Long term effects of early life maternal separation during tyrosine receptor kinase B (TrkB) knockdown

by

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Table of Contents

List of Figures

List of Tables

Abstract

Traditional antidepressants which target monoamine systems have significant side effects and a sizable proportion of patients are treatment-resistant. However, exciting new findings using low doses of the N-methyl-D-asparatate (NMDA) receptor antagonist, ketamine, have suggested that rapid modulation of glutamate systems which mediate neuroplasticity might hold promise for a new category of more efficacious antidepressants. Brain Derived Neurotrophic Factor (BDNF) is a neurotrophin that has been implicated in depression and is upregulated by low-dose ketamine with marked effects on synaptogenesis, synaptic strengthening, and dendritic spine outgrowth. BDNF plays a regulatory role in glutamatergic synapses and signals through tyrosine receptor kinase B (TrkB). Interestingly, early life stressors that can affect BDNF production are known to predispose individuals towards the later development of depression. We hypothesized that TrkB/BDNF signalling mediates neuroplastic changes during exposure to early stressors that can predispose individuals towards the later development of depression. Therefore, we utilized a knock-in transgenic mouse line ($TrkB^{F616A}$) with a mutation on the TrkB receptor, to reversibly block early postnatal BDNF/TrkB signalling on neurons during exposure to early life stress (maternal separation). In 3 month-old mice exposed to maternal separation, we found that TrkB knockdown impacted various aspects of their apparent resiliency to stress in adulthood. Moreover, these mice have very circumscribed variations in the expression of TrkB and BDNF within the brain. We speculate that BDNF might imprint early life stressor events, which ultimately influences the manifestation of depressive illness.

List of Abbreviations

1NM-PP1	1'-naphthylmethyl-4-amino-1-tert-butyl-3-(p-methylphenyl)pyrazolo[3,4-d]pyrimidine
5-HT	serotonin
AKT	protein kinase B
ATP	adenosine triphosphate
BDNF	brain-derived neurotrophic factor
CaM	calcium/calmodulin complex
CaMK	CaM-dependent kinase
cAMP	cyclic adenosine monophosphate
cAMP	cyclic adenosine monophosphate
CNS	central nervous system
CREB	cAMP response element binding protein
CREB	cAMP response element-binding protein
DAG	diacylglycerol
ERK	extracellular signal-regulated kinase
HET	heterozygote (+/-) of F616A mutant
IP3	inositol trisphosphate
KI	homozygous knock-in of F616A mutant allele
LTD	long-term depression
LTP	long-term potentiation
MAPK	mitogen-activated protein kinase
mBDNF	mature BDNF
MDD	major depressive disorder
mRNA	messenger ribonucleic acid
NAcc	nucleus accumbens
NGF	nerve growth factor
NT-3	neurotrophin-3
NT-4	neurotrophin-4
P75NTR	75 kDa neurotrophin receptor, also known as Low-affinity Nerve Growth Factor Receptor (LNGFR)
PD	postnatal day
PI3K	phosphatidylinositol 3-kinase
PLC-γ	phospholipase C-γ
SNRI	serotonin and norepinephrine reuptake inhibitor

SSRI	selective serotonin reuptake inhibitor
tPA	tissue plasminogen activator
Trk	tyrosine receptor kinase
TrkB.FL	full-length TrkB
TrkB.T1	truncated TrkB
WB	Western blotting
WT	wild type

Introduction

For the past three decades, research into the neurobiological basis of depression has focused primarily on understanding and modulating monoamine neurotransmitter systems such as serotonin and norepinephrine (for a historical review see Lopez-Munoz and Alamo, 2009). Indeed, this research has underpinned the development of the most commonly prescribed antidepressants, selective serotonin reuptake inhibitors (SSRIs) and selective serotonin and norepinephrine reuptake inhibitors (SNRIs). In the last decade, however, recent success with low-dose ketamine for treatment-resistant depression has put a focus on the role of glutamatergic systems and neurotrophic factors in mediating depressive disorders (Pittenger and Duman, 2008; DeWilde et al., 2015), and contributed to the development of a "neuroplasticity hypothesis of depression" (Sanacora et al., 2012). The most critical neurotrophin to emerge from this line of research is brain-derived neurotrophic factor (BDNF), which significantly influences neuronal development, survival, and neuroplastic processes (Björkholm and Monteggia, 2015).

The neurotrophins: NGF, NT-3, NT-4 and BDNF

BDNF is a member of the secretory neurotrophin family that includes nerve growth factor (NGF), neurotrophin-3 (NT-3) and neurotrophin-4/5 (NT-4). Each type of neurotrophin exists as a non-covalently bound dimer, which preferentially binds to a specific member of the tyrosine receptor kinase (TRK) family of membrane-bound proteins which include TrkA, TrkB, and TrkC. Amino acid homology between neurotrophins ranges from 50-55%, (Ip et al., 1992), which results in some overlap in ligand binding to Trk

receptors. Similarly, homology between the kinase domains of Trk receptors ranges between 71.9% and 78.3% (Bertrand et al., 2012). In addition to Trk receptor binding, all neurotrophins can bind, with lower affinity, to the p75 neurotrophin receptor (P75NTR) which is also known as the Low-affinity Nerve Growth Factor Receptor (LNGFR).

NGF interacts primarily with tyrosine receptor kinase A (TrkA) and is critical for neuronal development and differentiation, with highest expression during early brain development (Toyomoto et al., 2005). NT-3 is a promiscuous ligand that can bind weakly to TrkA and TrkB, but has the strongest affinity for TrkC. NT-3 is most abundant in early development and expression is greatly reduced as the brain matures, where it appears to play a role in mediating neuronal survival (Maisonpierre et al., 1990). NT-4 preferentially binds to TrkB receptors and is also highly abundant during early development, particularly in non-neuronal tissue (Ip et al., 1992). In adulthood, NT-4 has much lower expression, and is most important for maintenance of neuromuscular systems (Bothwell, 2014).

BDNF appears to play a more important role than NT-4 in neurodevelopment as $BDNF^{-/-}$ mice die after 2 to 3 weeks, whereas $NT\text{-}4^{-/-}$ are viable and fertile but lack some peripheral sensory neurons (Endres et al., 2000). NGF, NT-3 and NT-4 are constitutively released from neurons and neuroendocrine cells, whereas BDNF, (and to some extent NT-4), release is governed via regulatory and activity-dependent mechanisms (Maisonpierre et al., 1990; Hyman et al., 1994; Mowla et al., 1999). Accordingly, BDNF is an important regulator of synaptic plasticity at glutamatergic synapses (Lu, 2003; Cohen-Cory et al., 2010), where it is essential for neurodevelopment, synaptic growth and maintenance, and learning and memory (Itoh et al., 2016).

Production of Brain-derived neurotrophic factor (BDNF)

BDNF production peaks in the post-natal rodent brain at around day 20 (Patz and Wahle, 2006), highlighting its essential role in neurodevelopment. Activity-dependent production and release of BDNF occurs throughout the adult brain, with the highest levels in the hippocampus (Ernfors et al., 1990; Kawamoto et al., 1996; Berghuis et al., 2004). BDNF transcription is under the control of cyclic adenosine monophosphate (cAMP) response element-binding protein (CREB) (Tao et al., 1998) which can produce 8 different messenger ribonucleic acid (mRNA) transcripts, all of which produce identical proteins (Aid et al., 2007). BDNF mRNA is initially translated into a precursor peptide, preproBDNF. This precursor is cleaved in the endoplasmic reticulum to produce the 32kDa protein, proBDNF (Mowla et al., 2001). ProBDNF is then packaged into vesicles in the trans-golgi network, where some protein may be further cleaved by enzymes like furin to produce the a small amount of 28 kDa protein (pro28kDa-BDNF, or truncated proBDNF), and larger amounts of 14 kDa mature BDNF (mBDNF), or the 17 kDa BDNF pro-peptide (Dieni et al., 2012). Vesicles containing these proteins are released from neurons into the synapse in an activity-dependent manner. Cleavage can also occur in the extracellular milieu - proBDNF may be cleaved by extracellular proteases to produce more mBDNF or BDNF pro-peptide.

Neurons engage in calcium-dependent release of all forms of BDNF with release possible from both the presynaptic and postsynaptic membranes (Arai et al., 1996; Balkowiec and Katz, 2002; Yang et al., 2009). There is evidence that proBDNF tends to be secreted under low frequency tetanic stimulation, resulting in synaptic pruning and long term depression (LTD) (Orefice et al., 2016), in part due to proBDNF binding to presynaptic

P75NTR and sortilin (a membrane-bound sorting glycoprotein) which inhibits neurotransmitter release (Woo et al., 2005; Gibon et al., 2016). On the other hand, mBDNF is cleaved from proBDNF and secreted as a result of high frequency stimulation, and is responsible for synaptic growth and LTP (Nagappan et al., 2009). Furthermore, membrane depolarization can trigger co-release of ProBDNF and tissue plasmin activator (tPA) from the post-synaptic cell, where tPA then activates the protease plasmin, resulting in extracellular cleavage and production of mBDNF (Pang et al., 2004). Mature BDNF then binds to post-synaptic TrkB receptors in an autocrine manner, triggering signalling cascades that induce local synthesis of proteins essential to synapse development and dendritic spine growth (Lu, 2003; Elmariah et al., 2004; Luikart et al., 2005).

Pre-synaptic release of mBDNF can also signal through an autocrine loop with presynaptic TrkB receptors to enhance glutamate release (Kang and Schuman, 1995; Li et al., 1998). Moreover, presynaptic release of mBDNF is capable of paracrine signalling through post-synaptic TrkB receptors, triggering cascades that result in transcriptional up-regulation of synaptic density proteins, many of which are translated locally into proteins in dendritic spine compartments (Kang and Schuman, 1995; Monteggia et al., 2004). BDNF mRNA that is translated to protein in the neuronal soma can be transported to dendrites or axon terminals. Indeed, BDNF can be transported from one compartment to another in both anterograde and retrograde fashion (Elmariah et al., 2004). In the hippocampus, BDNF mRNA transcripts are also transported to distal dendrites of neurons, where they are rapidly translated into protein as a result of glutamatergic activation of AMPA receptors (Monteggia et al., 2013; Gonzalez et al., 2016).

Throughout the brain, the most abundant BDNF variant is proBDNF (Mowla et al., 2001), however, recent work suggests that there may be equal amounts of mBDNF and BDNF pro-peptide within the hippocampus, and that this concentration is ~10 fold more abundant than proBDNF (Dieni et al., 2012). The role of BDNF pro-peptide is not well understood, but there is some evidence that it mediates synaptic pruning and dendritic retraction through caspase-3 mediated mechanisms, however, the receptors it signals through have not been clearly identified (Guo et al., 2016). Little is known about the function of the less-abundant pro28kDa-BDNF, other than the fact that it is not a by-product in the production of mBDNF (Mowla et al., 2001).

It is important to note that BDNF release is not restricted to neurons. In addition to recycling BDNF (Vignoli et al., 2016), astrocytes also manufacture the peptide. Indeed, BDNF mRNA has be found in perisynaptic astrocytes in the midbrain, striatum and corpus callosum (Rubio, 1997; Toyomoto et al., 2005; Jean et al., 2008). Overexpression of astrocytic BDNF can induce neurogenesis along with anxiolytic and antidepressant effects (Quesseveur et al., 2013). Moreover, microglia also produce and release BDNF (Trang et al., 2011), suggesting that glia may play a role in mediating the response to antidepressants (for a review see Rial et al., 2015).

BDNF in Mental Health and Disease

Increases in cortical mBDNF are critical and essential for the synaptogenesis and mood improvements seen in both long-term treatment via traditional antidepressants, as well as the rapid response of low-dose ketamine (Duric and Duman, 2013; Björkholm and Monteggia, 2015). Single nucleotide polymorphisms in the BDNF gene, such as Val66Met, (where a methonine is substituted for a valine at codon 66 in the Pro domain, resulting in poor vesicle loading and transport), have been found to influence both predisposition to depression as well as antidepressant response in humans (Zou et al., 2010; Liu et al., 2012; Lee et al., 2013) and rodents (Yu et al., 2012). Only one study has claimed to see antidepressant effects from peripherally-administered BDNF (Schmidt and Duman, 2010), but it is generally accepted that BDNF does not readily cross the blood-brain-barrier due to its large molecular size (Poduslo and Curran, 1996). In any case, systemic administration of BDNF might have undesirable effects such as enhanced anxiety (Chou et al., 2014), cardiac arrhythmia (Fulgenzi et al., 2015) and enhanced response to neuropathic pain (Zhang et al., 2016). When BDNF is produced in peripheral tissues, it is found in abundance in platelets, and also can be detected in blood plasma and sera (Fujimura et al., 2002). Though some studies have found correlations between serum concentrations of BDNF, major depression and antidepressant efficacy (for a meta-analysis see Polyakova et al., 2015), there are many conflicting results and it is still unclear how peripheral BDNF relates to BDNF expression in the CNS.

Within the CNS, rodent studies have confirmed a strong correlation between BDNF dysfunction and mood disorders such as depression, bipolar disorders and anxiety

(Angelucci et al., 2005; Kerman, 2012; Lindholm and Castrén, 2014). Whereas up-regulation of mBDNF in the hippocampus and PFC is correlated with antidepressant efficacy and positive changes in mood, selective increases in uncleaved proBDNF can have opposing effects.

As mentioned earlier, proBDNF preferentially binds to P75NTR, whereas mBDNF preferentially binds to TrkB (Barker, 2009; Colle et al., 2015). However, P75NTR is often co-expressed with TrkB, and can form complexes which increase selectivity of the TrkB receptor for mBDNF over other neurotrophins (Bibel et al., 1999). Unfortunately, acute stress can interfere with cleavage of proBDNF to mBDNF (Tognoli et al., 2010), and excessive proBDNF can oppose the effects of mBDNF, thereby decreasing TrkB signalling (Zhou et al., 2013; Bai et al., 2016). Similarly, alterations in TrkB receptor expression can impact mBDNF signalling, and such alterations have been associated with psychiatric disorders such as depression, bipolar disorder, suicidal ideation (Pandey et al., 2008), anxiety, schizophrenia, alcohol dependence, and anorexia nervosa (for a review see Gupta et al., 2013).

Antidepressant treatment has been shown to increase BDNF and TrkB expression, as well as promote the proliferation of neuronal progenitor cells (Malberg et al., 2000; Santarelli, 2003; Castrén and Rantamäki, 2010). Although a reduction in neurogenesis does not necessarily directly predicate the development of depression, it can impact resiliency to stress, which is clearly linked to the disorder (Levone et al., 2015). In addition to the promotion of neurogenesis, the expression of BDNF and its TrkB receptor are also vital to

the long-term survival of newly differentiated neurons (Nibuya et al., 1995; Foltran and Diaz, 2016; Vilar and Mira, 2016).

The TrkB receptor

TrkB is part of the NTRK family of proteins which includes TrkA and TrkC. This receptor is expressed in the central and peripheral nervous systems, skeletal muscles, kidneys, pancreas and heart. The gene, Neurotrophic Receptor Tyrosine Kinase 2 (NTRK2), is located on chromosome 9 in humans and chromosome 13 in mice. Though promoter homology differs significantly, NTRK2 gene expression is highly conserved in humans and rodents (Shelton et al., 1995; Stoilov, Peter; Castren, Eero ; Stamm, 2002; Kingsbury, 2003), and thus mice are a highly suitable model to study this receptor system and its signalling cascades.

TrkB receptors are single-pass Type I membrane proteins that attach to the cell surface as unbound monomers, or non-covalently bound dimers (Shen and Maruyama, 2012). These proteins dimerize, phosphorylate and internalize to endosomes upon binding of BDNF, or NT-4, and to a lesser extent, NT-3. More than 100 possible splice variants for TrkB have been proposed, with 36 potential isoforms found in human tissue (Luberg et al., 2010), and 7 mRNA transcripts identified in the mouse brain (Klein et al., 1989). In addition to the full-length receptor protein, several TrkB receptors which lack some portion of the intracellular domain have been identified in human and mouse brains. The two most prominent TrkB isoforms are full length (TrkB.FL aka TrkB-FL, TrkB-TK+, gp145-TrkB) and Truncated (TrkB.T1 aka Trkb-T1, TrkB-TK-, gp95-TrkB). Both isoforms

share identical extracellular and transmembrane sequences, and can form both hetero and homodimers upon ligand binding (Baxter et al., 1997). Early work suggests that expression of TrkB within the CNS changes throughout development with TrkB.FL dominating prenatally and early in post-natal development, but TrkB.T1 dominating after postnatal day 20 (Fryer et al., 1996).

Truncated TrkB (TrkB.T1)

Though two other TrkB isoforms with reduced intracellular domains have been identified in the murine CNS, Trkb.T1 is the most widely distributed truncated form found in both humans and mice to date (Eide et al., 1996; Stoilov, Peter; Castren, Eero ; Stamm, 2002). The TrkB.T1 receptor consists of an extracellular binding domain and transmembrane segment that is identical to TrkB.FL, but it contains a unique sequence of just 13 amino acids on the intracellular portion. It completely lacks any known intracellular catalytic domains, and is highly conserved across species. Trkb.T1 is expressed by both neurons and astrocytes in the hippocampus and cortex (Armanini et al., 1995) and is the only TrkB form to be identified on mature astrocytes (Allen et al., 1994; Rose et al., 2003).

The function of TrkB.T1 is still not well-understood, but studies have determined that, on neurons, it acts as dominant negative regulator of TrkB.FL (Li et al., 1998; Carim-Todd et al., 2009), and P75NTR (Michaelsen et al., 2010). In glia, TrkB.T1 may play a role in sequestering excess BDNF and mediating Ca^{2+} signalling in astrocytes and microglia, (Rose et al., 2003; Mizoguchi et al., 2011) and regulating astrocyte morphology via the Rho-guanosine triphosphate enzyme family (Rho-GTPase) (Ohira et al., 2007). A small number of studies have suggested that TrkB truncated forms may also be involved in signal

transduction (Baxter et al., 1997; Huang and Reichardt, 2003), however, mechanisms are as yet unknown. Though neurotrophins and their receptors can be isolated from blood serum and leukocytes (Zhou et al., 2013), most studies of TrkB.T1 have been restricted to tissue analysis.

In post-mortem studies, increases in truncated isoforms of TrkB have been found in the prefrontal cortex of schizophrenics (Wong et al., 2013), and prolonged up-regulation of TrkB.T1 can be seen 8 weeks after traumatic penetrating brain injury (Rostami et al., 2014). Similarly, increased TrkB.T1 after stroke results in excitotoxicity and neuronal death (Vidaurre et al., 2012; Tejeda et al., 2016). While too much TrkB.T1 is linked to excitotoxicity, low cortical expression of TrkB.T1 has been found in post-mortem studies of suicide completers (Maussion et al., 2012), and this expression was not found to be correlated with a reduction in astrocyte numbers (Ernst et al., 2009).

In studies of TrkB.T1 knockout mice, the $T1^{-/-}$ mice are fertile and viable but display anxiety and abnormal dendritic morphology in the amygdala (Carim-Todd et al., 2009). As yet, there have been no studies published which identify the role of TrkB.T1 expression and signalling in murine models of depression. Hence, as will be discussed shortly, this is a primary focus of the present thesis.

A region-specific balance between TrkB.FL and TrkB.T1 may be as important as individual receptor expression (Kemppainen et al., 2012), as TrkB.T1 and TrkB.FL can form heterodimers which prevent auto-phosphorylation, and block TrkB.FL signalling (Eide et al., 1996). Indeed, the ratio of TrkB.FL/TrkB.T1 expression in striatum and SNpc may be

markers for Parkinson's disease (Fenner et al., 2014), and ratio decreases in the cortex may similarly be useful markers for Alzheimer's disease (Kemppainen et al., 2012).

Full Length TrkB (TrkB.FL)

TrkB.FL is the most studied TrkB isoform in the CNS, with 838 amino acids in total, including 384 amino acids on the intracellular portion. Within the cytoplasmic domain, this isoform has three catalytic regions: the tyrosine kinase autophosphorylation domain, the source homology-containing (Shc) domain, and the phospholipase C-gamma (PLC-γ) domain (Klein et al., 1990, 1991). Activation of TrkB.FL via ligand binding causes dimerization and internalization (Fryer et al., 1996). When TrkB.FL forms a ligand-bound homodimer, the receptor auto-phosphorylates at Tyrosines Y670, Y674, and Y675 (See Figure 1), and attracts adapter proteins which can activate multiple pathways including:

- ras-mitogen-activated protein kinase (MAPK) cascade, via phosphorylation of site Y515, which triggers extracellular signal–regulated kinase (ERK) and pathways for neuronal growth and differentiation;
- phosphatidylinositol 3-kinase (PI3K)-protein kinase B (Akt)-mammalian target of rapamycin (mTOR), via phosphorylation of site Tyr515, for cell growth and survival through anti-apoptotic mechanisms;
- and the PLC-γ pathway, via phosphorylation of Tyr816, which can signal through Inositol trisphosphate (IP_3) and calcium/calmodulin-dependent protein kinase (CAMK) to mediate CREB transcription and translation of proteins important to synaptic plasticity (Zirrgiebel et al., 1995). Phosphorylation of Y816 can also

activate a diacylglycerol (DAG) - Protein kinase C (PKC) pathway to induce synaptic protein translation.

Both the MAPK and PI3K pathways have been implicated in depression (Duman and Aghajanian, 2012), with activation of these pathways increasing synaptic protein transcription and translation, and receptor trafficking to the cell membrane (Minichiello, 2009). Despite a 50% homology between BDNF and NT-4, there is evidence to suggest that the BDNF causes more rapid ubiquination and degradation than NT-4, leading to more sustained signalling from NT-4, and activation of different pathways (Proenca et al., 2016). This differential activity can result in different populations of neurons relying on NT-4 or BDNF for survival (Minichiello et al., 1999), and suggest there may be preferential binding of ligands to specific TrkB isoforms (Fan et al., 2000). In addition to phosphorylation of Tyrosine domains, the receptor reportedly relies on phosphorylation of serine residues at amino acid 478 by cyclin dependent kinase 5 (Cdk5) to support hippocampal LTP and spatial memory formation (Lai et al., 2012). There is also some evidence to suggest that TrkB can be transactivated by the g-coupled protein receptors adenosine 2A (A_{2A}) and pituitary adenylate cyclase-activating polypeptide type I receptor (PAC_1) in the absence of neurotrophins (Lee and Chao, 2001).

To date, analysis of adult brain tissue has only found TrkB.FL in neurons (Rose et al., 2003; Givalois et al., 2004). Climent et al. (2000) identified TrkB.FL mRNA in developing astrocytes *in vitro*, however, the expression was significantly reduced when the astrocytes became confluent. Using Western blot, our own studies (unpublished), did not detect any TrkB.FL protein in mature, confluent astrocyte culture derived from C57 black mice (via

cryopreserved astrocytes from QBM Cell Science). In neurons, it has been established that signalling through TrkB.FL is essential for cell survival and differentiation (Zirrgiebel et al., 1995; Lowenstein and Arsenault, 1996), and neurite outgrowth (Zhou et al., 2012).

Although TrkB has been most heavily studied for its role in synaptic plasticity and memory formation (Madara and Levine, 2008), activation of TrkB.FL receptors on the post-synaptic membrane is essential to the prolonged antidepressant response of ketamine (Lepack et al., 2014; Carreno et al., 2015; Yang et al., 2015). This also promotes BNDF translation and release (Monteggia et al., 2013) and activation of the mTOR pathway leading to an increase in synaptic protein transcription and translation (Li et al., 2010). Mature BDNF signalling through TrkB also auto-regulates the expression of TrkB itself at the plasma membrane (Haapasalo et al., 2002).

Unlike TrkB.T1 knockouts, TrkB.FL knock-down mice have numerous motor neuron deficits, ganglion deficits and impaired cerebellar development. The fact that TrkB.FL$^{-/-}$ pups do not feed and die at P1 (Klein et al., 1993), highlights the essential activity of TrkB.FL during prenatal neurodevelopment. Similarly, constitutive BDNF$^{-/-}$ mice die within 4 weeks of birth due to significant losses in sensory neurons, and loss of cortical synaptic maintenance (Patterson et al., 1996; Gorski et al., 2003). Interestingly, the role of BDNF-mediated TrkB signalling is required for survival of cortical neurons, but not for pyramidal neurons in CA1 of the hippocampus. TrkB.FL is essential to development and maintenance of synapses in a large number of brain regions including the hypothalamus (Girardet et al., 2013), hippocampus (Elmariah et al., 2004; Luikart et al., 2005), and cortex (Shapiro et al., 2017), all of which play an important role in mediating the response to stress.

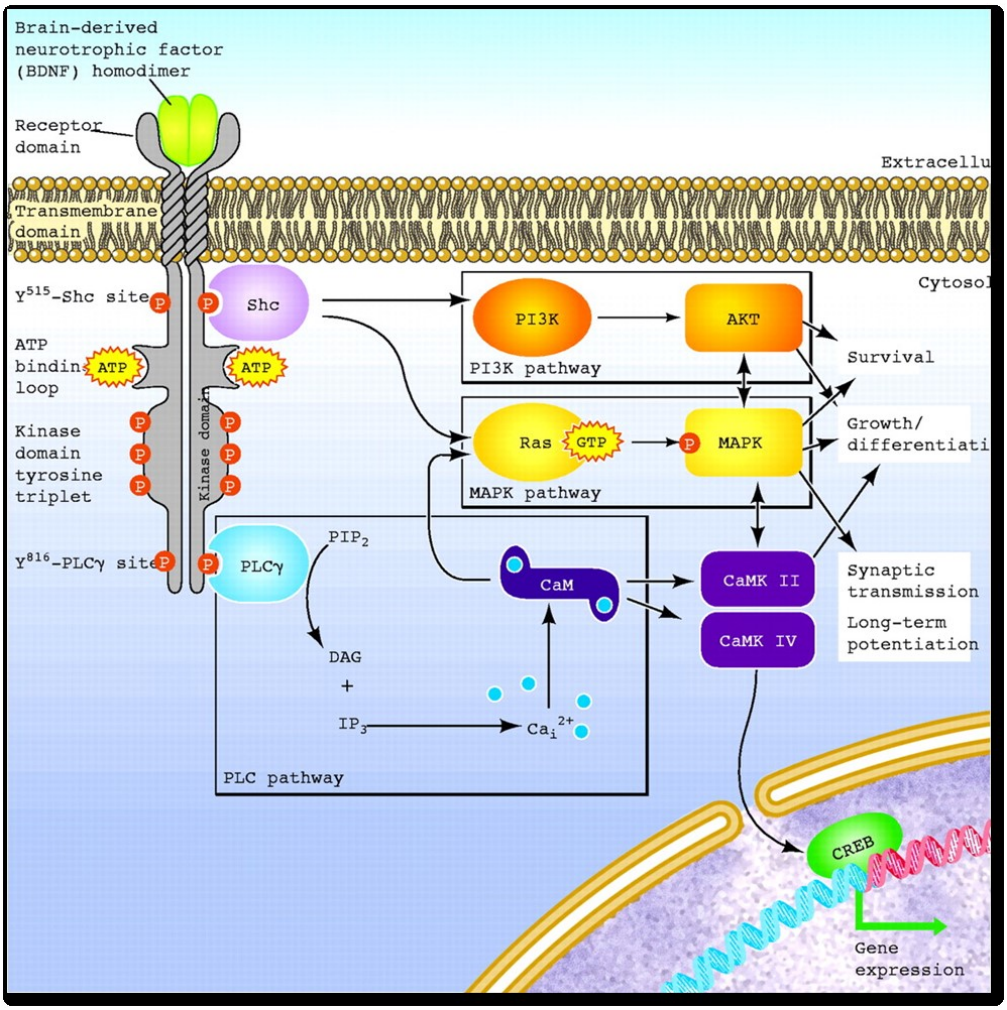

Figure 1. TrkB Phosphorylation domains and pathways
(used with permission from Blum and Konnerth, 2005) Abbreviations: adenosine triphosphate (ATP), calcium/calmodulin complex (CaM), CaM-dependent kinase (CaMK), phosphatidylinositol 3-kinase (PI3K), Protein kinase B (AKT), cyclic adenosine monophosphate (cAMP), cAMP response element-binding protein (CREB), IP3 (inositol trisphosphate), phospholipase C (PLC).

BDNF and HPA reactivity

The hippocampus is not only exquisitely sensitive to a variety of stressors but is an essential mediator of hypothalamic-pituitary-adrenocortical (HPA) activity, acting as a

negative feedback mechanism (for a review see Herman et al., 2016). Low-affinity glucocorticoid receptors (GR) in the mPFC and CA1 of the hippocampus are connected to circuits essential to inhibiting HPA axis output (Conrad, 2008; McEwen et al., 2012; McKlveen et al., 2013; Nicolaides et al., 2015). Acute activation of this circuit resulting in transient elevations of glucocorticoids can upregulate BDNF (Shi et al., 2010), and facilitate LTP, learning and memory (Kim and Yoon, 1998). Acute release of glucococorticoids can also enhance TrkB-mediated neuroprotection via the PI3K-Akt pathway, although this effect may be more beneficial to neurogenesis than mature neurons (Jeanneteau et al., 2008).

On the other hand, chronic stress leads to loss of GRs and thus disrupts the negative feedback loop arising from the hippocampus and mPFC, resulting in hyperreactivity in the HPA axis (Sapolsky et al., 1984; Mizoguchi et al., 2003). Hippocampal regulatory feedback also mediates glutamatergic signalling in areas of limbic system associated with stress reactivity (Flak et al., 2012; Zonjy et al., 2015). More specifically, both the mPFC and hippocampus can reduce hypothalamic induction of corticoid release through polysynaptic inhibitory circuits via the bed nucleus of the stria terminalis (BNST) and GABAergic inhibitory interneurons (Radley and Sawchenko, 2015). Conversely, enhanced activation of the amygdala can enhance corticoid release by suppressing inhibitory signals emanating from the BNST (Nestler and Carlezon, 2006; Price and Drevets, 2012; Herman, 2013) (See figure 2).

Dysregulated HPA activity stemming from loss of hippocampal regulatory control can lead to over-activation of glutamatergic synapses resulting in excitotoxicity, excessive

synaptic pruning, and loss of grey matter (Pittenger and Duman, 2008). At a structural level, chronic unpredictable stress results in a reduction in the number and complexity of dendritic spines in the hippocampus and prefrontal cortex (Radley, 2005; McEwen et al., 2012), but conversely can lead to increases in the number and density of spines in the amygdala and nucleus accumbens (NAcc) which may account for the increase in anxiety-like responses in some chronic stress paradigms (Qiao et al., 2016).

Normally associated with addiction and reward, the NAcc has more recently been implicated in mood disorders. Phasic firing of dopaminergic neurons from the ventral tegmental area (VTA) that project to the NAcc, were found to induce context-specific susceptibility to depression-like behaviours in repeated social defeat stress in mice (Chaudhury et al., 2012). Ultimately, chronic stress results in changes to brain morphology and neuroplasticity that disrupt critical cortical and limbic system circuits to adversely impact affective and cognitive outcomes (Fossati et al., 2004).

Early life stress and neuroplasticity

Since Wiesel and Hubel's (1963) first identification of a critical experience-driven postnatal period for visual development in cats, the identification of critical periods for plasticity have been extensively studied in non-human primates and rodents (for a review see Ismail et al., 2017). Both genetics and early life experiences are known to shape PFC and limbic system circuits within the brain, particularly when it comes to stress reactivity in adulthood (Sánchez et al., 2001; Lupien et al., 2009; Bock et al., 2014). Indeed, numerous studies have established that early life stress in humans, such as maltreatment, poverty,

war, or lack of maternal care, can alter HPA axis functioning, changing the trajectory of neurobiological development and hippocampal neuroplasticity, and predisposing individuals to mood disorders in adulthood (Hall and Hall, 1999; Pryce et al., 2005; Hackman et al., 2010; Essex et al., 2011; Daskalakis et al., 2015; Lucassen et al., 2015). However, trying to replicate the effect of early life stress in rodent models proved to be challenging.

The earliest studies by Levine (1967), using mild stressors and human handling on rats, identified what later became known as a stress hyporesponsive period (SHRP) for corticosterone from postnatal day (PD) 4 to PD14 (for a review, see Korosi and Baram, 2010). These early studies suggested that because of low corticosterone responses, rats did not have an equivalent susceptible neonatal period to early life stress. However, subsequent research using more severe and chronic stressors have found that using the right timing and type of stressor, can corroborate species-appropriate phenotypic findings relative to humans subjected to early life trauma. These studies have identified equivalent postnatal critical periods in rodents, the disruption of which can result in both molecular and neurodevelopmental changes as well as behavioural adaptations such as heightened reactivity in adulthood (Teicher et al., 2006; Santarelli et al., 2014; Bian et al., 2015).

One of the most commonly used rodent paradigms for early life stress is repeated maternal separation which has been shown to induce lasting behavioural, cognitive and neurochemical changes (Gracia-Rubio et al., 2016; McCoy et al., 2016; Tractenberg et al., 2016; Reincke and Hanganu-Opatz, 2017). The most robust responses using this protocol require daily removal of the dam from the pups at random times for a duration of 2-3

hours, with the pups separated from each other during the maternal separation period. While additional licking and grooming can make up for shorter intervals of separation, it does not compensate for longer intervals which exceed what would normally be experienced in the wild (Andersen, 2015). Randomizing the removal time and duration interferes with the dam developing anticipatory compensatory behaviours prior to the daily separation (Schmidt, 2010). Keeping the maternal separation duration to no more than 3 hours per day is important as dietary restriction can lead to an upregulation in BDNF and thus might confound results (Duan et al., 2001).

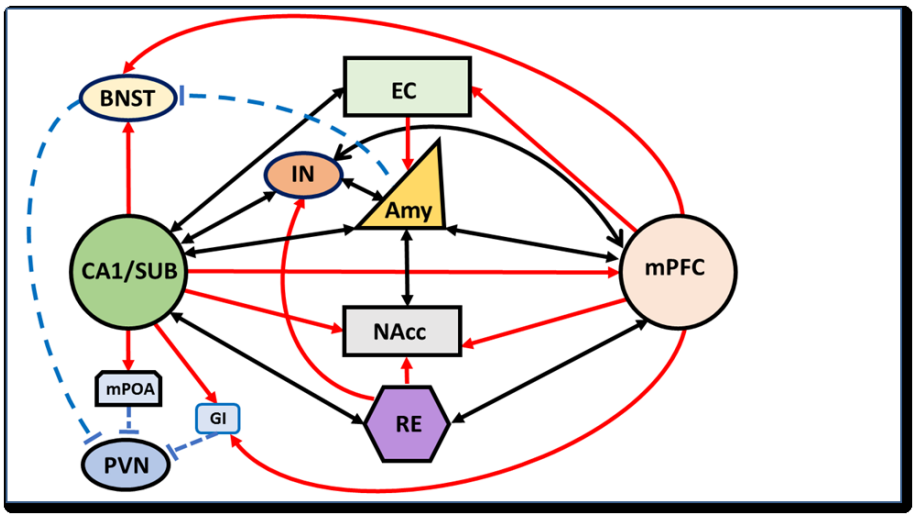

Figure 2. Simplified diagram depicting mood and memory-related circuitry of the medial prefrontal cortex (mPFC) and hippocampus (CA1/SUB).
Bidirectional pathways (in black) between the two regions exist for the nucleus accumbens (NAcc), medial thalamic reuniens (RE), insula (IN) and amygdala (Amy). The mPFC receives direct innervation from the CA1/SUB with reciprocal communication routed via the EC. Inhibitory projections (in blue) to the periventricular nucleus of the hypothalamus (PVN), which are believed to mediate the stress response, come primarily from the GABAergic projections from the medial preoptic area of the hypothalamus (mPOA), the bed nucleus of the stria terminalis (BNST) and GABAergic interneurons (GI). These inhibitory projections are activated via feedback from the mPFC and CA1/SUB. Conversely, activation of the amygdala can increase PVN activation via inhibition of the BNST. Note that for sake of simplicity, only inhibitory pathways to the hypothalamus are included. (Adapted from, and used under creative commons licence from Jin and Maren, 2015)

Initial work using the variations on the maternal separation paradigm focussed primarily on HPA axis functioning and hippocampal development in rats (Pihoker et al., 1993; Plotsky and Meaney, 1993; Gilles et al., 1996; Kalinichev et al., 2002). More recently, attention has turned to examining the influence of maternal separation on neuroplastic mechanisms, such as BDNF distribution. Unfortunately, studies utilizing mice still lag behind research on rats: At the time of this writing, a search for "maternal separation" AND "rats" in Pubmed, resulted in 762 matching documents, while a search for "maternal separation" AND "mice" resulted in 187 documents. When looking for outcomes that influence neuroplasticity, by including "AND BDNF", the PubMed search found 46 studies on rats and only 13 studies using mice.

From developmental studies on rats, it appears that the early ontogeny of BDNF expression in the CNS varies by brain region. In all regions, the lowest expression is immediately after birth and rises rapidly during infancy. In the hippocampus, septum, and cerebral cortex, BDNF expression rapidly increases between PD10 and PD20. Peak expression of BDNF in the septum and cortex is reached between PD20 and PD30, however, this peak does not occur until PD120 in the hippocampus. (Maisonpierre et al., 1990; Katoh-Semba et al., 2002). Data on mice is limited: in the mouse hippocampus, BDNF levels rise rapidly in the early postnatal period and peak at PD 15 (Ivanova and Beyer, 2001). In humans, limited data suggest that BDNF levels in the PFC are low in infancy and adolescence and peak in early adulthood (age 20-24) (Webster et al., 2002). The caveat with these results is that BDNF expression is typically examined using ELISA assays or PCR analysis of mRNA due to the low levels of protein expression in the early postnatal period.

Unfortunately, ELISAs cannot distinguish between the BDNF isoforms, and protein expression can differ significantly from mRNA expression during the lifespan (Rage et al., 2007).

Given that mBDNF potentiates synaptogenesis through TrkB receptors, and is important in the development of both excitatory and inhibitory synapses (Rico et al., 2002; Luikart et al., 2005), we wanted to investigate the role of experience-driven receptor-ligand interaction and expression in early development. Indeed, abnormal BDNF/TrkB signalling has been implicated in developmentally pathological conditions such as Fragile X syndrome, Autism, and Schizophrenia (Hoeffer and Klann, 2010; Wong et al., 2013; Castrén and Castrén, 2014; Martinez-Cengotitabengoa et al., 2015).

There is some evidence that BDNF expression during postnatal development is mediated by experience (Bracken and Turrigiano, 2009), yet little is known about how adverse early life experiences can shape TrkB distribution in the normally developing brain, and thus influence emerging neural circuits related to mood and stress reactivity. This is particularly important given that there are regional changes in TrkB.FL and TrkB.T1 distribution in rodent brains within the first 15 postnatal days (Fryer et al., 1996). However, most studies which have examined BDNF/TrkB signalling, have done so from the perspective of modulating BDNF (Daskalakis et al., 2015), or inhibiting TrkB via general kinase inhibitors such as K252a, which are not specific to TrkB (Koike et al., 2013). Unfortunately, manipulations that focus solely on modulating BDNF, do not address the fact that the TrkB receptor can also be activated by NT3 and NT4/5. Thus, we sought to examine developmental effects by manipulating the receptor function during maternal

separation, and to do so in a model that would allow us to assess stress resiliency later in life.

Chronic unpredictable stress

A significant body of work has established that humans who experience maltreatment in childhood are 2-3 times more likely to develop depression in adulthood, and are at double the risk for treatment-resistance (for a meta-analysis, see Nelson et al., 2017). But not all stress is bad – the "match/mismatch" hypothesis posits that neuroplasticity is a double-edged sword that can induce resiliency or "stress inoculation" if the contexts of adversity later in life mimic those experienced in childhood (Santarelli et al., 2014). Conversely, early life stress can induce maladaptive responses and emotional reactivity in adulthood, if the individual is faced with stressors and environmental contexts which are fundamentally different from those experienced in childhood and/or are inescapable (Schmidt, 2010; Homberg, 2012). Against this background, we were interested in the role of neuroplastic mechanisms in shaping limbic system circuits and behaviour when early life stress (via maternal separation) is followed by a mismatched set of inescapable stressors in adulthood.

Numerous models have been developed for inducing an ethologically relevant depressive-like phenotype in adult rodents. Repeated foot-shock, social defeat, and various forms of restraint stress have all been used (Martinez et al., 1998; Anisman and Matheson, 2005). It is well-established that chronic stress, particularly if it is unpredictable and uncontrollable, is a predisposing factor to mood disorders such as depression and anxiety

(for a review see Pittenger and Duman, 2008). The chronic unpredictable stress (CUS) paradigm subjects animals to a variety of different inescapable stressors (such as wet/no bedding, restraint, predator odors, and light cycle changes), which are applied at random times each day to avoid a habituation and coping response. The CUS paradigm has some advantages over other forms of chronic stress: it does not require additional mice as with social defeat; it is less physically harmful than footshock (and less likely to engender a PTSD-like phenotype); and the random nature impedes the development of coping mechanisms. If applied correctly, and for an appropriate duration, CUS consistently results in a murine depressive-like behavioural and molecular phenotype, with good predictive, construct and face validity (Willner, 1997). While mice cannot tell us if they are ruminating or suicidal, they do, by deviating from species-specific behaviours, exhibit measurable "depressive-like" symptoms when subjected to CUS for several weeks.

"Anhedonia", (the loss of interest in pleasurable things), is a key depressive symptom in humans. In mice, anhedonic behaviour is a primary outcome measure when assessing the effects of CUS, and can be measured through the sucrose preference test (SPT). Mice, like humans, like sweet, palatable food and drink and, after a short introduction period, will generally prefer a sucrose water solution over plain tap water, even when the sucrose solution is placed in a location that is different from their normal water bottle. When chronically stressed, mice revert to habitual behaviour and their preference and motivation to obtain sucrose over water declines (Willner et al., 1987). Mice also enjoy building nests, though there is debate about whether this is a hedonic, cognitive or social behaviour. Nevertheless, chronically stressed mice often develop

deficiencies in nest building, and this can be measured (Deacon, 2006). Weight changes are also a key symptom in human depression, with "typical" depression resulting in weight loss and "atypical" depression resulting in weight gain (Thase, 2007). Anxiety and depression are often co-morbid in humans, and accordingly, there are tests to measure anxious behaviour in mice, such as the open field test (OFT), Y-maze, or elevated plus maze. In these tests, less time spent exploring in open, lighted areas, or in the center of the open field, is consider a sign of anxious behaviour.

Study paradigm

To the best of our knowledge, no one has reversibly inhibited TrkB signalling in infancy in the context of a stressor, and then assessed the long-term consequences. Thus, this was the focus of the present thesis. To reiterate, the "match/mismatch" multi-hit hypothesis suggests that the combination of a severe and chronic early life stress can predispose an individual to greater stress reactivity and depression in the face of chronic stress in adulthood, if that later stress is substantively different from the early stressor. Conversely, early life stress can confer resiliency to chronic stress in adulthood, if that stress is fundamentally similar to the early life experience (Hill et al., 2014; Lesse et al., 2016).

We hypothesized that TrkB-mediated neuroplasticity in early life is required for these behavioural responses to result later in life. Similar to the proposition that impaired BDNF signalling in Met carriers of the Val66Met BDNF polymorphism might confer an advantage to those who experienced early life neglect (Caldwell et al., 2013), we

hypothesized that impairing BDNF signalling via the TrkB receptor during maternal separation might mitigate the stressor effects when confronted with fundamentally different challenges later in life. To test this hypothesis, our study inhibited TrkB.FL phosphorylation (thus inhibiting pathway activation by BDNF) during pups' nursing in combination with 10 days of random maternal separation, as this form of stress has been found to induce lasting changes in the HPA axis and hippocampal circuits in rodents (Sapolsky and Meaney, 1986; Suri et al., 2013; Nishi et al., 2014). We then later exposed mice to a well-accepted CUS paradigm in adulthood (Willner et al., 1987; Ibarguen-Vargas et al., 2008; Yoon et al., 2014), which is fundamentally different from the maternal separation, to determine if impairing neuroplasticity during early stress would reduce later life stress reactivity and its neurobiological correlates (See Figure 3 for study timeline).

To inhibit TrkB/BDNF signalling, our study utilized a $Ntrk2^{tm1Ddg/J}$ (aka $TrkB^{F616A}$) transgenic knock-in (KI) model mouse. These mice have a mixed C57BL/6J;C57BL/6N genetic background and carry a phenylalanine-to-alanine mutation (F-to-A) on the TrkB.FL receptor at amino acid 616 of the intracellular domain. The mutation allows the TrkB.FL receptor to function normally under basal conditions, but rapidly inhibits BDNF-induced autophosphorylation thus significantly knocking-down signalling through TrkB.FL when the small molecule inhibitor 1NM-PP1 is administered in drinking water (Chen et al., 2005). Normal function of TrkB.FL is restored when 1NM-PP1 has cleared the animal's system, which is 2-3 hours after acute administration, and approximately 2 days after chronic administration (Wang et al., 2003; Chen et al., 2005; Kaneko et al., 2008)). *In vivo* functioning of 1NM-PP1 as a TrkB signalling inhibitor via drinking water administration in

TrkB^{F616A} mutants has been verified previously, demonstrating that the drug has no effect on TrkB.FL/BDNF signalling in wild-type animals (Mantilla et al., 2014; Greising et al., 2015). In nursing dams, 1NM-PP1 has been documented to pass through breast milk and cross the blood-brain barrier to fully inhibit TrkB functioning in pups carrying the mutation, leaving wild-type unaffected (Wang et al., 2003; Byerly et al., 2013).

We hypothesized that the loss of plasticity induced by the transgenic knock-down of TrKB.FL signalling would reduce the "psychological imprint" of the maternal separation, thus making the full knock-in TrkB$^{F616A+/+}$ (KI) mice less reactive in the face of a set of novel unpredictable stressors in adulthood. We further hypothesized that these early life interventions would have lasting effects on BDNF and TrkB receptor isoform expression. Indeed, we believe this study of selective TrkB signalling down-regulation in early will provide important data regarding the developmental sensitivity of BDNF and TrkB-mediated neuroplasticity in brain circuitry related to stressor reactivity.

Methods

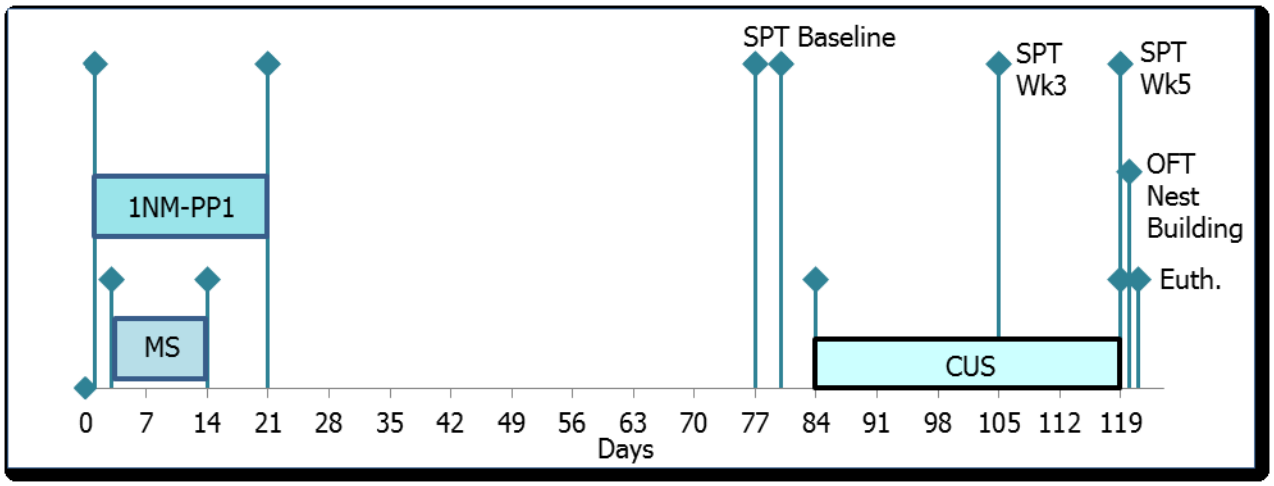

Figure 3. Study Timeline.
TrkB inhibitor administration from birth to weaning at 21 days (1NM-PP1); 10 days of maternal separation from post-natal day 3 to 14 (maternal separation (MS)); Chronic Unpredictable Stress for 35 days (CUS); sucrose preference test administration (SPT); Open field testing (OFT).

Animals

Adult heterozygous Ntrk2$^{tm1Ddg/J}$ (aka TrkB^{F616A}) mice (Charles River, Sherbrooke,QC) were bred to produce litters of pups for this experiment. Mice were housed for breeding with 2 males to 1 female, with nesting material and polypropylene houses in standard polypropylene cages (27 x 48 x 20 cm) until successful mating had occurred, at which point the males were removed. Multiple litters were produced in two cohorts, sufficient to provide at least 10 to 12 mice per genotype/treatment group, including both male and female animals. All dams were given 1NM-PP1 (Cayman Chemical, Ann Arbor, MI, USA, 100 nM) in drinking water (80 µM) from postnatal day (PD) 1 until weaning at 21 days. At weaning, all offspring were sexed and genotyped and moved to single housing with

standard bedding and nesting material. Environmental conditions included a 12-hour light/dark cycle with a room temperature of 21°C and humidity of 50%. Water and food (Harlan Mouse Chow, New Jersey) was provided *ad libitum*. All aspects of this experiment were approved by the Carleton University Committee for Animal Care and adhered to the CCAC ethical standards.

MATERNAL SEPARATION AND CHRONIC MILD UNPREDICTABLE STRESS PARADIGM

To measure the effects of early life stress followed by late-life stress, entire litters were randomly designated as either Stress or Control. From PD 3 to PD 14, Stress group pups were subjected to daily maternal separation for 2 to 3 hours at random intervals. Mothers were removed and housed separately in the same room while pups were separated from each other within the maternal cage on a 37°C heating pad. At 21 days, the pups were weaned, separated into individual cages, sexed and genotyped, and provided nesting material and no other enrichment. Both male and female animals from each litter were used for this study, and estrous cycle was not measured as prior research indicates that female behaviour in the C57 strain of mice does not vary over all 4 phases of the estrous cycle for the behavioural tests used in this study (Meziane et al., 2007).

At 12 weeks of age, pups in the stress group were subjected to 35 days of CUS involving a random series of twice-daily stressors. These stressors were delivered at random times and included: overnight illumination (24 hours of light), strobe light for 12 hours during dark cycle, 30 degree cage tilt, empty cage overnight, wet bedding overnight, 20 minutes of restraint, and exposure to strong odors (wintergreen, menthol) for 4-6 hours.

Animal weights were recorded and behavioural measures for anhedonia (Sucrose preference testing and nest building) and anxiety (Open Field testing) were performed. Via Western blot, we measured expression of TrkB.FL, TrkB.T1, and BDNF in the hippocampus and medial prefrontal cortex (mPFC) as these two regions are important in mediating the stress response (Price and Drevets, 2012). Tissue punches from the mPFC included the prelimbic, anterior cingulate, and infralimbic cortices. Tissue punches from the hippocampus included both dorsal and ventral regions of CA1, CA3 and the dentate gyrus. Tissue was also taken from the Nucleus Accumbens, to be evaluated for mBDNF expression. We also analyzed corticosterone from trunk blood taken at sacrifice to see if mice that had undergone stress had a heightened or blunted stress response relative to controls who had received 1NM-PP1 but were never subjected to stressors. Using immunofluorescence microscopy, we plan to analyze the distribution of TrkB receptor isoforms in the cortex and hippocampus, as well as double cortin (DCX), which is an indicator of neurogenesis.

Behavioural measures

Sucrose Preference Testing

When the mice reached adulthood, at 10.5 weeks of age, all animals were subjected to baseline sucrose preference testing through 4 days of exposure to a choice between 2% sucrose solution and tap water for 15 hours per day, 12 of which were during dark cycle. Our sucrose preference testing approach leverages motivation and side preference to ensure that mice are deliberately seeking out sucrose to drink, rather than drinking it out of habit (which can happen if the sucrose tube is where their water bottle usually resides). On day 1 of Baseline exposure, mice are introduced to both a 2% sucrose solution and

water, with the sucrose solution placed where their water bottle would normally reside. On day 2, the tubes are swapped, with sucrose in the non-water bottle location. On day 3, the sucrose is placed back in the water bottle location, and on day 4 of Baseline, the sucrose bottle is placed in the non-water bottle location again. Baseline sucrose preference values were computed from measures taken on the 4th day. All subsequent exposures to sucrose and water ensured that the sucrose solution was placed in the non-water bottle location. Water and sucrose tubes were weighed before placement and after removal and sucrose preference was computed as:

$$\frac{sucrose\ consumption\ in\ mL}{sucrose\ consumption + water\ consumption\ in\ mL} x\ 100\ .$$

As a reminder, mice were re-exposed to sucrose and water tubes at 21 days, and final sucrose preference measures were taken overnight on day 35 (Week 5) of the CUS regimen.

Weight

All mice were weighed at the start of sucrose preference (Baseline) and at day 21 (Week 3) of the CUS paradigm. Due to data loss, only weights for a random sample of 5-7 mice per treatment group/genotype were available at the end of the CUS regimen (Week5).

Nest Building Testing

At 9am on the day following completion of the CUS paradigm, animals from all groups (stress and control) had their nesting material removed and were given new cotton nestlet pads measuring 6x6x0.5cm. The latency to start tearing at the new pad, and the

quality of the nest were scored manually at intervals of 15, 30, 45, 60, and 180 minutes after receiving the nestlet pad based on the scoring paradigm developed by Deacon (2006).

OPEN FIELD TESTING

Three hours after completion of the nest building test (on the day following completion of CUS), all mice were given an Open Field test for 5 minutes in a box measuring 48cm x 48cm, made of wood with white walls and white floor, in a normally lighted room (approximately 100-200 luminous flux (LUX)) with an overhead camera. Automated scoring of video-recordings was computed Any-Maze™ software (Stoelting Co., Wood Dale, IL). The open field arena was virtually divided into central and peripheral regions with virtual grid lines intersecting the center section and a virtual center square measuring 24cm x 24cm. Each mouse was placed in the top left corner of the open field and allowed to freely explore the space for 5 minutes, with the experimenter not visible. Measurements taken were:

- Total Distance traveled (m)
- Number of Immobile episodes: Frequency with which mice stopped horizontal movement, but may still be grooming, sniffing, tail or ear twitching
- Number of entries to the Center Zone: Frequency with which the mice crossed with all four paws into the central square
- Time in Center Zone: Duration of time the mice spent in the central square (s)
- Distance travelled in Center Zone (m)
- Average speed in the Center Zone (m/s)
- Average speed over the 5 minute test period (m/s)

Biological measures

At completion of the CUS, all animals (both stress and control groups) were randomly assigned to either a rapid-decap or perfusion group, with 4-5 animals per genotype/treatment in each group. All animals were sacrificed in the morning, approximately 18 hours after the Open Field Test. Mice in the rapid-decap group (see Table 1) were humanely euthanized via rapid decapitation, and tissue sections from the medial prefrontal cortex (mPFC), hippocampus, and nucleus accumbens were taken for protein analyses. Trunk blood was also collected from this group for corticosterone analyses.

Mice in the perfusion group (See Table 1) were anesthetized with sodium pentobarbital (200mg/Kg,Euthasol®, Virbac USA) and perfused with saline followed by 4% paraformaldehyde. Whole brains were taken from the perfused animals and incubated in 10% solution for 4 hours, followed by incubation in 30% sucrose solution for 48 hours, after which they were flash frozen and stored at -80C until sliced for immunohistochemistry analyses.

	Rapid Decapitation			Perfusion		
Group	Males	Females	Total	Males	Females	Total
WT Control	2	4	6	1	5	6
WT Stress	3	3	6	5	1	6
HET Control	4	0	4	2	4	6
HET Stress	4	1	5	5	1	6
KI Control	4	0	4	1	3	4
KI Stress	2	3	5	3	2	5

Table 1 - Group Allocations by Genotype and Treatment

WESTERN BLOTTING

Protein quantification

Tissue samples were thawed, placed in a Ripa-Like Extraction Buffer (0.1% SDS, 1 mM Na ortho-vandate in 10 mM tris), and homogenized via sonication. Homogenized tissue was centrifuged for 10 minutes at 4C and 6000rpm, and the supernatant was extracted. The resulting supernatant was quantified using standard procedures (Pierce's BCA Protein Assay Kit). Once quantified, protein was mixed in a ratio of 4:1 with loading buffer, and denatured at 60°C for 5 minutes before being stored at -20°C.

TrkB analyses

Prior research has identified immaturely-glycoslated TrkB.FL overlapping with truncated TrkB proteins at approximately 105Kd in Western Blotting (Martin-Zanca et al., 1989; Rantamäki et al., 2011), which can make it difficult to discern the 95-100Kd band for TrkB.T1. Therefore, we took a novel approach and developed a protocol to remove glycosylation from protein samples prior to blotting for TrkB.FL and TrkB.T1, in order to achieve full separation of the two isoform bands. This technique was validated using 20µg and 40µg of whole brain lysate samples. Bands migrated approximately 21Kd lower as a result of degylcosylation, and we achieved a distinct separation of bands with a 20µg protein load. Though not used in our final assays, a TrkB.T1-specific antibody (Santa cruz, Sc-13) was used to verify that the truncated band did, in fact, migrate to ~66Kd, mirroring what we saw with the Pan-TrkB antibody from BD Biosciences (BD 610101) (See Figure 4).

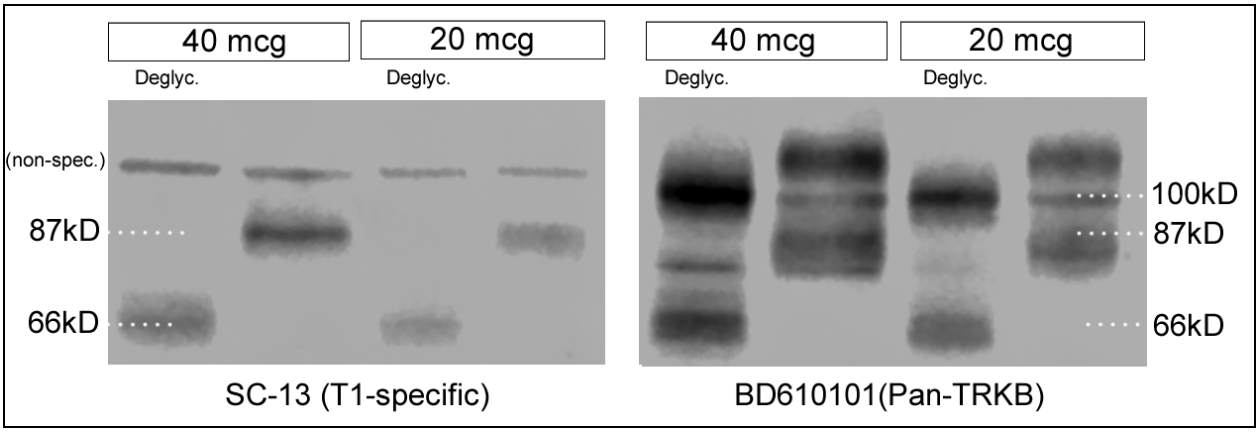

Figure 4. TrkB Deglycosylation Tests

Protein samples of 50µg were deglycosylated with 2µl PNGase F (Promega Cat #V4831), 2µl of Triton X and diluted with 1X loading buffer to 20µL. Samples were then incubated at 37°C for 2 hours and placed on ice for 10 minutes before loading into SDS-PAGE gels. Lanes were loaded with 8µL of sample containing 20µg of protein, and fractionated by SDS-polyacrylamide gel electrophoresis on 10% separating gels based on standard protocols. After electrophoresis, proteins were transferred to polyvinylidene difluoride (PVDF) membranes (Millipore) activated in methanol, and then dried overnight at room temperature. Prior to immunoblotting, membranes were reactivated in methanol for 30 seconds and then incubated at room temperature for 5 minutes with Revert Total Protein Stain (Li-Cor Biosciences, Cat# 926-1101). Membranes were imaged for 2 minutes at 700nm with a Li-Cor Odyssey Fc Imager to obtain total protein bands, and then washed in TBS and blocked for 1 hour in .5% fish gelatin (Sigma, cat #G7041) prior to antibody application. Antibodies were applied in the following dilutions: Primary mouse Anti-TrkB (1:1000; BD Biosciences Cat #610101) in 0.05% fish gelatin in TBST for 90 minutes, and

secondary anti-mouse IgG conjugated with 800nm Infrared Dye (1:5000; Li-Cor Biosciences, Cat# 925-32210) in a 0.5% fish gelatin solution containing 0.2% Tween-20 and 0.01% SDS for 1 hour. Protein bands were imaged using near-infrared at 800nm with the Li-Cor Odyssey Imager Fc for 5 minutes. TrkB.FL and TrkB.T1 bands were quantified using Western Blot densitometry analysis with Li-core Image Studio software (Li-Core Biosciences) and normalized to total protein via standard procedures (Taylor et al., 2013). Intermembrane variability was normalized to total membrane signal control.

BDNF Analyses

15% SDS-Page gels were prepared as previously described, with 15µg of protein per lane loaded. Immunoblotting was performed as previously described, including total protein measurements, with antibodies applied in the following dilutions: Primary mouse anti-BDNF (1:1,000; R&D Systems Anti-BDNF Cat# MAB248) and secondary anti-mouse IgG conjugated with 800nm Infrared Dye (1:15,000; Li-Cor Biosciences, Cat# 925-32210) in a 0.5% fish gelatin solution containing 0.2% Tween-20 and 0.01% SDS for 1 hour. Protein bands were imaged using near-infrared at 800nm with the Li-Cor Odyssey Imager Fc for 5 minutes. Mature BDNF bands for the mPFC, hippocampus and Nucleus Accumbens (NAcc) were quantified using Western Blot densitometry analysis with Li-core Image Studio software (Li-Core Biosciences) and normalized to total protein via standard procedures (Taylor et al., 2013).

CORTICOSTERONE DETERMINATION

Corticosterone was measured from trunk blood taken from animals euthanized by rapid-decapitation. Levels were determined by ELISA using the Enzo Life Sciences

Corticosterone Elisa kit (Cat #ADI-900-097). Results were quantified using a quantified on a Spectramax microplate reader set to 405 nm and to 570-590 nm for correction. Results were processed through Enzo Life Sciences Elisa Analysis at MyAssays.com, which calculates the concentration of corticosterone (pg/mL) in the samples using a Four Parameter Logistic curve.

IMMUNOHISTOCHEMISTRY

Perfused Brain tissue was cut at 15μm on a cryostat and serial sections were mounted on gelatin coated slides. Development of a TrkB staining protocol for frozen, perfused tissue is ongoing.

Statistical analysis

The Statistical package SPSS (Version 20) was used for all data analysis. Corticosterone results were analyzed using the univariate general linear analysis of variance (ANOVA), with follow-up using Bonferroni-adjusted pairwise comparisons. Weight and sucrose preference test results were analyzed using repeated measures ANOVA, with univariate contrast analyses and Bonferroni-adjusted pairwise comparisons for follow-up. Western Blot and open field test results were analyzed with a multivariate ANOVA, with univariate contrast analyses and Bonferroni-adjusted pairwise comparisons for follow-up. Data is presented in the form of marginal mean ± standard error of the mean (mean ± SEM), and differences were considered statistically significant when $p < 0.05$, unless otherwise noted.

Results

Behavioural measures

Both maternal separation and CUS produced anhedonic responses in the SPT

Only mice that exhibited a sucrose preference > 70% on day 4 of Baseline were used (n=54). A simple univariate ANOVA on Baseline measures (prior to start of CUS) revealed that mice in the Stress group had small but significantly lower sucrose preference than Control, ($F_{1,48}$=12.916, p=0.001). The main effect of Genotype in Baseline sucrose preference was also significant ($F_{2,48}$=3.81, p=0.050), but there was no significant interaction between Genotype and Treatment ($F_{2,48}$=2.262, p=0.115), indicating that the maternal separation alone had a anhedonic behavioural effect prior to CUS. A Bonferroni-adjusted pair-wise analysis ($p < 0.025$) found that the KI is only significantly different from the HET under the Stress condition (Mean difference = −6.470 ± 2.097, p=0.010), and that Stress was significantly different from Control only for the WT (Mean difference = −5.224 ± 2.110, p=0.017) and KI (Mean difference = −6.691 ± 2.161, p=0.002) animals. (Mean difference for HET was −0.890 ± 2.044, p=0.665). These findings are represented in Figure 5.

Week 5 (Final) SPT Measures

A three-way mixed repeated measures ANOVA was run to understand the effects of Genotype, Treatment and Time on Sucrose Preference. Five weeks of CUS resulted in a statistically significant three-way interaction between Time, Genotype and Treatment, ($F_{2,48}$ = 3.702, p = .032, partial η^2 = .134). As previously reported, there were no significant

two-way interactions between Treatment and Genotype at Baseline ($F_{2,48}$=2.263, P=0.115). Similarly, a univariate ANOVA did not reveal any significant 2-way interactions at Week 5 ($F_{2,48}$=2.071, P=0.137).

A univariate ANOVA on the difference between Week 5 and Baseline measures identified a significant interaction between Treatment and Genotype ($F_{2,48}$=3.072, p=0.032). Bonferroni-adjusted pairwise comparisons revealed that Stress WT had a significantly greater reduction in sucrose preference relative to Control WT (mean=−15.755 ± 4.155%, p<0.0005), as did Stress HET relative to Control HET (mean=−20.945 ± 4.025%, p<0.0005). The difference in sucrose preference for KI was not significant (mean=−5.215 ± 4.257%, p=0.226). These findings are represented in Figure 6.

Nest Building tests

In general, nest building scores improved over the 3-hour measurement period. A repeated measures ANOVA revealed no significant differences in nest building between Treatment groups or Genotypes, nor any interactions.

Both maternal separation and CUS influenced Weight

A three-way mixed repeated measures ANOVA was run using Baseline and Week 5 weight measures (where data was available, n=34) to understand the effects of Genotype, Treatment and Time on Weight. An initial univariate ANOVA of Baseline Weight found only a main effect of Treatment ($F_{2,28}$=7.073, p=0.013). Though there were no significant interactions between Treatment and Genotype ($F_{2,28}$=0.350, p=0.708), follow-up

Bonferroni-adjusted pairwise comparisons of Treatment by Genotype found that treatment was only statistically significant in KI mice ($F_{1,28}$=4.178, p=0.050).

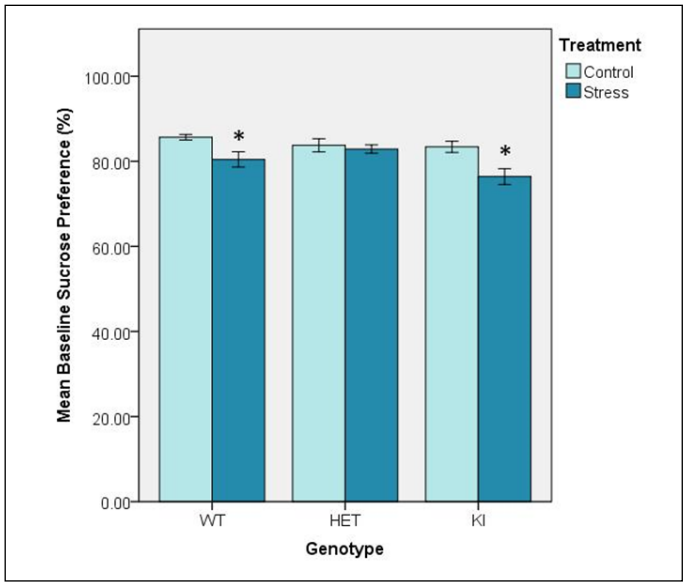

Figure 5. SPT: Baseline Sucrose Preference by Treatment and Genotype.
Stressed WT and KI mice started the 5 weeks of CUS with a reduced baseline relative to their Control counterparts

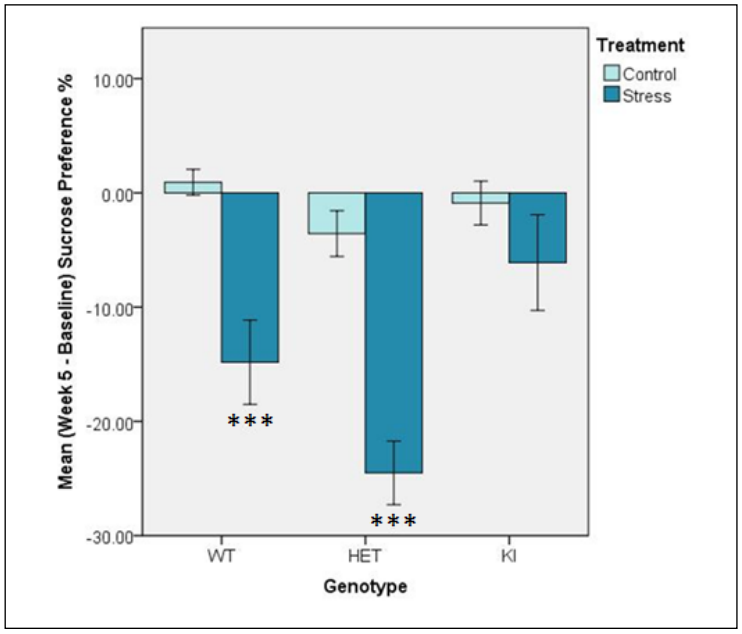

Figure 6 -SPT: Week 5 minus Baseline sucrose preference.
After 5 weeks of CUS, only the Stress HET and WT mice exhibited a statistically significant decline in sucrose preference, relative to their Controls.

Five weeks of CUS resulted in a statistically significant three-way interaction between Time, Genotype and Treatment ($F_{2,28}$ = 5.406, p=.010, partial η^2=0.279). A univariate analysis of Treatment at each time point (using an adjusted significance of p=0.025) found that Stress group mice had a significant marginal mean increase in weight over Controls both at Baseline (1.807 ± 0.679g, $F_{1,28}$=7.073, p=0.013), and at Week 5 of CUS (2.159± 0.669g, $F_{1,28}$=9.544, p=0.004). Univariate analysis at Week 5 did not find any interaction between Treatment and Genotype, however, further follow-up using Bonferroni-adjusted pairwise comparisons found that the only KI mice had a significant difference in weight between Stress and Controls at both Baseline (mean difference=2.540±1.243g, p=0.050), and at Week 5 (mean difference=3.040±1.278g, p=0.024) (See figure 6). A univariate analysis of the mean of individual weight differences between Week 5 and Baseline found that while not statistically significant, Stressed KI mice gained more than Controls (0.5±0.454g, p=0.280), whereas Stress HET mice gained less than Controls (-0.679±0.399g, p=0.100). However, by week 5 of the CUS, Stressed WT gained a significant amount more than Control WT (1.233±0.435g, $F_{1,28}$=8.056, p=0.008) (see figure 7).

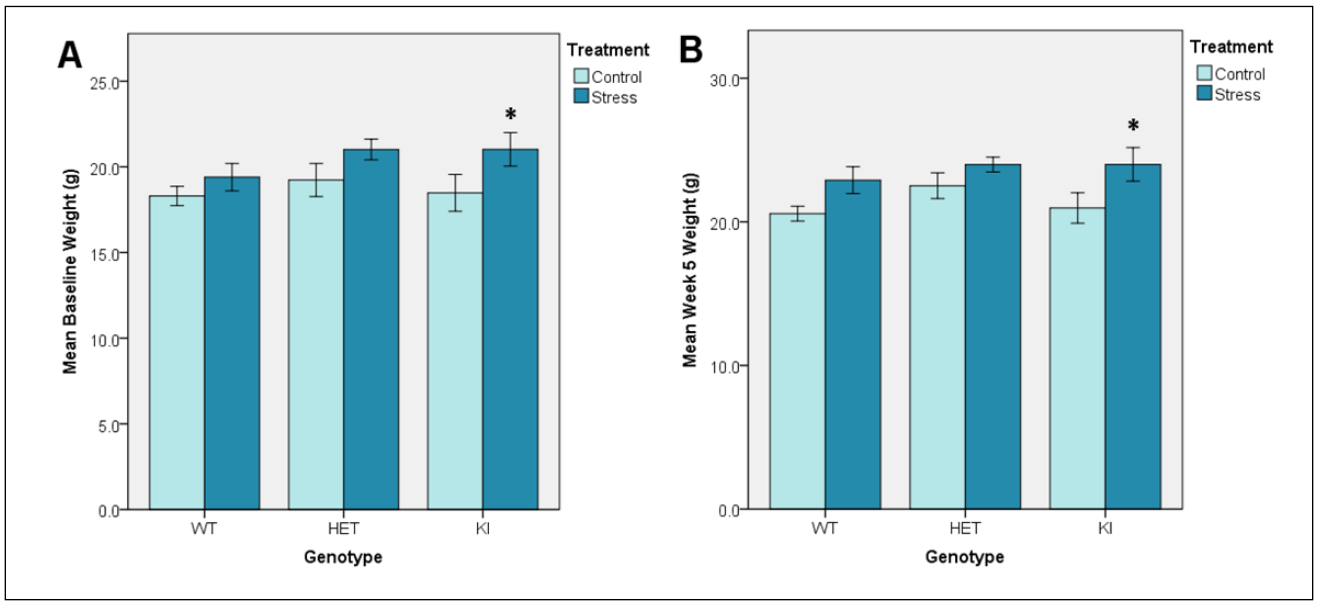

Figure 7. Weight: Baseline and Week 5 of CUS.
Marginal mean weight of Stress KI mice at Baseline (A) and at Week 5 (B) is significantly more than KI Controls.

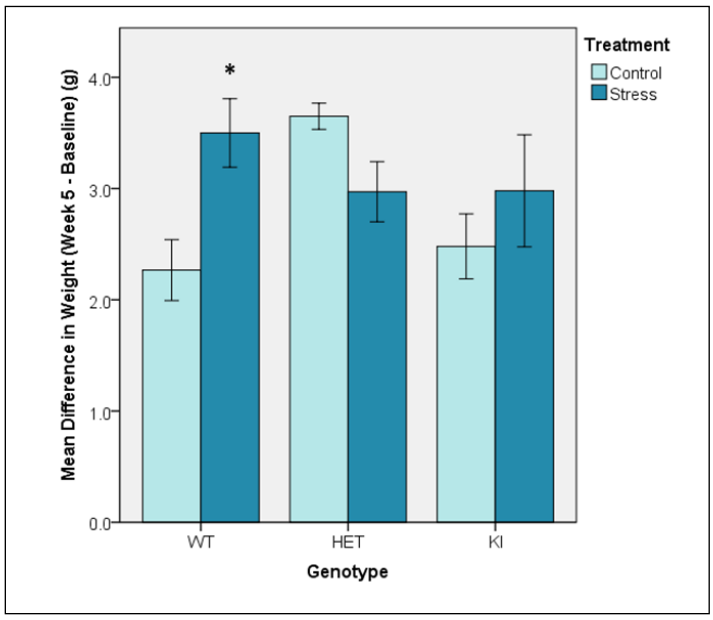

Figure 8. Weight: Difference in between Week 5 and Baseline Weight by Genotype and Treatment.
Five weeks of CUS induced a significant gain in the weight of WT mice relative to WT Controls. The average of individual weight change by Genotype/Treatment was not significant for HET or KI mice

ANXIETY-LIKE BEHAVIOUR IN THE OPEN FIELD TEST (OFT)

Open Field test data was analyzed using a Multivariate ANOVA, as the test parameters are functionally related. Pillai's trace value was used due to unequal sample sizes for treatment group/genotype. Multivariate tests indicated a significant effect of both Treatment ($F_{8,49}$=5.155, p<0.0005) and Genotype ($F_{16,100}$=1.753, p=0.049). The interaction between Genotype and Treatment bordered on significance ($F_{24,92}$=1.679, p=0.063). In a follow-up analysis of individual variables within the OFT using either Univariate contrasts or Bonferroni-adjusted pairwise comparisons, there were no significant main effects or interactions for Total Distance Travelled, suggesting that neither Genotype nor Treatment influenced overall locomotor function. Similarly, there were no significant differences in Average Speed, Number of Entries into the Center Zone nor in Distance Traveled in the Center Zone. Subsequent univariate contrast analysis on Time in Center Zone determined that Treatment had an effect on KI mice ($F_{1,56}$=6.078, p=0.017), and further Bonferroni-adjusted pairwise comparisons determined that Stressed KI mice spent significantly less Time in the Center Zone than their Control counterparts (−18.660±7.569s, p=0.017) (See figure 9). Less time spent in the center zone is generally considered a sign of anxious behaviour. Stressed KI mice also had fewer immobile episodes (−5.33±1.749, p=0.004), relative to KI Controls ($F_{1,56}$=9.296, p=0.004), (see Figure 10)

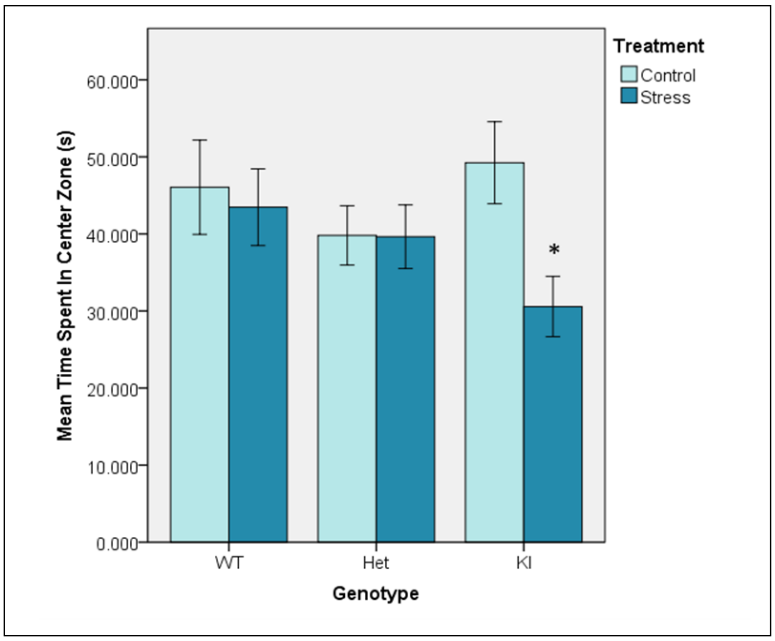

Figure 9. OFT: Mean time spent in center zone.
Only Stress KI mice spent significantly less time in the center zone than their Control counterparts (-18.660±7.569s, *p*=0.17)

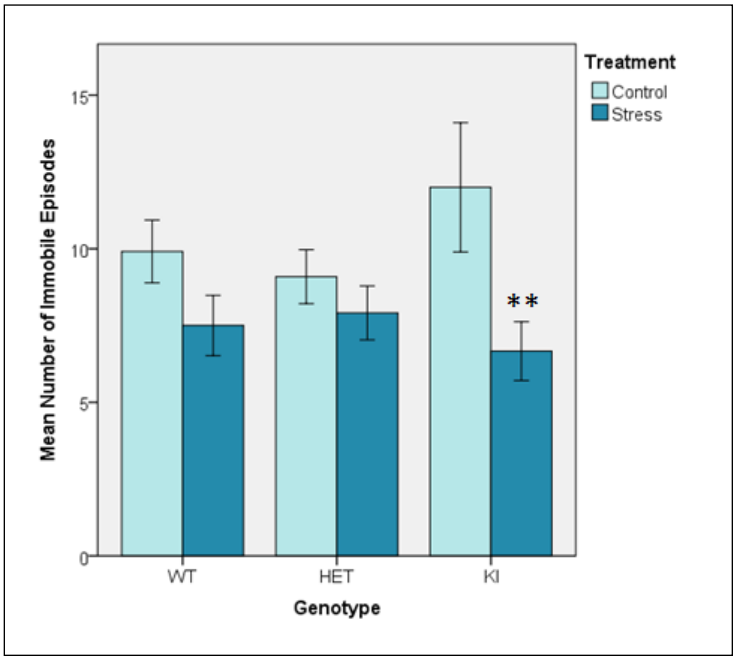

Figure 10. OFT: Number of Immobile episodes.
Stress had a significant effect on the total number of immobile episodes for KI mice relative to Control KI (−5.33±1.749, *p*=0.004)

Biological measures

CORTICOSTERONE

A univariate ANOVA on the natural log transform of Corticosterone concentration identified a significant main effect of Treatment ($F(1,23)=5.512$, $p=0.034$), but no significant effect of Genotype, nor any interaction. Corticosterone concentration was moderately negatively correlated with Sucrose Preference (see figures 11 & 12).

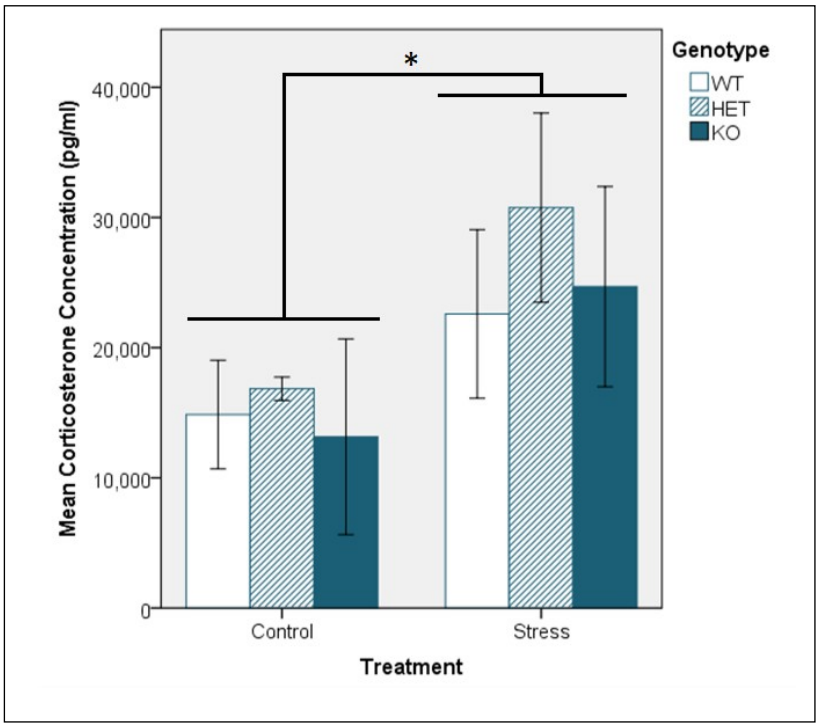

Figure 11. Corticosterone Concentration
Stressed mice had a significantly higher corticosterone concentration than Controls. There were no significant effects of Genotype.

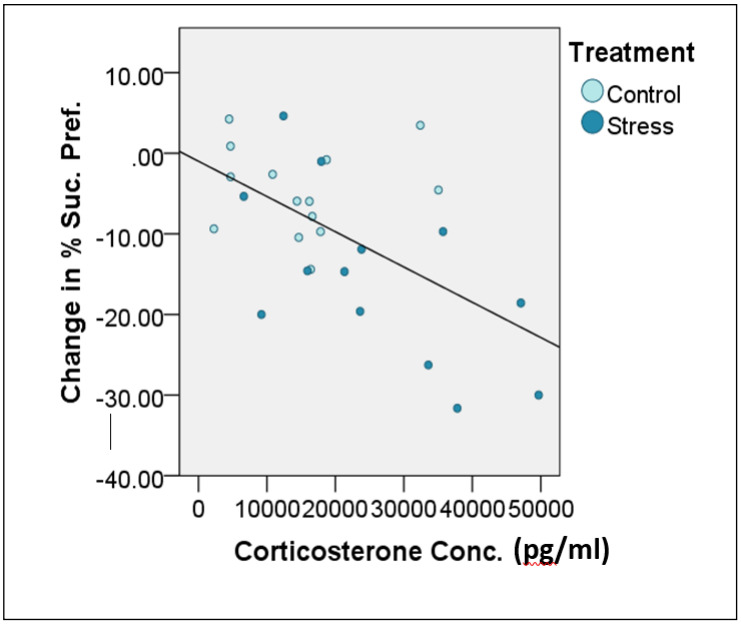

Figure 12. Corticosterone Concentration is negatively correlated with decline in sucrose preference.
Corticosterone levels were moderately negatively correlated with Final (Week 5 – Baseline) Sucrose Preference % (R=-0.572, p=0.001, 2-tailed).

TrkB.FL and TrkB.T1 protein expression in the Hippocampus and PFC

Western blot analyses of TrkB isoform protein levels and ratio are functionally related and thus were analyzed using a multivariate ANOVA. Multivariate tests indicated a significant main effect of both Treatment ($F_{3,44}$=4.194, p=0.011), and Region ($F_{3,44}$=61.647, p<0.0005), with a significant Region by Treatment interaction ($F_{3,44}$=2.837, p=0.049). There were no significant main effects of Genotype nor of Region by Genotype by Treatment interaction. Given the significance of the overall tests, subsequent univariate main and interaction effects of the 3 individual dependent variables were examined using p=0.017 (0.05/3) as the significance cut-off and following effects were identified:

- The main effect of Treatment was significant in TrkB.FL ($F_{1,46}$=7.964, p=0.007), and bordering on significance in TrkB.T1 ($F_{1,46}$=5.523, p=0.023)
- The main effect of Region was highly significant for both TrkB.FL ($F_{1,46}$=137.982, P<0.0005) and TrkB.T1($F_{1,46}$=125.179, p<0.0005)
- Region by Treatment interaction was bordering on significance only for the ratio of TrkB.FL to TrkB.T1 (TrkB.Ratio), $F_{1,46}$=5.938, p=0.019.

To further tease apart Treatment effects within Regions, a further independent multivariate analysis of each region, using an adjusted p-value=0.017 for significance (0.05/3) revealed:

- Within the PFC, Treatment is only significant for TrkB.FL ($F_{1,46}$=8.551, p=0.005) and TrkB.Ratio ($F_{1,46}$=6.765, P=0.012)
- Within the hippocampus, Treatment is significant for TrkB.T1 ($F_{1,46}$=7.437, p=0.009)

It was also interesting to note that the total TrkB.FL protein expression is significantly higher in the PFC than in the hippocampus (Bonferroni-adjusted mean difference=0.085, *p*<0.0005), as is the TrkB.Ratio (Bonferroni-adjusted mean difference=1.304, p< 0.0005) while the TrkB.T1 expression does not significantly differ by region. (See Figures 13-17).

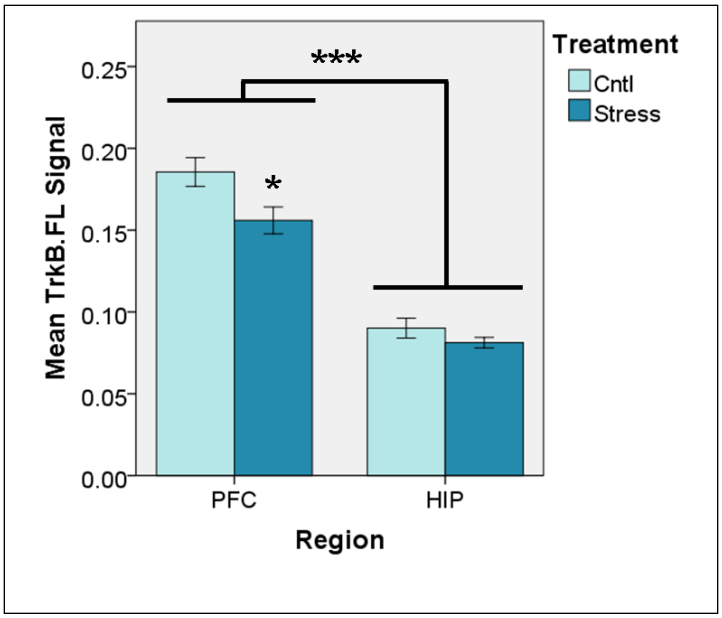

Figure 13. TrkB.FL by Region and Treatment.
TrkB.FL protein expression is significantly reduced in the Stress group in the PFC ($F_{1,46}$=8.551, p=0.005), and overall TrkB.FL expression is significantly higher in the PFC than it is in the hippocampus (HIP) (Bonferroni-adjusted mean difference=0.085, p<0.0005).

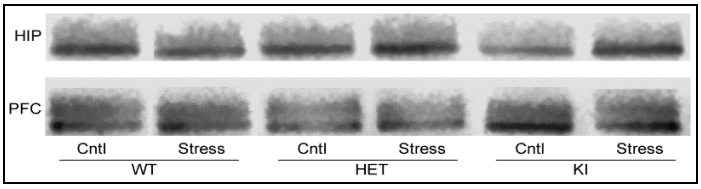

Figure 14. Western Blot, TrkB.FL
Western blots, TrkB.FL by region, genotype and treatment

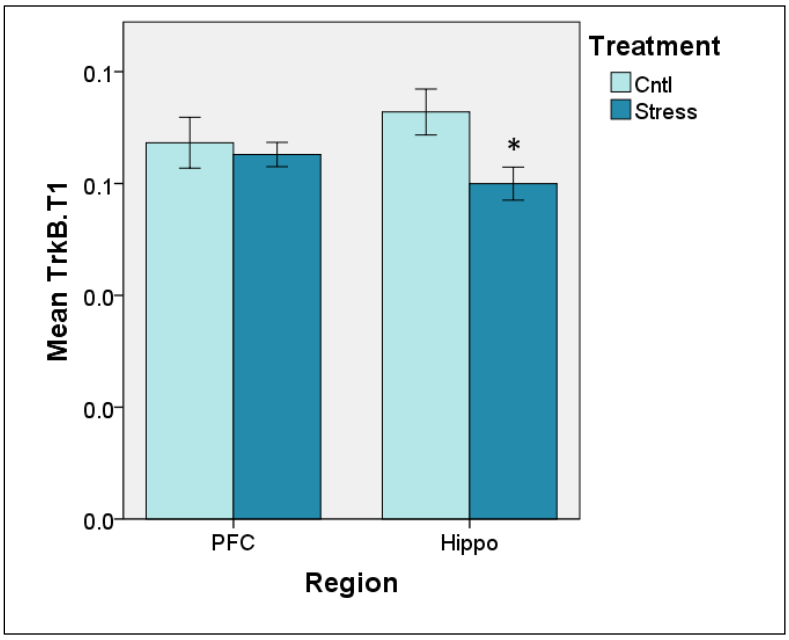

Figure 15. TrkB.T1 by Region and treatment.
There were no significant differences in TrkB.T1 protein expression across regions, however the Stress group had significantly lower expression of TrkB.T1 in the hippocampus (HIP) ($F_{1,46}$=7.437, p=0.009)

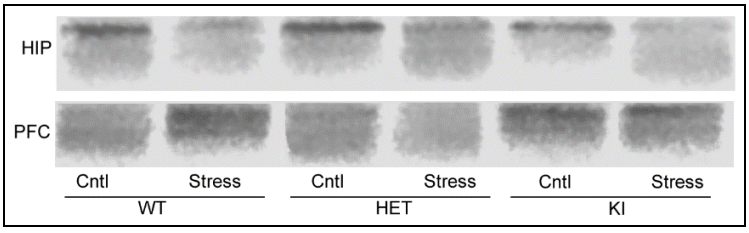

Figure 16. TrkB.T1 Western Blot
Western blot results of TrkB.T1 by region, genotype and treatment

Additional Univariate contrasts for TrkB.T1 in the Hippocampus, using a significance of p=0.017 (adjusted for 3 comparisons), found that there was a significant treatment effect only in HET animals (F1,23=7.045, p=0.014) (See Figure 18). Univariate contrasts of Genotype and Treatment for TrkB.FL in the PFC did not reveal any significant differences.

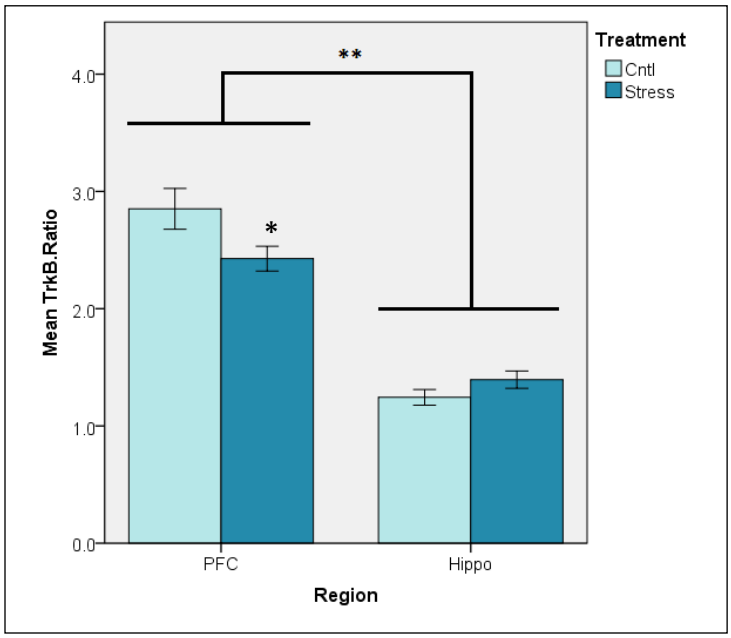

Figure 17. TrkB.Ratio by Region and Treatment.
The ratio of TrkB.FL to TrkB.T1 is significant in the PFC region for the Stress group ($F_{1,46}$=6.765, p=0.012). PFC: Cntl = 2.852±0.115, Stress= 2.389±0.111; Hippocampus: Cntl=1.244±0.115, Stress=1.390±0.111) Ratios remain unchanged in the hippocampus. Overall, the ratios are significantly different between regions $F_{1,46}$=125.179, p<0.0005)

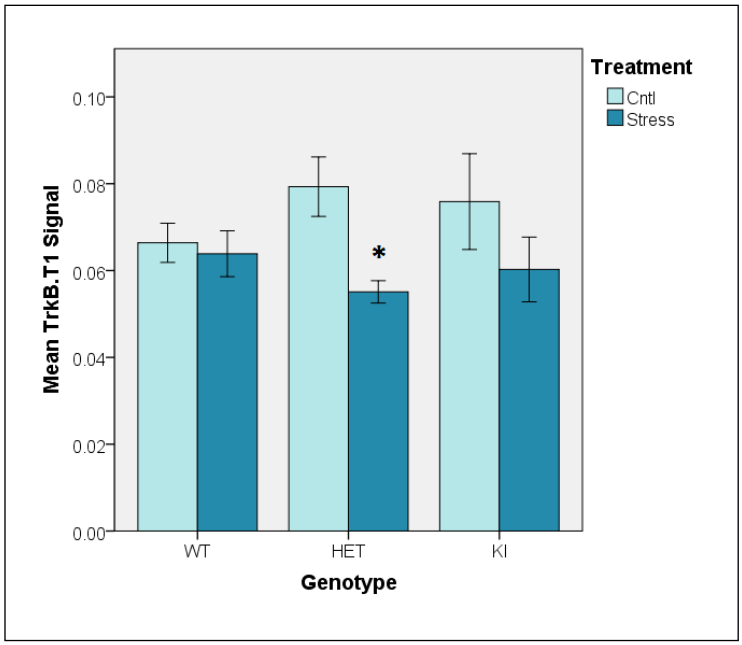

Figure 18. TrkB.T1 signal in the hippocampus by Genotype and Treatment.
Stressed HET mice show a significant reduction in TrkB.T1 expression relative to HET Controls.

BDNF PROTEIN EXPRESSION IN THE HIPPOCAMPUS, PFC AND NACC

Western blot analyses of mature BDNF protein expression was analyzed using a univariate ANOVA to explore regional differences in treatment and genotype in the PFC and hippocampus. Analysis revealed that there was a significant main effect of region ($F_{1,47}$=37.198, p<0.0005), with Treatment bordering on significance ($F_{1,47}$=3.910, p=0.54). While there is no main effect of Genotype on BDNF expression, there is a significant crossover interaction between Genotype and Treatment ($F_{2,47}$=6.248, p=0.004). Follow-up univariate contrasts at a significance cut-off of p=0.017, find that only BDNF expression for the KI Genotype is significantly reduced by Stress in both the hippocampus ($F_{1,23}$=7.224, p<0.013), and the PFC ($F_{1,24}$=7.183, p<0.013) (See Figures 19-21)

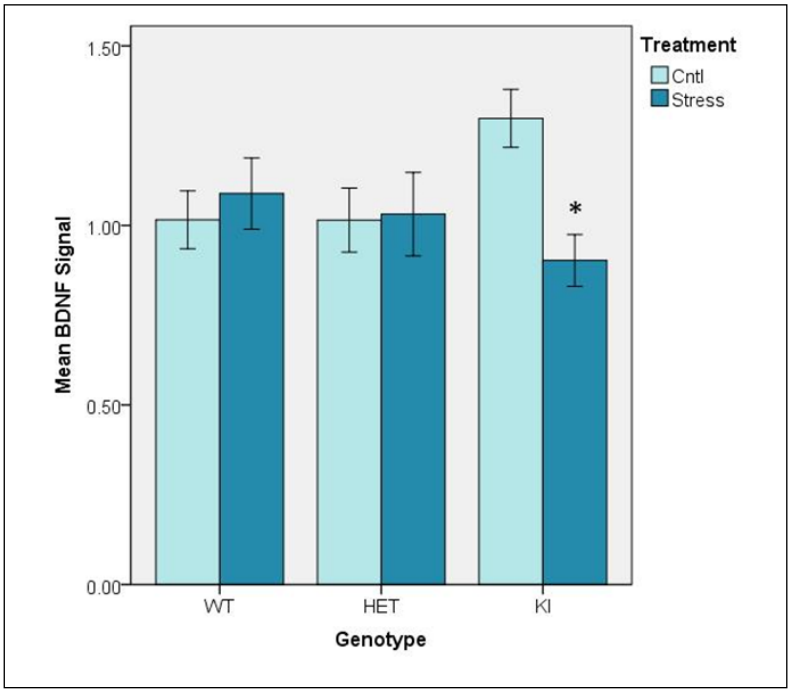

Figure 19. BDNF expression in the Hippocampus.
Treatment only had a significant effect on KI mice, resulting in a 30.4 ± 5.2% reduction in mature BDNF expression in the hippocampus.

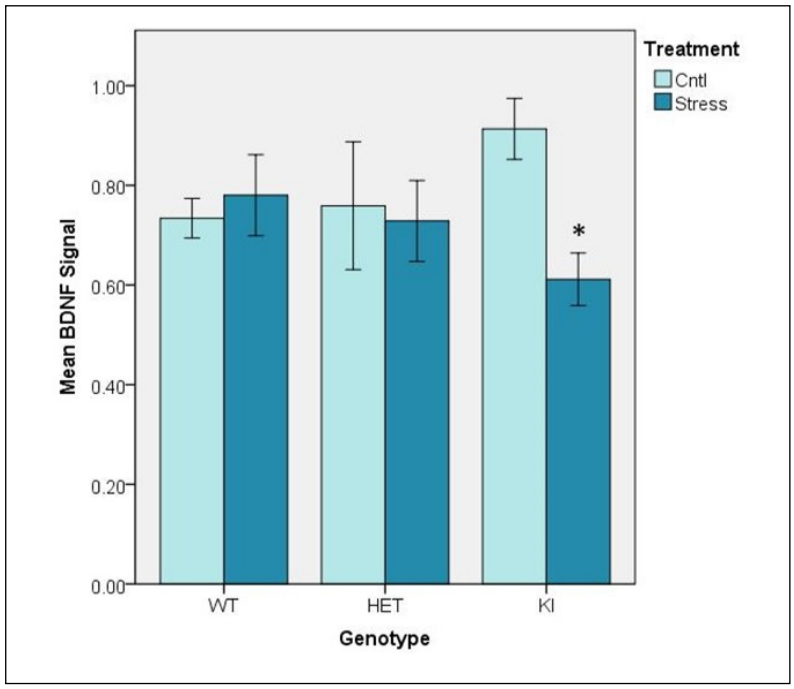

Figure 20. BDNF expression in the PFC.
Stressed KI mice exhibited a 33.1 ± 7.4% reduction in mature BDNF expression in the PFC.

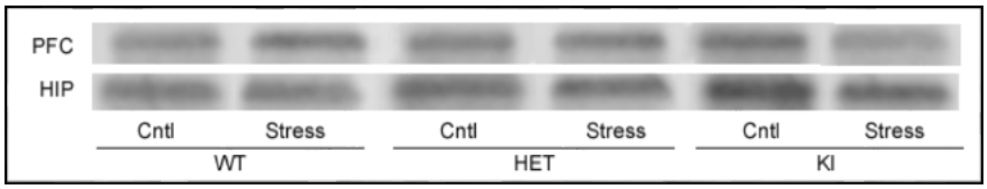

Figure 21. BDNF Western blot for PFC and hippocampus.
Western blot results for mature BDNF by region, genotype and treatment

The pattern of BDNF expression in the NAcc was fundamentally different from that of the PFC and hippocampus. There was a main effect of Genotype ($F_{2,20}$=4.628, p=0.022) but no main effect of Treatment, and no significant interaction. Follow-up univariate contrast tests found that Genotype was only significant within the Stress group

($F_{2,20}$=5.048, p=0.017). Bonferroni-adjusted pairwise comparisons of Genotype within Treatment found that BDNF expression in full KI animals was only significantly different from WT in the Stress group (mean difference = 0.321, p=0.020) (See figure 22)

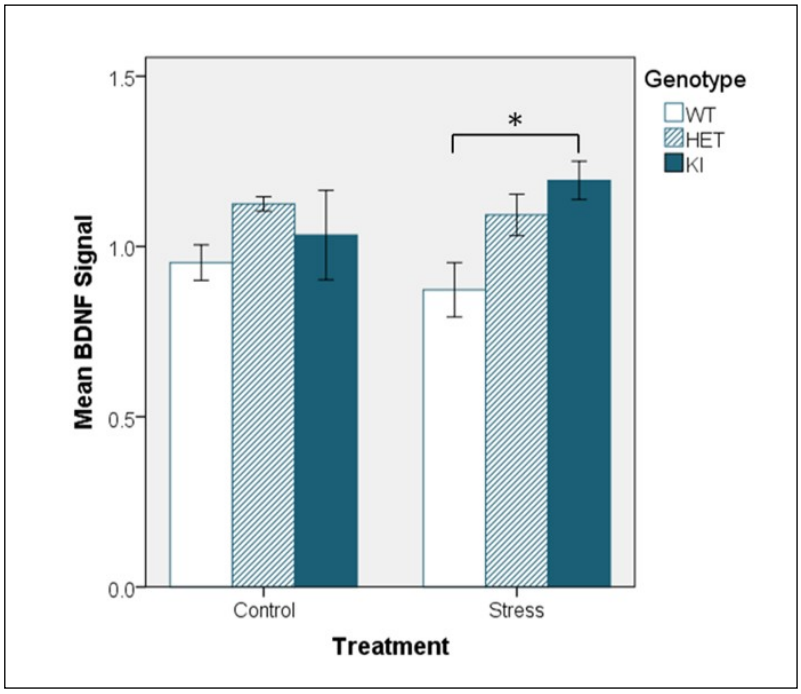

Figure 22. BDNF Expression in the Nucleus Accumbens
Full KI animals within the Stress Group had significantly greater BDNF expression than their WT counterparts (mean difference = 0.321, p=0.020).

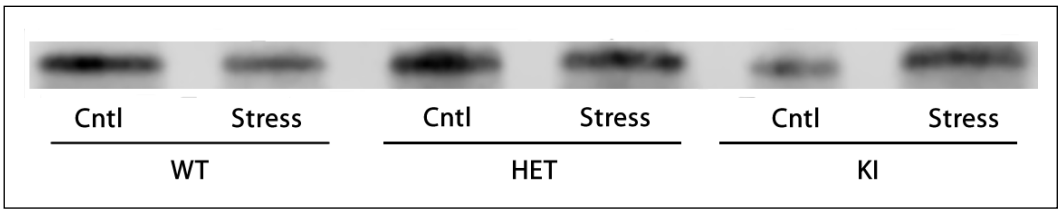

Figure 23. Western blot of BDNF expression in the Nucleus Accumbens

Discussion

This study evaluated the impact of TrkB.FL inhibition during maternal separation followed by chronic unpredictable stress in adulthood. Using the "match/mismatch" hypothesis as a frame of reference, we posited that inhibiting neuroplasticity during early maternal separation stress would result in blunted stress reactivity in adulthood when faced with a chronic unpredictable stressor that was fundamentally different from the early life stress. Measures of sucrose preference revealed that 10 days of maternal separation provoked a modest, but significant anhedonic effect (Figure 5). Importantly, the KI mice appeared to be somewhat protected from the CUS with respect to anhedonic behaviour, as only the WT and HET mice showed a significant decline in sucrose preference after five weeks of CUS (Figure 6). Yet, the stressor treatment reduced time in the center of the open field in the KI mice without affecting overall distance travelled, suggesting that some degree of anxiety or altered exploration was engendered by the TrKB knockdown (Figure 9).

The combination of stressors produced the expected rise in corticosterone; however, there were no significant effect of Genotype, suggesting that glucocorticoid release was not influenced by inhibition of BDNF signalling in early life (Figure 11). With respect to BDNF, there is some evidence to suggest that chronic stress should elicit an increase in BDNF in the nucleus accumbens (NAcc), but we found no significant overall treatment effects. It is interesting to note, however, that within the Stress treatment group, the full KI mice exhibited significantly higher BDNF expression than their WT counterparts

(see Figure 22). These findings are in keeping with the role of the NAcc in anxiety-like behaviours. Though the NAcc is most known for its role in mediating hedonic responses via the mesolimbic-accumbens reward pathway, it is also important in regulating avoidance behaviour (Levita et al., 2012) and responses to threat (Kalin et al., 2005). Studies using retrograde tracers in the amygdala, NAcc, striatum, and ventral tegmental area have suggested that the extended amygdala should include the NAcc shell (Zahm, 2000; Reichard et al., 2017). Indeed, deep brain stimulation to the NAcc in humans resulted in a marked reduction in anxiety ratings in high responders (Bewernick et al., 2010), and significant symptom reduction in patients with refractory obsessive-compulsive disorder (Ooms et al., 2014). Combined, these findings suggest that the increased BDNF in the NAcc of stressed KI mice could explain the enhanced anxiety-like behaviour of this group in the OFT.

In contrast to increased BDNF expression in the NAcc, Stressed KI mice exhibited a significant decline in mature BDNF in the PFC and hippocampus (See Figures 19, 21). Although we found no overall significant treatment effect, prior rodent studies using chronic stress paradigms have reported decreased BDNF protein expression in the PFC and hippocampus (Lakshminarasimhan et al., 2012; Makhathini et al., 2017), and similar reductions in BDNF from these regions have been reported in post-mortem tissue from depressed suicide completers (Hayley et al., 2015). Reduced BDNF expression has also been correlated with anxiety-like behaviour in mouse and rat studies. Transgenic mice that are homozygous for the Val66Met polymorphism have less BDNF, smaller hippocampal volumes, and spend less time in the center of the OFT and open arms of the elevated plus maze (Chen et al., 2006). Similarly, Wystar-Kyoto rats, which have smaller hippocampal

volumes and reduced BDNF signalling, exhibit a behavioural inhibition temperament and more anxiogenic phenotype, features of which can be rescued with bilateral infusion of BDNF into the dentate gyrus of the hippocampus (Janke et al., 2015). What is unclear, is if the BDNF in our Stressed KI mice was already diminished due to the maternal separation, or if the depletion was a result of mechanisms that were triggered by the stress in adulthood. In this context, it is interesting to note that Control KI mice appear to have higher basal levels of BDNF in the PFC and hippocampus than their WT counterparts. This higher basal level may be a compensatory mechanism due to the inhibition of signalling during infancy (as all mice were administered 1NM-PP1), however further studies are needed to confirm and elucidate potential mechanisms. There is some evidence to suggest that adverse biological and cognitive impacts of early stress may not present themselves until middle age (Suri et al., 2013). Our results suggest that BDNF expression later in life may be influenced by the combination of both stress and BDNF/TrkB signalling in early life.

Match/mismatch and BDNF signaling in early Life

Early life BDNF signalling is essential for maturation of hippocampal-infralimbic-amygdala circuits related to fear, and it has been established that reduced BDNF signalling in the hippocampus is associated with anxiety-related behaviours (McEwen, 2001). If we accept that the open field mirrors in some respect, aspects of the isolation experienced during maternal separation, then according to match/mismatch theories, one would expect maternal separation-exposed mice to be more resilient in the OFT where the stressor is considered a match to maternal separation (Bian et al., 2015) . Conversely, we would expect the maternal separation-exposed mice to be less resilient during CUS where the

stressors are fundamentally different from the isolation aspects of maternal separation (See Figure 24).

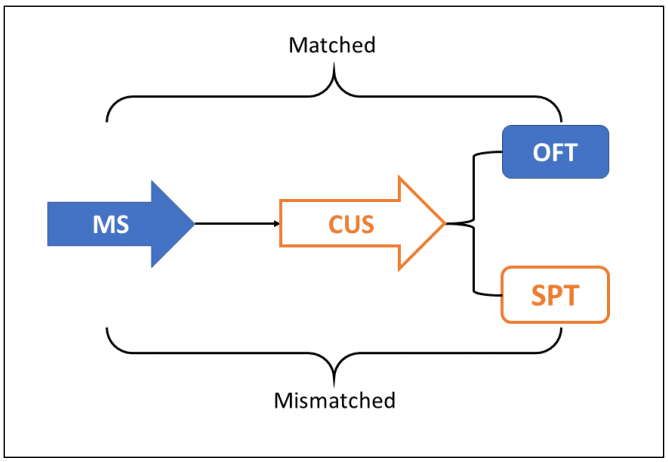

Figure 24. Match/Mismatch in study paradigm

Stress WT mice exhibit exactly this expected behaviour, with no anxious behaviours in the OFT, but a significant decline in sucrose preference. However, Stress KI mice exhibit the opposite behaviour to Stress WT, including enhanced anxiety-like behaviours in the OFT, which is consistent with Val66MET$^{+/+}$ mice where BDNF signalling is reduced (Chen et al., 2006).

It is possible that the reduced BDNF expression in the hippocampus and PFC of Stressed KI mice is influencing anxiety-like behaviours seen in the OFT, as these regions are important in regulating fear-related behaviours (Duval et al., 2015). Indeed, impairing BDNF signalling in the hippocampus has been found to reduce fear extinction and heighten anxiety-related behaviours in mice (Heldt et al., 2007). Combined, these results support our

hypothesis that synaptic plasticity mediated by BDNF signalling through TrkB receptors during infancy is essential to development of stress and anxiety-related circuitry.

Although we did not see BDNF alterations in all stressed animals, alterations in BDNF levels are not always concomitant with depressive phenotypes. In a study of human post-mortem tissue from depressed patients, there were no significant differences in BDNF mRNA transcripts from controls, however expression of the TrkB receptor was diminished, as was expression of parvalbumin (a marker of GABAergic interneurons) and glutamate decarboxylase 2 (necessary for production of GABA) (Tripp et al., 2012). While there are synapses that form in early development in the absence of neurotransmitters and activity (Verhage et al., 2000), it appears that TrkB plays an important role in activity-dependent synaptic regulation and development during critical periods, particularly in the hippocampus (Luikart et al., 2005), Visual cortex (Bracken and Turrigiano, 2009) , and cingulate cortex (Vandenberg et al., 2015) . This highlights the importance of looking beyond individual brain regions, to developmental influences on networks.

TrkB/BDNF signalling influences development of stress reactivity networks

The reduction in TrkB expression seen in stressed mice in our study (TrkB.FL in the medial PFC, and TrkB.T1 in the hippocampus) has precedents in human studies. Changes in receptor isoform distribution have been found in post-mortem studies of suicide-completers, with one group finding a significant reduction in TrkB.FL, but not TrkB.T1 mRNA in the hippocampus and Brodmann area 9 of the PFC (Dwivedi et al., 2003), and another finding downregulation of TrkB.T1 in Brodman areas 8 and 9 of the PFC (Ernst et

al., 2009). Thus, the changes seen in our study may be indicative of a deeper network dysregulation. In support of this theory, we find that there is an emerging body of work which has correlated depressive symptoms to changes in resting-state connectivity between brain regions.

An fMRI study of 51 patients with major depression found that resting global brain connectivity was significantly impaired in the ventromedial PFC, as well as in the subgenual cinculate cortex (Brodmann area 25) (Murrough et al., 2016). Mayberg and colleagues (2005), found that Brodmann area 25 is hyperactive in patients with treatment resistant depression. This region is a hub for functional network connectivity between the hippocampus, PFC, amygdala, insula thalamus and hypothalamus and may have a functional correlate in the dorsolateral PFC. In a study on the efficacy of transcranial magnetic stimulation, the researchers first identified that hypofunction of the left dorsolateral PFC (Brodmann 46) is associated with depression and its activity is inversely correlated with Area 25 activity and depressive symptoms (Fox et al., 2012). Stimulation of Area 46 activation via TMS correlates with reduced Area 25 activity, and alleviation of depressive symptoms, though the biological underpinnings of this effect are not yet understood. How TrkB influences synaptic development in these regions is an area of ongoing research.

The rapid antidepressant effects of low-dose ketamine have resulted in a surge in interest in glutamatergic synapses and depression, yet GABAergic synapses on interneurons and pyramidal cells develop first and are also likely critically important (Ben-Ari et al., 2004). Indeed, Carmona and colleagues (2006) studied $TrkB^{-/-}$ mice from PD2 to

PD9 and found that TrkB signalling in early life is essential to the formation of GABAergic synapses and the regulation of interneuron activity motifs in the hippocampus. These GABAergic interneurons control hippocampal synchronization and mediate excitability through calcium signalling (Zheng et al., 2011). TrkB activity is also necessary in development for the maturation of inhibitory interneurons in the cingulate cortex that synapse with pyramidal neurons and projections from the pons (Vandenberg et al., 2015). In fact, a growing body of work has demonstrated that BDNF/TrkB signalling during development may be more important for cell-specific maturation of inhibitory rather than excitatory synapses (Hong et al., 2008; Sakata et al., 2009; Vaz et al., 2011; Ghosal et al., 2017). This work has translational relevance as a number of studies point to a developmental imbalance between excitation and inhibition in depression (Sanacora et al., 2004; Gabbay et al., 2012).

Future work

Effects of maternal separation and sex on F616A mice

Breeding unfortunately led to an insufficient number of males and females for each Genotype/Treatment group to be able to analyze sex as a factor, and there is emerging evidence that sexual dimorphism in brain regions such as the nucleus accumbens (Hodes et al., 2015) may play a role in the etiology of mood disorders. (Notably, a recent meta-analysis of structural MRI volumes, corrected for total brain volume, found no sex differences in the size of the hippocampus (Tan et al., 2016)). Similarly, we had insufficient numbers to provide for sacrifice of animals to assess the biological effects of maternal separation alone. An additional factor, maternal behaviour on return to the home cage, was

not monitored, and it is possible that variations in maternal care may have had an influence. A follow-up study is being planned to monitor maternal care, analyze sex as a factor, and run molecular assays at 3 months after maternal separation. It is important to determine if the changes in BDNF and TrkB receptor distribution can be mediated by maternal separation alone, as prior work has established that this stressor can result in molecular and behavioural changes that vary from adolescence to adulthood.

GLYCOSYLATION INTERFERES WITH IMMUNOHISTOCHEMISTRY

Another caveat of this study was our inability to successfully produce an immunofluorescent stain for unphosphorylated TrkB in previously frozen perfused tissue. Further complicating this issue is the discontinuation of the only commercial antibody available for the truncated TrkB.T1 receptor, making visualization of individual receptor isoforms exceedingly challenging. We had hoped to be able to use immunofluorescence to co-label cells by using one antibody that targeted the intracellular domain (red), and another which targeted the extracellular domain (green). The goal was to be able to distinguish TrkB.T1 from TrkB.FL by subtracting regions that were fluorescing in the red channel from those fluorescing in the green channel. Unfortunately, we were not able to obtain sufficient antibody penetration despite trying three different protocols. We recently discovered that poor antibody penetration is apparently a known issue (private correspondence with Dr. David Kaplan of SickKids Neurosciences and Mental Health, Toronto, ON), and is related to the heavy N-glycosylation of the external residues of the TrkB receptor (Martin-Zanca et al., 1989) interfering with antibody binding. Next steps will involve attempts at deglycosylating tissue in an attempt to improve antibody binding

at the extracellular domain. We hope to obtain transgenic TrkB.T1-flagged mice from Dr. Lino Tessarolo at the National Cancer Institute of the NIH at a later date to further facilitate imaging studies.

The deglycosylation steps taken in our study before Western Blotting for TrkB have only a few precedents in existing literature for TrkB research (Cabelli et al., 1996; Puehringer et al., 2013; Liu et al., 2014). Moreover, no studies analyzing developmental receptor expression and distribution have used this technique to differentiate between TrkB.FL and TrkB.T1. This is perhaps why our results revealed more TrkB.FL than TrkB.T1 in mature brains, which conflicts with earlier studies (Armanini et al., 1995; Eide et al., 1996; Fryer et al., 1996). It is possible that our technique did not identify all TrkB; however, it is equally possible that prior studies using pan-TrkB antibodies may have misidentified immaturely-glycosylated TrkB.FL as TrkB.T1. It is interesting to note that Arango-Lievano and colleagues (2015), found very closely-spaced double phosphorylated bands of TrkB in cultured HEK-293 cells, which closely match the double bands seen in Pan-TrkB blots.

Developmental expression of TrkB isoforms

There appears to be a significant gap in our understanding of developmental TrkB receptor isoform expression in the mouse brain. Indeed, there are few studies that use quantitative techniques which unequivocally identify each isoform, and distinguish between mRNA transcription and protein expression. Further, developmental influences on isoform expression are not well understood: the first TrkB.FL$^{-/-}$ constitutive knockout

mutant showed a 50% reduction in truncated TrkB protein in both -/- and +/- mice, despite containing WT levels of truncated mRNA transcripts (Klein et al., 1993).

We believe this is the first work to employ a rodent model to examine the effect of stress on TrkB.FL/TrkB.T1 protein expression ratios in the hippocampus and PFC. As a follow-on to our current study, we hope to ascertain if the observed reduction in hippocampal TrkB.T1 expression is a result of the combination of stress and impaired TrkB.FL signalling in infancy. There is also a need to differentiate TrkB.T1 expression on neurons vs. astrocytes, so that developmental changes can be identified by cell type in light of recent work implicating glial cells in psychiatric disorders (Rajkowska and Miguel-Hidalgo, 2007; Imbe et al., 2012; Czeh et al., 2013; Bernstein et al., 2014). Expression of astrocytic glutamate transporters, in particular, have been linked to depressive-like behaviours in rodent models (Chen et al., 2014), and their expression has been associated with TrkB/BDNF signalling in the antidepressant response to ketamine (Liu et al., 2016). Thus, future studies will attempt to identify if early life stress differentially influences TrkB.T1 expression by cell type, and if that expression correlates with changes to key synaptic proteins implicated in depressive phenotypes.

THE TRKB^{F616A} MUTANT AS A MODEL FOR STUDYING BDNF SIGNALLING

Nineteen studies to date have utilized the TrkB^{F616A} mutants for analysis of TrkB signalling in studies on nociception (Renn et al., 2009; Wang et al., 2009), neuromuscular function (Greising et al., 2015), hypothalamic function (Bariohay et al., 2009; Byerly et al., 2013), inhibitory circuity maturation (Vandenberg et al., 2015), spinal cord injury (Zhan et al., 2013; Mantilla et al., 2014), traumatic brain injury (Aungst et al., 2013), motivated

behaviour (Johnson et al., 2008), antidepressant drug signalling (Rantamäki et al., 2011), circadian rhythms (Girardet et al., 2013), retinal circuitry (Kaneko et al., 2008; Majumdar et al., 2011), tests of TrkB agonists (Jang et al., 2010; Choi et al., 2012), receptor sequestration (Mou et al., 2011), glucocorticoid receptor interaction (Arango-Lievano et al., 2015, 2016), and most recently, the interaction of RhoGTPase signalling (Hedrick et al., 2016). This body of research demonstrates that this transgenic line of mice is a valuable model for analyzing the influence of TrkB.FL receptor function.

Our study results clearly show that knocking down BDNF signalling through TrkB.FL during infancy resulted in lasting behavioural and biological outcomes. Given that several other papers have validated the effectiveness of 1NM-PP1 inhibition of the $TrkB^{F616A}$ mice, and given our limited number of animals per cohort, we did not perform our own validation steps to ensure that phosphorylation of TrkB.FL was inhibited during drug administration. The original development of the mutant and early studies validated only that autophosphorylation at central tyrosine residues, and phosphorylation of the Y515 SHC binding site were inhibited during 1NM-PP1 administration (Chen et al., 2005; Johnson et al., 2008; Wang et al., 2009). More recently, in vitro work established the knockdown of phosphorylation at the tyrosine site Y816 (Arango-Lievano et al., 2015).

To the best of our knowledge, only Byerly and colleagues (2013) have looked at the developmental effects of transiently inhibiting BDNF/TrkB Signalling, with a focus on hypothalamic development and feeding behaviours. More detailed studies are needed to determine the full influence of TrkB.FL/BDNF signalling in the development of other brain regions. Finally, 1NM-PP1 does not inhibit TrkB.T1 signalling in $TrkB^{F616A}$ mutants, and

there is still a dearth of information on the functional role of the truncated receptor, particularly on astrocytes. We hope to pursue further work both *in vivo* and *in vitro*, utilizing $TrkB^{F616A}$ and TrkB.T1 knockout mutants, to map out early developmental distribution of both receptor isoforms, and to further elucidate the role of the TrkB.T1 receptor in normal development, synaptic growth and maintenance.

Taken together, the present data are consistent with the contention that early life events that impact neuroplasticity can influence the later development of depressive-like outcomes. Furthermore, the differential expression of TrkB isoforms that we found within the hippocampus and prefrontal cortex might be important for the long-term effects of early life stressors.

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