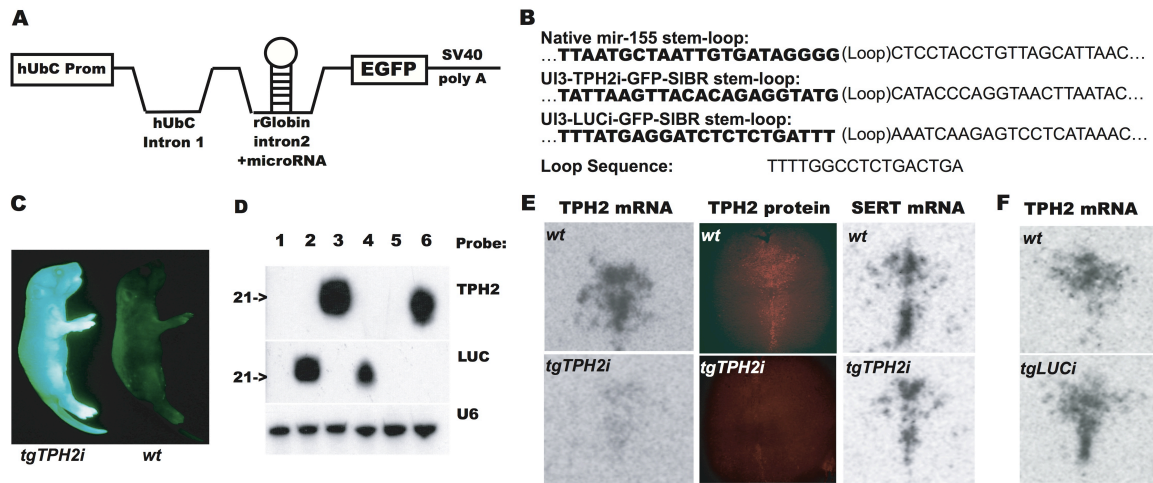
To knockdown TPH2 in mice, we designed an siRNA perfectly complementary to TPH2, using modified Dharmacon rules (302), and expressed them in UI3-GFP-SIBR, a previously described variant of the mir-155 based RNAi expression vector (303). The salient features of the resulting vector, UI3-TPH2i-GFP-SIBR, are shown in **Figure 5-1a**. Expression is driven by the human ubiquitin C promoter, followed by its native 1<sup>st</sup> intron and a synthetic intron 2 from the rabbit globin gene. Intron 2 was engineered with a ‘SIBR’ cassette containing minimal required sequences from mouse mir-155 (303). Mir-155 sequences, including the loop and mir-155\* strand (partially complementary to the mature miRNA, constituting the 2<sup>nd</sup> strand of the stem in the stem-loop structure) were replaced by sequences targeting TPH2 (**Fig. 5-1b**). A control construct, UI3-LUCi-GFP-SIBR, was engineered using the same vector expressing a siRNA tested previously (303) targeting nucleotides 1601-1622 of firefly luciferase in pGL3 (Promega).

Several different siRNA sequences against TPH2 were tested in reporter assays as previously described (303) and against overexpressed mouse TPH2 in HEK293 cells.

The single most potent of these, targeting nt. 1727 – 1748 in the 3'-UT of mouse TPH2 (Genbank# NM\_173391), was used to construct transgenic mice (*tgTPH2i*) in C57BL/6J x SJL/J background by pronuclear injection, while UI3-LUCi-GFP-SIBR was used to construct *tgLUCi* mice. Founders were backcrossed to C57BL/6J for a minimum of 3 generations before testing. Transgenic founders were identified by whole body detection of GFP fluorescence (**Fig. 5-1c**) and genotyping of tail DNA. All mice showing GFP fluorescence were confirmed positive by genomic PCR for the miRNA cassette. Subsequent generations were identified primarily by GFP fluorescence. Periodic genotyping confirmed that all GFP positive mice contained the siRNA transgene.

Northern analysis confirmed that the siRNA to TPH2 was expressed in *tgTPH2i* mice, but not in littermate controls or *tgLUCi* mice, and conversely, siRNA for luciferase was expressed in *tgLUCi* mice, but not in control or *tgTPH2i* mice (**Fig. 5-1d**). Results from transgenic mice matched expression in cultured mouse neuro-2a cells (**Fig. 5-1d**). TPH2 mRNA was decreased in raphe nuclei of *tgTPH2i* offspring as compared to their wildtype littermates (**Fig. 5-1e**). Quantitation of *in situ* hybridization (ISH) images revealed decreases from 42% to 93% in 4 founder lines tested. Two lines with the most robust TPH2 knockdown (>85%) exhibited poor reproductive fitness and failed to generate sufficient progeny for behavioral testing. The fourth line, detailed in this report, showed ~80% decrease in TPH2 mRNA in dorsal raphe (**Fig. 5-1e**), no differences in birth size or growth, and was successfully bred for behavioral analysis. TPH2 mRNA expression was unchanged in raphe of control (*tgLUCi*) mice (**Fig. 5-1f**), consistent with the expectation that TPH2 knockdown was specific to the siRNA and not related to a non-specific effect of the targeting construct. *TgLUCi* mice also reproduced normally. As

an alternate test of specificity, we examined the expression of two genes that are selectively expressed in raphe neurons: serotonin transporter (SERT) and serotonin 1b receptor (5-HT<sub>1b</sub>). SERT mRNA distribution by *in situ* hybridization (ISH) parallels expression of TPH2 and is not appreciably different in raphe of *tgTPH2i* mice (Fig. 5-1e).

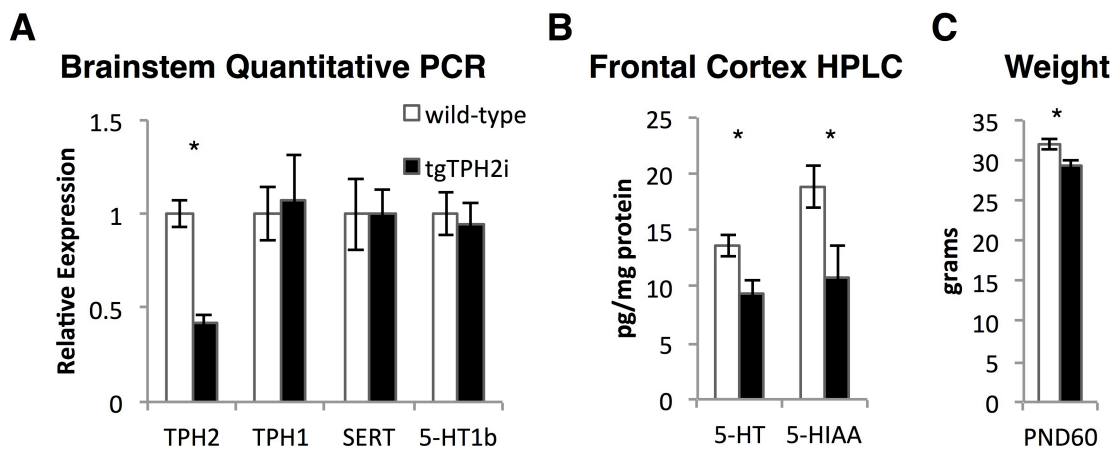


**Figure 5-1 Validation of TPH2 mRNA/protein knockdown and co-expression of GFP in transgenic mice.**

(A) Schematic of targeting construct showing human ubiquitinC promoter (hUbC) and location of mir-155 derived stem-loop SIBR cassette in rabbit globin (rGlobin) intron 2. (B) Synthetic stem-loop sequences targeting TPH2 (TPH2i) or luciferase (LUCi) as compared to native mir-155 stem-loop in UI3-GFP-SIBR. (C) Fluorescence for GFP in transgenic (*tgTPH2i*) vs. wildtype littermate (*wt*) controls. The image was over-exposed to visualize the *wt* pup which otherwise emit no fluorescence. (D) Northern analysis with probe against the mature TPH2 siRNA (top panel), the mature luciferase siRNA (middle panel) or the sample loading control U6 gene (bottom panel). Lanes 1-3 contain total RNA from mouse neuro-2a cells transfected with the empty UI3-GFP-SIBR vector (lane 1), UI3-LUCi-GFP-SIBR (lane 2) or UI3-TPH2i-GFP-SIBR (lane 3). Lanes 4-6 contain total RNA from brainstem of a *tgLUCi* mouse (lane 4), a *tgTPH2i* mouse (lane 6) and its *wt* littermate (lane 5). A 21 base synthetic RNA labeled in parallel served as a size standard. (E) *In situ* hybridization (ISH) for TPH2 mRNA (left panels), immunohistochemistry for TPH2 protein (middle panels), and ISH for serotonin transporter (SERT; right panels) in dorsal raphe of *wt* littermate (top panels) vs. *tgTPH2i* (bottom panels) mice. (F) ISH for TPH2 mRNA in dorsal raphe of *tgLUCi* and *wt* littermate mice.

Brainstem SERT and 5-HT<sub>1b</sub> expression were unchanged by quantitative real-time (qRT)-PCR (**Fig. 5-2A**), suggesting that the raphe cells were intact and healthy.

Consistent with changes observed by ISH, TPH2 mRNA by (qRT)-PCR was decreased greater than 60% in these samples. By contrast, mRNA for the paralogous gene TPH1, which is detectable at ~7 threshold cycles higher (14), was unchanged (**Fig. 5-2A**), indicating that the RNAi was specific for TPH2 and there was not a compensatory change in TPH1 expression in raphe. To determine if the observed knockdown of TPH2 was sufficient to decrease serotonin levels, frontal cortices from *tgTPH2i* transgenic and littermate control mice were processed for serotonin quantitation by HPLC. As shown in **Figure 5-2B**, 5-HT levels were decreased 32% and the primary serotonin metabolite 5-HIAA was decreased 43% in frontal cortex. As adults (PND60), *tgTPH2i* mice weighed ~8% less than wild-type littermates (**Fig. 5-2C**).

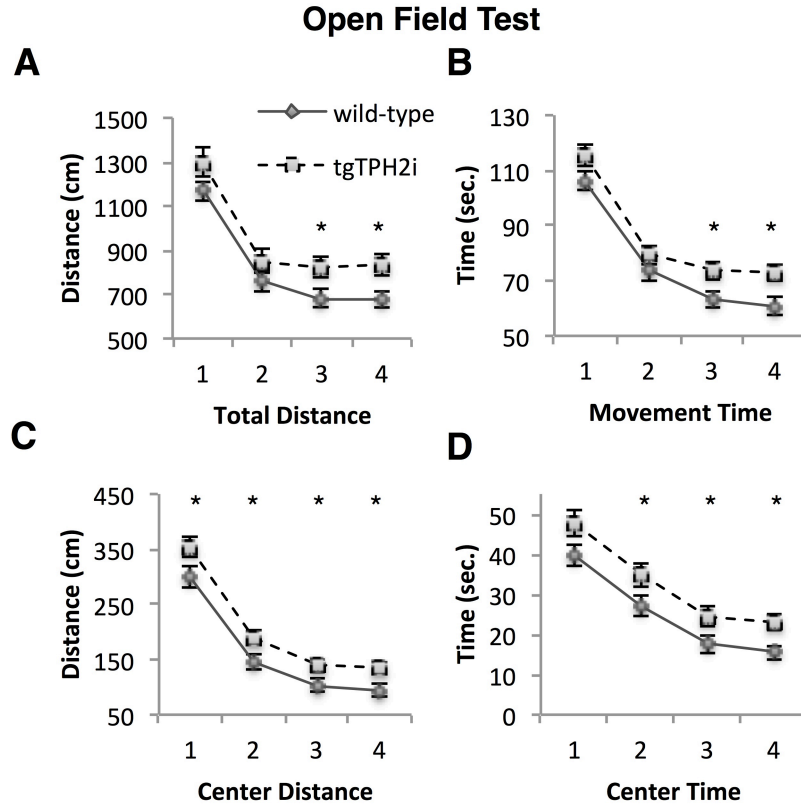


**Figure 5-2 Molecular characterization of *tgTPH2i* mice.**

(A) Quantitative reverse-transcriptase PCR for TPH2, TPH1, the serotonin transporter (SERT) and serotonin-1b receptor (5-HT<sub>1b</sub>) mRNA levels in brainstem of *tgTPH2i* and wild-type littermate controls. Within each run, expression was normalized against Hypoxanthine-guanine phosphoribosyltransferase (HPRT). Levels for each gene are normalized to levels in wild-type mice. (B) Serotonin (5-HT) and metabolite 5-

hydroxyindoleacetic acid (5-HIAA) levels in frontal cortex measured by HPLC. (C) Weight at postnatal 60. Significance denoted by  $p < .001$  (\*\*\*),  $p < .01$  (\*\*),  $p < .05$  (\*).

Having confirmed specific down-regulation of raphe TPH2 mRNA and brain serotonin levels, we explored behavior in paradigms commonly thought to measure anxiety. Raphe serotonin has long been proposed to underlie anxiety phenotypes, but its specific role has remained controversial (304). Conflict-based tests have proven effective metrics for anxiety related measures. The elevated plus maze (EPM), the open field test (OFT), and the Light/Dark (L/D) Box take advantage of the natural drive for safety (closed arms of the EPM, perimeter of the OFT, or dark section of L/D) vs. exploratory drive (open arms of the EPM, center of the OFT, or light section of L/D). Increased open arm time in the EPM, center time in the OFT, and time in the light section of the L/D are interpreted as decreased anxiety. In the OFT, in trials across multiple days, *tgTPH2i* mice demonstrated modestly increased total locomotion, statistically significant in later trials (**Fig. 5-3A, B**). More striking was increased distance and time covered in the center of the open field (**Fig. 5-3C, D**).



**Figure 5-3 Open-field behavior of tgTPH2i mice.**

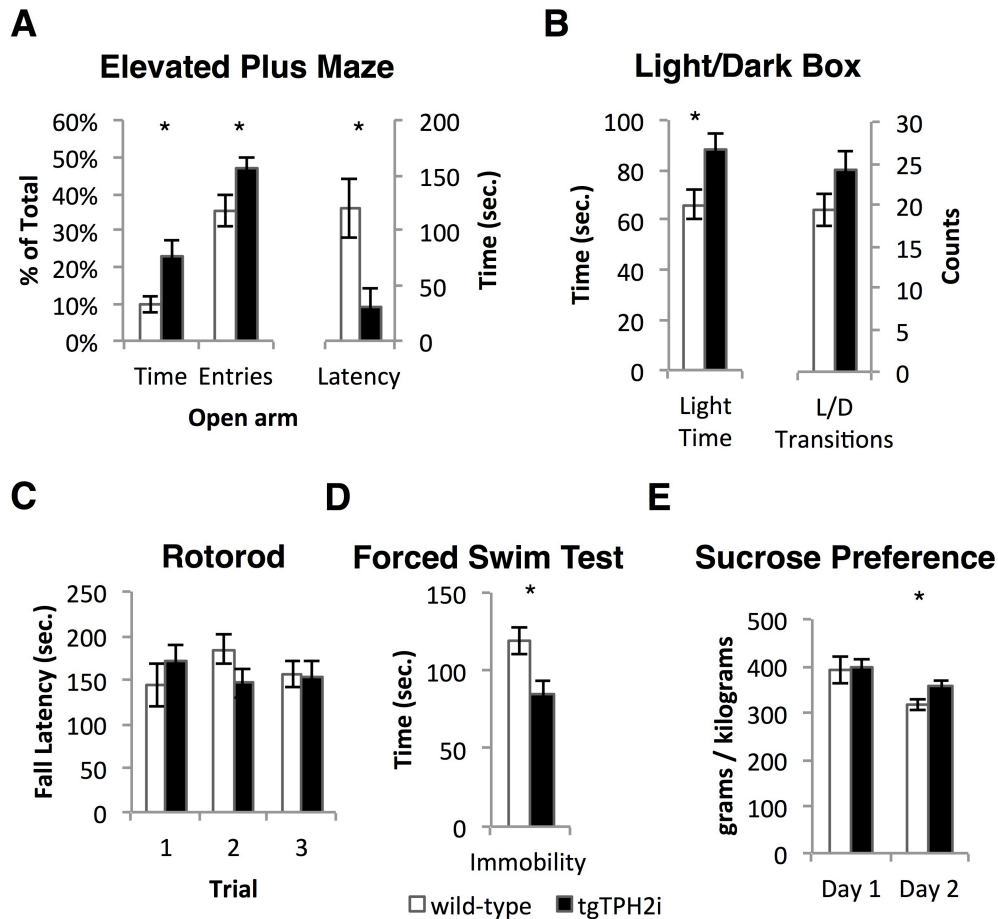
(A) Total distance traveled in the OFT. (B) Movement time in the OFT. (C) Distance traveled across the center in the OFT. (D) Total time in the center in the OFT. Significance denoted by  $p < .001$  (\*\*\*),  $p < .01$  (\*\*),  $p < .05$  (\*).

On the EPM, *tgTPH2i* mice demonstrated significantly increased percent time in open arms, increased percent entries into open arms, and decreased latency for open arm entry as compared to littermate controls (**Fig. 5-4A**). On the L/D box, *tgTPH2i* mice demonstrated increased time spent in the light time but unchanged L/D transitions (**Fig. 5-4B**). Results from these paradigms are consistent with decreased anxiety-like behavior, or increased novelty exploration in mice with decreased TPH2 expression. The comparable, if not greater, horizontal distance covered by *tgTPH2i* mice in the OFT confirms intact gross locomotor capacity. Moreover, *tgTPH2i* mice were not appreciably

different from controls in motor coordination or performance improvement with repeated trials in the rotorod test (**Fig. 5-4C**).

We investigated depression-related behavior in the sucrose preference paradigm and Porsolt forced swim test (FST). Designed to assess specific components of depression, reduced consumption in sucrose preference is interpreted as anhedonia, whereas increased immobility in FST is interpreted to reflect behavioral despair. In animal models of depression, treatment with anti-depressants, such as selective serotonin reuptake inhibitors, increase sucrose consumption (305) and decrease immobility time in the FST (289). Across a 2-day trial, sucrose consumption in *tgTPH2i* mice was increased relative to controls on day 2 (**Fig. 5-4B**). On the FST, *tgTPH2i* mice exhibited significantly decreased immobility time (**Fig. 5-4C**).



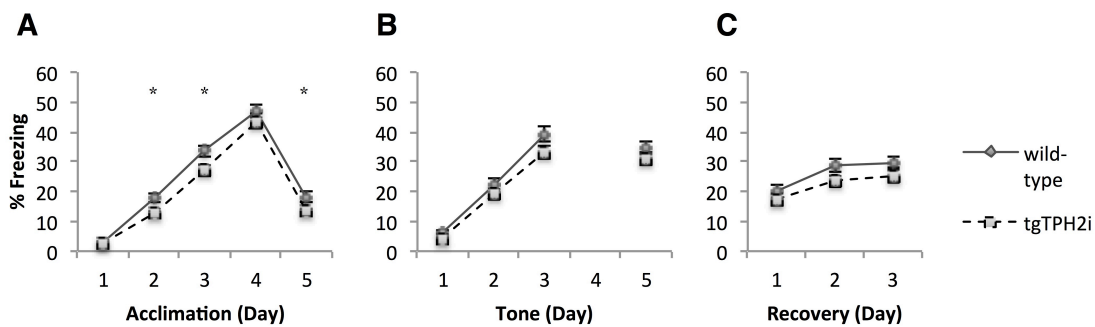


**Figure 5-4 Anxiety- and depression- like behavior in tgTPH2i mice.**

(A) Elevated Plus Maze activity. The left ordinate corresponds to open arm (OA) time and open arm entries, while the right ordinate corresponds to the latency measure. (B) Light/Dark (L/D) box activity. The left ordinate corresponds to time in the light while the right ordinate corresponds to number of light/dark transitions. (C) Time before falling in the automated rotorod test on three consecutive days (no group differences). (D) Immobility time in the in the Porsolt forced swim test. (E) Grams of sucrose consumed / kilogram of weight in the sucrose preference tests. Significance denoted by  $p < .001$  (\*\*\*),  $p < .01$  (\*\*),  $p < .05$  (\*).

We investigated associative learning in a cued and contextual fear conditioning paradigm. An acclimation (180 seconds), tone + shock (30 seconds), and recovery period (30 seconds) occurred over days 1-3. On day 1, freezing was similar between conditions during all periods. However, on days 2-3, percent time spent freezing was decreased

during acclimation, but no different during tone presentation and during recovery. On day 4, over a longer period of acclimation (300 seconds), percent time spent freezing was no different, although behavior trended similarly. On day 5, an acclimation period (120 seconds) was followed by a tone presentation period without shock (180 seconds). Percent time freezing was lower in *tgTPH2i* mice during the acclimation period but not different during the tone presentation (**Fig. 5-5 A-C**).

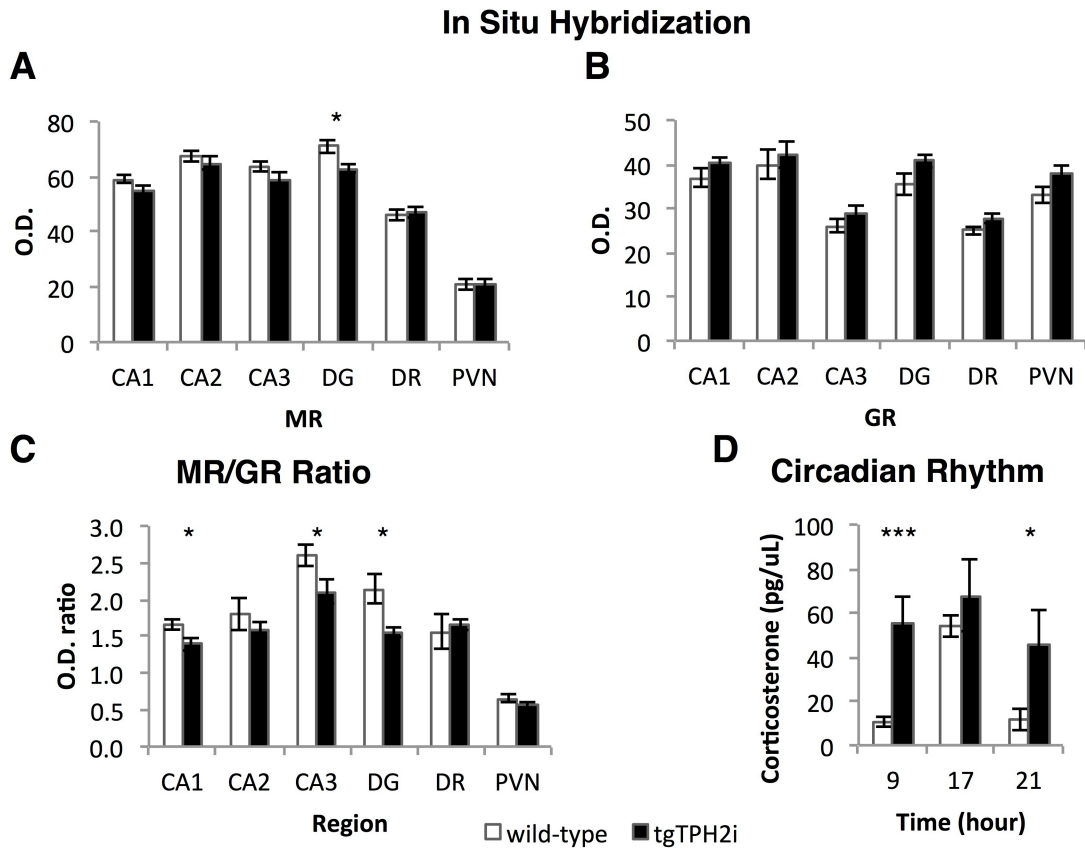


**Figure 5-5 Contextual and cued fear conditioning.**

(A) Percent freezing during the acclimation period. (B) Percent freezing during the tone + shock (days 1-3) or tone alone (day 5) period. (C) Percent freezing during the recovery period. Significance denoted by  $p < .001$  (\*\*\*),  $p < .01$  (\*\*),  $p < .05$  (\*).

We next investigated expression of corticosteroid receptors in *tgTPH2i* mice. An ISH of glucocorticoid receptor (GR) and mineralocorticoid receptor (MR) across multiple limbic structures, including hippocampus, raphe, hypothalamus, amygdala, and nucleus accumbens revealed a modest decrease in MR mRNA in the dentate gyrus of hippocampus (**Fig. 5-6A**), but unchanged GR mRNA (**Fig. 5-6B**). We found decreased MR/GR ratios in hippocampal CA1, CA2, and dentate gyrus subfields in *tgTPH2i* mice (**Fig. 5-6C**). Because MR and GR are implicated in corticosterone regulation, we assessed corticosterone levels at baseline. An ELISA for plasma corticosterone

demonstrated an elevated trough level and a resulting flattened circadian corticosterone rhythm in *tgTPH2i* mice (Fig. 5-6D).



**Figure 5-6 Stress axis expression and corticosterone circadian rhythm.**

(A) ISH optical density of mineralocorticoid receptor (MR) in hippocampus. (B) ISH optical density of glucocorticoid receptor (GR) in hippocampus. (C) Ratio of MR/GR O.D. in hippocampal subfields. (D) Corticosterone circadian rhythm. Light period from 0700 – 0700. Abbreviations: cornu Ammonis (CA), dentate gyrus (DG), dorsal raphe dorsalis / ventralis (DRd/DRv), and paraventricular nucleus (PVN). Significance denoted by  $p < .001$  (\*\*\*),  $p < .01$  (\*\*),  $p < .05$  (\*).

#### IV. Discussion

Despite numerous studies, the role of brain serotonin in complex behaviors remains controversial, potentially due to technical limitations (123,297). The discovery of TPH2 (14) highlighted ambiguity in previous studies, particularly those using para-

chlorophenylalanine, one of the more “selective” pharmacologic agents employed to interrogate the raphe system but which also inhibits pineal melatonin (306), presumably through TPH1, and affects levels of other monoamines (307). Genetic modulation of TPH2 offered the possibility to avoid this confound. Using a transgenic RNAi approach, we provide new data suggesting decreased raphe TPH2 expression, in the absence of any change in TPH1 expression, is associated with a decrease in anxiety- and depression-related behavior, increased locomotor activity, decreased contextual fear learning, a flattened circadian corticosterone rhythm and changes in the hippocampal MR/GR mRNA ratio. Consistent with the hypothesis that TPH2 is rate-limiting in serotonin biosynthesis, we find reduced serotonin and its metabolite levels in the brains of *tgTPH2i* mice.

Studies implicating low serotonin in the release of punished responding by minor tranquilizers in a conflict test (295) support its role in the pathogenesis of anxiety, but serotonin attenuation is not clinically effective in the treatment of anxiety disorders. In fact anxiety disorders are more commonly treated with serotonin augmenting agents, such as the serotonin reuptake inhibitors. Moreover, parallel studies implicate low serotonin in impulsivity (308,309), prompting the hypothesis that rather than mediating anxiety per se, a reduction in brain serotonin favors “active responding at the expense of behavioral suppression” (296). Pragmatically, active responding refers to a bias towards a motor response. Such a model is compatible with studies in which serotonin depletion is associated with an decrease in jump threshold to shock (310) and increased locomotion (311).

Consistent with a proposed role for raphe serotonin in behavioral inhibition (312), transgenic knockdown of TPH2 in our model led to increased open arm exploration and decreased latency to enter the open arm in the elevated plus maze, increased light time in the light/dark box, and increased center time as well as overall activity in the open-field, all of which may be interpreted as a decrease in threshold for motoric responding. The converse is reported from increased extracellular 5-HT in SERT KO mice (313), with less time spent in the center field on the open-field and in the open arm on the elevated zero maze (124). Increased activity in the open-field for *tgTPH2i* mice emerged primarily on later days of testing, suggesting diminished behavioral inhibition on motor activity with waning environmental novelty. This is consistent with evidence of serotonergic depletion altering open-field behavior predominantly in familiar environments (123), and would reconcile our findings with reports of unchanged open-field behavior in prior TPH2 knockout studies (38,40), in which repeated testing was not performed.

Our findings agree with prior characterizations of TPH2 KO transgenics suggesting varying degrees of disinhibited behavior across multiple domains. These include lower latencies to attack conspecifics, increased light time and longer latencies for initial entrance into the dark compartment in the L/D box, decreased latency to consume food in the novelty suppressed feeding test, non-discrimination in sexual behavior, and compulsion in marble burying and nest shredding, although elevated plus maze performance has been reported to be unchanged (38,40,41). This may reflect methodological differences in testing conditions, or else developmental differences from partial knockdown in our model versus complete knockout of TPH2 mRNA. Nonetheless, the convergence of findings employing an entirely different strategy

(shRNAi) to knockdown TPH2 expression lends strong support to the general model implicating brain serotonin in behavioral suppression.

*TgTPH2i* mice presented an anti-depressant phenotype, evinced by increased consumption in the sucrose preference test and decreased immobility time in the FST, consistent with behavior in TPH2 KO mice (40). Conversely, SERT KO transgenics demonstrate decreased sucrose consumption and greater immobility in the FST (313), indicating complementary behavioral effects from transgenic depletion or enhancement of global serotonergic tone. Yet, association of lowered 5-HT with an antidepressant phenotype is ostensibly inconsistent with the effectiveness of serotonin reuptake inhibitors (SSRIs) in treating major depression, which are posited to achieve therapeutic efficacy via increased extracellular 5-HT. This suggests alternate mechanisms may underlie behavior resembling an anti-depressant phenotype in the FST and sucrose preference.

One possibility is a distinction between postnatal vs. adult exposure to high levels of 5-HT. This is supported by evidence of prenatal or adolescent exposure to SSRIs linked to suicidal ideations, anxiety, agitation, and depression in pre-clinical studies. Moreover, these effects are mirrored by evidence of increased anxiety- and depression-like behavior in rodents exposed to SSRIs during early life, and paradoxical findings in which behavioral abnormalities induced by adolescent SSRI exposure can be treated by SSRIs in adult life (for review see Olivier, 2011) (42).

A second possibility, specific to FST behavior, is that swimming is not reflective of lower despair from low 5-HT per se, but rather a bias towards active responding. This may be related to reduced serotonergic regulation of structures implicated in modulating

escape behavior, effectively releasing behavioral response (314). Likewise, increased consumption in the sucrose preference paradigm may reflect decreased serotonergic inhibition of carbohydrate consumption regulated by medial hypothalamus (315,316) rather than reduced anhedonia. These interpretations help resolve inconsistencies between empirical treatment of major depression through augmenting 5-HT levels and the association of an anti-depressant phenotype with reduced 5-HT activity in *tgTPH2i* mice. Alternatively, because the mechanism by which SSRIs achieve therapeutic efficacy remains unclear, the long-term effect of SSRIs on serotonergic signaling may resemble those observed with decreased TPH2 expression. This is indirectly supported by evidence of increased TPH2 mRNA in depressed suicides (25) and in rodent models of adverse early life experience (24) and chronic stress (22,23). Of course, it is also possible that serotonin biology in rodents does not model the psychobiology of depression in humans.

On contextual and cued fear conditioning, *tgTPH2i* mice exhibited comparable performance to wild-type mice along most measures. These included progressive increases in freezing time with additional trials, intact cued learning when re-exposed to tone on day 5, and decreased contextual freezing on day 5 following omission of tone + shock on day 4. However, *tgTPH2i* mice also exhibited lower contextual freezing on days 2, 3, and 5 during acclimation, whereas cued freezing was undifferentiated from wild-type during periods of paired tone + shock or tone alone. This suggests deficits in contextual but not cued learning in *tgTPH2i* mice, which implies an impairment in hippocampal but not amygdala-based learning (317). Alternatively, the decreased contextual freezing may also reflect disinhibition from reduced serotonergic activity, in which *tgTPH2i* mice are biased towards making an active rather than passive response

upon exposure aversive context. This might result in an earlier escape from freezing, and is supported by a trend of lower *tgTPH2i* freezing in general across acclimation, tone, and recovery periods.

*TgTPH2i* mice demonstrated abnormalities in stress axis function, with blunting of the diurnal corticosterone rhythm and altered stress receptor expression. This has clinical implications, as ~50% of depressed patients also exhibit blunted circadian corticosterone rhythms with elevation of the diurnal trough (93,318). Decreased serotonin signaling to the suprachiasmatic nucleus (SCN) in *tgTPH2i* mice may underlie the observed changes, as chemical ablation of serotonergic innervation to the SCN was previously shown to flatten the circadian corticosterone rhythm and elevate the diurnal trough (95) and a hypofunctional allele of TPH2 in rhesus monkeys is associated with an increase in cortisol (319). In addition, decreases in hippocampal MR expression may contribute to a change in the corticosterone rhythm. MR receptors are implicated in the regulation of corticosterone levels during the nadir, due in part to its 10-fold higher binding affinity relative to GR. Decreases in MR expression have been associated with elevated basal corticosterone levels (320). Interestingly, decreases in hippocampal MR/GR ratio, as seen in *tgTPH2i* mice, have also been reported in animal models of chronic stress (22,23) and in suicide victims with a history of depression (67), supporting a functional relationship between low serotonin signaling and depression vulnerability through changes in stress axis biology (20).

While HPA axis dysregulation is consistent with models of vulnerability for mental illness, the anxiolytic- and antidepressant-like phenotype in *tgTPH2i* mice would, on the surface, seem contradictory. If, however, decreased serotonin signaling is



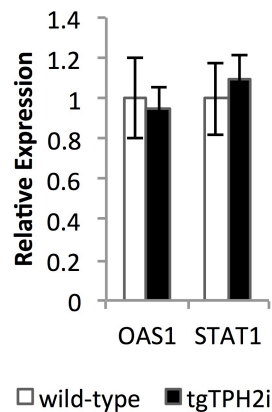
construed to facilitate behavioral disinhibition, a potentially more cogent model of vulnerability emerges. Disinhibition may contribute to negative urgency, a tendency to experience strong impulses under conditions of negative affect (144). Impulsive responding shortens the temporal interval for appraising risk. Together, the findings suggest low 5-HT may predispose to adverse outcomes and accompanying negative effects on mood. Additional work is necessary to clarify and accurately interpret the behavior resulting from transgenic knockdown of TPH2.

As an approach to modeling potential functional consequences from polymorphisms in TPH2, transgenic knockdown offers several advantages over acute manipulations from pharmacologic-based methods, considering that vulnerability to psychopathology from TPH2 polymorphisms would be expected to affect brain development (321) and serotonin turnover throughout the lifespan. It is encouraging that findings from our model, employing an entirely different approach to attenuate TPH2 expression, are consistent with those observed through homologous knockout of TPH2 (38,40). In the future, regional or temporal control of TPH2 expression will be of particular interest, given past evidence of a temporal dependency for 5-HT<sub>1a</sub> receptor ablation on anxiety- and depression- like behaviors (43).

There are limitations and risks associated with our shRNAi-mediated knockdown approach. These included potential off-target effects, activation of the interferon response, and competition with endogenous pathways. Concern for off-target effects is mitigated by the observation that the same behavioral findings were observed in 2 different transgenic lines. By driving expression from an RNA polymerase II promoter, we minimized risk for an interferon response previously reported with RNA polymerase

III driven constructs (322). Total mRNA levels for 2',5'-oligoadenylate synthetase 1 (OAS1) and signal transducer and activator of transcription 1 (STAT1), early indicator genes for activation of the double stranded RNA mediated interferon response (323), from *tgTPH2i* mouse brainstem were comparable to littermate controls (**Fig. 5-7**).

### Interferon Response



**Figure 5-7 Interferon response.**

Quantitative reverse-transcriptase PCR in mouse brainstem for the interferon response genes 2',5'-oligoadenylate synthetase 1 (OAS1) and signal transducer and activator of transcription 1 (STAT1) (no group differences). Significance denoted by  $p < .001$  (\*\*\*) ,  $p < .01$  (\*\*),  $p < .05$  (\*).

In addition, prior studies have suggested that expression driven by strong RNA polymerase III promoters induce toxicity (322), at least partly through competition for nuclear export with biologically essential silencing pathways (324). This was minimized by driving expression using an RNA polymerase II promoter, but we cannot exclude that these effects did not occur in *tgTPH2i* mice. Notwithstanding, our behavioral findings are consistent with results in TPH2 knockdowns using viral based approaches (38,40) and raphe lesion studies (123).

In summary, our findings provide further evidence, using an RNAi transgenic approach, of serotonergic depletion leading to behavioral disinhibition. These include decreases in anxiety- and depression- related behavior, and novel evidence of reduced contextual freezing in a fear conditioning paradigm. In addition, we provide novel evidence that transgenic knockdown of TPH2 mRNA results in abnormalities of the HPA stress axis. This includes an elevation in corticosterone circadian rhythm during the diurnal trough and a decrease in the ratio of hippocampal corticosteroid receptor expression. These findings further our understanding of the role of 5-HT in complex behaviors and stress biology.

## ***V. Materials and Methods***

**Clones.** UI3-TPH2i-GFP-SIBR and UI3-LUCi-GFP-SIBR (**Fig. 1a**) were constructed by synthesizing 64 base complementary oligonucleotides (Invitrogen, Carlsbad, CA) containing 60 base stem-loop structures (**Fig. 1b**) plus 4 base overhangs compatible with BbsI sites in UI3-GFP-SIBR, a precursor to UI4-GFP-SIBR described recently (303). UI3-GFP-SIBR contains the natural BbsI restriction site in the human ubiquitinC 1<sup>st</sup> intron that was deleted in UI4-GFP-SIBR to facilitate direct cloning of synthetic stem-loops. The constructs otherwise behave identically in cell culture reporter assays (unpublished). Sequences for the vectors are available online at <http://sitemaker.umich.edu/dlturner.vectors/home>.

**Transgenic mice.** Targeting constructs were released by restriction digestion of UI3-TPH2i-GFP-SIBR or UI3-LUCi-GFP-SIBR with PvuI and PvuII, and transgenic mice were made by standard pronuclear injection by the Transgenic Core Facility at the

University of Michigan. All manipulations, including behavior studies were approved by the University Committee on the Use and Care of Animals. Mice were genotyped by PCR of tail DNA and whole body fluorescence for green fluorescent protein (GFP) (**Fig. 1c**). PCR primers targeted rabbit globin intron 2 sequences flanking the SIBR cassette (5'-TCAGATTGTAAGATCCCATCG-3' and 5'-ACGCCAGCCAGAATTTATATG-3') or the 5'-untranslated and open reading frame of GFP (5'-GGCAACGTGCTGGTTATTGT-3' and 5'-CCGGACACGCTGAACTTGTG-3'). PCR was carried out with GoTaq (Promega, Carlsbad, CA) for 35 cycles of 20 seconds each at 94°C, 58°C, and 72°C. For GFP fluorescence, mice were illuminated with a xenon lamp (Superlux 175, Carl Zeiss, Inc., Brighton, MI) fitted with a 470 nm excitation filter (Chroma Tech. Corp., Rockingham, VT) and visualized through a SYBR filter (Scion Corp., Frederick, MD).

**Northern analysis for siRNA expression.** Neuro-2A cells at a density of  $5 \times 10^5$ /30 mm dish were transfected 16 hours later with 1 ug of targeting constructs using Expressfect (Denville Scientific, Metuchen, NJ) and harvested 24 hours later. Mouse brainstems were systematically cut in a dissecting block (Braintree Scientific, Braintree, MA) at bregma -3mm and at caudal cerebellum. Total RNA from neuro-2A cells and from brainstem, after stripping away cerebellum, was extracted with Trizol (Invitrogen).

Probes sequences complementary to mouse U6 snRNA (5'-ATTGCGTGTCATCCTTGCG-3'), TPH2i siRNA (5'-CATACCTCTGTGTA ACTTAATA-3'), and LUCi siRNA (5'-AAATCAGAGAGATCCTCATAAA-3') were radiolabeled by T4 polynucleotide kinase (Invitrogen), purified on Bio-gel P2 spin columns (Bio-Rad, Hercules, CA) and used to

probe 10 ug total RNAs run on an 18% polyacrylamide/urea gel as previously described (303). Blots were stripped between probings by boiling in 0.1x SSC/0.1% SDS for 10 minutes. Signal was obtained after exposure to x-ray film for 2 to 24 hours.

**Histochemistry.** *In situ* hybridization (ISH) and semi-quantitative analysis were carried out as previously described (325). A total of 18 (9 *tgTPH2i* and 9 *wild-type*) animals were used. Radiolabeled riboprobes were directed against mouse TPH2 (nt. 2277 to 2622 of Genbank NM\_173391), SERT (nt. 1723 to 2136 of Genbank NM\_010484), GR (nt. 1 to 596 of Genbank NM\_008173), or MR (nt. 940-1217 Genbank XM\_983321). TPH2 protein was visualized in perfusion fixed mouse brainstem processed according to the manufacturer's instructions for PH8 monoclonal primary antibody (Chemicon, Temecula, CA) at 1:5000 and Alexa Fluor 546 goat anti-mouse (Invitrogen) secondary antibody. At low titer, PH8 antibody is specific for tryptophan hydroxylase, and specificity for TPH2 in raphe is assured by the lack of TPH1 immunoreactivity in this region (299).

**Quantitative reverse transcriptase PCR.** Primers used (shown 5' → 3') were: TPH1 (CACAGTTCAGATCCCCTCTACA and GAACGTGGCCTAGGAGTTCA), TPH2 (TGTCCTTGGATTCTGCTGTG and GCCCACCAACTTCATTCTTC), SERT (CGTCGTGTCTTGGTTCTATGG and CAGATCCTCCAAAACCATCC), 5-HT1b (GTTGGCTGCCCTTCTTCAT and GAAACCAGCAGGCATCCTTA), OAS1 (CAAGCACTGGTACCAACTGTG and CAACTCTAGGGCGTACTGTGG), STAT1 (TTGTGTTGAATCCCGAACCT and TCGAACCACTGTGACATCCT), and the reference gene hypoxanthine-guanine phosphoribosyl transferase (HPRT; CAGTACAGCCCCAAAATGGT and GCGCTCATCTTAGGCTTTGT). First strand cDNA template was made from 1 ug of DNase-treated total RNA using iScript reverse

transcriptase, oligo(dT)/random hexamer mix, buffer and reaction conditions specified in the iScript cDNA synthesis kit (Bio-Rad). All primers were verified to amplify a single amplicon of the correct size and sequence. PCR reactions were run in triplicate on 8 subjects per group, and each result was replicated in a separate cohort. PCR conditions were 35 cycles of 94°C for 20 sec., 57°C for 15 sec., and 72°C for 15 sec. Ten-fold serial dilutions of verified amplicons were used to estimate amplification efficiencies, all of which were in the range of 1.94 to 2.01. Efficiency corrected relative expression levels were normalized to the expression of HPRT (326).

**Serotonin assay by high pressure liquid chromatography (HPLC).** Fresh frozen brains were sliced into 200  $\mu$ M sections at -4 to -6°C, thaw mounted onto glass slides and stored at -80°C until processed. Slides were warmed to -8°C on a cold plate. Frontal cortices were dissected with a scalpel and scraped into microtubes for sonication in 0.1% perchloric acid with 0.1 mM EDTA. Homogenates were pre-cleared by ultracentrifugation at 20,000g for 10 minutes. Pellets were dissolved in 1 N NaOH and proteins determined by Bradford assay (Bio-Rad). Supernatants were further clarified through PVDF 0.45  $\mu$ m filters (Millipore, Billerica, MA) and injected onto a C18 reverse phase analytical column (5  $\mu$ m, 250 x 4.6 mm; Biophase ODS, BAS, West Lafayette, IN) protected by a precolumn cartridge (5  $\mu$ m, 30 x 4.6 mm, BAS). The mobile phase was 0.05 M sodium phosphate/0.03 M Citrate (pH 2.8), 0.1 mM EDTA, 0.02% sodium octyl sulphate and 20% methanol. Serotonin and its metabolite were detected against standards, using a dual analytical electrode cell set at 0.02 V and -0.32 V, respectively (ESA, Bedford, MA) and quantitated by area under the curve. Five to 6 subjects per group were analyzed.

**Behavior Testing.** Activity in the Digiscan Open Field Activity chamber (AccuScan Instruments, Columbus, OH), mouse Rota-Rod (Ugo Basile/Stoelting, Chicago, IL), or elevated plus maze were carried out as previously described (327). The Digiscan and Rota-Rod generate automated data. The elevated plus maze was videotaped and scored manually using EPMscore (328). The Porsolt forced swim test was carried out as previously described (289). For sucrose preference, a 2% sucrose solution (along with *ad libitum* water) was provided for 30 minutes per day across a 2 day trial. All results are from 18-20 male subjects per group and were replicated in at least one separate cohort.

**Cued and Contextual Fear Conditioning.** Animals were assessed for percent freezing across 5 days in a conditioning chamber (Med Associates). For more information on the chamber, please see McKinney et al. (329). On days 1-3, an acclimation period of 180 seconds was followed by a 30 seconds tone presentation (5 kHz, 70 dB) that ended with a 2-second 0.50 mA foot shock. A 30 second recovery period followed. On day 4, animals were exposed to an acclimation period of 300 seconds with no tone or shock. On day 5, a 120 second acclimation period was followed by a 180 second period of tone (without shock) presentation.

**Corticosterone Levels.** To determine circadian corticosterone rhythm, trunk blood was collected at 0900 (control = 11; *tgTPH2i* = 10), 1700 (control = 6; *tgTPH2i* = 5), and 2100 hours (control = 6; *tgTPH2i* = 4). The light cycle was from 0700-1900 hours. Mice were anaesthetized using isoflurane and decapitated with a guillotine. Trunk blood was mixed with 50  $\mu$ l EDTA (0.5 M) + 2  $\mu$ l aprotonin (1 mg/mL). Serum corticosterone was measured using a DetectX colorimetric immunoassay (Arbor Assays). The sensitivity of

the kit was 18.6 pg/mL with a limit of detection of 16.9 pg/mL. The intra-assay variance was 6.9% and inter-assay variance was 7.9%.

**Statistical Tests:** Student's t-tests were run in Microsoft Excel. ANOVAs and post hoc tests were run in SPSS.



## Chapter 6 – Conclusion

### ***I. Primary findings: Role of early life experience on TPH2 expression & behavior***

Adverse early life experience (aELE) and serotonergic abnormalities are heavily implicated in the biology of mental illness. Understanding the role aELEs have in shaping postnatal serotonergic development is important for preventing and/or treating mental illness. Yet, research remained limited on how aELEs shape the serotonergic system and underlying mechanisms which mediate long-term vulnerability.

The original intent of this thesis work was to investigate epigenetic regulatory mechanisms underlying long-term changes in tryptophan hydroxylase 2 (TPH2) expression following MS, a well-validated and reliable rat model of aELE, in c57bl/6 mice. However, unexpected initial findings of shared decreases in TPH2 mRNA between MS15 and MS180 mice prompted a shift in focus towards clarifying the behavioral and molecular correlates of the mouse response to MS. This was necessary to illuminate the functional implications from decreases in TPH2 mRNA as well as for validating core findings of MS across species. Alternatively, if there was a unique response to MS in mice, clarifying the parameters underlying differences between species would assist in delineating what early life factors shape lifetime vulnerability or resiliency (for review of MS paradigm and its core findings, refer to **Box 6-1**).

**Box 6-1 – Review of maternal separation (MS) paradigm**

MS occurs over postnatal day 2-14, using 3 conditions to investigate the long-term impact of aLEs on behavior and molecular activity. These conditions included a 15 minute/day separation (MS15), a 180 minute/day separation (MS180), and an animal facility reared (AFR) control. The MS180 condition models adverse early life experience, whereas the MS15 condition is a handling control and AFR a non-handled control. In the original MS studies in rats, MS180s as adults typically exhibit an anxiogenic- and depression- like phenotype, with corresponding increases in stress hormone levels, impaired negative feedback on stress levels, increases in hypothalamic CRH and ACTH expression, and lower hippocampal GR expression. Conversely, MS15s as adults typically exhibit an anxiolytic- and anti-depressant-like phenotype, with corresponding decreases in stress hormone levels, enhanced negative feedback on stress levels, and complementary changes in HPA stress axis gene expression. In rats, MS180 is a model of early life induced vulnerability, whereas MS15 is a model of early life induced resiliency.

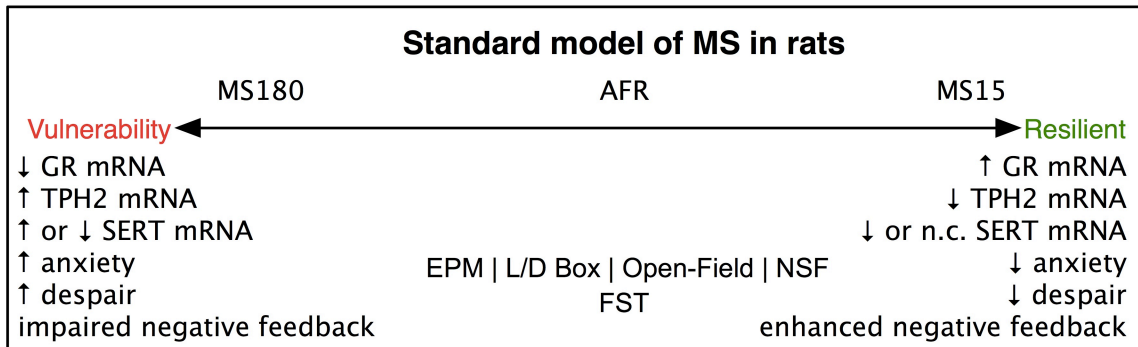
Analysis of the expression of related genes, serotonin transporter (SERT) and glucocorticoid receptor (GR), indicated MS15 and MS180 both decreased raphe somatodendritic SERT mRNA and increased hippocampal GR mRNA. Behavioral analysis indicated a common decrease in anxiety-like behaviors, corresponding to shared patterns of gene expression. These results suggested that MS15 and MS180 in c57bl/6 mice promoted resiliency irrespective of separation duration, in contrast to the expected pattern following MS in various rat strains (**Fig. 6-1A**).

The shared behavioral and molecular profile between MS conditions prompted further investigations into whether maternal mediation underlie resiliency to MS180. In support, an elevated level of post-separation maternal care was observed in MS15 and MS180 dams, corresponding to the duration of separation. Prior early life research suggested that enhanced maternal care and tactile stimulation underlies long-term resiliency. Hence, the transient but robust increase in post-separation combined licking / nursing / covering behavior strongly implicated an enhanced maternal response in mitigating early life stress and promoting long-term resiliency in c57bl/6 mice.

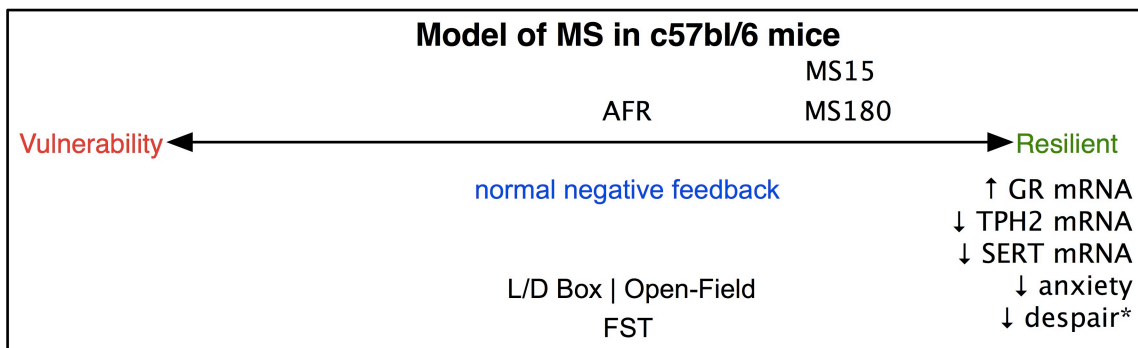
Converging lines of evidence, in which MS15 and MS180 increased hippocampal GR mRNA, decreased anxiety-like behavior, and enhanced post-separation maternal care, suggested that low TPH2 mRNA in MS mice had a functional role in establishing the anxiolytic-like phenotype. To clarify its role, a TPH2 transgenic knockdown line was developed and characterized. Complementing the findings from the MS studies, knockdown of TPH2 produced an even more robust decrease in anxiety- and depression-like behavior. This supported a role of MS-mediated decreases in TPH2 mRNA shaping long-term decreases in anxiety-like behaviors.

In summary, this thesis work has examined the effect of early life experience on TPH2 expression in c57bl/6 mice, providing supporting evidence of decreased TPH2 expression corresponding to molecular and behavioral changes which have been implicated in resiliency. It has clarified the effectiveness of MS in c57bl/6 mice, providing behavioral and molecular evidence of a unique response to MS in this mouse strain. This response entails increases in post-separation maternal care as a function of separation duration, suggesting that maternal mediation mitigates adverse effects of separation on pup homeostasis and promotes a profile of resiliency (**Fig. 6-1B**). The divergence in response to MS emphasizes the necessity of assessing maternal behavior in early life models to interpret and understand the implications of molecular and behavioral changes in adult life. Knockdown of TPH2 mRNA expression results in a corresponding lowering of anxiety-like behaviors, supporting a functional role of decreased TPH2 mRNA in shaping or modulating long-term behavior following maternal separation.

**A**



**B**



**Figure 6-1 Cross-species comparison of response to maternal separation.**

(A) Model of MS in rats corresponding to a differentiation of MS15 vs. MS180 behavior and expression. (B) Model of MS in c57bl/6 mice, in which MS15 and MS180 share a common pattern of behavior and expression. The MS15 and MS180 mouse profile are not as robust as those reported in rats. Negative feedback refers to HPA axis response to stress. The \* denotes MS180 alone exhibits lower despair.

## II. Limitations in linking MS & *tgTPH2i* behavior

<b>Anxiety Measures</b>	<b>MS15</b>	<b>MS180</b>	<b><i>tgTPH2i</i></b>
Elevated Plus Maze	unknown	unknown	↓
Elevated Zero Maze	no change	no change	unknown
Light/Dark Box	↓	↓	↓
Open-Field	↓	↓	↓
Novelty Suppressed Feeding	no change	no change	?
<b>Depression Measures</b>			
Sucrose Preference	unknown	unknown	↓
Forced Swim Test	no change	↓	↓

**Figure 6-2 Concordance between MS & *tgTPH2i* behavior.**

Arrows are relative to wild-type or AFR conditions. ↓ = decreases in anxiety- or depression- like behavior.

A decrease in TPH2 mRNA, from either transgenic knockdown or early life experience, corresponds to shared patterns of behavior in adult life (**Fig. 6-2**). The concordance in MS15, MS180, and *tgTPH2i* performance along anxiety-related measures, and to a lesser extent, depression-related measures, supports a role of decreased TPH2 in development of long-term resiliency. However, differences between MS and *tgTPH2i* mice also deserve consideration. First, TPH2 mRNA was knocked down throughout raphe nuclei by ~80% in adult *tgTPH2i* mice, whereas long-term decreases in MS15 and MS180 TPH2 mRNA were ~20% and restricted to the DRd and DRv sub-regions. This differentiates both magnitude and regional influence on serotonergic activity. Second, because protein levels in MS animals were undetermined, it remains to be clarified to what extent raphe TPH2 protein is decreased, as well as its effects on 5-HT availability. *TgTPH2i* mice exhibited a ~33% decrease in cortical 5-HT and 5-HIAA content relative to a ~80% drop in TPH2 mRNA, suggesting that 5-HT activity at baseline is to some extent buffered against deficits in raphe TPH2 mRNA. This implies the ~20% decrease in raphe TPH2 mRNA may have a lower effect on baseline 5-

HT activity in MS mice. Third, TPH2 mRNA was knocked down throughout the entirety of development in *tgTPH2i* mice, whereas it was decreased somewhere between PND2-60 for MS mice. The neurotrophic and modulatory role of 5-HT varies during fetal, postnatal, adolescent, and adult age, which may further differentiate the effects of lower TPH2 mRNA between MS15 and MS180 vs. *tgTPH2i* mice. However, despite these differences, the consequences on behavior from reductions in TPH2 mRNA do suggest a convergence towards a decrease in anxiety-like behaviors.

In the future, it will be necessary to delineate the temporal pattern of TPH2 mRNA and protein across age from early life experience. This will help clarify the functional role of early life-mediated decreases in TPH2 mRNA in shaping postnatal development and modulating behavior in adult life. In summary, the behavior of *tgTPH2i* mice supports a functional role of decreased TPH2 mRNA influencing behavior in MS mice, but more work will be necessary to clarify the mechanisms by which early life-mediated decreases in TPH2 mRNA alter behavior.

### ***III. Functional implications of sub-regional decreases in TPH2 expression***

Changes in raphe TPH2 mRNA in MS15 and MS180 mice were localized to midline dorsal raphe structures (DRd and DRv), whereas DRlw levels remained unchanged. The midline DR is the primary source of ascending afferents to cortical structures and is implicated in the higher-order integration of sensory, cognitive, and emotional input on behavior. In contrast, the DRlw is a primary source of innervations to subcortical and brainstem structures, implicated in modulating visceral responses to threat and autonomic and emotional states (for review, refer to **Box 6-2**). Therefore,

decreased TPH2 mRNA along midline DR structures suggests an impact on long-term tendencies to express anxiety responses (trait anxiety) rather than immediate anxiety or defense responses (state anxiety). Systems modulated by the DRlw are unaffected, which would leave responses to imminent or proximate threat, including “fight-or-flight,” panic, and visceral autonomic / emotional states, relatively intact.

**Box 6-2 – Review of midline vs. lateral dorsal raphe functional anatomy**

The DRd and DRv are the primary source of ascending afferents to cortical structures, including prefrontal, somatosensory, visual, and motor cortices, as well as motor structures including striatum and basal ganglia, which suggests involvement of midline DR in modulating higher-order processes. The midline DR receives projections from structures implicated in “top-down” regulation of anxiety states, including amygdala, infralimbic cortex, prelimbic cortex, lateral habenula, and BNST. Conversely, midline DR sends projections to various anxiety-related structures including amygdala, medial prefrontal cortex (mPFC), DLPAG, BNST, nucleus accumbens, and dorsal hypothalamic area (1). The innervation of the amygdala from the midline DR is particularly dense, suggesting a role in modulating attachment of emotional salience to experiences (2). This suggests a role in modulating long-term tendencies to express anxiety responses, i.e. trait anxiety, rather than immediate anxiety responses.

The DRlw receives and send innervations primarily to and from subcortical structures. These include descending innervations to structures involved in “fight-or-flight” response (3) such as the dorsolateral periaqueductal gray (DLPAG) and rostral ventrolateral medulla (RVLM), and ascending innervations to structures involved in autonomic & emotional states, including the amygdala, bed nucleus of the stria terminalis (BNST), medial preoptic area, and lateral hypothalamus (4), and amygdala (5).

The anatomical divergence of midline vs. lateral dorsal raphe structures coincides with divergent effects of decreased serotonergic inhibition on avoidance (general anxiety disorders) vs. escape behaviors (panic disorders). Structures innervated by the ascending pathway (midline DR) are implicated in avoidance behavior whereas structures innervated via the descending pathway (DRlw) are implicated in escape behavior.

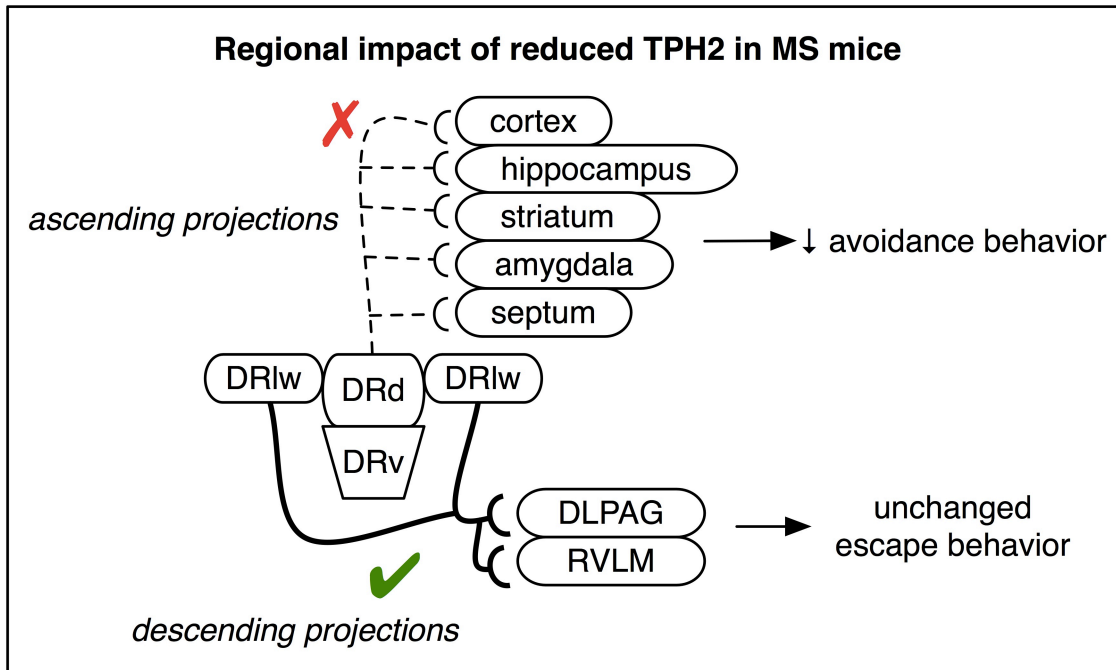
Importantly, evidence suggests decreased serotonergic activity in ascending projections reduces avoidance behavior, whereas decreased serotonergic activity in descending projections facilitates escape behavior (330). This implies a divergence in the consequence of reduced serotonergic influence on anxiety-related behaviors. Thus, localized decreases in TPH2 mRNA in MS15 and MS180 mice may selectively facilitate approach behaviors through reduced serotonergic signaling from midline dorsal raphe structures, consistent with MS15, MS180, and *tgTPH2i* performance on the L/D box, EPM, and open-field test.

Moreover, this implies escape behaviors mediated by the DRlw is unaffected in MS15 and MS180 mice, but increased in *tgTPH2i* mice. To elaborate, TPH2 mRNA was decreased globally in *tgTPH2i* mice, affecting serotonergic activity in both midline and lateral dorsal raphe structures whereas following MS in mice, TPH2 mRNA levels in DRlw were unchanged. In the Porsolt FST, the increase in *tgTPH2i* swim time suggests elevated escape-directed behavior, consistent with a facilitation of escape behavior due to reduced serotonergic inhibition from the DRlw to the DLPAG. In contrast, no change in DRlw TPH2 expression in MS15 and MS180 mice is consistent with unchanged escape behavior, i.e. swim time in the Porsolt FST.

In summary, this thesis work supports a functional effect of regional decreases in TPH2 mRNA on certain aspects of anxiety-related behavior. Specifically, MS mice exhibit reduced avoidance behavior and normal escape behavior, consistent with regional decreases in TPH2 mRNA in the DRd and DRv (**Fig. 6-3**), whereas *tgTPH2i* mice exhibit reduced avoidance and increased escape behavior, consistent with decreases in global TPH2 mRNA, including the DRd, DRv, and DRlw (**Fig. 6-4**). These findings suggest

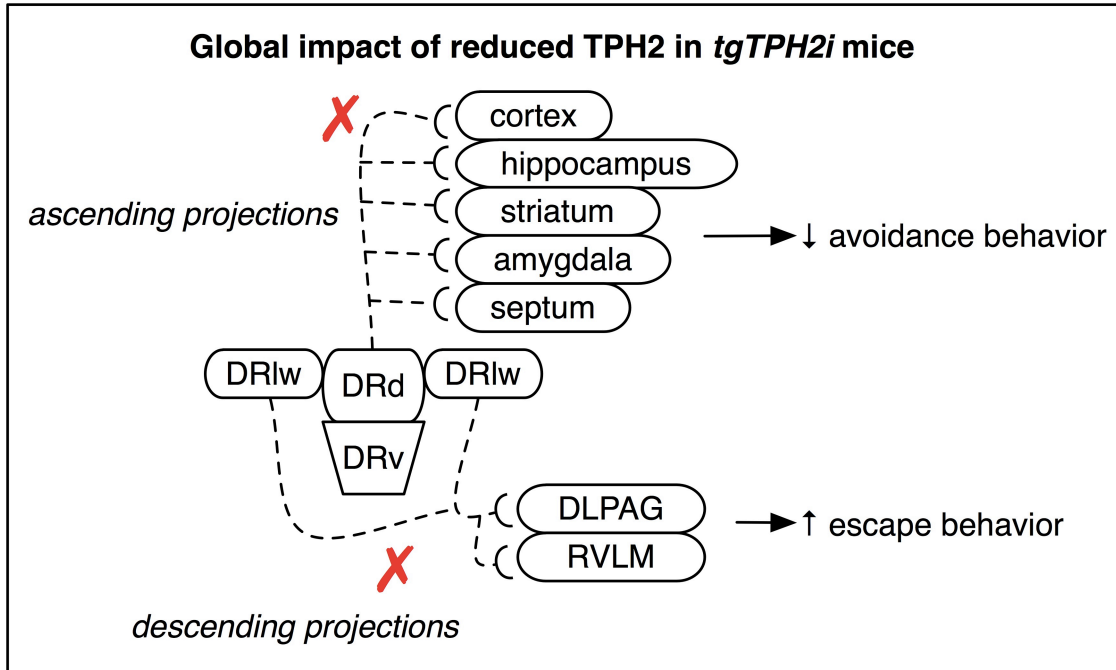


resiliency from early life may decrease avoidance behaviors, lowering anxiety-like responses through decreased TPH2 mRNA expression, without effecting panic-related responses. These findings support a dissociation between general anxiety vs. panic, and indicate regional differences in the control of these behaviors.



**Figure 6-3 Regional impact of reduced TPH2 in MS mice.**

The reduction in TPH2 mRNA decreases avoidance behavior via reduced 5-HT activity from midline DR structures while leaving 5-HT inhibition of escape behavior from lateral DR structures intact. Abbreviations: DRl = dorsal raphe lateral wings; DRd = dorsal raphe dorsalis; DRv = dorsal raphe ventralis; DLPAG = dorsolateral periaqueductal gray; RVLM = rostral ventrolateral medulla. Symbols: **X** = affected; **✓** = unaffected.



**Figure 6-4 Global impact of reduced TPH2 in *tgTPH2i* mice.**

Low TPH2 mRNA decreases avoidance behavior via reduced 5-HT activity from midline DR structures and increases escape behavior via reduced 5-HT activity from lateral DR structures. Abbreviations: DRlw = dorsal raphe lateral wings; DRd = dorsal raphe dorsalis; DRv = dorsal raphe ventralis; DLPAG = dorsolateral periaqueductal gray; RVLM = rostral ventrolateral medulla. Symbols: ✗ = affected; ✓ = unaffected.

#### ***IV. Maternal mediation in *c57bl/6* mice – promoting resiliency?***

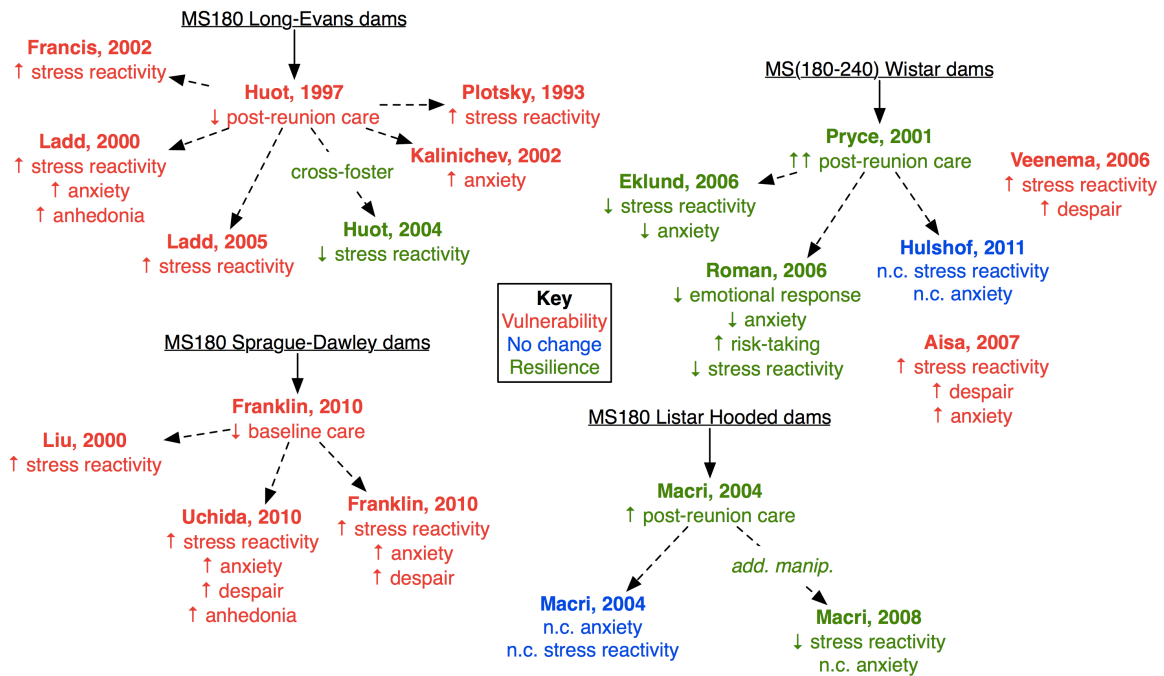
The post-separation increase in maternal care for MS180 dams, and to a lesser extent MS15 dams, suggests maternal mediation underlies the parallel changes in expression and behavior observed in MS15 and MS180 mice. Whereas maternal care significantly decreased as the lactation period waned in AFR dams, MS dams significantly increased licking / nursing / covering behavior. The transient bout of intense tactile stimulation may underlie developmental changes promoting later life resiliency. This is supported by studies in which artificial tactile stimulation in maternally deprived or separated pups minimized adverse development of HPA axis response to stress or even decreased anxiety-like behaviors in adults (272-274).

Moreover, these findings suggest that c57bl/6 mice exhibit a unique maternal response to the MS paradigm, in which MS15 and MS180 manipulations increase maternal care during the immediate period after MS. This is in contrast to the response in Long-Evans rats, the primary strain in which MS was developed, in which MS15 increases long-term maternal care (190) and MS180 results in delayed and disorganized care (201). This is supported by evidence that provision of foster litters during MS180 to Long Evans dams prevents development of vulnerability in separated offspring, instead shifting offspring behavior and stress response towards that resembling MS15s (202). In this context, the enhanced maternal response in c57bl/6 dams post-reunion most likely underlies mitigation of stress from prolonged separation and promotion of a positive outcome.

The relationship between maternal response to MS180 and offspring vulnerability and resiliency across various rat strains also supports the role of post-reunion care in mitigating the stress from separation. Although investigations into maternal response remain limited, there is evidence of a differential maternal response in Long-Evans, Sprague-Dawley, Wistar, and Listar-Hooded dams, correlating with differences in offspring outcome. For example, MS180 in Sprague-Dawley rats lowers baseline levels of dam care, and is associated with other studies that report an increase in offspring stress reactivity, anxiety-like state, anhedonia, and despair-like behaviors (229,331,332). This would be consistent with the association between long-term vulnerability and lower maternal care in Long-Evans rats previously discussed. In contrast, limited studies in Wistar and Listar-Hooded rat strains suggest that MS180 increases dam post-reunion maternal care (190,271), and associated studies report either no change (271,333) or

decreased stress reactivity, decreased anxiety-like state, increased risk taking, and lower emotional response in offspring (334,335). This suggests that enhanced post-reunion care following MS180 can either buffer against the stress of prolonged separation or even promote resiliency, similar to that observed in our studies (**Fig. 6-5**). In light of this past research across various rat strains, the increase in post-reunion maternal care in c57bl/6 dams is strongly suggestive of a similar role, in which enhanced maternal care mitigates stress from prolonged separation and promotes offspring resiliency (**Fig. 6-6**). This stresses the importance of investigating maternal care in early life experience studies for accurate interpretation of the long-term consequences of the experience.

The clinical implications of these findings suggest that early life intervention or treatment can be effective in preventing long-term vulnerability. For instances of childhood abuse, trauma, or neglect, a supportive caregiver influence may be capable of mitigating and promoting a positive long-term resolution despite adversity. Early life interventions, in which the appropriate caregiver response to childhood stress is established, may protect and even promote resiliency in later life.



**Figure 6-5 Maternal behavior and offspring profile in different rat strains.**

Rat studies separated by strain. Enhanced maternal behavior is associated with offspring resilience or buffering against the stress from separation. Abbreviations: n.c. = no change; up arrow = increased; down arrow = decreased.

## Maternal influence on resiliency / vulnerability



**Figure 6-6 Maternal influence on resiliency / vulnerability.**

In rats, MS15 enhanced long-term levels of maternal care, mitigating the stress from separation and promoting resiliency. Conversely MS180 results in disorganized maternal behavior, which in combination with a stress on pup homeostasis from prolonged separation, contributes to vulnerability. In mice, MS15 and MS180 confers a transient increase in post-separation maternal care, mitigating the stress from separation and promoting resiliency. The resiliency associated with increased tactile stimulation provided by the dams, effecting long-term changes in gene expression and behavior.

### **V. TPH2 DNA Methylation & Implications of IFAP Technique**

Although decreases in levels of raphe TPH2 mRNA were detected, DNA methylation of its 5' untranslated region and promoter remained unchanged, suggesting other regulatory regions or mechanisms underlie long-term changes in its expression. Reports of experience-induced epigenetic changes have remained sparse, despite substantial enthusiasm for epigenetics as a mechanism for developmental programming, suggesting early experience-dependent changes in DNA methylation may be less common than initially considered. However, the paucity of reports also reflects technical difficulties present in the analyses of DNA methylation. Specifically, difficulties in the

dissection of discrete brain nuclei, for which specificity and/or cost are core issues, have limited the number of studies investigating epigenetic mechanisms underlying changes in gene expression.

This thesis work has made a technical contribution towards lowering the cost and increasing specificity by developing the *ISH*-guided freeze matrix assisted punches (IFAP) technique for thin slide-mounted tissue extractions. A core advantage of this method is the streamlined approach of post-sectioning dissection, not interrupting standard histological workflow, while allowing for parallel analyses of expression. Although changes in methylation of the *TPH2* promoter were not detected following MS, the development of the IFAP technique furthers the field's capacity to rapidly and cheaply perform parallel analyses of expression and DNA methylation.

#### ***VI. Increases in hippocampal GR mRNA is not associated with a change in stress-induced corticosterone level in MS mice***

Despite increases in hippocampal GR mRNA in MS15 and MS180 mice, corticosterone levels at baseline, immediately following 30 minutes of restraint stress, and 30 minutes after stress termination were not different between MS15, MS180, and AFR mice. This suggests development of the stress response, at least to a 30-minute immobilization stress, is insensitive to early life experience in c57bl/6 mice. In contrast, increases in hippocampal GR mRNA following early life stress in various rat strains are associated with an attenuated stress response, in which baseline and/or peak levels are lower and negative feedback is enhanced.

Both normal and attenuated corticosterone responses to acoustic stress in MS15 and MS180 c57bl/6 mice have been reported. The response seems to be dependent on

whether MS occurred during the first or last 3 hours of the light phase, respectively (212). The normal response and separation experience corresponds to our study's experimental design, supporting our evidence of a normal corticosterone response. Yet, if MS has no effect on corticosterone response to stress, it raises questions as to the function of the increase in hippocampal GR mRNA.

Underlying the discrepancy in stress response may be differences in pattern and magnitude of change in hippocampal GR between species. In our c57bl/6 mouse studies, the increase in GR was limited to the hippocampal DG, whereas GR is reportedly increased in hippocampal DG, CA1, CA2, and CA3 regions in Long Evans rats. Additionally, the magnitude of increase in MS15 vs. MS180 or non-handled control rats is up to 2-fold higher, in comparison to the ~11-12% increase in c57bl/6 mice (183,336).

Several avenues of future investigation are of interest. Initially, it will be important to ascertain whether increased hippocampal GR mRNA corresponds to increased GR protein expression and function. It will be of interest to determine whether there is a differential role in chronic vs. acute stress. Possibly, an increased level of GR mRNA could buffer against depletion of surface GR from intracellular translocation of bound GR, acting to increase expression during periods of severe or prolonged stress. However, this remains to be determined. In summary, these findings suggest no change between increases in GR mRNA and corticosterone response to stress, supporting a unique effect of MS in c57bl/6 mice.



## ***VII. Role of low TPH2 in stress resiliency – prevention of prolonged 5-HT activity?***

The decrease in MS15 and MS180 raphe TPH2 mRNA is associated with decreased anxiety-like behavior and increased hippocampal GR mRNA, suggesting a role in resiliency. Our findings are in agreement with evidence of lower TPH2 mRNA in adult MS15 rats (24). There is evidence to suggest that TPH2 expression is sensitive to stress, with elevated TPH2 mRNA or protein found in models of vulnerability or mental illness. This includes following chronic exposure to stress hormones (22,23,337), immobilization stress (248,249,338), in MS180 adult rats exposed to social defeat stress (24), and in depressed suicides (25). Moreover, immobilization stress has also been reported to increase serotonergic turnover in the brainstem, frontal cortex, hypothalamus, and striatum (339). These studies suggest high TPH2 may be a marker for vulnerability, and that decreased TPH2 mRNA may confer resiliency against the influence of stress on abnormal 5-HT activity.

The functional implications of altered TPH2 mRNA following stress remains to be elucidated. One possible role of the reduction in TPH2 expression is to prevent sustained activation of serotonergic activity. This is supported by evidence of prolonged serotonergic activity post-stress in MS180 rats (198). If elevated TPH2 mRNA contributes to sustained serotonergic activation post-stress, it may accentuate imbalances in 5-HT signaling, contributing to vulnerability. This effect may be particularly pronounced during early postnatal life, where excessive 5-HT activity can have adverse long-term consequences on behavior and brain development (42). Fitting our data into this context, the decreased TPH2 mRNA in MS15 and MS180 mice could limit the

duration of post-stress serotonergic activity, minimizing potential adverse effects from sustained serotonergic signaling.

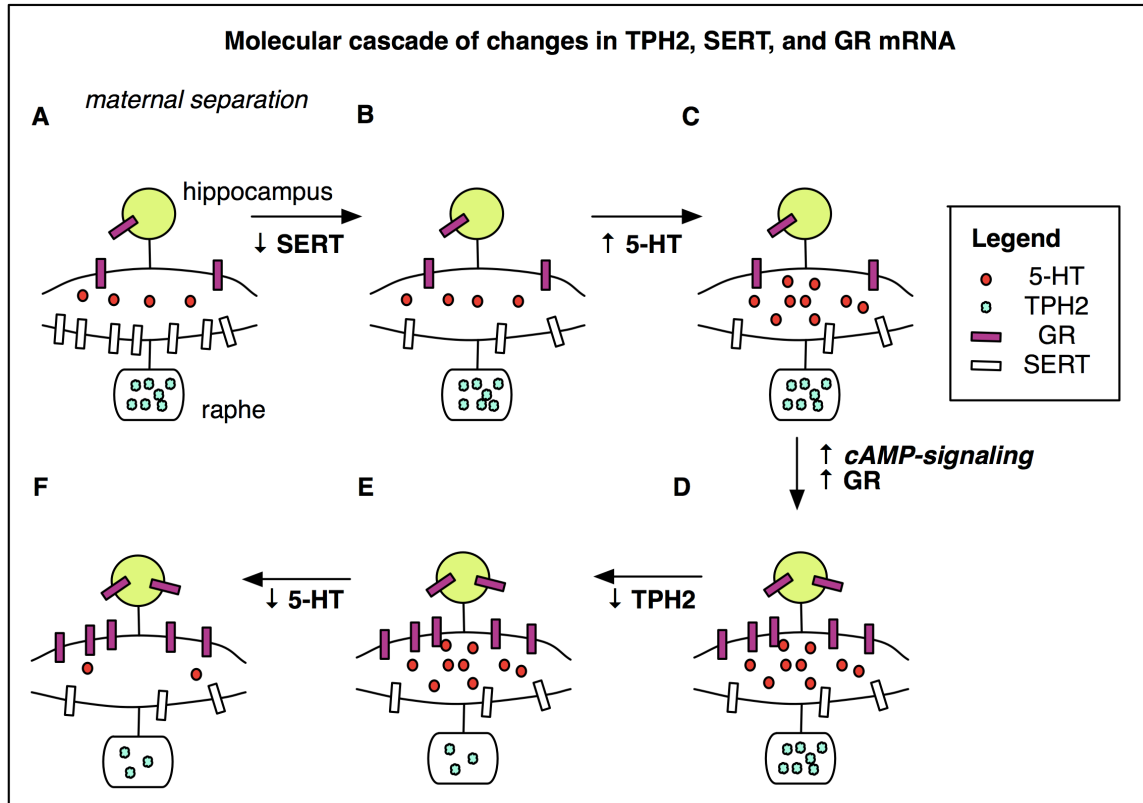
In the future, it will be of particular interest to clarify whether decreases in TPH2 mRNA confer a substantial resiliency to stress during postnatal and adult life, whether its expression shifts in response to stress, and the regional effects on 5-HT signaling. In summary, the evidence suggests early life-mediated decreases in TPH2 mRNA may confer resiliency, but future work will be necessary to clarify the implications of changes in its expression.

### ***VIII. Reconciling opposing effects of low TPH2 and SERT mRNA on 5-HT activity***

The theoretically opposing effects on 5-HT activity of decreased raphe TPH2 and SERT mRNA in MS mice requires reconciliation. Whereas decreased SERT would be predicted to elevate extracellular 5-HT by limiting reuptake, decreased TPH2 would be expected to lower 5-HT content by limiting the rate of synthesis. This would suggest a net null effect on 5-HT activity, assuming changes in their expression have a similar scale of effect on 5-HT activity. Alternatively, decreases in TPH2 and SERT may reflect a temporal sequence of events, in which an elevation or deficit in 5-HT activity, due to lower SERT or TPH2 mRNA, respectively, is compensated by changes in the expression of the other following an earlier life experience. This has been previously reported in the offspring of high maternal care dams, wherein 5-HT activity is elevated during early postnatal development, but is re-normalized or decreased in adult life. Interestingly, this is linked to development of the HPA axis, where 5-HT dependent elevations in cAMP-mediated signaling effect a long-term increase in hippocampal GR binding

(151,217,218), suggesting a possible role for temporally-dependent decreases in TPH2 and SERT mRNA.

It is possible that the MS-induced increase in maternal care might initiate a similar signaling cascade in *c57bl/6* mice. In this model, enhanced maternal care decreases SERT mRNA during early life to elevate extracellular 5-HT and establish an altered pattern of hippocampal GR expression. At a later age, 5-HT levels re-normalize or are further decreased by a compensatory decrease in TPH2 mRNA. This hypothetical model is illustrated in **Figure 6-7**. To validate this model in the future, a developmental study across several periods of postnatal life will be necessary. Moreover, it will be important to ascertain whether changes in mRNA correspond to protein expression and the extent to which 5-HT levels and activity are affected at a regional and global level.



**Figure 6-7 Model of a signaling cascade integrating long-term changes in TPH2, SERT, and GR expression.**

(A) Maternal separation increases maternal care, (B) decreasing SERT expression, (C) increasing 5-HT, and (D) stimulating 5-HT cAMP secondary messenger signaling to increase GR expression. At a later point, (E) TPH2 mRNA decreases and (F) decreases 5-HT levels. This decrease in 5-HT persists into adult life which in concert with increases in GR affects an anxiolytic-like phenotype.

### ***IX. Clinical implications: Reconciling the low TPH2 mRNA & SSRI efficacy on 5-HT activity & vulnerability***

In interpreting the clinical implication of this thesis work, a central issue is reconciling decreases in TPH2 mRNA and lower anxiety- and depression- like behavior with the framework of low serotonergic activity and efficacy of SSRIs in treating mood and anxiety disorders. One possibility is that rodents fail to adequately model the role of

serotonin in these human conditions. Animal behaviors interpreted as anxiety- or despair-like may reflect underlying mechanisms that are distinct from pathology in humans.

A second possibility is a distinction between early postnatal vs. adult exposure to elevated 5-HT activity, in which elevated 5-HT activity during early postnatal life has adverse effects on long-term development compared to its beneficial effects in adult life. This is supported by evidence that elevated 5-HT during early postnatal life, either from SERT knockout or postnatal SSRI exposure in mice, increases anxiety- and depression-like behavior (57,124), whereas it has an opposite effect in adult life (340). Similarly, adolescent exposure to SSRIs has an anxiogenic-like effect on adult behavior (341), which paradoxically can be rescued by SSRI treatment in adult life (63). Moreover, hippocampal and cortical rescue of 5-HT<sub>1a</sub> expression in 5-HT<sub>1a</sub> knockouts during early postnatal life re-establishes normal anxiety-related behavior, whereas it has no effect if rescued in adult life or if rescued only in the raphe (43). These behavioral changes are supplemented by evidence of molecular abnormalities, including reduced serotonergic cell count, area, and diameter (64) and clinical evidence of increased suicidal ideation and behavior, agitation, depression, and anxiety in children treated with SSRIs (51-53,55,56).

These findings suggest that excess 5-HT activity during early life can effect behavioral and molecular changes persisting into adult life. Conversely, this implies that lower TPH2 mRNA may serve a protective role against excess 5-HT activation by limiting stress or anxiety induced elevations in 5-HT synthesis during early life. In conclusion, the evidence suggests 5-HT has discrete effects during early vs. adult life. This developmental specificity helps reconcile our findings of low TPH2 mRNA

associated with decreased anxiety-like behavior with the clinical evidence of SSRI treatment elevating serotonergic activity to achieve therapeutic benefit.

A final point, the molecular mechanism underlying the therapeutic effectiveness of SSRIs, which require weeks to months of treatment, has yet to be elucidated. The SSRI's acute effect, i.e. blocking reuptake to increase extracellular 5-HT, is not directly linked to efficacy, and may initially even worsen symptoms of anxiety or depression. Chronic treatment with SSRIs has been shown to increase neurogenesis, de-sensitize somato-dendritic autoreceptors to alter 5-HT activity, and affect remodeling of the chromatin, all of which may underlie therapeutic mechanism of action. Early-life mediated decreases in TPH2 mRNA may be part of a larger sequence of postnatal developmental changes which produce a similar effect, but which remains to be explored. In the future, it will be essential to investigate the temporal pattern of TPH2 expression, its effects on end points altered by chronic SSRI treatment and on related brain systems, as well as the influence of early environmental factors on TPH2 expression and function. This will help elucidate early life-mediated changes of the serotonergic system on shaping vulnerability or resilience.

## ***X. Final thoughts***

This thesis work tested the influence of early life experience on long-term changes in TPH2, its epigenetic regulation, and its role in modulating behavior. Although the original intent was to delineate epigenetic mechanisms underlying long-term vulnerability to the serotonergic system, clear differences in the response to aELEs in the

mouse system shifted the focus of this work towards an investigation of the MS paradigm in c57bl/6 mice.

The MS experiments provide evidence of a unique long-term effect in c57bl/6 mice, in which MS15 and MS180 mice both exhibit increases in hippocampal GR mRNA, decreases in raphe TPH2 and SERT mRNA, a normal stress response, and a decrease in anxiety-like behaviors. These studies provide evidence of enhanced maternal care in MS dams, which likely mitigates the stress of separation and promotes long-term resilience in offspring. The transgenic experiments indicate that lowering raphe TPH2 mRNA decreases anxiety- and depression- like behaviors, supporting a functional role of lower TPH2 mRNA on anxiety-like behaviors in MS mice.

This thesis work also led to the development of IFAP, a novel tissue dissection technique to extract slide-mounted tissue for DNA methylation analyses. Because this technique allows for inexpensive and accurate dissection of discrete brain nuclei without interrupting histological workflow, it should enhance the field's capacity to more quickly assess early life-mediated changes in DNA methylation.

The clinical implications of this thesis work indicate that early life stress is not necessarily harmful nor always associated with increased vulnerability. Instead, genetic background, the type and severity of stress, and mitigating factors such as maternal care can determine the outcome of aELEs on long-term behavior. These findings emphasize the malleable nature of early postnatal life and its importance in shaping long-term behavior and vulnerability.

## References

1. **Stezhka, V.V. and T.A. Lovick.** 1997. Projections from dorsal raphe nucleus to the periaqueductal grey matter: studies in slices of rat midbrain maintained in vitro. *Neuroscience Letters* 230:57-60.
2. **Abrams, J.K., P.L. Johnson, A. Hay-Schmidt, J.D. Mikkelsen, A. Shekhar, and C.A. Lowry.** 2005. Serotonergic systems associated with arousal and vigilance behaviors following administration of anxiogenic drugs. *Neuroscience* 133:983-997.
3. **Lowry, C.A., A.K. Evans, P.J. Gasser, M.W. Hale, D.R. Staub, and A. Shekhar.** 2008. Topographic organization and chemoarchitecture of the dorsal raphe nucleus and the median raphe nucleus, p. 25-67. *In* J. Monti, S. Pandi-Perumal, B. Jacobs, and D. Nutt (Eds.), *Serotonin and Sleep: Molecular, Functional and Clinical Aspects*. Birkhauser-Verlag, Basel, Switzerland.
4. **Lee, H.S., Y.J. Eum, S.M. Jo, and B.D. Waterhouse.** 2007. Projection patterns from the amygdaloid nuclear complex to subdivisions of the dorsal raphe nucleus in the rat. *Brain Research* 1143:116-125.
5. **Rizvi, T.A., M. Ennis, M.M. Behbehani, and M.T. Shipley.** 1991. Connections Between The Central Nucleus Of The Amygdala And The Midbrain Periaqueductal Gray - Topography And Reciprocity. *Journal of Comparative Neurology* 303:121-131.
6. **Teicher, M.H.** 2000. Wounds That Time Won't Heal: The Neurobiology of Child Abuse. *Cerebrum: The Dana Forum on brain science* 2:50-67.
7. **Heim, C., M. Shugart, W.E. Craighead, and C.B. Nemeroff.** 2010. Neurobiological and psychiatric consequences of child abuse and neglect. *Developmental Psychobiology* 52:671-690.
8. **Bremner, J.D.** 2005. Effects of Traumatic Stress on Brain Structure and Function: Relevance to Early Responses to Trauma. *Journal of Trauma & Dissociation* 6:51-68.
9. **Carrion, V.G., C.F. Weems, S. Eliez, A. Patwardhan, W. Brown, R.D. Ray, and A.L. Reiss.** 2001. Attenuation of frontal asymmetry in pediatric posttraumatic stress disorder. *Biological Psychiatry* 50:943-951.



10. **Ito, Y., M.H. Teicher, C.A. Glod, and E. Ackerman.** 1998. Preliminary evidence for aberrant cortical development in abused children: A quantitative EEG study. *J. Neuropsychiatr. Clin. Neurosci.* *10*:298-307.
11. **Heim, C., D.J. Newport, S. Heit, Y.P. Graham, M. Wilcox, R. Bonsall, A.H. Miller, and C.B. Nemeroff.** 2000. Pituitary-adrenal and autonomic responses to stress in women after sexual and physical abuse in childhood. *Jama-Journal of the American Medical Association* *284*:592-597.
12. **Franklin, T.B., B.J. Saab, and I.M. Mansuy.** 2012. Neural Mechanisms of Stress Resilience and Vulnerability. *Neuron* *75*:747-761.
13. **Caspi, A., K. Sugden, T.E. Moffitt, A. Taylor, I.W. Craig, H. Harrington, J. McClay, J. Mill, et al.** 2003. Influence of life stress on depression: Moderation by a polymorphism in the 5-HTT gene. *Science* *301*:386-389.
14. **Walther, D.J., J.U. Peter, S. Bashammakh, H. Hortnagl, M. Voits, H. Fink, and M. Bader.** 2003. Synthesis of serotonin by a second tryptophan hydroxylase isoform. *Science* *299*:76.
15. **Patel, P.D., C. Pontrello, and S. Burke.** 2004. Robust and tissue-specific expression of TPH2 versus TPH1 in rat raphe and pineal gland. *Biol Psychiatry* *55*:428-433.
16. **Scheuch, K., M. Lautenschlager, M. Grohmann, S. Stahlberg, J. Kirchheiner, P. Zill, A. Heinz, D.J. Walther, and J. Priller.** 2007. Characterization of a functional promoter polymorphism of the human tryptophan hydroxylase 2 gene in serotonergic raphe neurons. *Biol Psychiatry* *62*:1288-1294.
17. **Chen, G.L., E.J. Vallender, and G.M. Miller.** 2008. Functional characterization of the human TPH2 5' regulatory region: untranslated region and polymorphisms modulate gene expression in vitro. *Hum Genet* *122*:645-657.
18. **Waider, J., N. Araragi, L. Gutknecht, and K.P. Lesch.** 2011. Tryptophan hydroxylase-2 (TPH2) in disorders of cognitive control and emotion regulation: A perspective. *Psychoneuroendocrinology* *36*:393-405.
19. **Warner-Schmidt, J.L. and R.S. Duman.** 2006. Hippocampal neurogenesis: Opposing effects of stress and antidepressant treatment. *Hippocampus* *16*:239-249.
20. **Lopez, J.F., H. Akil, and S.J. Watson.** 1999. Neural circuits mediating stress. *Biological Psychiatry* *46*:1461-1471.
21. **McEuen, J.G., S.G. Beck, and T.L. Bale.** 2008. Failure to mount adaptive responses to stress results in dysregulation and cell death in the midbrain raphe. *Journal of Neuroscience* *28*:8169-8177.
22. **Clark, M.S., R.A. McDevitt, B.J. Hoplight, and J.F. Neumaier.** 2007. Chronic low dose ovine corticotropin releasing factor or urocortin II into the rostral dorsal raphe alters exploratory behavior and serotonergic gene expression in specific subregions of the dorsal raphe. *Neuroscience* *146*:1888-1905.
23. **Donner, N.C., P.L. Johnson, S.D. Fitz, K.E. Kellen, A. Shekhar, and C.A. Lowry.** 2012. Elevated TPH2 mRNA expression in a rat model of chronic anxiety. *Depression and Anxiety* *29*:307-319.
24. **Gardner, K.L., M.W. Hale, S. Oldfield, S.L. Lightman, P.M. Plotsky, and C.A. Lowry.** 2009. Adverse experience during early life and adulthood interact to

- elevate TPH2 mRNA expression in serotonergic neurons within the dorsal raphe nucleus. *Neuroscience* 163:991-1001.
25. **Bach-Mizrachi, H., M. Dunderwood, A. Tin, S. Pellis, J.J. Mann, and V. Arango.** 2008. Elevated expression of tryptophan hydroxylase-2 mRNA at the neuronal level in the dorsal and median raphe nuclei of depressed suicides. *Molecular Psychiatry* 13:507-513.
  26. **Gutknecht, L., J. Waider, S. Kraft, C. Kriegebaum, B. Holtmann, A. Reif, A. Schmitt, and K.P. Lesch.** 2008. Deficiency of brain 5-HT synthesis but serotonergic neuron formation in Tph2 knockout mice. *Journal of Neural Transmission* 115:1127-1132.
  27. **Weaver, I.C.G., N. Cervoni, F.A. Champagne, A.C. D'Alessio, S. Sharma, J.R. Seckl, S. Dymov, M. Szyf, and M.J. Meaney.** 2004. Epigenetic programming by maternal behavior. *Nature Neuroscience* 7:847-854.
  28. **Murgatroyd, C., A.V. Patchev, Y. Wu, V. Micale, Y. Bockmuehl, D. Fischer, F. Holsboer, C.T. Wotjak, et al.** 2009. Dynamic DNA methylation programs persistent adverse effects of early-life stress. *Nature Neuroscience* 12:1559-U1108.
  29. **Csoka, A., A. Bahrack, and O.P. Mehtonen.** 2008. Persistent sexual dysfunction after discontinuation of selective serotonin reuptake inhibitors. *Journal of Sexual Medicine* 5:227-233.
  30. **Cassel, S., D. Carouge, C. Gensburger, P. Anglard, C. Burgun, J.B. Dietrich, D. Aunis, and J. Zwiller.** 2006. Fluoxetine and cocaine induce the epigenetic factors MeCP2 and MBD1 in adult rat brain. *Molecular Pharmacology* 70:487-492.
  31. **Tsankova, N.M., O. Berton, W. Renthal, A. Kumar, R.L. Neve, and E.J. Nestler.** 2006. Sustained hippocampal chromatin regulation in a mouse model of depression and antidepressant action. *Nat Neurosci* 9:519-525.
  32. **Covington, H.E., I. Maze, Q.C. LaPlant, V.F. Vialou, Y.N. Ohnishi, O. Berton, D.M. Fass, W. Renthal, et al.** 2009. Antidepressant Actions of Histone Deacetylase Inhibitors. *The Journal of Neuroscience* 29:11451-11460.
  33. **Korosi, A., M. Shanabrough, S. McClelland, Z.W. Liu, E. Borok, X.B. Gao, T.L. Horvath, and T.Z. Baram.** 2010. Early-Life Experience Reduces Excitation to Stress-Responsive Hypothalamic Neurons and Reprograms the Expression of Corticotropin-Releasing Hormone. *Journal of Neuroscience* 30:703-713.
  34. **Lucki, I.** 1998. The spectrum of behaviors influenced by serotonin. *Biological Psychiatry* 44:151-162.
  35. **Molliver, M.E.** 1987. Serotonergic Neuronal Systems - What Their Anatomic Organization Tells Us About Function. *J. Clin. Psychopharmacol.* 7:S3-S23.
  36. **Lowry, C.A., P.L. Johnson, A. Hay-Schmidt, J. Mikkelsen, and A. Shekhar.** 2005. Modulation of anxiety circuits by serotonergic systems. *Stress* 8:233-246.
  37. **Kriegebaum, C., N.-N. Song, L. Gutknecht, Y. Huang, A. Schmitt, A. Reif, Y.-Q. Ding, and K.-P. Lesch.** 2010. Brain-specific conditional and time-specific inducible Tph2 knockout mice possess normal serotonergic gene expression in the absence of serotonin during adult life. *Neurochem. Int.* 57:512-517.

38. **Angoa-Perez, M., M.J. Kane, D.I. Briggs, C.E. Sykes, M.M. Shah, D.M. Francescutti, D.R. Rosenberg, D.M. Thomas, and D.M. Kuhn.** 2012. Genetic depletion of brain 5HT reveals a common molecular pathway mediating compulsivity and impulsivity. *Journal of Neurochemistry* 121:974-984.
39. **Alenina, N., D. Kikic, M. Todiras, V. Mosienko, F. Qadri, R. Plehm, P. Boye, L. Vilianovitch, et al.** 2009. Growth retardation and altered autonomic control in mice lacking brain serotonin. *Proceedings of the National Academy of Sciences of the United States of America* 106:10332-10337.
40. **Savelieva, K.V., S.L. Zhao, V.M. Pogorelov, I. Rajan, Q. Yang, E. Cullinan, and T.H. Lanthorn.** 2008. Genetic Disruption of Both Tryptophan Hydroxylase Genes Dramatically Reduces Serotonin and Affects Behavior in Models Sensitive to Antidepressants. *PLoS One* 3:3301-3313.
41. **Liu, Y., Y.a. Jiang, Y. Si, J.-Y. Kim, Z.-F. Chen, and Y. Rao.** 2011. Molecular regulation of sexual preference revealed by genetic studies of 5-HT in the brains of male mice. *Nature* 472:95-99.
42. **Olivier, J.D.A., T. Blom, T. Arentsen, and J.R. Homberg.** 2011. The age-dependent effects of selective serotonin reuptake inhibitors in humans and rodents: A review. *Progress in Neuro-Psychopharmacology and Biological Psychiatry* 35:1400-1408.
43. **Gross, C., X.X. Zhuang, K. Stark, S. Ramboz, R. Oosting, L. Kirby, L. Santarelli, S. Beck, and R. Hen.** 2002. Serotonin(1A) receptor acts during development to establish normal anxiety-like behaviour in the adult. *Nature* 416:396-400.
44. **Bonari, L., N. Pinto, E. Ahn, A. Einarson, M. Steiner, and G. Koren.** 2004. Perinatal Risks of Untreated Depression During Pregnancy. *Canadian Journal of Psychiatry-Revue Canadienne De Psychiatrie* 49:726-735.
45. **Oberlander, T.F., P. Reebye, S. Misri, M. Papsdorf, J. Kim, and R.E. Grunau.** 2007. Externalizing and attentional behaviors in children of depressed mothers treated with a selective serotonin reuptake inhibitor antidepressant during pregnancy. *Archives of Pediatrics & Adolescent Medicine* 161:22-29.
46. **Oberlander, T.F., R.E. Grunau, C. Fitzgerald, M. Papsdorf, D. Rurak, and W. Riggs.** 2005. Pain reactivity in 2-month-old infants after prenatal and postnatal serotonin reuptake inhibitor medication exposure. *Pediatrics* 115:411-425.
47. **Casper, R.C., B.E. Fleisher, J.C. Lee-Ancajas, A. Gilles, E. Gaylor, A. DeBattista, and H.E. Hoyme.** 2003. Follow-up of children of depressed mothers exposed or not exposed to antidepressant drugs during pregnancy. *Journal of Pediatrics* 142:402-408.
48. **Vorhees, C.V., K.D. Acuffsmith, M.A. Schilling, J.E. Fisher, M.S. Moran, and J. Buelkesam.** 1994. A Developmental Neurotoxicity Evaluation Of The Effects Of Prenatal Exposure To Fluoxetine In Rats. *Fundamental and Applied Toxicology* 23:194-205.
49. **Van den Hove, D.V.A., C.E. Blanco, A. Scheepens, L. Desbonnet, A.-M. Myint, B.E. Leonard, J. Prickaerts, and H.W.M. Steinbusch.** 2008. Prenatal maternal paroxetine treatment and neonatal mortality in the rat: A preliminary study. *Neonatology* 93:52-55.

50. **Lisboa, S.F.S., P.E. Oliveira, L.C. Costa, E.J. Venancio, and E.G. Moreira.** 2007. Behavioral evaluation of male and female mice pups exposed to fluoxetine during pregnancy and lactation. *Pharmacology* 80:49-56.
51. **Gibbons, R.D., C.H. Brown, K. Hur, S.M. Marcus, D.K. Bhaurnik, J.A. Erkens, R.M.C. Herings, and J.J. Mann.** 2007. Early evidence on the effects of regulators' suicidality warnings on SSRI prescriptions and suicide in children and adolescents. *American Journal of Psychiatry* 164:1356-1363.
52. **Hammad, T.A., T. Laughren, and J. Racoosin.** 2006. Suicidality in pediatric patients treated with antidepressant drugs. *Arch. Gen. Psychiatry* 63:332-339.
53. **March, J., S. Silva, S. Petrycki, J. Curry, K. Wells, J. Fairbank, B. Burns, M. Domino, et al.** 2004. Fluoxetine, cognitive-behavioral therapy, and their combination for adolescents with depression - Treatment for adolescents with depression study (TADS) randomized controlled trial. *Jama-Journal of the American Medical Association* 292:807-820.
54. **Olfson, M., S.C. Marcus, and D. Shaffer.** 2006. Antidepressant drug therapy and suicide in severely depressed children and adults - A case-control study. *Arch. Gen. Psychiatry* 63:865-872.
55. **Usala, T., A. Clavenna, A. Zuddasa, and M. Bonati.** 2008. Randomised controlled trials of selective serotonin reuptake inhibitors in treating depression in children and adolescents: A systematic review and meta-analysis. *European Neuropsychopharmacology* 18:62-73.
56. **Whittington, C.J., T. Kendall, P. Fonagy, D. Cottrell, A. Cotgrove, and E. Boddington.** 2004. Selective serotonin reuptake inhibitors in childhood depression: systematic review of published versus unpublished data. *Lancet* 363:1341-1345.
57. **Ansorge, M.S., M. Zhou, A. Lira, R. Hen, and J.A. Gingrich.** 2004. Early-Life Blockade Of The 5-HT Transporter Alters Emotional Behavior In Adult Mice. *Science* 306:879-881.
58. **Hansen, H.H., C. Sanchez, and E. Meier.** 1997. Neonatal administration of the selective serotonin reuptake inhibitor Lu 10-134-C increases forced swimming-induced immobility in adult rats: A putative animal model of depression? *Journal of Pharmacology and Experimental Therapeutics* 283:1333-1341.
59. **Maciag, D., L. Williams, D. Coppinger, and I.A. Paul.** 2006. Neonatal citalopram exposure produces lasting changes in behavior which are reversed by adult imipramine treatment. *Eur. J. Pharmacol.* 532:265-269.
60. **de Castro, R.M., J.M.B. Medeiros, C.M. da Silva, L.M.P. Ferreira, R.C.A. Guedes, J.E. Cabral, and J.J.A. Costa.** 2001. Reduction of intraspecific aggression in adult rats by neonatal treatment with a selective serotonin reuptake inhibitor. *Brazilian Journal of Medical and Biological Research* 34:121-124.
61. **Popa, D., C. Lena, C. Alexandre, and J. Adrien.** 2008. Lasting syndrome of depression produced by reduction in serotonin uptake during postnatal development: Evidence from sleep, stress, and behavior. *Journal of Neuroscience* 28:3546-3554.

62. **Mirmiran, M., N.E. Vandepoll, M.A. Corner, H.G. Vanoyen, and H.L. Bour.** 1981. Suppression Of Active Sleep By Chronic Treatment With Chlorimipramine During Early Postnatal-Development - Effects Upon Adult Sleep And Behavior In The Rat. *Brain Research* 204:129-146.
63. **Karpova, N.N., J. Lindholm, P. Pruunsild, T. Timmusk, and E. Castren.** 2009. Long-lasting behavioural and molecular alterations induced by early postnatal fluoxetine exposure are restored by chronic fluoxetine treatment in adult mice. *European neuropsychopharmacology* 19:97-108.
64. **da Silva, C.M., L. Goncalves, R. Manhaes-de-Castro, and M.I. Nogueira.** 2010. Postnatal fluoxetine treatment affects the development of serotonergic neurons in rats. *Neuroscience Letters* 483:179-183.
65. **Stahl, S.M.** 1998. Mechanism of action of serotonin selective reuptake inhibitors: Serotonin receptors and pathways mediate therapeutic effects and side effects. *Journal of affective disorders* 51:215-235.
66. **Herman, J.P. and W.E. Cullinan.** 1997. Neurocircuitry of stress: Central control of the hypothalamo-pituitary-adrenocortical axis. *Trends in neurosciences* 20:78-84.
67. **Lopez, J.F., D.T. Chalmers, K.Y. Little, and S.J. Watson.** 1998. Regulation of Serotonin1A, Glucocorticoid, and Mineralocorticoid Receptor in Rat and Human Hippocampus: Implications for the Neurobiology of Depression. *Biological Psychiatry* 43:547-573.
68. **Nemeroff, C.B.** 1999. The preeminent role of early untoward experience on vulnerability to major psychiatric disorders: the nature-nurture controversy revisited and soon to be resolved. *Molecular Psychiatry* 4:106-108.
69. **McEwen, B.S. and E. Stellar.** 1993. Stress and the individual: Mechanisms leading to disease. *Archives of Internal Medicine* 153:2093-2101.
70. **Kathol, R.G., R.S. Jaeckle, J.F. Lopez, and W.H. Meller.** 1989. Pathophysiology of HPA axis abnormalities in patients with major depression: An update. *American Journal of Psychiatry* 146:311-317.
71. **Charney, D.S., A.Y. Deutch, J.H. Krystal, S.M. Southwick, and M. Davis.** 1993. Psychobiologic mechanisms of posttraumatic stress disorder. *Arch. Gen. Psychiatry* 50:294-303.
72. **Gold, P.W., D.L. Loriaux, A. Roy, M.A. Kling, J.R. Calabrese, C.H. Kellner, L.K. Nieman, R.M. Post, et al.** 1986. Responses To Corticotropin-Releasing Hormone In The Hypercortisolism Of Depression And Cushings-Disease - Pathophysiologic And Diagnostic Implications. *New England Journal of Medicine* 314:1329-1335.
73. **Plotsky, P.M., E.T. Cunningham Jr, and E.P. Widmaier.** 1989. Catecholaminergic modulation of corticotropin-releasing factor and adrenocorticotropin secretion. *Endocrine Reviews* 10:437-458.
74. **Davis, M.** 1992. The role of the amygdala in fear and anxiety. *Annual Review of Neuroscience* 15:353-375.
75. **Allen, J.P. and C.F. Allen.** 1974. Role of the amygdaloid complexes in the stress induced release of ACTH in the rat. *Neuroendocrinology* 15:220-230.
76. **Cullinan, W.E.** 1995. Pattern and time course of immediate early gene expression in rat brain following acute stress. *Neuroscience* 64:477-505.

77. **Feldman, S., N. Conforti, and E. Melamed.** 1987. Paraventricular nucleus serotonin mediates neurally stimulated adrenocortical secretion. *Brain Research Bulletin* 18:165-168.
78. **Sapolsky, R.M.** 2000. Glucocorticoids and hippocampal atrophy in neuropsychiatric disorders. *Arch. Gen. Psychiatry* 57:925-935.
79. **Sapolsky, R.M.** 1996. Why stress is bad for your brain. *Science* 273:749-750.
80. **Diorio, D., V. Viau, and M.J. Meaney.** 1993. The role of the medial prefrontal cortex (cingulate gyrus) in the regulation of hypothalamic-pituitary-adrenal responses to stress. *Journal of Neuroscience* 13:3839-3847.
81. **Dobrakovova, M., R. Kvetnansky, T. Torda, and K. Murgas.** 1982. Changes of plasma and adrenal catecholamines and corticosterone in stressed rats with septal lesions. *Physiology and Behavior* 29:41-45.
82. **Whitnall, M.H.** 1993. Regulation of the hypothalamic corticotropin-releasing hormone neurosecretory system. *Progress in Neurobiology* 40:573-629.
83. **Makara, G.B. and E. Stark.** 1974. Effect of gamma aminobutyric acid (GABA) and GABA antagonist drugs on ACTH release. *Neuroendocrinology* 16:178-190.
84. **Hillhouse, E.W. and N.G.N. Milton.** 1989. Effect of noradrenaline and gamma aminobutyric acid on the secretion of corticotrophin-releasing factor-41 and arginine vasopressin from the rat hypothalamus in vitro. *Journal of Endocrinology* 122:719-723.
85. **Jacobson, L. and R. Sapolsky.** 1991. The role of the hippocampus in feedback regulation of the hypothalamic-pituitary-adrenocortical axis. *Endocrine Reviews* 12:118-134.
86. **Herman, J.P., M.K.H. Schafer, E.A. Young, R. Thompson, J. Douglass, H. Akil, and S.J. Watson.** 1989. Evidence for hippocampal regulation of neuroendocrine neurons of the hypothalamo-pituitary-adrenocorticoid axis. *Journal of Neuroscience* 9:3072-3082.
87. **Herman, J.P., W.E. Cullinan, M.I. Morano, H. Akil, and S.J. Watson.** 1995. Contribution of the ventral subiculum to inhibitory regulation of the hypothalamo-pituitary-adrenocortical axis. *J. Neuroendocrinol.* 7:475-482.
88. **Sapolsky, R.M., S. Zola-Morgan, and L.R. Squire.** 1991. Inhibition of glucocorticoid secretion by the hippocampal formation in the primate. *Journal of Neuroscience* 11:3695-3704.
89. **Liberzon, I., J.F. Lopez, S.B. Flagel, D.M. Vazquez, and E.A. Young.** 1999. Differential regulation of hippocampal glucocorticoid receptors mRNA and fast feedback: Relevance to post-traumatic stress disorder. *J. Neuroendocrinol.* 11:11-17.
90. **Plotsky, P.M. and M.J. Meaney.** 1993. Early, postnatal experience alters hypothalamic corticotropin-releasing factor (CRF) mRNA, median eminence CRF content and stress-induced release in adult rats. *Molecular Brain Research* 18:195-200.
91. **Owens, M.J. and C.B. Nemeroff.** 1991. Physiology and pharmacology of corticotropin-releasing factor. *Pharmacological Reviews* 43:425-473.
92. **Price, M.L., A.L. Curtis, L.G. Kirby, R.J. Valentino, and I. Lucki.** 1998. Effects of Corticotropin-Releasing Factor on Brain Serotonergic Activity. *Neuropsychopharmacology* 18:492-502.

93. **Holsboer, F.** 2001. Stress, hypercortisolism and corticosteroid receptors in depression: implications for therapy. *Journal of affective disorders* 62:77-91.
94. **Banky, Z., B. Halasz, and G. Nagy.** 1986. Circadian corticosterone rhythm did not develop in rats seven weeks after destruction with 5,7-dihydroxytryptamine of the serotonergic nerve terminals in the suprachiasmatic nucleus at the age of 16 days. *Brain Research* 369:119-124.
95. **Williams, J.H., V.M. Miallallen, M. Klinowski, and E.C. Azmitia.** 1983. Effects Of Microinjections Of 5,7-Dihydroxytryptamine In The Suprachiasmatic Nuclei Of The Rat On Serotonin Reuptake And The Circadian Variation Of Corticosterone Levels. *Neuroendocrinology* 36:431-435.
96. **Szafarczyk, A., G. Alonso, G. Ixart, F. Malaval, J. Nougier-Soule, and I. Assenmacher.** 1980. Serotonergic system and circadian rhythms of ACTH and corticosterone in rats. *American Journal of Physiology - Endocrinology And Metabolism* 239:E482-E489.
97. **Seckl, J.R., K.L. Dickson, and G. Fink.** 1990. Central 5,7-Dihydroxytryptamine Lesions Decrease Hippocampal Glucocorticoid and Mineralocorticoid Receptor Messenger Ribonucleic Acid Expression. *J. Neuroendocrinol.* 2:911-916.
98. **Jorgensen, H., U. Knigge, A. Kjaer, T. Vadsholt, and J. Warberg.** 1998. Serotonergic involvement in stress-induced ACTH release. *Brain Research* 811:10-20.
99. **Kuroda, Y., Y. Watanabe, D.S. Albeck, N.B. Hastings, and B.S. McEwen.** 1994. Effects Of Adrenalectomy And Type-I Or Type-Ii Glucocorticoid Receptor Activation On 5-Ht1A And 5-Ht2 Receptor-Binding And 5-Ht Transporter Messenger-Rna Expression In Rat-Brain. *Brain Research* 648:157-161.
100. **McKittrick, C.R., D.C. Blanchard, R.J. Blanchard, B.S. McEwen, and R.R. Sakai.** 1995. Serotonin Receptor-Binding In A Colony Model Of Chronic Social Stress. *Biological Psychiatry* 37:383-393.
101. **Berton, O., H.E. Covington, III, K. Ebner, N.M. Tsankova, T.L. Carle, P. Ulery, A. Bhonsle, M. Barrot, et al.** 2007. Induction of Delta FosB in the periaqueductal gray by stress promotes active coping responses. *Neuron* 55:289-300.
102. **Hale, M.W., A. Hay-Schmidt, J.D. Mikkelsen, B. Poulsen, J.A. Bouwknecht, A.K. Evans, C.E. Stamper, A. Shekhar, and C.A. Lowry.** 2008. Exposure To An Open-Field Arena Increases C-Fos Expression In A Subpopulation Of Neurons In The Dorsal Raphe Nucleus, Including Neurons Projecting To The Basolateral Amygdaloid Complex. *Neuroscience* 157:733-748.
103. **Martinez, M., P.J. Phillips, and J. Herbert.** 1998. Adaptation in patterns of c-fos expression in the brain associated with exposure to either single or repeated social stress in male rats. *Eur. J. Neurosci.* 10:20-33.
104. **Roche, M., K.G. Commons, A. Peoples, and R.J. Valentino.** 2003. Circuitry underlying regulation of the serotonergic system by swim stress. *Journal of Neuroscience* 23:970-977.
105. **Matsuo, M., Y. Kataoka, S. Mataka, Y. Kato, and K. Oi.** 1996. Conflict situation increases serotonin release in rat dorsal hippocampus: in vivo study with microdialysis and Vogel test. *Neuroscience Letters* 215:197-200.

106. **Shimizu, N., S. Take, T. Hori, and Y. Oomura.** 1992. In vivo measurement of hypothalamic serotonin release by intracerebral microdialysis: Significant enhancement by immobilization stress in rats. *Brain Research Bulletin* 28:727-734.
107. **Kirby, L.G., K.C. Rice, and R.J. Valentino.** 2000. Effects of corticotropin-releasing factor on neuronal activity in the serotonergic dorsal raphe nucleus. *Neuropsychopharmacology* 22:148-162.
108. **Gross, C. and R. Hen.** 2004. The developmental origins of anxiety. *Nat Rev Neurosci* 5:545-552.
109. **American Psychiatric, A. and D.-I. American Psychiatric Association. Task Force on.** 2000. Diagnostic and statistical manual of mental disorders : DSM-IV-TR. American Psychiatric Association, Washington, DC.
110. **Kessler, R., W. Chiu, O. Demler, and E. Walters.** 2005. Prevalence, severity, and comorbidity of 12-month dsm-iv disorders in the national comorbidity survey replication. *Arch. Gen. Psychiatry* 62:617-627.
111. **Schwartz, C.E., N. Snidman, and J. Kagan.** 1999. Adolescent Social Anxiety as an Outcome of Inhibited Temperament in Childhood. *Journal of the American Academy of Child and Adolescent Psychiatry* 38:1008-1015.
112. **Kagan, J. and N. Snidman.** 1999. Early childhood predictors of adult anxiety disorders. *Biological Psychiatry* 46:1536-1541.
113. **Lesch, K.P., D. Bengel, A. Heils, S.Z. Sabol, B.D. Greenberg, S. Petri, J. Benjamin, C.R. Muller, et al.** 1996. Association of anxiety-related traits with a polymorphism in the serotonin transporter gene regulatory region. *Science* 274:1527-1531.
114. **Greenberg, B.D., Q. Li, F.R. Lucas, S. Hu, L.A. Sirota, J. Benjamin, K.P. Lesch, D. Hamer, and D.L. Murphy.** 2000. Association between the serotonin transporter promoter polymorphism and personality traits in a primarily female population sample. *American Journal of Medical Genetics* 96:202-216.
115. **Gilbertson, M.W., M.E. Shenton, A. Ciszewski, K. Kasai, N.B. Lasko, S.P. Orr, and R.K. Pitman.** 2002. Smaller hippocampal volume predicts pathologic vulnerability to psychological trauma. *Nature Neuroscience* 5:1242-1247.
116. **Bremner, J.D., P. Randall, E. Vermetten, L. Staib, R.A. Bronen, C. Mazure, S. Capelli, G. McCarthy, et al.** 1997. Magnetic resonance imaging-based measurement of hippocampal volume in posttraumatic stress disorder related to childhood physical and sexual abuse - A preliminary report. *Biological Psychiatry* 41:23-32.
117. **Driessen, M., J. Herrmann, K. Stahl, M. Zwaan, S. Meier, A. Hill, M. Osterheider, and D. Petersen.** 2000. Magnetic resonance imaging volumes of the hippocampus and the amygdala in women with borderline personality disorder and early traumatization. *Arch. Gen. Psychiatry* 57:1115-1122.
118. **Stein, M.B., C. Koverola, C. Hanna, M.G. Torchia, and B. McClarty.** 1997. Hippocampal volume in women victimized by childhood sexual abuse. *Psychological Medicine* 27:951-959.
119. **McNaughton, N.** 1997. Cognitive Dysfunction Resulting from Hippocampal Hyperactivity: Possible Cause of Anxiety Disorder? *Pharmacology Biochemistry and Behavior* 56:603-611.



120. **Igelström, K.M. and P.M. Heyward.** 2012. Inhibition of hippocampal excitability by citalopram. *Epilepsia* 53:2032-2042.
121. **Davidson, R.J., W. Irwin, M.J. Anderle, and N.H. Kalin.** 2003. The neural substrates of affective processing in depressed patients treated with venlafaxine. *American Journal of Psychiatry* 160:64-75.
122. **Bhagwagar, Z., M. Wylezinska, M. Taylor, P. Jezard, P.M. Matthews, and P.J. Cowen.** 2004. Increased brain GABA concentrations following acute administration of a selective serotonin reuptake inhibitor. *American Journal of Psychiatry* 161:368-370.
123. **Soubrie, P.** 1986. Reconciling the role of central serotonin neurons in human and animal behavior. *Behavioral and Brain Sciences* 9:319-335.
124. **Olivier, J.D.A., M.G.C. Van Der Hart, R.P.L. Van Swelm, P.J. Dederen, J.R. Homberg, T. Cremers, P.M.T. Deen, E. Cuppen, et al.** 2008. A study in male and female 5-HT transporter knockout rats: An animal model for anxiety and depression disorders. *Neuroscience* 152:573-584.
125. **Kelley, A.E.** 2004. Memory and Addiction: Shared Neural Circuitry and Molecular Mechanisms. *Neuron* 44:161-179.
126. **Holstege, G.** 1995. The Basic, Somatic, And Emotional Components Of The Motor System In Mammals, p. 137-154. *In* G. Paxinos (Ed.), *The Rat Nervous System*. Academic Press, San Diego.
127. **Graeff, F.G., F.S. Guimaraes, T.G.C.S. De Andrade, and J.F.W. Deakin.** 1996. Role of 5-HT in stress, anxiety, and depression. *Pharmacology Biochemistry and Behavior* 54:129-141.
128. **Wogar, M.A., C.M. Bradshaw, and E. Szabadi.** 1993. Effects of lesions of the ascending 5-hydroxytryptaminergic pathways on choice between delayed reinforcers. *Psychopharmacology* 111:239-243.
129. **Amat, J., M.V. Baratta, E. Paul, S.T. Bland, L.R. Watkins, and S.F. Maier.** 2005. Medial prefrontal cortex determines how stressor controllability affects behavior and dorsal raphe nucleus. *Nature Neuroscience* 8:365-371.
130. **Lidov, H.G.W. and M.E. Molliver.** 1982. An Immunohistochemical Study Of Serotonin Neuron Development In The Rat - Ascending Pathways And Terminal Fields. *Brain Research Bulletin* 8:389-430.
131. **Acsady, L., D. Arabadzisz, I. Katona, and T.F. Freund.** 1996. Topographic distribution of dorsal and median raphe neurons with hippocampal, septal and dual projection. *Acta Biologica Hungarica* 47:9-19.
132. **Andrade, T., C.E.A. Macedo, H. Zangrossi, and F.G. Graeff.** 2004. Anxiolytic-like effects of median raphe nucleus lesion in the elevated T-maze. *Behav. Brain Res.* 153:55-60.
133. **Zaichenko, M., G. Vanetsian, and G. Merzhanova.** 2011. Differences in behavior of impulsive and self-controlled rats in the open-field and light-dark tests. *Zh Vyssh Nerv Deiat Im I P Pavlova* 61:340-350.
134. **Carli, M., T.W. Robbins, J.L. Evenden, and B.J. Everitt.** 1983. Effects of lesions to ascending noradrenergic neurons on performance of a 5-choice serial reaction time task in rats - implications for theories of dorsal noradrenergic bundle function based on selective attention and arousal. *Behav Brain Res* 9:361-380.

135. **Fletcher, P.J.** 1995. Effects of combined or separate 5,7-dihydroxytryptamine lesions of the dorsal and median raphe nuclei on responding maintained by a DRL 20s schedule of food reinforcement. *Brain Res* 675:45-54.
136. **Roberts, D.C., E.A. Loh, G.B. Baker, and G. Vickers.** 1994. Lesions of central serotonin systems affect responding on a progressive ratio schedule reinforced either by intravenous cocaine or by food. *Pharmacol Biochem Behav* 49:177-182.
137. **John Mann, J. and K.M. Malone.** 1997. Cerebrospinal fluid amines and higher-lethality suicide attempts in depressed inpatients. *Biological Psychiatry* 41:162-171.
138. **Sheehan, K., N. Lowe, A. Kirley, C. Mullins, M. Fitzgerald, M. Gill, and Z. Hawi.** 2005. Tryptophan hydroxylase 2 (TPH2) gene variants associated with ADHD. *Mol Psychiatry* 10:944-949.
139. **Zepf, F.D., C. Stadler, L. Demisch, M. Schmitt, M. Landgraf, and F. Poustka.** 2008. Serotonergic functioning and trait-impulsivity in attention-deficit/hyperactivity-disordered boys (ADHD): influence of rapid tryptophan depletion. *Human Psychopharmacology: Clinical and Experimental* 23:43-51.
140. **Zimmermann, M., M. Grabemann, C. Mette, M. Abdel-Hamid, J. Ueckermann, M. Kraemer, J. Wiltfang, B. Kis, and F.D. Zepf.** 2012. The Effects of Acute Tryptophan Depletion on Reactive Aggression in Adults with Attention-Deficit/Hyperactivity Disorder (ADHD) and Healthy Controls. *PLoS One* 7.
141. **Dayan, P. and Q.J.M. Huys.** 2008. Serotonin, Inhibition, and Negative Mood. *PLoS Comput Biol* 4:e4.
142. **Crockett, M.J., L. Clark, A.M. Apergis-Schoute, S. Morein-Zamir, and T.W. Robbins.** 2012. Serotonin Modulates the Effects of Pavlovian Aversive Predictions on Response Vigor. *Neuropsychopharmacology* 37:2244-2252.
143. **Deakin, J.F.W.** 1983. Roles of serotonergic systems in escape, avoidance and other behaviours. *Theory in Psychopharmacology Vol 2*:149-193.
144. **Whiteside, S.P. and D.R. Lynam.** 2001. The Five Factor Model and impulsivity: using a structural model of personality to understand impulsivity. *Personality and Individual Differences* 30:669-689.
145. **Kendler, K.S., L.M. Karkowski, and C.A. Prescott.** 1999. Causal relationship between stressful life events and the onset of major depression. *American Journal of Psychiatry* 156:837-841.
146. **Arborelius, L., M.J. Owens, P.M. Plotsky, and C.B. Nemeroff.** 1999. The role of corticotropin-releasing factor in depression and anxiety disorders. *Journal of Endocrinology* 160:1-12.
147. **Willner, P.** 1997. Validity, reliability and utility of the chronic mild stress model of depression: a 10-year review and evaluation. *Psychopharmacology* 134:319-329.
148. **Dranovsky, A. and R. Hen.** 2006. Hippocampal neurogenesis: Regulation by stress and antidepressants. *Biological Psychiatry* 59:1136-1143.
149. **Rajkowska, G., J.J. Miguel-Hidalgo, J. Wei, G. Dilley, S.D. Pittman, H.Y. Meltzer, J.C. Overholser, B.L. Roth, and C.A. Stockmeier.** 1999.

- Morphometric evidence for neuronal and glial prefrontal cell pathology in major depression. *Biological Psychiatry* 45:1085-1098.
150. **Rajkowska, G.** 2002. Cell pathology in bipolar disorder. *Bipolar Disorders* 4:105-116.
  151. **Meaney, M.J., J. Diorio, D. Francis, S. Weaver, J. Yau, K. Chapman, and J.R. Seckl.** 2000. Postnatal handling increases the expression of cAMP-inducible transcription factors in the rat hippocampus: The effects of thyroid hormones and serotonin. *Journal of Neuroscience* 20:3926-3935.
  152. **Weaver, I.C.G., P. La Plante, S. Weaver, A. Parent, S. Sharma, J. Diorio, K.E. Chapman, J.R. Seckl, et al.** 2001. Early environmental regulation of hippocampal glucocorticoid receptor gene expression: characterization of intracellular mediators and potential genomic target sites. *Molecular and Cellular Endocrinology* 185:205-218.
  153. **Drevets, W., J. Price, and M. Furey.** 2008. Brain structural and functional abnormalities in mood disorders: implications for neurocircuitry models of depression. *Brain Structure and Function* 213:93-118.
  154. **Gos, T., D. Krell, R. Brisch, H. Bielau, K. Trubner, J. Steiner, H.-G. Bernstein, and B. Bogerts.** 2008. Demonstration of decreased activity of dorsal raphe nucleus neurons in depressed suicidal patients by the AgNOR staining method. *Journal of affective disorders* 111:251-260.
  155. **Cowen, P.J., M. Parry-Billings, and E.A. Newsholme.** 1989. Decreased plasma tryptophan levels in major depression. *Journal of affective disorders* 16:27-31.
  156. **M, A., T. L., and T. P.** 1976. 5-hiaa in the cerebrospinal fluid: A biochemical suicide predictor? *Arch. Gen. Psychiatry* 33:1193-1197.
  157. **Healy, D. and B.E. Leonard.** 1987. Monoamine transport in depression: Kinetics and dynamics. *Journal of affective disorders* 12:91-103.
  158. **Meltzer, H., B. Umberkoman-Wiita, A. Robertson, T.B. Jo, M. Lowry, and R. Perline.** 1984. Effect of 5-hydroxytryptophan on serum cortisol levels in major affective disorders: I. enhanced response in depression and mania. *Arch. Gen. Psychiatry* 41:366-374.
  159. **Bell, C., J. Abrams, and D. Nutt.** 2001. Tryptophan depletion and its implications for psychiatry. *The British Journal of Psychiatry* 178:399-405.
  160. **Smith, K.A., C.G. Fairburn, and P.J. Cowen.** 1997. Relapse of depression after rapid depletion of tryptophan. *The Lancet* 349:915-919.
  161. **Blier, P. and C. de Montigny.** 1994. Current advances and trends in the treatment of depression. *Trends in Pharmacological Sciences* 15:220-226.
  162. **Stockmeier, C.A.** 2003. Involvement of serotonin in depression: evidence from postmortem and imaging studies of serotonin receptors and the serotonin transporter. *J. Psychiatr. Res.* 37:357-373.
  163. **Laruelle, M., A. Abidargham, M.F. Casanova, R. Toti, D.R. Weinberger, and J.E. Kleinman.** 1993. Selective Abnormalities Of Prefrontal Serotonergic Receptors In Schizophrenia - A Postmortem Study. *Arch. Gen. Psychiatry* 50:810-818.
  164. **Leake, A., A.F. Fairbairn, I.G. McKeith, and I.N. Ferrier.** 1991. Studies On The Serotonin Uptake Binding-Site In Major Depressive Disorder And Control

- Postmortem Brain - Neurochemical And Clinical Correlates. *Psychiatry Research* 39:155-165.
165. **Lawrence, K.M., F. Depaermentier, S.C. Cheetham, M.R. Crompton, C.L.E. Katona, and R.W. Horton.** 1990. Brain 5-HT Uptake Sites, Labeled With H-3 Paroxetine, In Antidepressant-Free Depressed Suicides. *Brain Research* 526:17-22.
166. **Little, K.Y., D.P. McLaughlin, J. Ranc, J. Gilmore, J.F. Lopez, S.J. Watson, F.I. Carroll, and J.D. Butts.** 1997. Serotonin transporter binding sites and mRNA levels in depressed persons committing suicide. *Biological Psychiatry* 41:1156-1164.
167. **Fuster, J.M.** 1995. Memory and planning - Two temporal perspectives of frontal lobe function, p. 9-20. *In* H.H. Jasper, S. Riggio, and P.S. Goldman-Rakic (Eds.), *Epilepsy and the Functional Anatomy of the Frontal Lobe*.
168. **Owen, A.M.** 1997. The Functional Organization of Working Memory Processes Within Human Lateral Frontal Cortex: The Contribution of Functional Neuroimaging. *Eur. J. Neurosci.* 9:1329-1339.
169. **Fuster, J.M.** 2000. Prefrontal neurons in networks of executive memory. *Brain Research Bulletin* 52:331-336.
170. **Arango, V., M.D. Underwood, M. Boldrini, H. Tamir, S.A. Kassir, S.C. Hsiung, J.J.X. Chen, and J.J. Mann.** 2001. Serotonin 1A receptors, serotonin transporter binding and serotonin transporter mRNA expression in the brainstem of depressed suicide victims. *Neuropsychopharmacology* 25:892-903.
171. **Stockmeier, C.A., L.A. Shapiro, G.E. Dilley, T.N. Kolli, L. Friedman, and G. Rajkowska.** 1998. Increase in serotonin-1A autoreceptors in the midbrain of suicide victims with major depression - Postmortem evidence for decreased serotonin activity. *Journal of Neuroscience* 18:7394-7401.
172. **Hart, J., M. Gunnar, and D. Cicchetti.** 1995. Salivary cortisol in maltreated children: Evidence of relations between neuroendocrine activity and social competence. *Development and Psychopathology* 7:11-26.
173. **De Bellis, M.D., L. Lefter, P.K. Trickett, and F.W. Putnam.** 1994. Urinary Catecholamine Excretion in Sexually Abused Girls. *Journal of the American Academy of Child and Adolescent Psychiatry* 33:320-327.
174. **Kaufman, J., B. Birmaher, J. Perel, R.E. Dahl, P. Moreci, B. Nelson, W. Wells, and N.D. Ryan.** 1997. The Corticotropin-Releasing Hormone Challenge in Depressed Abused, Depressed Nonabused, and Normal Control Children. *Biological Psychiatry* 42:669-679.
175. **Weller, E., R. Weller, M. Fristad, and J. Bowes.** 1990. Dexamethasone suppression test and depressive symptoms in bereaved children: A preliminary report. *J Neuropsychiatry Clin Neurosci.* 2:418-421.
176. **Goenjian, A.K., R. Yehuda, R.S. Pynoos, A.M. Steinberg, M. Tashjian, R.K. Yang, L.M. Najarian, and L.A. Fairbanks.** 1996. Basal cortisol, dexamethasone suppression of cortisol, and MHPG in adolescents after the 1988 earthquake in Armenia. *American Journal of Psychiatry* 153:929-934.

177. **Stein, M.B., R. Yehuda, C. Koverola, and C. Hanna.** 1997. Enhanced dexamethasone suppression of plasma cortisol in adult women traumatized by childhood sexual abuse. *Biological Psychiatry* 42:680-686.
178. **Heim, C., D.J. Newport, R. Bonsall, A.H. Miller, and C.B. Nemeroff.** 2001. Altered pituitary-adrenal axis responses to provocative challenge tests in adult survivors of childhood abuse. *American Journal of Psychiatry* 158:575-581.
179. **Vanderkolk, B.A., D. Dreyfuss, M. Michaels, D. Shera, R. Berkowitz, R. Fisler, and G. Saxe.** 1994. Fluoxetine In Posttraumatic-Stress-Disorder. *Journal of Clinical Psychiatry* 55:517-522.
180. **Martin, A., J. Kaufman, and D. Charney.** 2000. Pharmacotherapy of early-onset depression - Update and new directions. *Child and Adolescent Psychiatric Clinics of North America* 9:135-157.
181. **Bennett, A.J., K.P. Lesch, A. Heils, J.C. Long, J.G. Lorenz, S.E. Shoaf, M. Champoux, S.J. Suomi, et al.** 2002. Early experience and serotonin transporter gene variation interact to influence primate CNS function. *Molecular Psychiatry* 7:118-122.
182. **Weller, E.B., R.A. Weller, M.A. Fristad, and J.M. Bowes.** 1990. Dexamethasone suppression test and depressive symptoms in bereaved children: a preliminary report. *The Journal of neuropsychiatry and clinical neurosciences* 2:418-421.
183. **Ladd, C.O., R.L. Huot, K.V. Thrivikraman, C.B. Nemeroff, and P.M. Plotsky.** 2004. Long-term adaptations in glucocorticoid receptor and mineralocorticoid receptor mrna and negative feedback on the hypothalamo-pituitary-adrenal axis following neonatal maternal separation. *Biological Psychiatry* 55:367-375.
184. **Levine, S.** 2000. Influence of psychological variables on the activity of the hypothalamic, pituitary, adrenal axis. *Eur. J. Pharmacol.* 405:149-160.
185. **Meaney, M.J. and D.H. Aitken.** 1985. The effects of early postnatal handling on hippocampal glucocorticoid receptor concentrations - temporal parameters. *Developmental Brain Research* 22:301-304.
186. **Panagiotaropoulos, T., S. Pondiki, A. Papaioannou, F. Alikaridis, A. Stamatakis, K. Gerozisis, and F. Stylianopoulou.** 2004. Neonatal handling and gender modulate brain monoamines and plasma corticosterone levels following repeated stressors in adulthood. *Neuroendocrinology* 80:181-191.
187. **Anisman, H., M.D. Zaharia, M.J. Meaney, and Z. Merali.** 1998. Do early-life events permanently alter behavioral and hormonal responses to stressors? *International Journal of Developmental Neuroscience* 16:149-164.
188. **Smotherman, W. and R. Bell.** 1980. *Maternal mediation of early experience.* Spectrum Publications, New York.
189. **Liu, D., J. Diorio, B. Tannenbaum, C. Caldji, D. Francis, A. Freedman, S. Sharma, D. Pearson, et al.** 1997. Maternal Care, Hippocampal Glucocorticoid Receptors, and Hypothalamic-Pituitary-Adrenal Responses to Stress. *Science* 277:1659-1662.

190. **Pryce, C.R., D. Bettschen, and J. Feldon.** 2001. Comparison of the effects of early handling and early deprivation on maternal care in the rat. *Developmental Psychobiology* 38:239-251.
191. **Levine, S.** 1994. Maternal behavior as a mediator of pup adrenocortical function. *Ann NY Acad Sci*:260-275.
192. **Levine, S.** 1967. Maternal and environmental influences on adrenocortical response to stress in weanling rats. *Science* 156:258-260.
193. **D'amato, F.R. and S. Cabib.** 1987. Chronic Exposure To A Novel Odor Increases Pups Vocalizations, Maternal-Care, And Alters Dopaminergic Functioning In Developing Mice. *Behavioral and Neural Biology* 48:197-205.
194. **D'Amato, F.R., S. Cabib, R. Ventura, and C. Orsini.** 1998. Long-term effects of postnatal manipulation on emotionality are prevented by maternal anxiolytic treatment in mice. *Developmental Psychobiology* 32:225-234.
195. **Champagne, F.A., D.D. Francis, A. Mar, and M.J. Meaney.** 2003. Variations in maternal care in the rat as a mediating influence for the effects of environment on development. *Physiol. Behav.* 79:359-371.
196. **Liu, D., J. Diorio, J.C. Day, D.D. Francis, and M.J. Meaney.** 2000. Maternal care, hippocampal synaptogenesis and cognitive development in rats. *Nature Neuroscience* 3:799-806.
197. **Lee, J.-H., H.J. Kim, J.G. Kim, V. Ryu, B.-T. Kim, D.-W. Kang, and J.W. Jahng.** 2007. Depressive behaviors and decreased expression of serotonin reuptake transporter in rats that experienced neonatal maternal separation. *Neuroscience Research* 58:32-39.
198. **Daniels, W.M.U., C.Y. Pietersen, M.E. Carstens, and D.J. Stein.** 2004. Maternal separation in rats leads to anxiety-like behavior and a blunted ACTH response and altered neurotransmitter levels in response to a subsequent stressor. *Metabolic Brain Disease* 19:3-14.
199. **Caldji, C., D. Francis, S. Sharma, P.M. Plotsky, and M.J. Meaney.** 2000. The effects of early rearing environment on the development of GABA(A) and central benzodiazepine receptor levels and novelty-induced fearfulness in the rat. *Neuropsychopharmacology* 22:219-229.
200. **Ladd, C.O., R.L. Huot, K.V. Thrivikraman, C.B. Nemeroff, M.J. Meaney, and P.M. Plotsky.** 2000. Long-term behavioral and neuroendocrine adaptations to adverse early experience. *Biological Basis for Mind Body Interactions* 122:81-103.
201. **Huot, R.L., D.A. Smith, and P.M. Plotsky.** 1997. Alterations of maternal-infant interaction as a result of maternal separation in Long Evans rats and its behavioral and neuroendocrine consequence. *In* I.S. Psychoneuroendocrinology (Ed.), XXVIIIth Congress, San Francisco, CA.
202. **Huot, R.L., M.E. Gonzalez, C.O. Ladd, K.V. Thrivikraman, and P.M. Plotsky.** 2004. Foster litters prevent hypothalamic-pituitary-adrenal axis sensitization mediated by neonatal maternal separation. *Psychoneuroendocrinology* 29:279-289.
203. **Lehmann, J., C.R. Pryce, D. Bettschen, and J. Feldon.** 1999. The Maternal Separation Paradigm and Adult Emotionality and Cognition in Male and Female Wistar Rats. *Pharmacology Biochemistry and Behavior* 64:705-715.

204. **Ladd, C.O., K.V. Thrivikraman, R.L. Huot, and P.M. Plotsky.** 2005. Differential neuroendocrine responses to chronic variable stress in adult Long Evans rats exposed to handling-maternal separation as neonates. *Psychoneuroendocrinology* 30:520-533.
205. **Bhansali, P., J. Dunning, S.E. Singer, L. David, and C. Schmauss.** 2007. Early life stress alters adult serotonin 2C receptor pre-mRNA editing and expression of the alpha subunit of the heterotrimeric G-protein Gq. *Journal of Neuroscience* 27:1467-1473.
206. **Millstein, R.A. and A. Holmes.** 2007. Effects of repeated maternal separation on anxiety- and depression-related phenotypes in different mouse strains. *Neurosci. Biobehav. Rev.* 31:3-17.
207. **Savignac, H.M., T.G. Dinan, and J.F. Cryan.** 2011. Resistance to Early-Life Stress in Mice: Effects of Genetic Background and Stress Duration. *Frontiers in Behavioral Neuroscience* 5:1-12.
208. **Veenema, A.H., R. Bredewold, and I.D. Neumann.** 2007. Opposite effects of maternal separation on intermale and maternal aggression in C57BL/6 mice: Link to hypothalamic vasopressin and oxytocin immunoreactivity. *Psychoneuroendocrinology* 32:437-450.
209. **Navailles, S., R. Zimnisky, and C. Schmauss.** 2010. Expression of Glucocorticoid Receptor and Early Growth Response Gene 1 during Postnatal Development of Two Inbred Strains of Mice Exposed to Early Life Stress. *Dev. Neurosci.* 32:139-148.
210. **George, E.D., K.A. Bordner, H.M. Elwafi, and A.A. Simen.** 2010. Maternal separation with early weaning: a novel mouse model of early life neglect. *BMC Neurosci.* 11:14.
211. **Meagher, M.W., A.N. Sieve, R.R. Johnson, D. Satterlee, M. Belyavskiy, W. Mi, T.W. Prentice, T.H. Welsh, Jr., and C.J.R. Welsh.** 2010. Neonatal Maternal Separation Alters Immune, Endocrine, and Behavioral Responses to Acute Theiler's Virus Infection in Adult Mice. *Behavior Genetics* 40:233-249.
212. **Parfitt, D.B., J.R. Walton, E.A. Corriveau, and D.L. Helmreich.** 2007. Early life stress effects on adult stress-induced corticosterone secretion and anxiety-like behavior in the C57BL/6 mouse are not as robust as initially thought. *Horm. Behav.* 52:417-426.
213. **Parfitt, D.B., J.K. Levin, K.P. Saltstein, A.S. Klayman, L.M. Greer, and D.L. Helmreich.** 2004. Differential early rearing environments can accentuate or attenuate the responses to stress in male C57BL/6 mice. *Brain Research* 1016:111-118.
214. **Veenema, A.H. and I.D. Neumann.** 2009. Maternal separation enhances offensive play-fighting, basal corticosterone and hypothalamic vasopressin mRNA expression in juvenile male rats. *Psychoneuroendocrinology* 34:463-467.
215. **Wei, Q., X.-Y. Lu, L. Liu, G. Schafer, K.-R. Shieh, S. Burke, T.E. Robinson, S.J. Watson, et al.** 2004. Glucocorticoid receptor overexpression in forebrain: A mouse model of increased emotional lability. *Proceedings of the National Academy of Sciences of the United States of America* 101:11851-11856.

216. **Raineki, C., M.A. De Souza, R.E. Szawka, M.L. Lutz, L.F.T. De Vasconcellos, G.L. Sanvitto, I. Izquierdo, L.R. Bevilaqua, et al.** 2009. Neonatal Handling And The Maternal Odor Preference In Rat Pups: Involvement Of Monoamines And Cyclic Amp Response Element-Binding Protein Pathway In The Olfactory Bulb. *Neuroscience* 159:31-38.
217. **Hellstrom, I.C., S.K. Dhir, J.C. Diorio, and M.J. Meaney.** 2012. Maternal licking regulates hippocampal glucocorticoid receptor transcription through a thyroid hormone, serotonin, NGFI-A signalling cascade. *Philosophical Transactions of the Royal Society B: Biological Sciences* 367:2495-2510.
218. **Smythe, J.W., W.B. Rowe, and M.J. Meaney.** 1994. Neonatal handling alters serotonin (5-HT) turnover and 5-HT<sub>2</sub> receptor-binding in selected brain-regions - Relationship to the handling effect on glucocorticoid receptor expression. *Developmental Brain Research* 80:183-189.
219. **Oreland, S., C. Pickering, C. Gokturk, L. Oreland, L. Arborelius, and I. Nylander.** 2009. Two repeated maternal separation procedures differentially affect brain 5-hydroxytryptamine transporter and receptors in young and adult male and female rats. *Brain Research* 1305:S37-S49.
220. **Matthews, K., J.W. Dalley, C. Matthews, T.H. Tsai, and T.W. Robbins.** 2001. Periodic maternal separation of neonatal rats produces regional and gender-specific effects on biogenic amine content in post-mortem adult brain. *Synapse* 40:1-10.
221. **Andersen, S.L., P.J. Lyss, N.L. Dumont, and M.H. Teicher.** 1999. Enduring neurochemical effects of early maternal separation on limbic structures, p. 756-759. *In* J.F. McGinty (Ed.), *Advancing from the Ventral Striatum to the Extended Amygdala - Implications for Neuropsychiatry and Drug Abuse: in Honor of Lennart Heimer*. New York Acad Sciences, New York.
222. **Jahng, J.W., V. Ryu, S.B. Yoo, S.J. Noh, J.Y. Kim, and J.H. Lee.** 2010. Mesolimbic dopaminergic activity responding to acute stress is blunted in adolescent rats that experienced neonatal maternal separation. *Neuroscience* 171:144-152.
223. **Konno, K., M. Matsumoto, H. Togashi, T. Yamaguchi, T. Izumi, M. Watanabe, T. Iwanaga, and M. Yoshioka.** 2007. Early postnatal stress affects the serotonergic function in the median raphe nuclei of adult rats. *Brain Research* 1172:60-66.
224. **Arborelius, L., B.W. Hawks, M.J. Owens, P.M. Plotsky, and C.B. Nemeroff.** 2004. Increased responsiveness of presumed 5-HT cells to citalopram in adult rats subjected to prolonged maternal separation relative to brief separation. *Psychopharmacology* 176:248-U245.
225. **Kosten, T.A., X.Y. Zhang, and P. Kehoe.** 2004. Infant rats with chronic neonatal isolation experience show decreased extracellular serotonin levels in ventral striatum at baseline and in response to cocaine. *Developmental Brain Research* 152:19-24.
226. **Bordner, K.A., E.D. George, B.C. Carlyle, A. Duque, R.R. Kitchen, T.T. Lam, C.M. Colangelo, K.L. Stone, et al.** 2011. Functional genomic and proteomic analysis reveals disruption of myelin-related genes and translation in a mouse model of early life neglect. *Frontiers in psychiatry / Frontiers Research Foundation* 2:18.



227. **De Bellis, M.D., M.S. Keshavan, D.B. Clark, B.J. Casey, J.N. Giedd, A.M. Boring, K. Frustaci, and N.D. Ryan.** 1999. Developmental traumatology Part II: Brain development. *Biological Psychiatry* 45:1271-1284.
228. **Jackowski, A.P., H. Douglas-Palumberi, M. Jackowski, L. Win, R.T. Schultz, L.W. Staib, J.H. Krystal, and J. Kaufman.** 2008. Corpus callosum in maltreated children with posttraumatic stress disorder: A diffusion tensor imaging study. *Psychiatry Research-Neuroimaging* 162:256-261.
229. **Uchida, S., K. Hara, A. Kobayashi, H. Funato, T. Hobara, K. Otsuki, H. Yamagata, B.S. McEwen, and Y. Watanabe.** 2010. Early Life Stress Enhances Behavioral Vulnerability to Stress through the Activation of REST4-Mediated Gene Transcription in the Medial Prefrontal Cortex of Rodents. *Journal of Neuroscience* 30:15007-15018.
230. **Patel, P.D., D.A. Bochar, D.L. Turner, F. Meng, H.M. Mueller, and C.G. Pontrello.** 2007. Regulation of tryptophan hydroxylase-2 gene expression by a bipartite RE-1 silencer of Transcription/Neuron restrictive silencing factor (REST/NRSF) binding motif. *Journal of Biological Chemistry* 282:26717-26724.
231. **Jones, P.A. and S.B. Baylin.** 2002. The fundamental role of epigenetic events in cancer. *Nat Rev Genet* 3:415-428.
232. **Jaenisch, R. and A. Bird.** 2003. Epigenetic regulation of gene expression: how the genome integrates intrinsic and environmental signals. *Nature Genet.* 33:245-254.
233. **Mill, J., T. Tang, Z. Kaminsky, T. Khare, S. Yazdanpanah, L. Bouchard, P. Jia, A. Assadzadeh, et al.** 2008. Epigenomic Profiling Reveals DNA-Methylation Changes Associated with Major Psychosis. *The American Journal of Human Genetics* 82:696-711.
234. **Bernard, R., I.A. Kerman, F. Meng, S.J. Evans, I. Amrein, E.G. Jones, W.E. Bunney, H. Akil, et al.** 2009. Gene expression profiling of neurochemically defined regions of the human brain by in situ hybridization-guided laser capture microdissection. *Journal of Neuroscience Methods* 178:46-54.
235. **Palkovits, M. and M. Brownstein.** 1983. *Microdissection of brain areas by the punch technique.* Wiley, New York.
236. **Vazquez, D.M., C.R. Neal Jr, P.D. Patel, N. Kaciroti, and J.F. Lopez.** 2012. Regulation of corticoid and serotonin receptor brain system following early life exposure of glucocorticoids: Long term implications for the neurobiology of mood. *Psychoneuroendocrinology* 37:421-437.
237. **Maniatis, T., E. Fritsch, and J. Sambrook.** 1983. *Molecular cloning.* . Headington Hill Hall, New York.
238. **Walther, D.J., J.-U. Peter, S. Bashammakh, H. Hortnagl, M. Voits, H. Fink, and M. Bader.** 2003. Synthesis of Serotonin by a Second Tryptophan Hydroxylase Isoform. *Science* 299:76.
239. **Blakely, R.D., H.E. Berson, R.T. Fremeau, M.G. Caron, M.M. Peek, H.K. Prince, and C.C. Bradley.** 1991. Cloning and expression of a functional serotonin transporter from rat brain. *Nature* 354:66-70.

240. **Sidor, M.M., A. Amath, G. MacQueen, and J.A. Foster.** 2010. A developmental characterization of mesolimbocortical serotonergic gene expression changes following early immune challenge. *Neuroscience* 171:734-746.
241. **Chen, G.L., M.A. Novak, J.S. Meyer, B.J. Kelly, E.J. Vallender, and G.M. Miller.** 2010. The effect of rearing experience and TPH2 genotype on HPA axis function and aggression in rhesus monkeys: A retrospective analysis. *Horm. Behav.* 57:184-191.
242. **de Kloet, E.R., R.M. Sibug, F.M. Helmerhorst, and M. Schmidt.** 2005. Stress, genes and the mechanism of programming the brain for later life. *Neurosci. Biobehav. Rev.* 29:271-281.
243. **Gardner, K.L., M.W. Hale, S.L. Lightman, P.M. Plotsky, and C.A. Lowry.** 2009. Adverse early life experience and social stress during adulthood interact to increase serotonin transporter mRNA expression. *Brain Research* 1305:47-63.
244. **Romeo, R.D., A. Mueller, H.M. Sisti, S. Ogawa, B.S. McEwen, and W.G. Brake.** 2003. Anxiety and fear behaviors in adult male and female C57BL/6 mice are modulated by maternal separation. *Horm. Behav.* 43:561-567.
245. **Paxinos, G.** 2001. *The mouse brain in stereotaxic coordinates* / George Paxinos, Keith B.J. Franklin. Academic, San Diego, Calif. ; London .
246. **Caldji, C., D. Liu, S. Sharma, J. Diorio, D. Francis, M.J. Meaney, and P.M. Plotsky.** 2010. Development of Individual Differences in Behavioral and Endocrine Responses to Stress: Role of the Postnatal Environment. *In* A.P. Society (Ed.), *Comprehensive Physiology*. John Wiley & Sons, Inc.
247. **Francis, D.D., J. Diorio, P.M. Plotsky, and M.J. Meaney.** 2002. Environmental enrichment reverses the effects of maternal separation on stress reactivity. *Journal of Neuroscience* 22:7840-7843.
248. **Chamas, F., L. Serova, and E.L. Sabban.** 1999. Tryptophan hydroxylase mRNA levels are elevated by repeated immobilization stress in rat raphe nuclei but not in pineal gland. *Neuroscience Letters* 267:157-160.
249. **Chamas, F.M., M.D. Underwood, V. Arango, L. Serova, S.A. Kassir, J.J. Mann, and E.L. Sabban.** 2004. Immobilization stress elevates tryptophan hydroxylase mRNA and protein in the rat raphe nuclei. *Biological Psychiatry* 55:278-283.
250. **Boadlebiber, M.C., K.C. Corley, L. Graves, T.H. Phan, and J. Rosecrans.** 1989. Increase in the activity of tryptophan-hydroxylase from cortex and midbrain of male fischer-344 rats in response to acute or repeated sound stress. *Brain Research* 482:306-316.
251. **Flanigan, T.J. and M.N. Cook.** 2011. Effects of an Early Handling-Like Procedure and Individual Housing on Anxiety-Like Behavior in Adult C57BL/6J and DBA/2J Mice. *PLoS One* 6.
252. **Pinheiro, M.L., V. Ferraz-de-Paula, A. Ribeiro, M. Sakai, M.M. Bernardi, and J. Palermo-Neto.** 2011. Long-Term Maternal Separation Differentially Alters Serum Corticosterone Levels and Blood Neutrophil Activity in A/J and C57BL/6 Mouse Offspring. *Neuroimmunomodulation* 18:184-190.

253. **Macri, S., F. Chiarotti, and H. Wurbel.** 2008. Maternal separation and maternal care act independently on the development of HPA responses in male rats. *Behav. Brain Res.* 191:227-234.
254. **Levine, S.** 2001. Primary social relationships influence the development of the hypothalamic, Pituitary, Adrenal axis in the rat. *Physiol. Behav.* 73:255-260.
255. **Schmidt, M., L. Enthoven, M. van der Mark, S. Levine, E.R. de Kloet, and M.S. Oitzl.** 2003. The postnatal development of the hypothalamic, pituitary, adrenal axis in the mouse. *International Journal of Developmental Neuroscience* 21:125-132.
256. **Ognibene, E., W. Adriani, A. Caprioli, O. Ghirardi, S.F. Ali, L. Aloe, and G. Laviola.** 2008. The effect of early maternal separation on brain derived neurotrophic factor and monoamine levels in adult heterozygous reeler mice. *Progress in Neuro-Psychopharmacology & Biological Psychiatry* 32:1269-1276.
257. **Cohen, J. and R.S. Bridges.** 1981. Retention of maternal-behavior in nulliparous and primiparous rats - effects of duration of previous maternal experience. *Journal of Comparative and Physiological Psychology* 95:450-459.
258. **Hao Y Fau - Huang, W., D.A. Huang W Fau - Nielsen, T.A. Nielsen Da Fau - Kosten, and T.A. Kosten.** 2011. Litter gender composition and sex affect maternal behavior and DNA methylation levels of the oprm1 gene in rat offspring. *Front Psychiatry* 2.
259. **Jahanshahi, A., E. Le Maitre, Y. Temel, L. Lanfumey, M. Hamon, K.-P. Lesch, R.M. Tordera, J. Del Rio, et al.** 2011. Altered expression of neuronal tryptophan hydroxylase-2 mRNA in the dorsal and median raphe nuclei of three genetically modified mouse models relevant to depression and anxiety. *Journal of Chemical Neuroanatomy* 41:227-233.
260. **Laplante, P., J. Diorio, and M.J. Meaney.** 2002. Serotonin regulates hippocampal glucocorticoid receptor expression via a 5-HT7 receptor. *Developmental Brain Research* 139:199-203.
261. **Peyron, C., J.M. Petit, C. Rampon, M. Jouvett, and P.H. Luppi.** 1997. Forebrain afferents to the rat dorsal raphe nucleus demonstrated by retrograde and anterograde tracing methods. *Neuroscience* 82:443-468.
262. **Jasinska, A.J., C.A. Lowry, and M. Burmeister.** 2012. Serotonin transporter gene, stress and raphe raphe interactions: a molecular mechanism of depression. *Trends in neurosciences* 35:395-402.
263. **Lechin, F., B. van der Dijs, and G. Hernandez-Adrian.** 2006. Dorsal raphe vs. median raphe serotonergic antagonism. Anatomical, physiological, behavioral, neuroendocrinological, neuropharmacological and clinical evidences: Relevance for neuropharmacological therapy. *Progress in Neuro-Psychopharmacology and Biological Psychiatry* 30:565-585.
264. **Rozeboom, A.M., H. Akil, and A.F. Seasholtz.** 2007. Mineralocorticoid receptor overexpression in forebrain decreases anxiety-like behavior and alters the stress response in mice. *Proceedings of the National Academy of Sciences of the United States of America* 104:4688-4693.

265. **Own, L.S. and P.D. Patel.** 2012. ISH-guided freeze-matrix assisted punches (IFAP): Technique for extracting punches from thin slide-mounted tissues for DNA methylation analysis. *Biotechniques* 53:175-180.
266. **Aisa, B., R. Tordera, B. Lasheras, J. Del Rio, and M.J. Ramirez.** 2008. Effects of maternal separation on hypothalamic, pituitary, adrenal responses, cognition and vulnerability to stress in adult female rats. *Neuroscience* 154:1218-1226.
267. **Maciag, C.M., G. Dent, P. Gilligan, L.Q. He, K. Dowling, T. Ko, S. Levine, and M.A. Smith.** 2002. Effects of a non-peptide CRF antagonist (DMP696) on the behavioral and endocrine sequelae of maternal separation. *Neuropsychopharmacology* 26:574-582.
268. **Wang, L., J. Jiao, and S. Dulawa.** 2011. Infant maternal separation impairs adult cognitive performance in BALB/cj mice. *Psychopharmacology* 216:207-218.
269. **Venerosi, A., F. Cirulli, F. Capone, and E. Alleva.** 2003. Prolonged perinatal AZT administration and early maternal separation: effects on social and emotional behaviour of periadolescent mice. *Pharmacology Biochemistry and Behavior* 74:671-681.
270. **Wei, L., A. David, R.S. Duman, H. Anisman, and A. Kaffman.** 2010. Early life stress increases anxiety-like behavior in Balbc mice despite a compensatory increase in levels of postnatal maternal care. *Horm. Behav.* 57:396-404.
271. **Macrí, S., G.J. Mason, and H. Würbel.** 2004. Dissociation in the effects of neonatal maternal separations on maternal care and the offspring's HPA and fear responses in rats. *Eur. J. Neurosci.* 20:1017-1024.
272. **Suchecki, D., P. Rosenfeld, and S. Levine.** 1993. Maternal regulation of the hypothalamic-pituitary-adrenal axis in the infant rat: the roles of feeding and stroking. *Developmental Brain Research* 75:185-192.
273. **van Oers, H.J., E.R. de Kloet, T. Whelan, and S. Levine.** 1998. Maternal deprivation effect on the infant's neural stress markers is reversed by tactile stimulation and feeding but not by suppressing corticosterone. *The Journal of neuroscience : the official journal of the Society for Neuroscience* 18:10171-10179.
274. **Imanaka, A., S. Morinobu, S. Toki, S. Yamamoto, A. Matsuki, T. Kozuru, and S. Yamawaki.** 2008. Neonatal tactile stimulation reverses the effect of neonatal isolation on open-field and anxiety-like behavior, and pain sensitivity in male and female adult Sprague Dawley rats. *Behav. Brain Res.* 186:91-97.
275. **Azevedo, M.S.d., F.L.d. Souza, M.V.F. Donadio, A.B. Lucion, and M. Giovenardi.** 2010. Interventions in the neonatal environment in rats and their relationship to behavior in adulthood and maternal behavior. *Psychology & Neuroscience* 3:73-78.
276. **Levine, S., D. Glick, and P.K. Nakane.** 1967. Adrenal and Plasma Corticosterone and Vitamin A in Rat Adrenal Glands During Postnatal Development. *Endocrinology* 80:258-260.
277. **Ryu, V., J.H. Lee, S.B. Yoo, X.F. Gu, Y.W. Moon, and J.W. Jahng.** 2008. Sustained hyperphagia in adolescent rats that experienced neonatal maternal separation. *Int J Obes* 32:1355-1362.

278. **Fabricius, K., G. Wörtwein, and B. Pakkenberg.** 2008. The impact of maternal separation on adult mouse behaviour and on the total neuron number in the mouse hippocampus. *Brain Structure and Function* 212:403-416.
279. **Weber, E.M., B. Algers, H. Würbel, J. Hultgren, and I.A.S. Olsson.** 2012. Influence of Strain and Parity on the Risk of Litter Loss in Laboratory Mice, Reproduction in Domestic Animals. Blackwell Publishing Ltd, Oxford.
280. **Shoji, H. and K. Kato.** 2006. Maternal behavior of primiparous females in inbred strains of mice: A detailed descriptive analysis. *Physiol. Behav.* 89:320-328.
281. **Shanks, N., J. Griffiths, S. Zalcman, R.M. Zacharko, and H. Anisman.** 1990. Mouse strain differences in plasma corticosterone following uncontrollable footshock. *Pharmacology Biochemistry and Behavior* 36:515-519.
282. **Anisman, H.** 2001. Psychogenic, neurogenic, and systemic stressor effects on plasma corticosterone and behavior: mouse strain-dependent outcomes. *Behav. Neurosci.* 115:443-454.
283. **Pedersen, C., S. Vadlamudi, M. Boccia, and S. Moy.** 2011. Variations in maternal behavior in C57BL/6J mice: behavioral comparisons between adult offspring of high and low pup-licking mothers. *Frontiers in Psychiatry* 2:1-9.
284. **George, E., K. Bordner, H. Elwafi, and A. Simen.** 2010. Maternal separation with early weaning: a novel mouse model of early life neglect. *BMC Neurosci.* 11:1-14.
285. **Alleva, E., A. Caprioli, and G. Laviola.** 1989. Litter gender composition affects maternal behavior of the primiparous mouse dam (*Mus musculus*). *J. Comp. Psychol.* 103:83-87.
286. **Huot, R.L., K.V. Thirivikraman, M.J. Meaney, and P.M. Plotsky.** 2001. Development of adult ethanol preference and anxiety as a consequence of neonatal maternal separation in Long Evans rats and reversal with antidepressant treatment. *Psychopharmacology* 158:366-373.
287. **Stern, J.M.** 1996. Somatosensation and Maternal Care in Norway Rats, p. 243-294. *In* J. Rosenblatt, and C. Snowdon (Eds.), *Advances in the Study of Behavior*. Academic Press.
288. **Patel, P.D. and A.F. Seasholtz.** 2006. Computer-assisted scoring of the elevated plus maze. *Biotechniques* 41:700-704.
289. **Lucki, I.** 1997. The forced swimming test as a model for core and component behavioral effects of antidepressant drugs. *Behavioural Pharmacology* 8:523-532.
290. **Castagné, V., P. Moser, S. Roux, and R.D. Porsolt.** 2001. Rodent Models of Depression: Forced Swim and Tail Suspension Behavioral Despair Tests in Rats and Mice, *Current Protocols in Pharmacology*. John Wiley & Sons, Inc., New York.
291. **Burrows, H.L., M. Nakajima, J.S. Lesh, K.A. Goosens, L.C. Samuelson, A. Inui, S.A. Camper, and A.F. Seasholtz.** 1998. Excess corticotropin releasing hormone-binding protein in the hypothalamic-pituitary-adrenal axis in transgenic mice. *The Journal of Clinical Investigation* 101:1439-1447.
292. **Cohen, J.** 1988. *Statistical power analysis for the behavioral sciences*. Erlbaum, Hillsdale, NJ.

293. **Blier, P. and C. de Montigny.** 1999. Serotonin and drug-induced therapeutic responses in major depression, obsessive-compulsive and panic disorders. *Neuropsychopharmacology* 21:91S-98S.
294. **Roth, B.L.** 2006. *The Serotonin Receptors: From Molecular Pharmacology to Human Therapeutics.* Humana Press, Inc., New Jersey.
295. **Wise, C.D., B.D. Berger, and L. Stein.** 1972. Benzodiazepines: anxiety-reducing activity by reduction of serotonin turnover in the brain. *Science* 177:180-183.
296. **Soubrie, P.** 1986. Reconciling the role of central serotonin neurons in human and animal behavior. *The Behavioral and Brain Sciences* 9:319-335.
297. **Lorens, S.A.** 1978. Some behavioral effects of serotonin depletion depend on method: a comparison of 5,7-dihydroxytryptamine, p-chlorophenylalanine, p-chloroamphetamine, and electrolytic raphe lesions. *Ann N Y Acad Sci* 305:532-555.
298. **Delgado, P.L., H.L. Miller, R.M. Salomon, J. Licinio, J.H. Krystal, F.A. Moreno, G.R. Heninger, and D.S. Charney.** 1999. Tryptophan-depletion challenge in depressed patients treated with desipramine or fluoxetine: implications for the role of serotonin in the mechanism of antidepressant action. *Biol Psychiatry* 46:212-220.
299. **Sakowski, S.A., T.J. Geddes, D.M. Thomas, E. Levi, J.S. Hatfield, and D.M. Kuhn.** 2006. Differential tissue distribution of tryptophan hydroxylase isoforms 1 and 2 as revealed with monospecific antibodies. *Brain Res* 1085:11-18.
300. **Zhang, X., J.M. Beaulieu, R.R. Gainetdinov, and M.G. Caron.** 2006. Functional polymorphisms of the brain serotonin synthesizing enzyme tryptophan hydroxylase-2. *Cell Mol Life Sci* 63:6-11.
301. **Tenner, K., F. Qadri, B. Bert, J.r.-P. Voigt, and M. Bader.** 2008. The mTPH2 C1473G single nucleotide polymorphism is not responsible for behavioural differences between mouse strains. *Neuroscience Letters* 431:21-25.
302. **Reynolds, A., D. Leake, Q. Boese, S. Scaringe, W.S. Marshall, and A. Khvorova.** 2004. Rational siRNA design for RNA interference. *Nat Biotechnol* 22:326-330.
303. **Chung, K.H., C.C. Hart, S. Al-Bassam, A. Avery, J. Taylor, P.D. Patel, A.B. Vojtek, and D.L. Turner.** 2006. Polycistronic RNA polymerase II expression vectors for RNA interference based on BIC/miR-155. *Nucleic Acids Res* 34:e53.
304. **Treit, D., A. Robinson, S. Rotzinger, and C. Pesold.** 1993. Anxiolytic effects of serotonergic interventions in the shock-probe burying test and the elevated plus-maze test. *Behav Brain Res* 54:23-34.
305. **Strekalova, T., N. Gorenkova, E. Schunk, O. Dolgov, and D. Bartsch.** 2006. Selective effects of citalopram in a mouse model of stress-induced anhedonia with a control for chronic stress. *Behavioural Pharmacology* 17:271-287.
306. **Niles, L.P., G.M. Brown, J.W. Chambers, and S.F. Pang.** 1984. Effects of P-chlorophenylalanine on pineal and endocrine function in the rat. *Pharmacol Res Commun* 16:851-864.

307. **Tagliamonte, A., P. Tagliamonte, G.U. Corsini, G.P. Mereu, and G.L. Gessa.** 1973. Decreased conversion of tyrosine to catecholamines in the brain of rats treated with p-chlorophenylalanine. *J Pharm Pharmacol* 25:101-103.
308. **Asberg, M., L. Traskman, and P. Thoren.** 1976. 5-HIAA in the cerebrospinal fluid. A biochemical suicide predictor? *Arch Gen Psychiatry* 33:1193-1197.
309. **Linnoila, M., M. Virkkunen, M. Scheinin, A. Nuutila, R. Rimon, and F.K. Goodwin.** 1983. Low cerebrospinal fluid 5-hydroxyindoleacetic acid concentration differentiates impulsive from nonimpulsive violent behavior. *Life Sci* 33:2609-2614.
310. **Tenen, S.S.** 1967. The effects of p-chlorophenylalanine, a serotonin depletor, on avoidance acquisition, pain sensitivity and related behavior in the rat. *Psychopharmacologia* 10:204-219.
311. **Fibiger, H.C. and B.A. Campbell.** 1971. The effect of para-chlorophenylalanine on spontaneous locomotor activity in the rat. *Neuropharmacology* 10:25-32.
312. **Bechtholt, A.J., T.E. Hill, and I. Lucki.** 2007. Anxiolytic effect of serotonin depletion in the novelty-induced hypophagia test. *Psychopharmacology* 190:531-540.
313. **Homberg, J.R., J.D.A. Olivier, B.M.G. Smits, J.D. Mul, J. Mudde, M. Verheul, O.F.M. Nieuwenhuizen, A.R. Cools, et al.** 2007. Characterization of the serotonin transporter knockout rat: A selective change in the functioning of the serotonergic system. *Neuroscience* 146:1662-1676.
314. **Graeff, F.G., F.S. Guimaraes, T. DeAndrade, and J.F.W. Deakin.** 1996. Role of 5-HT in stress, anxiety, and depression. *Pharmacology Biochemistry and Behavior* 54:129-141.
315. **Blundell, J.E.** 1992. Serotonin and the biology of feeding. *The American Journal of Clinical Nutrition* 55:155S-159S.
316. **Leibowitz, S.F. and G. Shorposner.** 1986. Brain-Serotonin And Eating Behavior. *Appetite* 7:1-14.
317. **Phillips, R.G. and J.E. Ledoux.** 1992. Differential Contribution Of Amygdala And Hippocampus To Cued And Contextual Fear Conditioning. *Behav. Neurosci.* 106:274-285.
318. **Belanoff, J.K., M. Kalehzan, B. Sund, S.K.F. Ficek, and A.F. Schatzberg.** 2001. Cortisol activity and cognitive changes in psychotic major depression. *American Journal of Psychiatry* 158:1612-1616.
319. **Chen, G.L., M.A. Novak, S. Hakim, Z. Xie, and G.M. Miller.** 2006. Tryptophan hydroxylase-2 gene polymorphisms in rhesus monkeys: association with hypothalamic-pituitary-adrenal axis function and in vitro gene expression. *Molecular Psychiatry* 11:914-928.
320. **De Kloet, E.R., E. Vreugdenhil, M.S. Oitzl, and M. Joels.** 1998. Brain corticosteroid receptor balance in health and disease. *Endocrine Reviews* 19:269-301.
321. **Nakamura, K. and H. Hasegawa.** 2007. Developmental role of tryptophan hydroxylase in the nervous system. *Mol Neurobiol* 35:45-54.
322. **Cao, W., R. Hunter, D. Strnatka, C.A. McQueen, and R.P. Erickson.** 2005. DNA constructs designed to produce short hairpin, interfering RNAs in transgenic

- mice sometimes show early lethality and an interferon response. *Journal of applied genetics* 46:217-225.
323. **Sledz, C.A., M. Holko, M.J. de Veer, R.H. Silverman, and B.R. Williams.** 2003. Activation of the interferon system by short-interfering RNAs. *Nat Cell Biol* 5:834-839.
324. **Grimm, D., K.L. Streetz, C.L. Jopling, T.A. Storm, K. Pandey, C.R. Davis, P. Marion, F. Salazar, and M.A. Kay.** 2006. Fatality in mice due to oversaturation of cellular microRNA/short hairpin RNA pathways. *Nature* 441:537-541.
325. **Patel, P.D., J.F. Lopez, D.M. Lyons, S. Burke, M. Wallace, and A.F. Schatzberg.** 2000. Glucocorticoid and mineralocorticoid receptor mRNA expression in squirrel monkey brain. *J Psychiatr Res* 34:383-392.
326. **Pfaffl, M.W.** 2001. A new mathematical model for relative quantification in real-time RT-PCR. *Nucleic Acids Res* 29:e45.
327. **McKinney, B.C., J.S. Schneider, G.L. Schafer, J.L. Lowing, S. Mohan, M.X. Zhao, M.Y. Heng, R.L. Albin, et al.** 2008. Decreased locomotor activity in mice expressing tTA under control of the CaMKII $\alpha$  promoter. *Genes, Brain and Behavior* 7:203-213.
328. **Patel, P.D., A.F. Seasholtz, and P.D. Patel.** 2006. Computer-assisted scoring of the elevated plus maze. *Biotechniques* 41:700-704.
329. **McKinney, B.C. and G.G. Murphy.** 2006. The L-Type voltage-gated calcium channel Cav1.3 mediates consolidation, but not extinction, of contextually conditioned fear in mice. *Learning & Memory* 13:584-589.
330. **Graeff, F.G., M.B. Viana, and P.O. Mora.** 1997. Dual role of 5-HT in defense and anxiety. *Neurosci. Biobehav. Rev.* 21:791-799.
331. **Franklin, T.B., H. Russig, I.C. Weiss, J. Graeff, N. Linder, A. Michalon, S. Vizi, and I.M. Mansuy.** 2010. Epigenetic Transmission of the Impact of Early Stress Across Generations. *Biological Psychiatry* 68:408-415.
332. **Liu, Caldji, Sharma, Plotsky, and Meaney.** 2000. Influence of Neonatal Rearing Conditions on Stress-Induced Adrenocorticotropin Responses and Norepinephrine Release in the Hypothalamic Paraventricular Nucleus. *J. Neuroendocrinol.* 12:5-12.
333. **Hulshof, H.t.J., A. Novati, A. Sgoifo, P.G.M. Luiten, J.A. den Boer, and P. Meerlo.** 2011. Maternal separation decreases adult hippocampal cell proliferation and impairs cognitive performance but has little effect on stress sensitivity and anxiety in adult Wistar rats. *Behav. Brain Res.* 216:552-560.
334. **Eklund, M.B. and L. Arborelius.** 2006. Twice daily long maternal separations in Wistar rats decreases anxiety-like behaviour in females but does not affect males. *Behav. Brain Res.* 172:278-285.
335. **Roman, E., L. Gustafsson, M. Berg, and I. Nylander.** 2006. Behavioral profiles and stress-induced corticosteroid secretion in male Wistar rats subjected to short and prolonged periods of maternal separation. *Horm. Behav.* 50:736-747.
336. **Odonnell, D., S. Larocque, J.R. Seckl, and M.J. Meaney.** 1994. Postnatal handling alters glucocorticoid, but not mineralocorticoid messenger RNA



- expression in the hippocampus of adult rats *Molecular Brain Research* 26:242-248.
337. **Clark, J.A., R.B. Flick, L.Y. Pai, I. Szalayova, S. Key, R.K. Conley, A.Y. Deutch, P.H. Hutson, and E. Mezey.** 2007. Glucocorticoid modulation of tryptophan hydroxylase-2 protein in raphe nuclei and 5-hydroxytryptophan concentrations in frontal cortex of C57/Bl6 mice. *Mol Psychiatry* 13:498-506.
338. **Chamas, F. and E.L. Sabban.** 2002. Role of the 5' untranslated region (UTR) in the tissue-specific regulation of rat tryptophan hydroxylase gene expression by stress. *Journal of Neurochemistry* 82:645-654.
339. **Browne, C.A., G. Clarke, T.G. Dinan, and J.F. Cryan.** 2011. Differential stress-induced alterations in tryptophan hydroxylase activity and serotonin turnover in two inbred mouse strains. *Neuropharmacology* 60:683-691.
340. **Sánchez, C. and E. Meier.** 1997. Behavioral profiles of SSRIs in animal models of depression, anxiety and aggression. *Psychopharmacology* 129:197-205.
341. **Oh, J.-e., B. Zupan, S. Gross, and M. Toth.** 2009. Paradoxical Anxiogenic Response of Juvenile Mice to Fluoxetine. *Neuropsychopharmacology* 34:2197-2207.