Truncated Tyrosine Kinase Receptor B (TrkB.T1) plays a role in
susceptibility to chronic stress

by

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Abstract

Brain-derived neurotrophic factor (BDNF) and its receptor, tyrosine kinase B (TrkB), have been implicated in depressive and anxiety disorders. In fact, the efficacy of antidepressant agents may be related to BDNF-TrkB signaling fueling important neuroplastic changes. Interestingly, besides the full-length version of TrkB, the receptor can also be alternatively processed into a truncated form, TrkB.T1, which can act in an inhibitory capacity. However, there is reason to believe that TrkB.T1 might also have other novel signaling effects that might be relevant for dealing with stressors. To this end, using mice lacking TrkB.T1 and wild type littermates exposed to a chronic stressor regimen, we assessed behavioral and TrkB expression profiles, as well as gut microbial profile (given its recent links with anxiety). Our results showed that while the repeated different stressor regimen did markedly alter open-field performance, this was unaffected by the TrkB.T1 knockout. A sex effect was apparent, however in that male mice showed the greatest exploration abnormalities. Moreover, TrkB expression within the amygdala also varied as a function of sex; yet, there were no observed difference in the gut microbiota among the groups. These data do not support a major role for TrkB.T1 in modulating anxiety phenotype, but there were many limitations of this thesis study. Further experiments will focus more comprehensively upon the phenotype of TrkB.T1 mice, particularly with regards to depressive-like pathology.

List of Abbreviations

5-HT	Serotonin
ACTH	Adrenocorticotrophic hormone
ANOVA	Analysis of variance
AVP	Arginine-vasopressin
BDNF	Brain derived neurotrophic factor
BLA	Basolateral amygdala
BOLD	Blood-oxygen-level-dependent
Brd-U	Bromodeoxyuridine / 5-bromo-2'-deoxyuridine
Ca ²⁺	Calcium
cAMP	Cyclic adenosine monophosphate
CBT	Cognitive behavioural therapy
CeA	Central nucleus of the amygdala
CORT	corticosterone
CREB	cAMP response element-binding protein
CRH	Corticotrophin releasing hormone
CUS	Chronic unpredictable stress
DNA	Deoxyribonucleic Acid
ENS	Enteric nervous system
EPO	Erythropoietin
EPOR	Erythropoietin receptor
fMRI	Functional magnetic resonance
GABA	Gamma aminobutyric acid
GF	Germ-free
GSK-3	Glycogen synthesis kinase 3
HPA	Hypothalamic-pituitary-adrenal
IL-6	Interleukin-6
JB-1	Lactobacillus rhamnosus
KO	Knockout
LTD	Long term depression

LTP	Long term potentiation
MAPK	Mitogen-activated protein kinase
mRNA	Messenger ribonucleic acid
NMDA	N-methyl-D-aspartate
p75NTR	p75 neurotrophin receptor
PFC	Prefrontal cortex
PIK-3	Phosphoinositide 3-kinases
PKC	Protein kinase C
PLC-y	Phospholipase C-y
PTSD	Post-traumatic stress disorder
SAM	Sympathetic-adrenal medullary
SNP	Single nucleotide polymorphism
SNRI	Selective norepinephrine reuptake inhibitors
SP	Substance protein
SPF	Specific pathogen free
SSRI	Selective serotonin reuptake inhibitor
tPA	tissue plasminogen activation
Trk	Tropomyosin receptor kinases
TrkB	Tyrosine kinase B
WT	Wild type

Truncated Tyrosine Kinase Receptor B (TrkB.T1) plays a role in susceptibility to chronic stress

Introduction

Background

The monoamine treatment approach to depression and anxiety centers around the use of selective serotonin reuptake inhibitors (SSRIs). Various studies and current research indicate that SSRIs may not be as effective in treating depression and anxiety as previously thought. Medical practitioners have utilized various strategies such as switching to another SSRI if tolerance develops, although the following “switch” does not demonstrate effective results. Therefore, for effective results after tolerance has developed in an individual, the best current practice is to switch to another class of antidepressants that is not an SSRI (Papakostas et al., 2008). Selective norepinephrine reuptake inhibitors (SNRIs) are another class of antidepressants used. Many monoamine treatment approaches seem to be ineffective in some depressed individuals, a term known as SSRI or SNRI refractory. It has been suggested that SSRI/SNRI refractory depressed individuals may be irresponsive due to higher levels of the pro-inflammatory cytokine, interleukin-6 (IL-6) (Yoshimura et al., 2009), which may create an inflammatory milieu that could negatively impact monoamine utilization, as well as adversely affect homeostatic neuroplasticity. In one study looking at a

single dosage (0.75mg.kg) of setraline (substance of SSRI), it was evident that there is a decrease of IL-6 mRNA expression (Sitges et al., 2014). Interestingly, a drop of IL-6 seem to be an indicator of improvement (Galecki et al., 2018). In order to have an effective treatment of anxiety or depression, multiple factors such as anti-inflammatory processes must be kept in mind. Brain derived neurotrophin factor (BDNF) has been shown through multiple studies to play a major role in the efficacy of anxiety and depression treatment. It has been demonstrated that an individual suffering from depression displays changes in brain derived neurotrophin factor (BDNF) levels as depressed patients (SSRI/SNRI responsive as well as refractory) exhibit decreased levels of BDNF (Yoshimura et a., 2009).

Ketamine is an N-methyl-D-aspartate (NMDA) antagonist that displays rapid response as an antidepressant. Due to NMDA's rapid bioavailability in individuals, antidepressant effects are seen within 1-2 hours after ketamine intake. Though regarding its route of administration, studies have demonstrated that for a more effective response, ketamine should be administered intravenously at a slow pace, likely owing to its unique pharmacokinetic properties (Singh et al., 2016). Normally, NMDA inhibits the BDNF receptor (TrkB, Tropomyosin receptor kinase B), but upon antagonizing the NMDA receptor, the monotonic inhibition on TrkB will be released. This causes the activation of BDNF ligand specific TrkB receptor. By activating TrkB receptor, BDNF levels increase due to increase of protein translation (Yoshii & Constantine-Paton, 2010). Though ketamine has positive effects on depression, it does not directly affect the hypothalamic-pituitary adrenal (HPA) axis (Brachman et al., 2016). Interestingly, Ketamine also does not have any influence on the neurochemical impact of acute behavioral stressors (Yamada & Jinno,

2019). Similar to ketamine, erythropoietin (EPO) seems to also stimulate the BDNF robustly (Osborn et al., 2013).

Ketamine is classified as an ionotropic glutamate NMDA receptor antagonist. Though the mechanism behind ketamine's fast-acting responses towards depressive symptoms is proposed to be by elevating BDNF translation as well as secretion (Garcia et al., 2008). Ketamine not only has effects on BDNF expression, but it also seems to have an effect on inhibiting glycogen synthesis kinase 3 (GSK-3). Furthermore, studies have indicated that by inhibiting GSK-3 in certain regions of the brain such as the hippocampus and the prefrontal cortex (PFC), depressive symptoms are alleviated (Ma et al., 2013). With that being said, the dosage at which ketamine is administered can be very critical in regard to how long ketamine effects last and how rapid its effects commence. It is suggested that ketamine is most effective when administered at low doses (0.5mg/kg) through intravenous methods. By giving 0.5mg/kg to a patient, the individual can experience relief from depressive symptoms within two hours and such relief can last of to two weeks (Shwartz et al., 2016).

In addition to novel treatment options for depression/anxiety, erythropoietin (EPO) has been introduced as a new target pathway due to its similar effects to ketamine (Osborn et al., 2013). EPO is a hematopoietic growth factor that is mainly involved in effecting the hematopoietic system, but evidence has demonstrated that it has an effect on multiple cells including various immune cells. One main effect of EPO is that upon binding to erythropoietin receptor (EPOR), it demonstrates neurotrophic as well as neuroprotective

effects (Ma et al., 2016). Interestingly, studies have demonstrated that the induction of EPO through electroconvulsive seizures in an animal model resulted in an increased expression of BDNF particularly in the hippocampus. Furthermore, the following increased expression in BDNF in the hippocampus demonstrated antidepressant like effects (Viviani et al., 2005). Though the following research findings may seem promising, other research studies question the efficacy in treating depressive-like behavior particularly in animal models (Leconte et al., 2011). Regardless of the conflicting results in examining EPO's efficacy in treating depression, one human study indicates that using EPO in treatment resistant depressed patients results in increased plasma levels of BDNF (Vinberg et al., 2015). In addition to mood alleviation, EPO treated patients also displayed an increased blood-oxygen-level-dependent (BOLD) signal via functional magnetic resonance (fMRI) in the left amygdala indicating the amygdala proper activation and response to all kinds of responses including disgust, happy, fearful, and neutral faces (Miskowiak et al., 2008).

Role of neurotrophic factors on anxiety

BDNF is observed in many regions of the brain such as the prefrontal cortex, hippocampus, thalamus, amygdala, and various other regions. The ligand specific receptors for BDNF are TrkB and p75 neurotrophin receptor (p75NTR), but TrkB has higher affinity to BDNF ($\sim 10^{-11}$ M in dissociation constant (Kd)). The p75NTR on the other hand has lower affinity to BDNF ($\sim 10^{-9}$ M in Kd) (Razavi et al., 2015; Sakuragi et al., 2013). The precursor peptide is known as pro-BDNF which then transforms into mature BDNF through the trans-golgi apparatus (Greenberg et al., 2009). Through many animal experimental studies, it has been proposed that BDNF may promote antidepressant/anti-

anxiogenic effects on rodents who undergo learned helplessness and inescapable shock. Levels of BDNF are normally downregulated through undergoing chronic stressors, but the alleviation of depressive-like symptoms are noted when BDNF levels increase in various regions of the brain such as the hippocampus. The mechanistic action of ketamine is that it is a non-competitive NMDA antagonist. Upon the activation of NMDA receptor, ketamine enters the NMDA receptor channel and blocks the flow of cations. Normally, the overactivation of NMDA receptors creates an overflow of calcium (Ca^{2+}) influx which causes excitotoxicity within the neuron. Therefore, ketamine works great due to the blockage of overflow of cations such as Ca^{2+} ions (Liu et al., 2001). Ketamine's high affinity receptor is TrkB, which is located presynaptically and post-synaptically. Therefore, the activation of such receptors stimulates neurotransmitter release as well as postsynaptic responses (Björkholm & Monteggia, 2016).

Effects of BDNF on anxiety-like disorders

As mentioned earlier, BDNF has been shown to play a major role in cell survival and neuroplasticity. Abnormal levels of this neurotrophic factor are suggested to induce depressive-like and anxiety-like symptoms (Janke et al., 2015). Further to BDNF's relationship with depressive-like and anxiety-like symptomatology, BDNF is also associated with structural changes in the brain. The following is shown as abnormal decrease in levels of BDNF are demonstrated to have an effect on the hippocampal volume as it decreases the overall volume of the hippocampus (Rizos et al., 2011). Low levels of BDNF as well as impaired secretion of BDNF via Val66Met single nucleotide polymorphism (SNP) are also associated with anxiety-like behaviors as well as mood disorders such as

depression and obsessive-compulsive disorder (Janke et al., 2015). More interestingly, SNPs in the BDNF gene, Val66Met, has been shown to elevate anxiety-like behaviors in mouse models as well as make them resistant to the efficacy of fluoxetine, a typical SSRI (Bath et al., 2012). Though a large portion of contemporary research on BDNF is focused on specific regions of the brain, such as the hippocampus and prefrontal cortex, BDNF also plays a role in other brain regions. The amygdala is one brain region that completes the limbic system and plays a role in emotional responses. The serotonin system seems to be modulated by BDNF signaling as dysfunction in the BDNF signaling could result in a dysfunction in the amygdala. This mainly has been introduced in the context of post-traumatic stress disorder (PTSD) development (Green et al., 2013). In rodent studies, the amygdala region of the brain is shown to be increasingly affected by chronic stress exposure, particularly in rats, as it has been shown that there is a decrease in volume particularly in the basolateral amygdala (BLA) due to chronic stress exposure (Yang et al., 2006). While on the other hand, chronic stress exposure also resulted in an increase in the dendritic arborization as well as spine density in the BLA (Vyas et al., 2006).

As noted by multiple research studies, the HPA axis plays a large role in stress responses. Stress corticotrophin releasing hormone (CRH) from the hypothalamus can affect BDNF. In a rat study investigating the effects of chronic stress (3h restraint/day) on HPA and BDNF, it is demonstrated that rats who underwent chronic stress exhibited dysregulation in HPA axis as the baseline mRNA and peptide content of CRH and arginine-vasopressin (AVP) were significantly increased. The plasma concentrations of adrenocorticotrophic hormone (ACTH) and corticosterone (CORT) were also increased.

Interestingly, BDNF basal content was also modified as it was increased in multiple brain regions including the hippocampus, hypothalamus and pituitary in chronically stressed mice. On this note, pathological fear states are suggested to result from a chronic increase in glucocorticoids which seem to influence the regulation of BDNF expression (Gourley et al., 2009).

In addition to the relation between BDNF and HPA axis, the amygdala is prominently responsible for consolidating fear memory. After pairing a conditioned stimulus (sound of a tone) with unconditioned stimulus (electric foot shot), an increase in BDNF mRNA as well as TrkB phosphorylation was exhibited in the basolateral amygdala (Rattiner et al., 2004). Also, it seems that there is a positive correlation between amygdala BDNF levels and anxiety-like behavior in the elevated plus maze (Yee et al., 2006). Upon fear conditioning, activation of TrkB by BDNF is prevalent in the amygdala and this activation results in activating mitogen-activated protein kinase (MAPK) as well as phosphoinositide 3-kinases (PI3K) (LC & PW, 2006). The activation of MAPK cascade signaling results in the consolidation of fear memory as well as synaptic plasticity (Schafe et al., 2000).

It is suggested that BDNF interacts with multiple processes effects the amygdala in fear memory consolidation. One factor that BDNF seems to highly and strongly effect is the gamma aminobutyric acid (GABAergic) neurons present in the amygdala. One example, GABAergic receptors are rapidly decreased in fear conditioning. This prominent decrease is shown to be in the amygdala (Heldt & Ressler, 2007). This decrease of GABAergic receptors

indicates a hyperexcitability of the membrane. Also, this internalization of GABA(A) receptors seem to be BDNF-TrkB dependent as the activation of BDNF-TrkB in memory consolidation is activated/proceeded by the activation of protein kinase C (PKC) (Mou et al., 2011). In addition to memory consolidation, PKC also plays a major role in cyclic adenosine monophosphate (cAMP) response element-binding protein (CREB) transcription as well as neuroplasticity due to the fact that activation of BDNF-TrkB leads to the activation of phospholipase C- γ (PLC- γ) pathway (Bjorkhlom & Monteggia, 2016). Activation of the PLC- γ pathway through BDNF-TrkB leads to increased plasticity in its respective region, hence stronger activation of within the region.

Effects of BDNF on long-term potentiation and synaptic plasticity

NMDA is one type of ionotropic glutamate receptor that is located on the presynaptic terminal. It plays a major role in the regulation of Ca^{2+} influx. The NMDA receptor is at least partly modulated by levels of BDNF, since as elevated levels of the trophic factor was associated with increased NMDA and intracellular Ca^{2+} levels (Bjorkholm & Monteggia, 2016). Further, long-term potentiation (LTP) and synaptic plasticity on the presynaptic terminal can be strengthened by the modulation of BDNF levels. The above phenomenon was introduced and described by a study conducted on BDNF knockout neurons in mouse neocortical glial cells. In this study, the researchers investigated the effects of BDNF on presynaptic long-term plasticity in regard to NMDA receptor activation. They observed the strength of active cycling presynaptic vesicles through styryl dye FM4-6. BDNF knockout neocortical neurons seemed to have significant decrease in the fluorescence intensity (Walz et al., 2006).

Implications in the dendritic spine density particularly in the hippocampus seems to play a crucial role in depression and anxiety related disorders. Upon exposure to chronic stress, there seems to be dendritic retraction particularly in the CA3 region of the hippocampus (Adamec et al., 2011). In addition, a reduction in the overall CA3 hippocampal volume seems to be significant when exposed to chronic stress (Donohue et al., 2006). Long term depression (LTD) and LTP have been a topic of interest in regard to BDNF. The difference on how LTP and LTD would rise is due to the different cleaving of BDNF. Prior to BDNF synthesis, BDNF has a precursor called proBDNF. ProBDNF is cleaved proteolytically through the activation of tissue plasminogen activation (tPA), which eventually produces mature BDNF (Musumeci & Minichiello, 2019). LTP is initiated upon cleaving of proBDNF by tPA process to generate mature BDNF (Pang et al., 2004). On the other hand, LTD is facilitated by the proBDNF-p75 neurotrophin receptor signalling (Woo et al., 2005). LTD is accomplished due to the activation of p75 NTR receptors via proBDNF. That being said, it should be noted that proBDNF is suggested to promote apoptosis (leading to LTD) while BDNF promotes cell survival via TrkB receptors (leading to LTP) (Bjorkholm & Monteggia, 2016). With all this information on p75NTR, it should not be insinuated that it only promotes cell death as many other research studies indicated that p75NTR has multiple functions on neurons as well as glial cell. For example, in Schwann cells, BDNF binding to this receptor promoted remyelination post-injury. Depending on the cell cortex, p75NTR can either facilitate or attenuate proliferation (Meeker & Williams, 2015). With all that being said, it can be insinuated that BDNF can facilitate both LTP and LTD depending on the mechanistic of action it pursues. LTP is achieved by either enhancing

neurotransmitter release presynaptically, or by sustained blockage of NMDA receptors at rest (Erinn et al., 2014).

TrkB (FL and T1) and Anxiety

There are three predominant tyrosine kinase receptors (Trk), TrkA, TrkB, and TrkC. The TrkB receptor is a specific ligand receptor particularly related to BDNF (Dienhardt & Chao, 2014). For a vast majority of time it has been thought that TrkB is present only in one form, but it is now acknowledged that there are a few variations in isoforms that are created through the cleaving of the main TrkB receptor, TrkB.FL, TrkB.T1 and TrkB.Shc receptor isoform. The primary role of TrkB.T1 receptor is to act as a dominate negative regulator (or inhibitor) of the function of TrkB.FL (full length). It helps regulate BDNF signaling through the regulation of the extracellular TrkB.FL. It should also be noted that TrkB.T1 lacks the intracellular domain found on the full-length form (Carim-Todd et al., 2009).

Previous studies looking at TrkB.T1 and anxiety related disorders are relatively scarce, as only one study on record exists in regard to selectively deleting the TrkB.T1 receptor to examine its effects on the brain. Carim-Todd et al. (2009) is one of the few researchers who examined the pathological aspects of knocking out the TrkB.T1 receptor. In their study, Carim-Todd et al. discovered deleting the TrkB.T1 but retaining the TrkB.FL receptor resulted in pathological abnormality of neurites of neurons regarding the length

and complexity in the BLA. The following goes hand in hand with the behavioral observations noted as TrkB.T1 knockout (KO) mice exhibited an increased anxiety phenotypic behavior in the open field test and elevated plus maze, compared to wild type. It is important to note that no morphological changes were present in the hippocampal region, nor were any behavioral changes present (although deficits in LTP were observed).

Remarkably, TrkB.T1 is the only isoform that is expressed on astrocytes. Yet, there is a paucity of studies that have investigated the effects of TrkB.T1 on these glial cells. Although the in-depth function of TrkB.T1 on astrocytes is not yet well understood, it was suggested that TrkB.T1 activation stimulates intracellular Ca^{2+} signaling in glia cells (Rose et al., 2003). A recent study investigating the role of TrkB.T1 on astrocytes revealed that the global deletion of TrkB.T1 in mice model subjects resulted in a decreased volume of astrocytes. Interestingly, it was also evident that the peri-synaptic genes which are important in astrocyte functioning were dysregulated (Holt et al., 2019).

Gut microbiota on BDNF-TrkB and Anxiety

In recent years, research has demonstrated an increased relationship between the gut microbiota and neurological disorders. Specifically, disorders such as anxiety and depression have been a focus of study, as studies conducted by Nardone & Compare, 2014; Vuong et al., 2017; Rogers et al., 2016 explain the complex relationship between gut microbiota with anxiety and depressive related symptomatology. With the above researchers in mind, it is demonstrated that dysregulation of gut microbiota can lead to anxiety-like and depressive-like symptomatology in rodents. For example, an intrinsic look

at how gut bacteria can affect mouse behavior was analyzed by Sudo et al. (2004) where it is demonstrated that germ free (GF) mice exhibit increased response of ACTH and levels of CORT when they underwent chronic stress, compared to house specific pathogen free (SPF) mice. Although these results may not disclose the exact mechanisms involved in how gut microbiota affect behaviour, they are indicative that the gut bacteria have a relationship with certain neurological disorders such as anxiety and depression.

When examining communicative pathways between the gut and the brain, one should pay close attention to the vagus nerve. The vagus nerve is thought to be the main communicative pathway between the gut and the brain, and this pathway is also suggested to be bidirectional (Forsythe et al., 2014). Recently, researchers have examined how disruption of the vagus nerve affects neurotransmitter presence in specific regions of the brain. The following has been conducted in an effort to demonstrate how gut bacteria can affect neurological processes. For example, a study conducted by O'Leary et al. (2017) suggests disruption of the vagus nerve can result in a decrease in BDNF mRNA expression within the CA1, CA3 and dentate gyrus of the hippocampus. Furthermore, measurements of bromodeoxyuridine / 5-bromo-2'-deoxyuridine (BrdU)-positive cells per section indicate vagotomised mice express a decreased survival rate of newly born cells in the dorsal and ventral hippocampus (O'Leary et al., 2017). The following is extremely important when understanding how gut microbiota can affect neurological processes as O'Leary et al. indicate disruption of communicative pathways between the gut and the brain result in significant differences in BDNF mRNA and cell survival rate within the hippocampus. With

the following provided, one can begin to understand the implicit effects the gut can have on neurological function.

Understanding the effects of gut microbiota on the brain is increasingly important when understanding how the gut can affect neurological changes within specific regions of the brain. Heijtz et al. (2011) examine the following while comparing BDNF mRNA expression between GF and SPF mice. Within the following study, it is demonstrated that GF mice exhibit decreased BDNF mRNA expression within the amygdala, hippocampus and cingulate cortex when compared to SPF mice. Furthermore, neurological difference between GF and SPF mice are supported by behavioural related symptoms. For example, Heijtz et al., 2011 performed a light/dark preference test on both GF and SPF groups, where they examined group preferences on light or dark settings given that the GF mice had been exposed to gut microbiota early in life. Compared to control SPF mice, GF mice spent significantly more time in the light compartments than dark, indicating an increased anxiolytic state. Additionally, BDNF mRNA expression is shown to be decreased in various regions of the brain in GF mice compared to SPF mice. The following includes having decreased BDNF mRNA expression in orbital frontal cortex, anterior olfactory region, CA1, BLA, and cingulate cortex. The following would suggest that colonization of gut flora could influence the underlying neural circuitry for anxiety and fear.

As we continue to examine how the gut microbiota effects neurological processes within the brain, it becomes increasingly clear that BDNF plays a crucial role in this relationship. Not only does gut microbiota effects BDNF mRNA in regions of the brain such

as amygdala, hippocampus and cingulate cortex, but BDNF also seems to have an important role to play in enteric nervous system (ENS) function and balance. Various studies look into the presence of BDNF in the ENS specifically in colonic epithelial cells as well as enteric glia cells (Wang et al., 2015 & Steinkamp et al., 2012). The expression of BDNF and TrkB immunoreactivity in the gut has been shown in guinea pig ileum and is expressed in various regions. These regions include the expression of both BDNF and TrkB in the submucosal plexus, myenteric plexus, myenteric ganglia, and interganglionic connectives. Furthermore, BDNF immunoreactivity is shown in mucosal epithelial cells, mucosal villi, and subsets of myenteric neurons. TrkB immunoreactivity is shown prominently in the mucosa. BDNF and TrkB is also evident in cultures of myenteric neurons (Boesmans et al., 2008).

When looking at the mechanistic of action of BDNF and TrkB in the gut, it seems that BDNF does not have a direct effect on Ca^{2+} signalling as it does in the hippocampus (Kafitz et al., 1999). Although BDNF does exhibit indirect effects on Ca^{2+} signalling within the enteric nervous system (ENS). The following is shown as BDNF in cultured myenteric neurons influences Ca^{2+} signalling in a time-dependent manner through serotonin (5-HT) and substance protein (SP). This is facilitated as 5-HT and SP gradually increased after several hours of exposure to BDNF which then results in increased Ca^{2+} signalling. The relationship between BDNF and indirect mechanisms leading to Ca^{2+} signalling was examined further by Boesmans et al. (2008) where TrkB receptors in the gut were blocked. As a result of blocking TrkB receptors, Ca^{2+} signalling ceased entirely which demonstrates BDNF's indirect effects on Ca^{2+} signalling within the ENS.

Evidence has emerged regarding synaptic transmission in the ENS as peristaltic reflex in the rat colon is shown to be augmented by BDNF. In this process, BDNF stimulates the release of 5-HT and calcitonin gene related peptide that facilitates mucosal activity (Grider et al., 2003). Therefore, it is demonstrated that BDNF plays a significant role in the normal function of gut peristaltic reflex.

Anxiety and gut bacteria are largely influenced by each other as various studies indicate a disbalance in the gut bacteria can lead to anxiety like symptoms, and the anxiety like symptoms can lead to a disbalance in the gut bacteria (Savignac et al., 2014; Park et al., 2013, Kelly et al., 2013). Four major gut phyla include Firmicutes, Bifidobacteria, Actinobacteria, and Proteobacteria (Khanna & Tosh, 2014). Among the following major gut phyla, an imbalance can result in anxiety like symptoms (See review: Clapp et al., 2017). For example, in a study conducted investigating the role of 2 bifidobacteria strain (*B.longum* 1714 and *B.breve* 1205) and escitalopram (an SSRI) in innately anxious mice (BALB/c mice), both bifidobacteria strains showed lower anxiety-like symptoms in marble burying test. Interestingly, escitalopram has also showed reduced anxiety levels in the marble burying test. *B. longum* 1714 showed reduced stress induced hyperthermia and reduced anxiety like behavior in tail suspension test. *B. breve* 1205 showed lower anxiety levels in the elevated plus maze (Savignac et al., 2014). In addition to the following, Lactobacillus strain in the intestine play a role in depressive and anxiety disorders as it is shown to influence brain regions associated with depressive and anxiety disorders. Such brain regions include the amygdala, hippocampus and prefrontal cortex. Lactobacillus

bacterial strain was suggested as a probiotic to help the relieve of anxiety and depressive like symptoms. Chronic treatment of *Lactobacillus rhamnosus* (JB-1) in BALB/c mice showed a reduction of GABA-B1b mRNA expression in amygdala, hippocampus and locus coeruleus. Alternatively, GABA.A $\alpha 2$ mRNA expression is shown to be increased in the amygdala and prefrontal cortex but decreased in the hippocampus. Regarding anxiety-behavioral measures, JB-1 treated mice showed an anxiolytic effect in the open field test as these mice showed increased number of entries compared to controls (broth fed mice). Additionally, JB-1 treated mice showed significant less time being immobile in forced swim test (depressive-behavioral measure) compared to controls. Corticosterone (CORT) levels are shown to be lower in JB-1 treated mice compared to controls (Bravo et al., 2011). CORT is considered to be a stress hormone in rodents and is normally produced following stimulation of the HPA axis. Therefore, the measure of CORT is normally evaluated as a measure of stress in rodents (Prickaerts & Steckler, 2005). This decreased CORT in the JB-1 treated mice suggests that the probiotic lactobacillus displayed anxiolytic effects in innately anxious mice. In order to test whether or not probiotic lactobacillus effects the brain directly, vagotomised mice were used for this investigation. Vagotomised mice prevented JB-1 treated mice to exhibit anxiolytic effects in the open field test as they have shown to spend less time in the center zone compared to sham surgery mice. In regard to GABA mRNA expression in the brain, vagotomised mice displayed decreased levels of GABA.A $\alpha 2$ in BLA and central nucleus of the amygdala (CeA) of the amygdala compared to sham surgery mice. (Bravo et al., 2011).

Thesis Aim

This study aims to investigate the effects of the TrkB.T1 receptor on anxiety-like pathology. In the past, researchers have paid prominent attention to BDNF and its specific ligand receptor TrkB.FL on its role in anxiety-like disorders, but little to no attention has been placed on the effects of the receptor's isoform, TrkB.T1.

Therefore, the functional aspect of TrkB.T1 will be examined using behavioral measures as well as anatomical measures. In addition, the gut microbiota will be analyzed due to its remarkable connectivity with depression and anxiety. When relating gut bacteria to functional aspects within the brain, it has been demonstrated that BDNF and gut microbiota seem to be strongly connected. In fact, as demonstrated previously, gut microbiota seems to influence the expression of BDNF mRNA in certain brain regions.

The following research study will assess the impact of chronic unpredictable stress (CUS) (35 days of CUS) in adult mice (8-10 weeks of age). Chronic unpredictable stress is a paradigm that is used for rodents. It is composed of exposing the rodent to repeated exposure of stressors that are unpredictable. These stressors last for weeks depending on the stressors used for the rodents (Sequeira-Cordero et al., 2019). Our adult mice will vary in genotype (TrkB.T1 knockout versus wild type), sex (male versus female), and treatment (stress versus control). We assessed this CUS impact using the behavioral test known as the "open field test". The open field test measures anxiety-like symptoms by looking at the movement of the mice in an open field apparatus. We hypothesize to observe control mice being less anxious-like compared to stress. We also hypothesize the genotypic difference in

relation to anxiety-like behavior as by theory, TrkB.T1 knockout (KO) mice should show heightened anxiogenic behavior compared to wild-type mice (WT). Additionally, this thesis also involves the investigation of the expression of TrkB.FL in the amygdala region as the amygdala is mainly involved in anxiety and fear responses. With respect to the gut flora, we aim to explore the gut flora changes/differences in treatment groups, sex differences, and genotypic differences. We hypothesize that the control group will not have any significant changes in microbiota, while the stress group will exhibit significant changed/deviated scale of the microbiota.

Aim 1

To determine if 35 days of chronic unpredictable stress with 2X daily manipulations will provide anxiogenic-like behavior mice of different treatment (control vs stress), sex (male vs female), and genotype (wild type vs TrkB.T1 knockout). Open field test is the behavioral measure we will pursue in order to examine the anxiogenic-like symptoms of the mice.

Aim 2

To investigate the TrkB.FL receptor expression in the amygdala after the mice have been exposed to the CUS paradigm. It should also be noted that we will also investigate the TrkB.FL protein expression in all mouse groups.

Aim 3

We aim to determine if any changes are present in the gut flora due to chronic stress exposure between different groups (treatment x genotype x sex).

Hypothesis

We hypothesize that an increased anxiogenic phenotype would be evident in the stress group compared to controls. Additionally, we hypothesize within-group differences as stress KO mice will display increased anxiogenic phenotype compared to stress WT mice. Regarding TrkB.FL expression in the amygdala, we hypothesize stress KO mice will display an increased expression, compared to stress WT mice, of the TrkB.FL receptor due to the functional aspect of TrkB.T1 receptor on full length (as TrkB.T1 is shown to have dominant negative inhibition on TrkB.FL). Lastly, in regards to analyzing the effects of stress on gut flora, we hypothesize that chronic stress will result in a decrease of bifidobacteria (*B.longum* 1714 strain and *B.breve* 1205 strain) in stressed WT mice compared to control WT mice. Additionally, *Lactobacillus rhamnosus* is hypothesized to be reduced in stress WT mice compared to control WT mice. Regarding genotypic differences and gut flora, we expect to see a significant decrease of bifidobacteria (*B.longum* 1714 strain and *B.breve* 1205 strain) and *Lactobacillus rhamnosus* in stress KO mice compared to stress WT. We expect alterations in these species due to the fact that they have been shown to be decreased in the gut after mice have exhibited depressive as well as anxiety related symptoms

Methods

Animals and experimental design

Subjects in the following research study are laboratory mice strain C57BL/6J. They will be either TrkB.T1 knockout or wildtype mice, and both genders will undergo the experiment. Three cohorts will be used having a total number of 140 mice (Cohort 1= 24, Cohort 2= 55, & Cohort 3= 61). Prior to behavioral measures, mice will be cohoused until 8-10 weeks of age and will be randomly separated into stress and control treatment groups after this period. They will be singly housed in standard cages upon entering the experiment. Prior to the initiation of CUS, all mice will undergo 5 days of behavioral baseline tests, a sucrose preference test, *refer to Table 1*. In addition, fecal boli samples will be collected from both groups. Following the behavioral baseline test, mice will undergo the CUS for 35 days including 2x daily manipulations, *refer to Table 2 on page 28*. Following 35 CUS, all mice will be tested using the behavioral baseline test, and sucrose preference test. Behavioral tests such as the open field test as well as the alternate Y-maze will be used following the last sucrose preference test. In addition, fecal boli samples will be collected post chronic stress. In order to investigate the neuroanatomical changes, half of the mice per group will be euthanized via rapid decapitation for Western blot measures. The second half of the mice per group will be euthanized via 100mg/kg sodium pentobarbital and perfused with paraformaldehyde for immunohistochemistry measures. Western blotting will be used to look at TrkB FL receptor (by looking at pTrkB-Y816 and unphosphorylated TrkB.FL) in the amygdala. As mentioned earlier, in order to investigate the microbiota of the following mice, fecal boli will be collected pre & post-chronic unpredictable stress.

Table 1: Timeline table.

Days	1	2	3	4	35 Days	36	37	38	39	40	41	42
	Suc Pref	Suc Pref	Suc Pref	Suc Pref	CUS	CTL Suc Pref	STR Suc Pref	BBK	OF - CTL	OF - STR	Alt Y m - CTL	Alt Y m - STR
							BBK					

Table 2: Chronic unpredictable stress schedule:

Restraint 15 minutes
Restraint 20 minutes
Restraint 30 minutes
Cinnamon scent
Peppermint scent
wintergreen scent
Eucalyptus scent
Lights on - all day
Lights off - all day
Lights on 12 hours
Lights off 12 hours
Cage tilt
Wet bedding
Soaked bedding
Empty cage

Behavioral measure: Open field test

Following CUS sucrose preference test, all mice were examined for anxiogenic-like symptoms. This involved the behavioral measure open field test. The first day after sucrose preference test, control mice were measured anxiety-like symptoms. On the second day of post sucrose preference test, stress mice were then examined in the open field test.

Sacrifice

Half of each treatment group is euthanized via rapid decapitation. The rapid decapitation euthanasia process is for protein analysis, TrkB.FL expression in the amygdala. In short, the mice were rapidly decapitated through the cervical vertebrae via surgical scissors. Multiple regions of the brain were extracted including the prefrontal cortex, hippocampus, amygdala, and the nucleus accumbens. These brain regions were collected using a chilled micro-dissecting block with 0.5mm slots. Then using a micro-punch, the brain specific regions were collected. The tissues were then placed in vials on ice for Western blot usage. The other half of each treatment group were euthanized via 100mg/kg sodium pentobarbital and perfused with 4% paraformaldehyde for brain tissue analysis via immunohistochemistry. The brain was extracted and rapidly after flushing the blood out using 5ml saline, the brain tissue was placed in a 4% paraformaldehyde vial and placed on ice. The brain tissue was then freeze at -20C°.

Western blot

The expression of TrkB.FL was examined in the amygdala using Western blot. The gels were created with 7.5% separating gel comprised which comprised of 3.75ml of Acrylamide and 100μL of 10% APS and stacking gel. Prior to loading the proteins in the gel electrophoresis, sodium dodecyl sulfate was added to the protein to allow the protein to unfold creating a linear shape as well as coat the chain with a negative charge to allow the natural migration of the proteins from a negative charge to a positive charge. The gels were then placed on the running gel. The gels then were transferred onto blocking membranes. Following transfer, the membranes were left overnight to dry. On the next day, blocking,

primary (1:1000 pTrkB ABN1381 Rabbit & 1:1000 TrkB BD610101), and secondary (1:15000 680nm LiCor Rabbit for pTrkB & 1:1500 800nm LiCor for TrkB Mouse) antibody were used to determine phosphorylated TrkB.FL (pTrkB.FL) (ABN 1381). After imaging the pTrkB.FL, the membranes were stripped then blocked for 30 minutes. After 30 minutes of block, the primary antibody for the unphosphorylated TrkB.FL (BD 10102) was placed for overnight. On the 3rd day, the secondary antibody for the unphosphorylated TrkB.FL was placed following imaging of the membranes at 700nm channel and 800nm channel using LICOR.

Gut Microbiota

Fecal boli samples were taken prior to the start of the CUS, then recollected after the 35 days of CUS. Regarding the fecal boli samples being collected prior to CUS, both treatment groups (stress and control) mice were placed in empty cages for 10 minutes to collect the fecal boli samples. Fecal samples were placed in vile tubes and placed on dry ice. Both treatment group fecal samples were collected in order to maintain the consistency of undergoing techniques. Following the 35 days of CUS, mice fecal boli samples were collected from the open field apparatus box after the mice have been retuned into their standard housing cage. DNA extraction was performed on all fecal boli samples using Norgen stool kit (Cat No 27600). The DNA concentration was then measured and documented. The only fecal extracted DNA samples were used are the ones greater than 10ng/ul for sufficient analysis of the gut flora. The DNA samples were then sent to the integrated microbiome resource in Dalhousie University for analyzing the profile of all the gut microbiota.

Statistical analysis

The data collected for TrkB.FL protein expression as well as open field test were analyzed using 2x2x2 ANOVA (treatment, sex, genotype) through SPSS (version #25). Significant differences were considered at p-value <0.05. GraphPad Prism 5 was used to generate a 3-way interaction graph.

Results

Open Field Test

Using a Multivariate ANOVA to analyze the open field test, it was shown that there was a significant treatment effect regarding the Small Center Frequency as the Stress group had higher Small Center Frequency compared to Control group, ($F_{(1,126)}=36.417$, $p<0.001$), refer to Figure 1. Additionally, the Stress group took significantly shorter time to enter the Small Center compared to the Control group, ($F_{(1,126)}=7.316$, $p=0.008$) (Figure 2). This was not only evident in the Small Center region, but also in the Large Center region of the open field test as the Stress group had significantly higher Large Center Frequency and lower latency to enter Large Center, ($F_{(1,83.43)}=55.826$, $p<0.001$) and ($F_{(1,1.94)}=4.064$, $p=0.046$) respectively. Figures 3 and 4 illustrates the differences. A 3-way interaction between Sex, Genotype and Treatment was shown to be significant in regards to Large Center Cumulative Duration $F_{(1,126)}=3.960$, $p=0.049$). Using Bonferroni-adjusted pairwise comparisons as follow-up analysis, we found that male Control KO mice spent less time in

the Large Center compared to female Control KO mice $F_{(1,126)}=4.882$, $p=0.029$, refer to Figure 5.

Figure 1. OFT: Small Center Frequency.

Stress group had significantly higher small center frequency compared to Control group ($F_{(1,126)}=36.417$, $p < 0.001$).

Figure 2. OFT: Small Center Latency to Enter.

The stress group took significantly shorter time to first enter the small center zone compared to Control group ($F_{(1,126)}=7.316$, $p=0.008$).

Figure 3. OFT: Large Center Frequency.

Stress group high significantly higher frequency in the large center compared to Control group ($F_{(1,83.43)}=55.826$, $p<0.001$).

Figure 4. OFT: Large Center Latency to Enter.

Stress group took significantly shorter time to first enter the large zone ($F_{(1,1.94)}=4.064$, $p=0.046$).

Figure 5. OFT: Large Center Cumulative Duration.

Only Control male KO mice had significantly lower cumulative duration in the large center zone compared to Control female KO $F_{(1,126)}=4.882$, $p=0.029$.

Additionally, there is also a Treatment effect in regard to the Corner Frequency $F_{(1,126)}=60.995$, $p<0.001$ as the Stress group had higher frequency compared to Control group (Figure 6). There was also a 3 way interaction between Treatment, Sex, Genotype and Corner Frequency $F_{(1,126)}=3.283$, $p=0.072$. Bonferroni-adjusted pairwise comparison as follow-up analysis indicated that Stress female KO and WT mice had higher corner frequency compared to their Control counterparts, $F_{(1,126)}=15.721$, $p<0.001$ and $F_{(1,126)}=53.60$, $p=0.022$ respectively. Stress male KO and WT also had higher Corner frequency compared to their Control counterparts, $F_{(1,126)}=14.621$, $p<0.001$ and $F_{(1,126)}=29.929$, $p<0.000$ respectively (Figure 6).

Figure 6. OFT: Corner Frequency.

Both Stress female KO mice and Stress female WT mice are shown to have had significantly higher Corner Frequency compared to their Control counterparts $F_{(1,126)}=15.721$, $p<0.001$ and $F_{(1,126)}=5.360$, $p=0.022$, respectively. Both Stress male KO and Stress male WT mice are also shown to have had higher Corner Frequency compared to their Control counterparts $F_{(1,126)}=14.621$, $p<0.001$ and $F_{(1,126)}=29.929$, $p<0.001$, respectively.

Through a multivariate ANOVA, there was a Treatment effect on the Total Distance travelled as the Stress mice traveled greater distance compared to Control mice $F_{(1,126)}=78.025$, $p<0.001$, refer to Figure 7. Additionally, there was a borderline significant 3-way interaction (Treatment, Genotype, Sex) on the Total Distance travelled $F_{(1,126)}=3.829$, $p=0.053$. To tease this difference apart further using Bonferroni-adjusted pairwise comparison, revealed that female Stress KO mice had greater Total Distance compared to female Control KO mice $F_{(1,126)}=20.416$, $p<0.001$. This was also evident in female Stress WT mice as they have shown to have traveled greater distances compared to female Control WT mice $F_{(1,126)}=7.312$, $p=0.008$. Male Stress KO mice as well as male Stress WT mice had also shown to have greater Total Distance compared to their Control counterparts,

$F_{(1,126)}=18.783$, $p<0.001$ and $F_{(1,126)}=36.726$, $p<0.001$ respectively. Please refer to Figure 7 for a visual representation of the Bonferroni-adjusted pairwise comparison follow-up analysis. Additionally, through Bonferroni-adjusted pairwise comparison, female Control WT mice is shown to have greater Total Distance compared to male Control WT mice $F_{(1,126)}=8.480$, $p=0.004$, refer to figure 8.

Figure 7. OFT: Total Distance (cm).

Both Stress female KO mice and Stress female WT mice had significantly greater total distance compared to their Control counterparts $F_{(1,126)}=20.416$, $p<0.001$ and $F_{(1,126)}=7.312$, $p=0.008$ respectively. Stress male KO mice and Stress male WT mice also had significantly greater total distance compared to their Control counterparts $F_{(1,126)}=18.783$, $p<0.001$ and $F_{(1,126)}=36.726$, $p<0.001$ respectively.

Figure 8. OFT: Total Distance (cm).

There is a sex difference, such that Control female WT mice had significantly greater Total Distance compared to Control male WT mice $F_{(1,126)}=8.480$, $p<0.004$.

Additionally, there was a Treatment effect in regard to the velocity as the Stress group is shown to have higher velocity compared to the Control group $F_{(1,126)}=78.022$, $p<0.001$, refer to Figure 9. There was also a 3-way interaction between Treatment, Sex, Genotype and velocity $F_{(1,126)}=3.825$, $p=0.053$. Following Bonferroni-pairwise comparison as following up analysis, it was shown that Stress female KO and WT have higher velocity than their Control counterparts, $F_{(1,126)}=20.413$, $p < 0.001$ and $F_{(1,126)}=7.313$, $p=0.008$ respectively. Also, the same was found in the males as the Stress male KO and WT are shown to have higher velocity compared to their Control counterparts $F_{(1,126)}=18.786$, $p<0.001$ and $F_{(1,126)}=36.719$, $p<0.001$ respectively.

Figure 9. OFT: Velocity (cm/s).

Both Stress female KO and Stress female WT mice had significantly higher velocity compared to their Control counterparts $F_{(1,126)}=20.413$, $p<0.000$ and $F_{(1,126)}=7.313$, $p=0.008$, respectively. Stress male KO mice and Stress male WT mice also had significantly higher velocity than their Control counterparts $F_{(1,126)}=18.786$, $p<0.001$ and $F_{(1,126)}=36.719$, $p<0.001$, respectively.

TrkB.FL expression in the Amygdala

TrkB.FL protein levels as well as ratio between the phosphorylated TrkB (pTrkB.FL) and unphosphorylated TrkB were analyzed using multivariate ANOVA. A significant main effect was evident between Sex and fully glycosylated TrkB ($F_{(1,32)}=6.418$, $p=0.016$) as well as partially glycosylated TrkB ($F_{(1,32)}=4.829$, $p=0.035$). The following indicates that the TrkB receptor is significantly higher in females than males, refer to Figures 10 and 11. There was no main effect regarding Treatment groups (Stress and Control) nor Genotype (KO and WT). Interestingly, there was a significant main effect between Sex and the ratio of pTrkB.FL and TrkB.FL, as the top band (fully glycosylated) and the bottom band (partially glycosylated) were shown to be significant, ($F_{(1,32)}=5.270$, $p=0.028$) and ($F_{(1,32)}=4.608$, $p=0.040$) respectively. A further assessment of the ratios between pTrkB and TrkB using

Bonferroni-adjusted pairwise comparisons, it showed that female Stress WT mice have lower pTrkB:TrkB ratio (partially glycosylated receptor) compared to male Stress WT mice ($F_{(1,32)}=4.891$, $p=0.034$). Additionally, Stress male WT mice had significantly higher ratio compared to Control male WT mice ($F_{(1,32)}=8.562$, $p=0.006$), refer to Figure 15. This was also evident in the fully glycosylated pTrkB:TrkB ratio as female Stress WT mice was shown to be lower in ratio compared to male Stress WT mice ($F_{(1,32)}=4.113$, $p=0.051$).

Figure 10. Fully glycosylated TrkB.FL by Sex and Treatment.

Females had significantly higher fully glycosylated TrkB.FL in Amygdala compared to males ($F_{(1,32)}=6.418$, $p=0.016$).

Figure 11. Partially glycosylated TrkB.FL by Sex and Treatment.

Females had significantly higher partially glycosylated TrkB.FL compared to males $F_{(1,32)}=4.829$, $p=0.035$.

Figure 12. Western blot of TrkB.FL (BD610101 antibody).

Figure 13. Ratio of fully glycosylated p.TrkB.FL and TrkB.FL by Sex and Treatment.

Females had significantly lower ratio of fully glycosylated pTrkB.FL and TrkB.FL compared to males $F_{(1,32)}=5.270$, $p=0.028$.

Figure 14. Ratio of partially glycosylated pTrkB.FL and TrkB.FL by Sex and Treatment.

Females had significantly lower ratio of partially glycosylated pTrkB.FL and TrkB.FL compared to males $F_{(1,32)}=4.608$, $p=0.040$.

Figure 15. Ratio of partially glycosylated pTrkB.FL and TrkB.FL by Treatment, Genotype and Sex.

Stress WT females had significantly lower pTrkB.FL:TrkB.FL ratio compared to Stress WT males $F_{(1,32)}=4.891$, $p=0.034$. Stress male WT mice had higher pTrkB.FL:TrkB.FL ratio compared to Control male WT mice $F_{(1,32)}=8.562$, $p=0.006$.

Figure 16. Ratio of fully glycosylated pTrkB.FL and TrkB.FL by Treatment, Genotype and Sex.

Stress female WT mice had significantly lower pTrkB.FL:TrkB.FL ratio compared to Stress male WT mice $F_{(1,32)}=4.113$, $p=0.051$.

Figure 17. Western blot of Unphosphorylated TrkB.FL (left image – 1:1000 BD610101 Rabbit antibody) and Phosphorylated TrkB.FL (right image – 1:1000 ABN1381 Mouse).

Discussion

While BDNF and its primary receptor, TrkB, have received substantial attention with regards to depression and anxiety (Martinowich et al., 2007), little work has focused upon the truncated, TrkB.T1 form of the receptor. Consequently, the purpose of this study is to evaluate the impact of knocking out TrkB.T1 receptor following with chronic unpredictable stress and evaluate its effect on the gut microbiota configuration and anxiety behavior. Curiously, the stressor exposure increased exploration of the center of an open

field arena. Accordingly, to the typical interpretation of this test, this outcome suggests a *reduction* in anxiety with the stressor exposure, but the stress group also had greater total distance compared to controls, suggesting hyperlocomotion. It could be that the stressed mice had a “manic-like” behavior as they were shown to be hyperactive. Similarly, hyperactivity was seen in a study conducted to block TrkB^{CaMKII-CRE} in the forebrain in which these mice exhibited hyperlocomotion as well as impulsive behavior rather than seeing a typical depression-like symptom where there would be a decrease in the locomotion as well as impulsive behavior (Zörner et al., 2003). Interestingly, male mice exhibited these behavioral changes to a greater extent than females, indicates sex-dependent modulation of the stressor impact. Yet, genotype had no effect, indicating that our data do not support a major role for TrkB.T1 in anxiety responses to a chronic unpredictable stressor regimen. Though many research studies argue that a typical anxiety/depressive like symptom are reflected in a lack of entries to the center zone of the open field apparatus, there also may be atypical symptoms associated with depression and anxiety in which the opposite symptoms would rise. Again, the velocity at which the mice travelled were also shown to be higher in the stressed mice, which is a marker for hyperactivity. There might also be another explanation, such that our choice of stressor might impact habituation levels to the open field. Indeed, the control mice may have experienced the open field itself as a stressful event, whereas the stress group habituated to experiencing chronic stress that the open field test was not as stressful compared to the other stressors they have experienced.

The expression of the TrkB.FL was also investigated in an attempt to assess how it is influenced by the chronic stress exposure, as well as investigate if TrkB.T1 knockout will

influence its expression in the amygdala. We focused upon the amygdala given its important role in mediated anxiety and also the fact that previous reports indicated that BDNF influence signaling in this brain region (Yee et al., 2007; Marle et al., 2009; Etkin & Wager, 2007; Lonsdorf et al., 2010; Chhatwal et al., 2006; Rattiner et al., 2004; Andero et al., 2011). The majority of the studies that have assessed BDNF in the amygdala, have found that the receptor TrkB increases in phosphorylation specifically during the consolidation period (which is suggested to be due to the release of BDNF) in response to Pavlovian fear conditioning stressors (Rattiner et al., 2004, 2005; Yee et al., 2006). Also, interestingly, prelimbic cortex BDNF levels have downstream effects through TrkB receptor on memory consolidation specifically in the amygdala but has no effect in the extinction of fear. Specifically, prelimbic BDNF knockdown mice had attenuated response to fear after fear-conditioning, but when exposed to systemic TrkB agonist, 7,8-dihydroxyflavone, the attenuation of fear was alleviated (Choi et al., 2012). Additionally, TrkB receptor increases in phosphorylation during consolidation period when exposed to Pavlovian fear conditioning. This suggests that the activation of the TrkB receptor is subsequent to BDNF release in the amygdala (Rattiner et al., 2004). This may be relevant for the present study in that understanding how different TrkB isoforms can have different functions and different effects on the fear related aspect of the amygdala. Our present study knocked out TrkB.T1 receptor isoform to see its impact on the TrkB.FL receptor. We also had a stress group that did not have the TrkB.T1 receptor knocked out to see if the CUS will have a solo impact on the TrkB.FL expression in the amygdala.

Intriguingly, we found sex differences in TrkB.FL levels, such that females had significantly higher TrkB.FL expression in the amygdala compared to male mice (Figure 13). This was also evident in the partially glycosylated TrkB.FL receptor expression as the females were shown to have higher protein content than males (Figure 11). It should also however be noted that stressed mice had lower expression levels than controls. This is somewhat consistent with another study performed by Bland et al., 2005 as they showed sex differences in regard to acute uncontrollable/inescapable stress in rat model as their control group showed higher BDNF levels in the prefrontal subregions (anterior cingulate, prelimbic, infralimbic and ventral orbital) in females compared to male rats. On the other hand, their male rats showed higher BDNF levels in the prefrontal subregions when exposed to inescapable stress then immediately sacrificed (Bland et al., 2005). Additionally, maltreatment including stress delivered in depression models provoked an increase in BDNF expression in female but not male rats in the amygdala region (Hill et al., 2014). It also seems that estrogen may play a role in gender related differences as estrogen is suggested to induce BDNF transcription through enhancing CREB activity as well as elevating the methylation of BDNF promoter (Zhou et al., 2005). The CREB activity is suggested to be one major transcription factor to influence BDNF availability in neurons through Ca^{2+} extracellular influx (Tao et al., 1998). In addition, BDNF seems to be affected by estradiol as the induction of estradiol seemed to be elevated BDNF mRNA levels in the hippocampus (CA3, CA4 and dentate gyrus). This suggests that estradiol hormone is required for BDNF expression in certain regions (Singh et al., 1995).

Our findings could be at least, in part, due to the interaction of GABA_A receptors as it has been shown that TrkB is one of the main pathways that lead to the internalization of GABA_A receptors which plays a role in the hyperactivity of amygdala during fear memory reconsolidation (Andero et al., 2014). A decrease in the TrkB protein is suggested to decrease the tonic/phasic inhibition in amygdala which results in a heightened excitatory drive. Another study also suggested that the consolidation fear memory period in the amygdala occurs due to the increase in TrkB phosphorylation (activated) expression (Rattiner et al., 2004). With this said, it could be that the lack of the TrkB.T1 receptor in the present study played a role in the internalization of GABA_A receptors. Yet it is not fully understood as to why the WT mice did not show an increased TrkB receptor expression.

Besides absolute TrkB.FL levels, it also is useful to look at the ratio between its phosphorylated and non-phosphorylated forms. Indeed, Woo et al., 2012 found lower pTrkB:TrkB ratio in females with 5-HT1A^{-/-} mice as well as 5-HT1A^{-/-}/BDNF^{+/-} mice in the hippocampus, but not in male mice. Phosphorylation at different sites on the TrkB receptor seem to play different roles. For example, phosphorylation at Y515 residue was shown to activate MAPK and PI3K pathways. Point mutations in the Y515 residue was shown to produce issues/deficits in regard to the consolidation of fear conditioning. On the other hand, phosphorylation at Y816 residue was shown to activate CREB signaling pathways. Point mutations in the Y816 residue showed deficits in regard to the acquisition of fear conditioning (Mahan & Ressler, 2012). This indicates that different phosphorylation sites in the TrkB receptor may have different effects on amygdala fear related processes. Our study specifically investigates the Y816 of the TrkB FL receptor. We looked at the ratio between

phosphorylated Y816 receptor as well as the unphosphorylated TrkB FL receptor. To this end, we found that the females had significantly higher TrkB receptor expression compared to male mice, and females had lower pTrkB:TrkB ratio compared to males. Sex differences have been shown in several studies, as females have higher TrkB receptor expression in amygdala and forebrain compared to males while males have higher TrkB expression in the hippocampus (Liu et al., 2014; Snigdha et al., 2011; Bakos et al., 2009).

In regard to the microbiota configuration pre and post chronic unpredictable stress, there were no significant differences between any of the groups. This was somewhat surprising since many studies found that *Lactobacillus rhamnosus*, *Bifidobacterium longum*, and *Bifidobacterium breve* all seem to be affected by anxiety and depressive models (Osadchiy et al., 2019; Froster & Neufled, 2013; Clapp et al., 2017; Park et al., 2013). Further research has shown that SNPs (single-nucleotide polymorphisms) is what has been shown to influence the gut microbiota configuration (Bleckman et al., 2015). In this regard, C57BL/6j mice are shown to have an inactive toll-like receptor-10 (TLR-10), as C57BL/6j mice have been shown to have a pseudogene rather than an active gene which is shown to be an active gene in humans (Mestas et al., 2004). TLR-10 is an immune receptor which is shown to have an anti-inflammatory response rather than pro-inflammatory response on human primary cells as it was shown to suppress B cell proliferation as well as cytokine production (Hess et al., 2017). Studies involving stress paradigms with depression and anxiety-like related behaviors and how the gut microbiota influenced these have been conducted mainly using BALB/c strain mice and GF versus SPF mice rather than the C57BL/6j mice that we currently used (Zheng et al., 2016; Dinel et al., 2019). Therefore,

perhaps using one of these strains (BALB/c or GF/SPF mice) would have shown to produce sufficient gut microbiota shift due to chronic stress. The changes in the bacterial strains such as bifidobacteria, lactobacillus, and streptococcus are mainly shown on GF mice or BALB/c mice rather than C57BL/6j mice (Desbonnet et al., 2008; Lyte et al., 2011; McVey Neufeld et al., 2018). Therefore, it would be suggested to use BALB/c mice rather than C57BL/6j mice in order to test how the gut microbiota is influenced from chronic stress.

To the best of our knowledge, this is the first study that assessed how chronic unpredictable stress influences anxiety outcomes in TrkB.T1 KO mice. In particular, we provide evidence that TrkB.T1 KO mice do not differ in their anxiety-like behavior nor was the microbiome or amygdaloid TrkB.FL levels altered. However, we did demonstrate that female mice had higher TrkB.FL expression in the amygdala compared to male mice, but the ratio between pTrkB.FL & TrkB was shown to be lower in females than males. Further studies are suggested to investigate the effect of TrkB.T1 KO on additional indices of anxiety and depression.

Appendix A

Gut microbiota configuration

The gut microbiota was examined in order to see if chronic unpredictable stress has an impact on the gut flora of the Stress mice versus Control mice. Additionally, genotypic differences and sex differences were also taken into account. It seems that there is no significant difference in any of the treatment groups, genotypic groups nor sex differences. As illustrated in figure 19, the bar-plot does not show any patterns that would naturally be shown if there is a switch in the gut flora between all variable groups.

Figure 18: Taxa Bar-plot (Taxonomic level 6).

A Bar-plot of the gut microbiota showing no patterns. Neither Treatment, Genotype, nor Sex seem to be shown to have an influenced the gut flora in this strain of mice.

	D_0_Bacteria;D_1_Bacteroidetes;D_2_Bacteroidia;D_3_Bacteroidales;D_4_Muribaculaceae;D_5_uncultured_bacterium
	D_0_Bacteria;D_1_Bacteroidetes;D_2_Bacteroidia;D_3_Bacteroidales;D_4_Muribaculaceae;D_5_uncultured_Bacteroidales_bacter
	D_0_Bacteria;D_1_Bacteroidetes;D_2_Bacteroidia;D_3_Bacteroidales;D_4_Muribaculaceae;D_5_Muribaculum
	D_0_Bacteria;D_1_Firmicutes;D_2_Clostridia;D_3_Clostridiales;D_4_Lachnospiraceae;D_5_Lachnospiraceae_NK4A136_group
	D_0_Bacteria;D_1_Bacteroidetes;D_2_Bacteroidia;D_3_Bacteroidales;D_4_Bacteroidaceae;D_5_Bacteroides
	D_0_Bacteria;D_1_Bacteroidetes;D_2_Bacteroidia;D_3_Bacteroidales;D_4_Muribaculaceae;D_5_Ambiguous_taxa
	D_0_Bacteria;D_1_Firmicutes;D_2_Bacilli;D_3_Lactobacillales;D_4_Lactobacillaceae;D_5_Lactobacillus
	D_0_Bacteria;D_1_Bacteroidetes;D_2_Bacteroidia;D_3_Bacteroidales;D_4_Prevotellaceae;D_5_Prevotellaceae_UCG-001
	D_0_Bacteria;D_1_Firmicutes;D_2_Erysipelotrichia;D_3_Erysipelotrichales;D_4_Erysipelotrichaceae;D_5_Faecalibaculum
	D_0_Bacteria;D_1_Bacteroidetes;D_2_Bacteroidia;D_3_Bacteroidales;D_4_Prevotellaceae;D_5_Alloprevotella
	D_0_Bacteria;D_1_Bacteroidetes;D_2_Bacteroidia;D_3_Bacteroidales;D_4_Rikenellaceae;D_5_Alistipes
	D_0_Bacteria;D_1_Bacteroidetes;D_2_Bacteroidia;D_3_Bacteroidales;D_4_Muribaculaceae;D_5_Muribaculum
	D_0_Bacteria;D_1_Firmicutes;D_2_Clostridia;D_3_Clostridiales;D_4_Lachnospiraceae;D_5_uncultured
	D_0_Bacteria;D_1_Bacteroidetes;D_2_Bacteroidia;D_3_Bacteroidales;D_4_Rikenellaceae;D_5_Rikenellaceae_RC9_gut_group
	D_0_Bacteria;D_1_Firmicutes;D_2_Clostridia;D_3_Clostridiales;D_4_Ruminococcaceae;D_5_Caproiciproducens
	D_0_Bacteria;D_1_Bacteroidetes;D_2_Bacteroidia;D_3_Bacteroidales;D_4_Tannerellaceae;D_5_Parabacteroides
	D_0_Bacteria;D_1_Firmicutes;D_2_Deferribacteres;D_3_Deferribacteriales;D_4_Deferribacteraceae;D_5_Mucispirillum
	D_0_Bacteria;D_1_Bacteroidetes;D_2_Bacteroidia;D_3_Bacteroidales;D_4_Prevotellaceae;D_5_Paraprevotella
	D_0_Bacteria;D_1_Firmicutes;D_2_Clostridia;D_3_Clostridiales;D_4_Lachnospiraceae;D_5_Tyzzerella_3
	D_0_Bacteria;D_1_Firmicutes;D_2_Clostridia;D_3_Clostridiales;D_4_Lachnospiraceae;D_5_Lachnoclostridium
	D_0_Bacteria;D_1_Firmicutes;D_2_Clostridia;D_3_Clostridiales;D_4_Ruminococcaceae;D_5_Ruminococcaceae_UCG-001
	D_0_Bacteria;D_1_Firmicutes;D_2_Clostridia;D_3_Clostridiales;D_4_Lachnospiraceae;D_5_Lachnospiraceae_UCG-001
	D_0_Bacteria;D_1_Bacteroidetes;D_2_Bacteroidia;D_3_Bacteroidales;D_4_Marinifilaceae;D_5_Odoribacter
	D_0_Bacteria;D_1_Firmicutes;D_2_Clostridia;D_3_Clostridiales;D_4_Ruminococcaceae;D_5_Ruminococcaceae_UCG-014
	D_0_Bacteria;D_1_Firmicutes;D_2_Clostridia;D_3_Clostridiales;D_4_Ruminococcaceae;D_5_Oscillibacter
	D_0_Bacteria;D_1_Bacteroidetes;D_2_Bacteroidia;D_3_Bacteroidales;D_4_Rikenellaceae;D_5_Rikenella
	D_0_Bacteria;D_1_Firmicutes;D_2_Clostridia;D_3_Clostridiales;D_4_Lachnospiraceae;D_5_Eubacterium_xylanophilum_group
	D_0_Bacteria;D_1_Firmicutes;D_2_Clostridia;D_3_Clostridiales;D_4_Ruminococcaceae;D_5_Ruminoclostridium_9
	D_0_Bacteria;D_1_Epsilonbacteraeota;D_2_Campylobacteria;D_3_Campylobacteriales;D_4_Helicobacteraceae;D_5_Helicobacter
	D_0_Bacteria;D_1_Proteobacteria;D_2_Gammaproteobacteria;D_3_Betaproteobacteriales;D_4_Burkholderiaceae;D_5_Parasutteria
	D_0_Bacteria;D_1_Firmicutes;D_2_Clostridia;D_3_Clostridiales;D_4_Lachnospiraceae;D_5_Roseburia
	D_0_Bacteria;D_1_Firmicutes;D_2_Clostridia;D_3_Clostridiales;D_4_Ruminococcaceae;D_5_Ruminoclostridium
	D_0_Bacteria;D_1_Firmicutes;D_2_Clostridia;D_3_Clostridiales;D_4_Lachnospiraceae;D_5_Lachnospiraceae_UCG-006
	D_0_Bacteria;D_1_Firmicutes;D_2_Clostridia;D_3_Clostridiales;D_4_Clostridiales_vadinBB60_group;D_5_uncultured_bacterium
	D_0_Bacteria;D_1_Proteobacteria;D_2_Deltaproteobacteria;D_3_Desulfovibrionales;D_4_Desulfovibrionaceae;D_5_uncultured
	D_0_Bacteria;D_1_Firmicutes;D_2_Clostridia;D_3_Clostridiales;D_4_Clostridiales_vadinBB60_group;D_5_Ambiguous_taxa
	D_0_Bacteria;D_1_Proteobacteria;D_2_Deltaproteobacteria;D_3_Desulfovibrionales;D_4_Desulfovibrionaceae;D_5_Desulfovibrio
	D_0_Bacteria;D_1_Firmicutes;D_2_Clostridia;D_3_Clostridiales;D_4_Clostridiales_vadinBB60_group;D_5_Roseburia
	D_0_Bacteria;D_1_Actinobacteria;D_2_Actinobacteria;D_3_Bifidobacteriales;D_4_Bifidobacteriaceae;D_5_Bifidobacterium
	D_0_Bacteria;D_1_Firmicutes;D_2_Clostridia;D_3_Clostridiales;D_4_Peptococcaceae;D_5_uncultured
	D_0_Bacteria;D_1_Firmicutes;D_2_Erysipelotrichia;D_3_Erysipelotrichales;D_4_Erysipelotrichaceae;D_5_Turicibacter
	D_0_Bacteria;D_1_Firmicutes;D_2_Clostridia;D_3_Clostridiales;D_4_Clostridiales_1;D_5_Clostridium_sensu_stricto_1
	D_0_Bacteria;D_1_Firmicutes;D_2_Clostridia;D_3_Clostridiales;D_4_Peptostreptococcaceae;D_5_Romboutsia
	D_0_Bacteria;D_1_Firmicutes;D_2_Clostridia;D_3_Clostridiales;D_4_Lachnospiraceae;D_5_ASF356
	D_0_Bacteria;D_1_Firmicutes;D_2_Clostridia;D_3_Clostridiales;D_4_Ruminococcaceae;D_5_Ruminoclostridium_5
	D_0_Bacteria;D_1_Firmicutes;D_2_Clostridia;D_3_Clostridiales;D_4_Lachnospiraceae;D_5_Blautia
	D_0_Bacteria;D_1_Firmicutes;D_2_Clostridia;D_3_Clostridiales;D_4_Lachnospiraceae;D_5_Eubacterium_ventriosum_group
	D_0_Bacteria;D_1_Firmicutes;D_2_Clostridia;D_3_Clostridiales;D_4_Ruminococcaceae;D_5_Intestinimonas
	D_0_Bacteria;D_1_Firmicutes;D_2_Erysipelotrichia;D_3_Erysipelotrichales;D_4_Erysipelotrichaceae;D_5_Erysipelatoclostridium
	D_0_Bacteria;D_1_Firmicutes;D_2_Clostridia;D_3_Clostridiales;D_4_Ruminococcaceae;D_5_Ruminococcaceae_NK4A214_group
	D_0_Bacteria;D_1_Firmicutes;D_2_Clostridia;D_3_Clostridiales;D_4_Ruminococcaceae;D_5_Anaerotruncus
	D_0_Bacteria;D_1_Actinobacteria;D_2_Coribacteria;D_3_Coribacteriales;D_4_Eggerthellaceae;D_5_Enterorhabdus
	D_0_Bacteria;D_1_Firmicutes;D_2_Clostridia;D_3_Clostridiales;D_4_Clostridiales_1;D_5_Candidatus_Arthromitus
	D_0_Bacteria;D_1_Firmicutes;D_2_Clostridia;D_3_Clostridiales;D_4_Lachnospiraceae;D_5_A2
	D_0_Bacteria;D_1_Firmicutes;D_2_Clostridia;D_3_Clostridiales;D_4_Lachnospiraceae;D_5_GCA-900066575
	D_0_Bacteria;D_1_Firmicutes;D_2_Clostridia;D_3_Clostridiales;D_4_Lachnospiraceae;D_5_Acetatifactor
	D_0_Bacteria;D_1_Firmicutes;D_2_Clostridia;D_3_Clostridiales;D_4_Ruminococcaceae;D_5_Ruminococcaceae_UCG-013
	D_0_Bacteria;D_1_Firmicutes;D_2_Clostridia;D_3_Clostridiales;D_4_Lachnospiraceae;D_5_Tyzzerella
	D_0_Bacteria;D_1_Firmicutes;D_2_Clostridia;D_3_Clostridiales;D_4_Family_XII;D_5_Family_XIII_UCG-001
	D_0_Bacteria;D_1_Firmicutes;D_2_Clostridia;D_3_Clostridiales;D_4_Ruminococcaceae;D_5_Eubacterium_coprostanoligenes_gn
	D_0_Bacteria;D_1_Firmicutes;D_2_Clostridia;D_3_Clostridiales;D_4_Ruminococcaceae;D_5_uncultured
	D_0_Bacteria;D_1_Proteobacteria;D_2_Alphaproteobacteria;D_3_Rhizobiales;D_4_Rhizobiaceae;D_5_Allorhizobium-Neorhizobium
	D_0_Bacteria;D_1_Firmicutes;D_2_Erysipelotrichia;D_3_Erysipelotrichales;D_4_Erysipelotrichaceae;D_5_Candidatus_Stoquefichu
	D_0_Bacteria;D_1_Firmicutes;D_2_Clostridia;D_3_Clostridiales;D_4_Family_XII;D_5_Eubacterium_brachy_group
	D_0_Bacteria;D_1_Firmicutes;D_2_Clostridia;D_3_Clostridiales;D_4_Ruminococcaceae;D_5_Butyricicoccus
	D_0_Bacteria;D_1_Firmicutes;D_2_Clostridia;D_3_Clostridiales;D_4_Family_XII;D_5_Eubacterium_nodatum_group
	D_0_Bacteria;D_1_Firmicutes;D_2_Clostridia;D_3_Clostridiales;D_4_Ruminococcaceae;D_5_Ruminococcaceae_UCG-010
	D_0_Bacteria;D_1_Actinobacteria;D_2_Coribacteria;D_3_Coribacteriales;D_4_Atopobiaceae;D_5_Coribacteriaceae_UCG-002
	D_0_Bacteria;D_1_Firmicutes;D_2_Erysipelotrichia;D_3_Erysipelotrichales;D_4_Erysipelotrichaceae;D_5_Roseburia
	D_0_Bacteria;D_1_Firmicutes;D_2_Clostridia;D_3_Clostridiales;D_4_Ruminococcaceae;D_5_Ruminococcus_1
	D_0_Bacteria;D_1_Firmicutes;D_2_Clostridia;D_3_Clostridiales;D_4_Lachnospiraceae;D_5_Anaerostipes
	D_0_Bacteria;D_1_Firmicutes;D_2_Clostridia;D_3_Clostridiales;D_4_Ruminococcaceae;D_5_GCA-900066225
	D_0_Bacteria;D_1_Firmicutes;D_2_Clostridia;D_3_Clostridiales;D_4_Ruminococcaceae;D_5_Ruminococcaceae_UCG-003
	D_0_Bacteria;D_1_Firmicutes;D_2_Clostridia;D_3_Clostridiales;D_4_Peptococcaceae;D_5_Peptococcus
	D_0_Bacteria;D_1_Firmicutes;D_2_Clostridia;D_3_Clostridiales;D_4_Peptostreptococcaceae;D_5_Clostridioides
	D_0_Bacteria;D_1_Firmicutes;D_2_Clostridia;D_3_Clostridiales;D_4_Lachnospiraceae;D_5_Marvinbryantia
	D_0_Bacteria;D_1_Proteobacteria;D_2_Deltaproteobacteria;D_3_Desulfovibrionales;D_4_Desulfovibrionaceae;D_5_Bilophila
	D_0_Bacteria;D_1_Firmicutes;D_2_Clostridia;D_3_Clostridiales;D_4_Lachnospiraceae;D_5_Lachnospiraceae_FCS020_group
	D_0_Bacteria;D_1_Firmicutes;D_2_Bacilli;D_3_Lactobacillales;D_4_Streptococcaceae;D_5_Streptococcus
	D_0_Bacteria;D_1_Firmicutes;D_2_Clostridia;D_3_Clostridiales;D_4_Ruminococcaceae;D_5_Ruminoclostridium_6
	D_0_Bacteria;D_1_Firmicutes;D_2_Clostridia;D_3_Clostridiales;D_4_Ruminococcaceae;D_5_Ruminococcus_2
	D_0_Bacteria;D_1_Cyanobacteria;D_2_Melainabacteria;D_3_Gastranaerophilales;D_4_Ambiguous_taxa;D_5_Ambiguous_taxa
	D_0_Bacteria;D_1_Proteobacteria;D_2_Gammaproteobacteria;D_3_Pseudomonadales;D_4_Pseudomonadaceae;D_5_Pseudomonas
	D_0_Bacteria;D_1_Proteobacteria;D_2_Gammaproteobacteria;D_3_Betaproteobacteriales;D_4_Burkholderiaceae;D_5_Rhodofera
	D_0_Bacteria;D_1_Firmicutes;D_2_Clostridia;D_3_Clostridiales;D_4_Ruminococcaceae;D_5_Harryflintia
	D_0_Bacteria;D_1_Firmicutes;D_2_Bacilli;D_3_Lactobacillales;D_4_Enterococcaceae;D_5_Enterococcus
	D_0_Bacteria;D_1_Firmicutes;D_2_Bacilli;D_3_Lactobacillales;D_4_Leuconostocaceae;D_5_Weissella
	D_0_Bacteria;D_1_Firmicutes;D_2_Bacilli;D_3_Bacillales;D_4_Staphylococcaceae;D_5_Staphylococcus
	D_0_Bacteria;D_1_Tenericutes;D_2_Mollicutes;D_3_Mycoplasmatales;D_4_Mycoplasmataceae;D_5_Ureaplasma
	D_0_Bacteria;D_1_Firmicutes;D_2_Clostridia;D_3_Clostridiales;D_4_Ruminococcaceae;D_5_Flavonifactor

Figure 19: List of bacteria and their color coordinate.

The following is a list of the bacterial strains with their specific color that is shown in the bar-plot shown in figure 19.

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