ROLE OF TYROSINE-RELATED KINASE B INHIBITION IN THE MESOCORTICOLIMBIC STRESS AND REWARD CIRCUITRIES OF THE ADOLESCENT AND ADULT BRAIN FOLLOWING A HETEROTYPIC STRESS REGIMEN

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ABSTRACT

The mesocorticolimbic system is involved in fundamental processes that drive motivational behaviors essential for survival (feeding, reproduction and sexual behavior, etc.), as well as neurochemical activity involved in mood regulation. Stressful life events are an important cause of dysregulated psychological functioning, which in some leads to a pathophysiology of mood disorders. A source of such disorder could be, among other underlying factors, an impairment of synaptic plasticity induced by alterations in the levels of neurotrophins and/or aberrant glucocorticoid responses. The role of the brain derived neurotrophic factor (BDNF) and its high affinity receptor tyrosine-related kinase B (TrkB) in the mesocorticolimbic reward circuitry has been largely studied in adulthood, yet a possible role of this system in mediating memory and emotional responses induced by stress during the juvenile, adolescence period has not been elucidated. The proposed set of thesis studies are designed to investigate the roles of BDNF and TrkB signaling, via the selective and non-competitive TrkB antagonist, ANA-12 (N-[2-[[(Hexahydro-2-oxo-1H-azepin-3-yl)amino]carbonyl]phenyl]-benzo[b]thiophene-2-carboxamide), in the expression of stress-induced changes in the brain stress circuitry (including the medial prefrontal cortex (mPFC), hypothalamic-pituitary-adrenal (HPA) axis, and hippocampus) and reward signaling systems of the brain (including the nucleus accumbens (NAc) and ventral tegmental area (VTA)). In addition, experiments aim to determine behavioral changes following stress exposure in male and female Wistar rats. Finally, the possible interplay between BDNF, dopamine, glutamate and orexins in response to repeated stress is examined. Articles 1 and 2, aimed to assess the biochemical and behavioral effects of direct ANA-12 infusion (0.25 μg/ 0.5μl) into the nucleus accumbens shell during exposure to a 10-day heterotypic stress paradigm in male rats. Specifically, Article 1 demonstrated a key role for BDNF/TrkB signaling to regulate stress-induced effects. Notably, the impact of ANA-12 to attenuate anxiety-like behavior in repeatedly stressed rats while increasing anxiety behavior in non-stress rats suggest an interesting behavioral and neurochemical state-dependent process induced by TrkB receptor signaling. Article 2 supports the key role for BDNF secretion in basal and stress-induced behaviors in rats suggesting an influence of TrkB in sociability, motivation and passive avoidance. Furthermore, this role of TrkB extended to increased

expression of orexin A in the Perifornical area (PfA) and a decrease in the ventral CA1 of the hippocampus, and in stress-induced elevations in orexinergic projections to the VTA, of which reductions were observed in non-stress groups treated with ANA-12. Article 3 demonstrated gender-specific behavioral and biochemical responses in different developmental periods and the impact of TrkB activation, dependent on stress exposure, to affect the regulation of TrkB receptor isoforms (full length and truncated TrkB, TrkB.FL and TrkB.T1, respectively) in adulthood. Results revealed increased CORT responses in adolescent females relative to males and attenuated CORT secretions in both genders by TrkB inhibition. Elevated activity levels in young adult females and increased passive coping behavior in the forced swim in stress-naïve females were also noted, in addition to novel observations on brain region and sex differences in TrkB receptor isoforms. Taken together, thesis findings derived from applications of ANA-12, shall foster knowledge on the contribution of BDNF in regulation of mood upon stress exposure at times when the brain is undergoing important maturation and remodelling, as well as on the relationship of stress exposure during adolescence and lasting brain and behavioral disorders in adulthood.

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LIST OF ABBREVIATIONS

ANA-12: *N*-[2-[[(Hexahydro-2-oxo-1*H*-azepin-3-yl)amino]carbonyl]phenyl]benzo[b]thiophene-2-carboxamide

ANS: ANA-12 + no stress

AS: ANA-12 + stress

BDNF: Brain-derived neurotrophic factor

BLA: Basolateral amygdala

BNST: Bed nucleus of the stria terminalis

BSA: bovine serum albumin

cAMP: Cyclic adenosine monophosphate

CREB: cAMP response element binding protein

CA1: Cornu ammonis 1

CA3: Cornu ammonis 3

CeA: Central nucleus of the amygdala

CNS: Central nervous system

CRH: Corticotropin-releasing hormone

D1: Dopamine receptor 1

D2: Dopamine receptor 2

DA: Dopamine

ΔFosB: delta FBJ murine osteosarcoma viral oncogene homolog B

EPM: Elevated plus maze

ERα: Estrogen receptor alpha

ERß: Estrogen receptor beta

FS: Forced swim

$GABA_A$: Gamma-aminobutyric acid receptor A

GR: Glucocorticoid receptors

HCRT: Hypocretin

HPA: Hypothalamic-pituitary-adrenal

IHC: Immunohistochemistry

LH: Lateral hypothalamus

MAPK: Ras/ mitogen-activated protein kinase

mPFC: Medial prefrontal cortex

MSN: Medium spiny neurons

MR: Mineralocorticoid receptors

mRNA: messenger ribonucleic acid

NAc: Nucleus accumbens

NGF: Nerve growth factor

NMDA: *N*-methyl-*D*-aspartate

NT-3: Neurotrophin 3

NT-4/5: Neurotrophin 4/5

OFT: Open field test

OX_1R: Orexin receptor 1

OX_2R: Orexin receptor 2

$p75^{NTR}$: Pan 75 neurotrophin receptor

PBS: Phosphate buffered saline

pCREB: phosphorylated CREB

PfA: Perifornical area

PI3-K: Phosphatidylinositol 3- Kinase

PND: Postnatal day

PNS: Peripheral nervous system

PVN: Paraventricular nucleus of the hypothalamus

RS: Restraint stress

SIT: Social interaction test

TH: Tyrosine hydroxylase

Trk: Tropomyosin-related kinase receptor family

TrkA: Tyrosine-related kinase A

TrkB: Tyrosine-related kinase B

TrkB.FL: Full-length TrkB receptor isoform

TrkB.T1: Truncated TrkB receptor isoform

VGCCs: Voltage gated Ca^{2+} channels

VGluT2: Vesicular glutamate transporter 2

VNS: Vehicle + no stress

VS: Vehicle + stress

VTA: Ventral tegmental area

YMCPP: Y maze conditioned place preference

YMPAT: Y maze passive avoidance task

General introduction

Depression is a prevalent mental disorder that affects up to one-sixth of the active population at least once in their lifetime (Kozisek, Middlemas, & Bylund, 2008; Masi & Brovedani, 2011), with a 5:2 female to male prevalence ratio, however it does not show an age bias (i.e. from childhood to old age) (Bondy, 2002). It is characterized by adverse changes in mood, anhedonia, worthlessness, agitation and suicidal ideation (Stahl, 2008), and is known to involve more than one etiology (Bondy, 2002). Due to lack of individualized anti-depressant medications, about half of the population are unable to show an improvement in their clinical symptoms and the state of dysregulation in brain processes is associated with a delay between the initiation of treatment and attenuation of symptoms. This may be due to sex specificity in the body's response to medication (e.g. hormones, metabolism, body composition). As such, more research is being conducted to explain predispositions to depression and complex processes of remission. Currently, strong evidence has been provided for cell atrophy, loss of glia and neurons as contributing factors to the pathophysiology of several mood disorders comorbid with depression, such as anxiety and other stress related illnesses (See review (Duman & Li, 2012)), which also implicate reduced levels of the monoamines noradrenaline and serotonin at the synaptic cleft.

It has been proposed that long-term adaptations are necessary to the therapeutic actions of chronic treatments (Duman, Heninger, & Nestler, 1994), including cognitive behavioral therapy and synergistic non/pharmacological options. Among such adaptations, the regulation of neurotrophins, such as the brain-derived neurotrophic factor (BDNF) (Kozisek et al., 2008) in both presynaptic and postsynaptic mechanisms, has led to the neurotrophic hypothesis of depression (Duman, 2004), especially when depression is associated with stress (Chao, 2003). This hypothesis proposes that depression is a consequence of decreased neurotrophic support, e.g. BDNF and neurotrophin-3 (NT-3), which leads to neuronal atrophy, reduced hippocampal neurogenesis and loss of glia that may be blocked or reversed with the use of antidepressant treatment (Duman & Aghajanian, 2012; Duman & Voleti, 2012; Hosang, Shiles, Tansey, McGuffin, & Uher, 2014). For example, reduced BDNF levels have been implicated in the behavioral dysfunctions consistent with serotonergic

abnormalities (Autry & Monteggia, 2012; Martinowich & Lu, 2008) and trophic effects on noradrenergic neurons (Chao, 2003; Fawcett et al., 1998; Saarelainen et al., 2003). Notably, exogenous administration of BDNF to the hippocampus of rats induced antidepressant behavioral effects in animal models of depression, comparable to the effects of chronic pharmacological treatment with antidepressants (Chao, 2003; Shirayama, Chen, Nakagawa, Russell, & Duman, 2002). In addition, normal signaling of BDNF's primary receptor, tyrosine-related kinase B (TrkB), is required for the behavioral effects typically induced by antidepressants, thereby implicating activation of the TrkB receptor in the mechanism of action of antidepressant drugs (Saarelainen et al., 2003). Thus, antidepressant treatments are reported to elevate adaptive responses of neuronal systems and activity-dependent neuronal plasticity by activating BDNF and gradually improving network function and mood (Kozisek et al., 2008). In this context, the use of animal models provides a comparable approach of elucidating the mechanisms that lead to vulnerability and adaptability to depression in humans (i.e. what we learn in one species may help to understand responses in the other species).

Along these lines, pharmacological manipulations involving administration of the selective TrkB receptor antagonist, ANA-12, is of interest in this thesis with regards to the effects of repeated stress exposure on neurochemical, physiological and behavioral changes in Wistar rats (See Fig. 1 for chemical structure of ANA-12). The derivation of ANA-12 through in silico screening and in-depth pharmacological assessments confirmed the high efficacy (full inhibition) and potency (submicromolar) of the molecule on the extracellular domain of TrkB receptors, demonstrating a 2-site mode of action by preventing activation by BDNF, non-competitively, in high (specific to neurons) and low (TrkB only-expressing cells) affinity sites (Cazorla et al., 2011). As such, ANA-12 is reported to inhibit processes downstream of TrkB without altering TrkA and TrkC functions, and is shown to induce anxiolytic and anti-depressant effects in rodents exposed to various tests predictive of anxiety responses in humans (Cazorla et al., 2011). Therefore, ANA-12 may show efficacy as a therapeutic in the treatment of mood disorders.

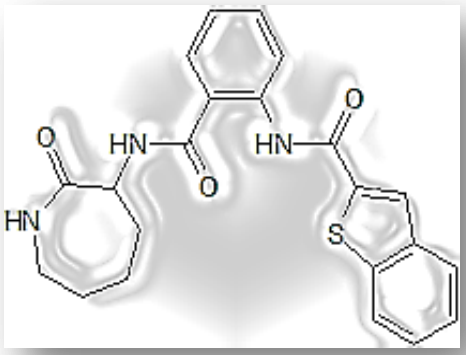

Figure 1. Chemical structure of ANA-12. Synonym: *N*-[2-[[(Hexahydro-2-oxo-1*H*-azepin-3-yl)amino]carbonyl]phenyl]benzo[b]thiophene-2-carboxamide. (Purchased from Sigma-Aldrich).

In the following sections of the introduction, section 1 presents an overview of neurotrophins with a focus on BDNF synthesis and expression, and activation of its receptors TrkB and pan75 neurotrophin receptor ($p75^{NTR}$). Section 2 discusses the interaction of BDNF with the neuroendocrine system, beginning with HPA axis responsivity to stress, influence of neuropeptides such as orexins and CRH in the activation of the axis, and the link between glucocorticoids and BDNF's role in stress adaptation. The interplay between BDNF and dopamine transmission on stress-related signaling is also discussed. In addition, the site-specific actions of BDNF, following stress, on the brain stress (paraventricular nucleus of the hypothalamus (PVN), hippocampus, prefrontal cortex (PFC) and amygdala) and reward systems (nucleus accumbens (NAc) and ventral tegmental area (VTA)) are explored. This section also considers sex dimorphism in BDNF signaling and the impact of stress responsivity on developmental periods. Finally, section 3 outlines the objectives for this thesis and briefly summarizes the series of thesis experiments.

1. An overview of regulatory actions of neurotrophins

Neurotrophins are among the many growth factors that are widely studied for their mediatory role in the adult central nervous system (CNS). They are secreted by neurons in both a constitutive (i.e. Ca^{2+}-independent) and activity -dependent (i.e. Ca^{2+}-dependent) manner (Al-Qudah & Al-Dwairi, 2016; Blöchl & Thoenen, 1996; Goodman et al., 1996; B. Lu, 2003). Adaptive responses of an organism consist of a variety of refinements in the morphology and function of neurons that can lead to strengthening or weakening of synapses, which may

alter presynaptic neurotransmitter release and postsynaptic neuron responsiveness (Tao, Finkbeiner, Arnold, Shaywitz, & Greenberg, 1998).

Among neurotrophins considered as genuine molecular mediators of synaptic development and plasticity, BDNF, a highly conserved protein, exerts diverse effects on neuronal survival (i.e. inhibited apoptosis of developing neurons), differentiation, synapse formation, maturation and function of neurons throughout the CNS and peripheral nervous system (PNS) (Huang & Reichardt, 2001; Kafitz, Rose, Thoenen, & Konnerth, 1999; Lommatzsch et al., 1999; Park & Poo, 2013; Ukropec, Ukropcova, Kurdiova, Gasperikova, & Klimes, 2008), in addition to its actions as a mediator of behavioral interactions between an organism and its external environment (Tyler, Alonso, Bramham, & Pozzo-Miller, 2002). It is synthesized in subregions of the hypothalamus, such as the lateral hypothalamus (LH), paraventricular nucleus (PVN), and ventromedial- and dorsomedial- hypothalamic nucleus (VMN and DMN, respectively) (Conner, Lauterborn, Yan, Gall, & Varon, 1997). Furthermore, the 14 kDa neurotrophin is abundantly expressed in the developing and mature mammalian CNS with high levels observed in the hippocampus, amygdala and cerebral cortex (Alcantara et al., 1997; Hofer, Pagliusi, Hohn, Leibrock, & Barde, 1990; Murakami, Imbe, Morikawa, Kubo, & Senba, 2005; Phillips, Hains, Laramee, Rosenthal, & Winslow, 1990). Via its high-affinity transmembrane receptor TrkB, BDNF regulates gene expression, ion channel function, morphogenesis, neuronal structure, and synaptic plasticity (Andero, Choi, & Ressler, 2014; Schaich, Wellman, Koi, & Erdos, 2016). Co-expression of BDNF and TrkB in neurons and vasculature of the CNS suggest that BDNF acts as paracrine (local action on nearby cells) or autocrine (direct action on the cell in which it was produced) factors to modulate function (Cohen-Cory, Escandón, & Fraser, 1996; Goodman et al., 1996; H. Kim, Li, Hempstead, & Madri, 2004; Kokaia et al., 1993). BDNF and TrkB have been identified in presynaptic axon terminals, indicative of anterograde axonal transport by afferent systems including central nucleus of the amygdala (CeA) and bed nucleus of the stria terminalis (BNST), and in postsynaptic dendritic compartments of neurons, suggesting their ability to induce bidirectional release and activity (Conner et al., 1997; Noble, Billington, Kotz, & Wang, 2011; Tyler et al., 2002).

In the mammalian brain, the expression, release and/or actions of BDNF are directly and tightly regulated by neural activity (Park & Poo, 2013; Hans Thoenen, 1991) in a Ca^{2+} and neuron-selective manner (Tao, West, Chen, Corfas, & Greenberg, 2002). For instance, the induction of BDNF exon III transcription is Ca^{2+} dependent, and shown to occur in neurons but not in other cell types (e.g. following membrane depolarization of PC12 cells), suggesting the restrictive range of cell types and stimuli that may induce transcription. Furthermore, elevated intracellular levels of cyclic AMP (cAMP) has little to no influence on transcription, although BDNF expression is in part regulated by the transcription factor cAMP response element binding protein (CREB), which is implicated in the regulatory control of activity-dependent adaptive responses of neurons (Tao et al., 2002). This phenomenon suggests that BDNF expression is driven by a coordinated activation of transcription factors. In this context, membrane depolarization by high potassium (K^+) triggers Ca^{2+} influx via L-type voltage gated Ca^{2+} channels (VGCCs) and non- N-methyl-*D*-aspartate (NMDA)-type glutamate receptors, which promotes the binding of transcription factors such as Ca^{2+} response factor (CaRF) and phosphorylation of CREB, thereby inducing BDNF transcription, mRNA expression and secretion of the protein (Al-Qudah & Al-Dwairi, 2016; Ghosh, Carnahan, & Greenberg, 1994; Park & Poo, 2013; Tao et al., 1998, 2002; Zafra, Hengerer, Leibrock, Thoenen, & Lindholm, 1990; Zheng et al., 2009). Interestingly, while induction of BDNF mRNA transcription is mediated by non-NMDA glutamate receptors, activation of NMDA receptors determines the basal levels of BDNF mRNA, as noted by a rapid inhibition of BDNF mRNA expression following administration of the non-competitive NMDA receptor antagonist, MK-801, in hippocampal cultures and *in vivo* (Castrén, Berzaghi, Lindholm, & Thoenen, 1993; Zafra, Castrén, Thoenen, & Lindholm, 1991).

Other members of the neurotrophin family of proteins include the nerve growth factor (NGF), neurotrophin 3 (NT-3) and NT-4/5. Sequences of these proteins share about 50% amino acid homology with BDNF (Hohn, Leibrock, Bailey, & Barde, 1990; Leibrock et al., 1989; Maisonpierre et al., 1990), indicating structural similarities that are involved in neuronal specificity. The biological activity of these neurotrophins depends primarily upon the activation of specific high-affinity Tropomyosin-related kinase family of receptors

(Trk) (Vesa, Kruttgen, & Shooter, 2000). These structurally related receptors have similar intrinsic protein-tyrosine kinase activities, but different ligand binding properties (Greene & Kaplan, 1995; Jiang et al., 2005; Klein et al., 1991; Lamballe, Klein, & Barbacid, 1991; Rodriguez-Tebar, Dechant, & Barde, 1990). Thus, BDNF and NT-4/5 have a high affinity for TrkB, which has three known isoforms, a full length transmembrane receptor and two truncated receptors that lack the tyrosine kinase domain (TrkB.FL, TrkB.T1 and TrkB.T2) (Eide et al., 1996; Fryer, Kaplan, & Kromer, 1997). In contrast, NGF and NT-3 binds preferentially to TrkA and TrkC, respectively, although they show secondary interactions with TrkB in certain cell culture systems (Barbacid, 1994; Boulle et al., 2012; Cazorla et al., 2011; Jiang et al., 2005; Segal, 2003). The binding of BDNF to TrkB receptors can lead to the activation of downstream intracellular signaling cascades, including the phospholipase C-γ (PLC-γ), phosphatidylinositol 3- Kinase (PI3-K) and Ras/ mitogen-activated protein kinase (MAPK), following trans-autophosphorylation of their tyrosine residues (Chao, 2003). The Trk receptors are required for neuronal survival and neurite outgrowth.

Neurotrophins can also bind to the proteolysis and apoptosis promoter, pan 75 neurotrophin receptor ($p75^{NTR}$, a member of the tumor necrosis factor family (TNF-CD40-Fas receptor family)) (Lu, Cheng, Lim, Khoshnevisrad, & Poo, 2010), although with different rate constants (Rodriguez-Tebar et al., 1990). Interactions between Trk family members and $p75^{NTR}$, and the ratio of receptor expression, can lead to conformational changes in the binding affinity for neurotrophins during competition for limited concentrations of trophic factors (Chao, 2003; Chao & Hempstead, 1995), determining both their responsiveness and specificity (Chao, 2003; Chao, Rajagopal, & Lee, 2006), as well as the numbers of surviving cells during development. For example, co-expression of TrkB with $p75^{NTR}$ may be necessary to enhance selectivity for BDNF (Chao & Hempstead, 1995). Of note, mature BDNF (considered the biologically active form) signals through TrkB, while its precursor pro-BDNF preferentially activates $p75^{NTR}$, resulting in activation of signaling cascades with opposite modulatory effects on neuronal survival, growth cones decisions to inhibitory axon-guidance molecules, and synaptic plasticity (Chao, 2003; Olsen, Kaas, Schwartz, Nykjaer, & Glerup, 2013). Lack of $p75^{NTR}$ or pro-BDNF/BDNF is shown to increase memory in an inhibitory avoidance task (Olsen et al.,

2013). Conversely, elevated $p75^{NTR}$ expression in the CNS has been reported following ischemia (Kokaia, Andsberg, Martinez-Serrano, & Lindvall, 1998), traumatic injury (Martinez-Murillo, Fernandez, Bentura, & Rodrigo, 1998) and stress (Dobrowsky & Carter, 2000), presumably owing to a change in environmental conditions. Therefore, it is thought that the activation of $p75^{NTR}$ plays a role in mediation of cell death and functional impairments in a variety of neurodegenerative disorders, whereas BDNF's activation of TrkB receptors may promote neuroprotection through beneficial effects on cell survival and synaptic transmission (Hennigan, O'Callaghan, & Kelly, 2007).

Understanding of the differential activation of these discrete receptors in the adult brain is highly complex and remains to be elucidated. Furthermore, changes in transcription of neurotrophins can cause long term alterations in the functionality of adult neurons, and as a result this may produce the delay observed in therapeutic actions of antidepressant treatments (Chao, 2003). Of interest is the proposed role of BDNF and TrkB in the pathophysiology of mood disorders, such as anxiety.

2. Interaction of BDNF with the neuroendocrine system

2.1. The hypothalamic-pituitary-adrenal axis response to stress

Normal hypothalamic-pituitary-adrenal (HPA) axis activity is important for the regulation of internal homeostasis and survival during times of stress. Stress is defined as any disruption of homeostasis (Miller & O'Callaghan, 2002). The central control stations of the stress system is mainly comprised of the dorsomedial parvocellular corticotropin releasing hormone (CRH) and arginine – vasopressin (AVP) neurons of the paraventricular nucleus (PVN) of the hypothalamus and the locus coeruleus/Norepinephrine (LC/NE) autonomic systems and their peripheral effectors, the pituitary-adrenal axis and the limbs of the autonomic system (Tsigos & Chrousos, 2002). Activation of the HPA axis is facilitated through anatomical connections between brain regions such as the amygdala, hippocampus and hypothalamus (Fig. 2). Activation of the amygdala in response to stressors of any kind triggers the release of CRH in the PVN of the hypothalamus within minutes of stressor onset, followed by the induced secretion of adrenocorticotropic hormone (ACTH) from the anterior lobe of the pituitary gland by the hypothalamus, and the subsequent stimulated synthesis and

secretion of glucocorticoids (cortisol in humans and corticosterone in rodents) by the adrenal gland. Glucocorticoids are downstream effectors of the HPA axis and they feedback at the level of the anterior pituitary, hypothalamus, prefrontal cortex and hippocampus and binds to two types of nuclear corticosteroid receptors that act as transcriptional regulators (Type I mineralocorticoid receptors (MR) and Type II glucocorticoid receptors (GR)) in order to dampen excess activation of the HPA axis and terminate the stress response (Herman, McKlveen, Solomon, Carvalho-Netto, & Myers, 2012; Tsigos & Chrousos, 2002). GR, exhibiting highest density in the PVN and in limbic regions that modulate PVN function, is also localized in various stress-responsive brain regions, whereas MR has a more restrictive binding pattern in the brain and is suggested to regulate the HPA axis during basal periods of secretion (Ratka, Sutanto, Bloemers, & de Kloet, 1989; Reul & de Kloet, 1986). GR is thought to mediate memory consolidation, whereas MR processes appraisal and novelty responses (Srinivasan, Shariff, & Bartlett, 2013). Within the hippocampus, the balance and optimum function of MR and GR regulate the level of glucocorticoids in the serum by inhibitory feedback, especially inhibitory inputs derived from GR-expressing neurons, which is the dominant receptor subtype regulating stress response. This negative feedback mechanism to inhibit the CRH system allows an organism to adjust to dynamic environments, and enhance adaptability to stressful stimuli throughout life (Sinclair, Purves-Tyson, Allen, & Weickert, 2014).

Research suggests that the immediate, adaptive responses to physical or emotional stressors have some specificity towards the different classes of stressors that generate them (Tsigos & Chrousos, 2002), e.g. processive (requiring interpretation by higher brain structures with respect to previous experience) or systemic (physiologic threat) (Herman & Cullinan, 1997; Herman, Prewitt, & Cullinan, 1996). However, this response can become maladaptive as the severity of the stressor increases and result in abnormal changes in brain plasticity that may impair the ability of the brain to appropriately regulate and respond to subsequent stressors (Radley & Morrison, 2005). In a chronic stress condition, inappropriate activation of the amygdala leads to hyperactivity of the HPA axis, resulting in enhanced levels of glucocorticoids (Fig. 2). Prolonged exposure to increased concentrations of this corticosteroid, e.g. in the hippocampus, leads to atrophy and neuronal loss, and

weakens or disrupts the negative feedback mechanism. For instance, chronic activation of GR in the hippocampus in response to stress is shown to downregulate and alter the function of the receptor, and therefore this relative lack of hippocampal GR may reduce HPA axis inhibitory feedback and lead to an over-activity and hypersecretion of glucocorticoids (Anacker, Zunszain, Carvalho, & Pariante, 2011; Conrad, 2008). Furthermore, this dysregulation of the HPA axis can compromise neuronal integrity, and impair cognitive and behavioral functions. The outcome of the system in response to glucocorticoids depends on the severity, duration and context of the stressor, the age and sex of the organism, species, and the hypo- or hyper-responsiveness of the axis (Aisa, Tordera, Lasheras, Del Río, & Ramírez, 2007; Chen, Zhou, Bai, Zhou, & Chen, 2015; Ostrander, Ulrich-Lai, Choi, Richtand, & Herman, 2006; Sapolsky, 2015).

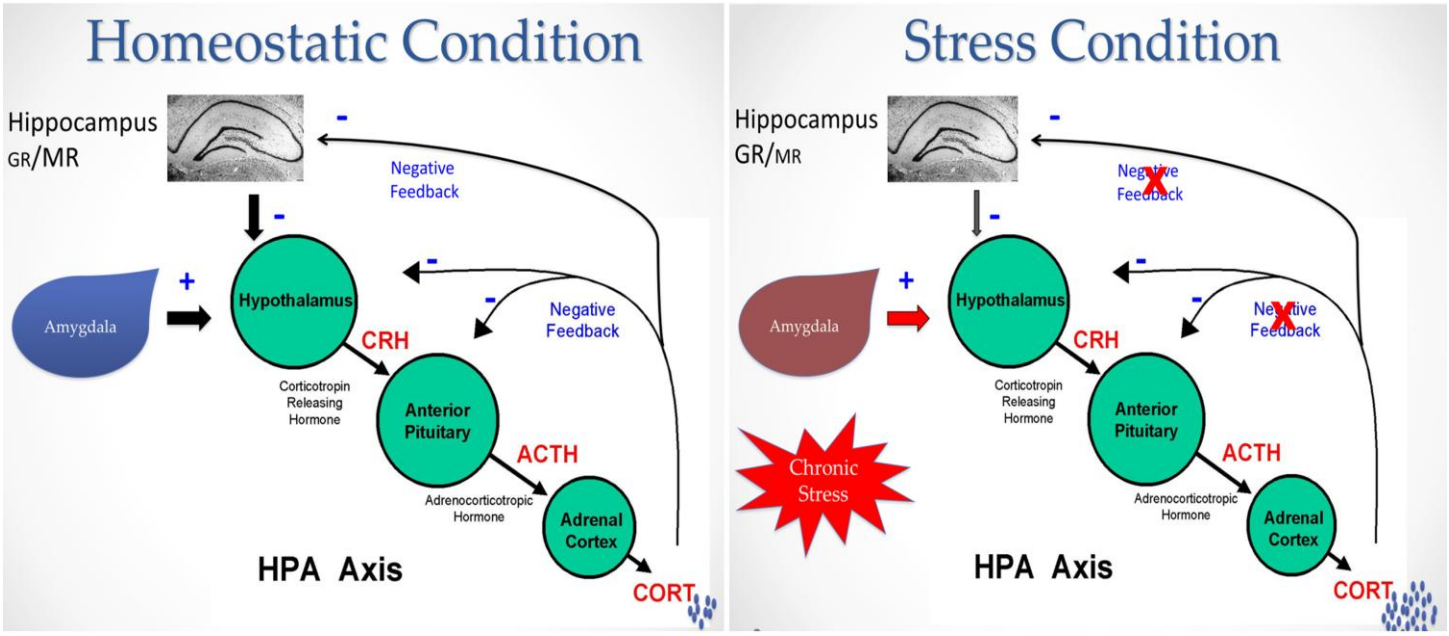

Figure 2. Schematic diagram of the HPA axis – homeostatic and stress conditions. In a homeostatic condition, activation of the amygdala promotes the eventual release of glucocorticoids (CORT, cortisol in humans and corticosterone in animals) through the HPA axis. Glucocorticoids then feedback at the level of the hippocampus, hypothalamus and pituitary to dampen excess activation of the HPA axis, thus promoting resilience and survival. In a chronic stress condition, inappropriate activation of the amygdala extensively stimulates the HPA axis, resulting in high levels of CORT in the body. Prolonged exposure to high concentrations of glucocorticoids in the hippocampus leads to neuronal loss and morphological atrophy, and thus the negative feedback becomes weakened or disrupted.

2.2. Hormones, neuropeptides and transmitter activation of the HPA axis, role in stress and interaction with BDNF

2.2.1. Role of BDNF/TrkB in mood disorders (glucocorticoids and stress adaptation)

Glucocorticoids play an important role in the regulation of basal and stress-related homeostasis within the HPA axis and extra-hypothalamic regions and are thus implicated in the promotion or prevention of stress adaptability (Srinivasan et al., 2013). Therefore, it is noted that an individual's ability to cope with stress in the environment may depend on their HPA axis reactivity and can predict their capacity to recover quickly from adverse experiences or to succumb to a stress-related disorder such as anxiety and depression. Glucocorticoids also provide feedback loops for axis regulation and exhibit diurnal fluctuations, with the extent of release corresponding with a peak at the onset of the active period of the day and nadir at the end of the active phase (Chung, Son, & Kim, 2011). This natural circadian rhythm can be impacted following acute or chronic exposure to stressful environmental stimuli.

Research has shown that stress can affect the ability to experience pleasure and impair decision-making processes (Wanat, Bonci, & Phillips, 2013). The reduction in reward-seeking behaviors is associated with changes in dopamine transmission and heightened stress response evidenced by a prolonged activation of the HPA axis. In addition, the BDNF/TrkB pathway has been implicated in several psychiatric and mood disorders including PTSD, phobia, anxiety and panic disorder (Andero et al., 2014; Autry & Monteggia, 2012). Recently, the interplay between glucocorticoids and BDNF/TrkB-mediated signaling was discussed (Anacker et al., 2011; Arango-Lievano et al., 2016; Jeanneteau & Arango-Lievano, 2016; Numakawa et al., 2010), associating glucocorticoids and BDNF with the pathophysiology of depression.

GR mediates the adaptation to stress as the principle regulator of the feedback inhibition mechanism of the HPA Axis (Gądek-Michalska, Spyrka, Rachwalska, Tadeusz, & Bugajski, 2013), effecting dynamic changes in stress systems, synaptic plasticity and cognitive functions. The translocation of GR into the nucleus of neurons stimulated with BDNF is reported to require the presence of glucocorticoids, providing a mechanism through which the synergistic actions of BDNF and glucocorticoids prime the activation of GR's genomic response and promote functional attributes required for adaptive plasticity of neurons to stress and response to

antidepressants (Arango-Lievano et al., 2015). This suggests that glucocorticoid signaling and adaptation to stress can change as a function of BDNF signaling (Arango-Lievano et al., 2016; Lambert et al., 2013). In this regard, BDNF via TrkB activation, as well as the effects of stress, are reported to directly impact GR transcriptional regulation on functionally distinct gene expression (Lambert et al., 2013).

GR and TrkB are co-expressed in the cortex, hippocampus and PVN, providing further support for the possible modulatory role of BDNF/TrkB signaling to phosphorylate and regulate GR activity (Lambert et al., 2013). Treatment with dexamethasone (a synthetic glucocorticoid) was shown to activate TrkB in the hippocampus and promote cell survival in neurons deprived of trophic support via a mechanism of genomic GR actions (Jeanneteau, Garabedian, & Chao, 2008). In addition, co-treatment of primary neuronal cultures with mifepristone (a GR antagonist) reduced TrkB phosphorylation, indicating promotion of TrkB signaling by GR activation. Moreover, behavioral studies have reported BDNF-induced alterations in glucocorticoid responses in cortico-limbic circuits involved in goal-directed decision-making, forced swim performance, fear extinction and contextual fear memory (Gourley et al., 2012; Gourley, Kedves, Olausson, & Taylor, 2009; Gourley, Kiraly, Howell, Olausson, & Taylor, 2008; Revest et al., 2014). Chronic oral administration of corticosterone in rodents induced long term decreases in hippocampal BDNF and phosphorylated CREB (pCREB) as well as increased amotivational performance in a progressive ratio task and forced swim immobility, effects which were reversed following acute BDNF micro-infusion into the hippocampus (Gourley et al., 2008). Such molecular link between the BDNF and glucocorticoid systems highlights their actions in both neuroprotection and stress-associated pathophysiology in the nervous system.

2.2.2. Role of the orexin system in the regulation of HPA axis activation

Many neuropeptides and hormones regulate autonomic and neuroendocrine functions, influencing wakefulness and vigilance states. Of interest are the hypocretin family of neuropeptides (also known as orexins), which are mainly expressed in the lateral hypothalamus (LH), perifornical area (PfA) and posterior hypothalamus (Cluderay, Harrison, & Hervieu, 2002; Takeshi Sakurai, 2014), but have extensive CNS projections. Orexin A and orexin B are derived from a common precursor prepro-orexin gene and both elicit

physiological actions via two types of orphan G-protein-coupled receptors, orexin receptor-1 (OX_1R) and orexin receptor-2 (OX_2R) (Cluderay et al., 2002; Sakamoto, Yamada, & Ueta, 2004; Tsunematsu & Yamanaka, 2012). Binding of orexin A to OX_1R is one order of magnitude greater than orexin B binding, whereas binding of both orexin A and orexin B to OX_2R are of similar affinities. It has been proposed that orexin neurons in the LH play a role in motivated behaviors such as reward seeking, feeding and emotionality, whereas orexin neurons in the PfA and posterior hypothalamus mainly regulate arousal and adaptation to stress (Harris & Aston-Jones, 2006; Takeshi Sakurai, 2014; Srinivasan et al., 2013), a response possibly mediated by BDNF signaling.

The orexin system is a key component in stress response, orexins and their receptors being expressed in the PVN, pituitary, adrenal cortex and extra-hypothalamic structures (Spinazzi, Andreis, Rossi, & Nussdorfer, 2006). Acute stress appears to increase orexin neuron activation (Furlong, Vianna, Liu, & Carrive, 2009; Ida et al., 2000). For instance, 1 h of immobilization stress in 2-month old rats and 30 min of cold stress in 6-month old rats were reported to increase orexin mRNA levels in the LH (Ida et al., 2000). Studies also showed that ICV or systemic administration of orexin A can increase Fos protein expression in CRH cells, enhance CRH and AVP release from the PVN and CeA and activate the HPA axis, leading to the secretion of ACTH and glucocorticoids (Al-Barazanji, Wilson, Baker, Jessop, & Harbuz, 2001; Ida et al., 2000; Jászberényi, Bujdosó, Pataki, & Telegdy, 2000; Kuru et al., 2000; Nowak et al., 2000; Sakamoto et al., 2004). In contrast, chronic stress inhibits orexin activity (Nocjar, Zhang, Feng, & Panksepp, 2012), downregulating orexin A in the LH (Lutter et al., 2008), but upregulating expression in the ventral portion of the hippocampus (Nollet et al., 2012). The latter has been observed in the cerebral spinal fluid of suicidal patients with major depressive disorder (Brundin, Björkqvist, Petersén, & Träskman-Bendz, 2007). These findings suggest that orexins play a role in the HPA axis response to a variety of stressful stimuli.

2.2.3. Impact of stress on BDNF/ TrkB signaling: interplay between CRH, BDNF and the dopamine transmitter systems

The mesocorticolimbic dopamine (DA) system has been implicated in stress-induced effects on the CNS (Enrico et al., 2013). The system plays important roles in executive function, reward, motivation, cognition,

learning, memory, and movement (Yamaguchi, Wang, Li, Ng, & Morales, 2011). Activation of the mesolimbic dopamine pathway (comprised of the dopaminergic neurons projecting from the ventral tegmental area (VTA) to the nucleus accumbens (NAc) (Koob & Kreek, 2007)) allows an organism to engage in emotional salience of stimuli in the environment, whereas activation of the mesocortical dopamine pathway (which connects the VTA to the prefrontal cortex) plays an important role in affective and cognitive functions as well as emotional arousal (Berton et al., 2006; Imperato, Puglisi-Allegra, Casolini, & Angelucci, 1991; Moal & Simon, 1991; Westerink, Enrico, Feimann, & Vries, 1998). Importantly, the VTA responds to both rewarding (Almela et al., 2012; Di Chiara & Imperato, 1988) and aversive (Anstrom & Woodward, 2005) stimuli through differential regulation of dopamine release (Matsumoto & Hikosaka, 2009) and improves learned associations with novel stimuli via different sensory modalities to appetitive and aversive outcomes (Kim, Matthews, & Moghaddam, 2010). Additionally, activity of the VTA-NAc reward circuit can be influenced by orexins (E. J. Nestler & Carlezon, 2006). The VTA expresses both OX_1R and OX_2R, with a predominant expression of OX_1R in DA neurons (Takeshi Sakurai, 2014). Orexins innervate the VTA and are expressed in DA cell bodies, potently exciting these neurons and triggering their release in the NAc and PFC (Bubser & Schmidt, 1990; Korotkova, Sergeeva, Eriksson, Haas, & Brown, 2003; Vittoz & Berridge, 2006), suggesting that they play a role in modulating DA activity and behavioral state dependent processes.

Recently, CRH coactivation of CRHR1 and CRHR2 receptors is shown to modulate dopamine release in the NAc of stress naïve mice (Lemos et al., 2012), which if released at low concentrations can promote appetitive response to stimuli such as social bonding (Lim et al., 2007). However, unilateral dopamine depletion with the catecholaminergic-neuron-selective neurotoxin, 6-OHDA, prior to ipsilateral CRH infusion into the NAc blocked appetitive responding in a conditioned place preference. Furthermore, exposure to severe stress (e.g. repeated forced swimming for two days) acted to switch emotional response to CRH action in the NAc from appetitive to aversive, as observed in conditioned place aversion and reduced novel object exploration, and blocked the ability of CRH to induce dopamine release (Lemos et al., 2012), indicating a biological mechanism for the shift in affective states characterized in stress-induced depressive disorders. The authors note that this

loss of CRH-dopamine interaction within the NAc could be prevented by the GR antagonist, RU-486, implicating a critical role of glucocorticoids in this interaction. Note: RU-486 is also a pure progesterone antagonist, and with use, sex-specific assessments become important. As part of its role in regulating motivational vs. aversive properties of various stimuli, a dysregulation of mesocorticolimbic DA signaling has been implicated in the pathophysiology and etiology of depression and mood disorders.

Neurotrophins are reported to influence DAergic neurons in an autocrine or paracrine manner, and the distribution of neurons expressing BDNF and NT-3 mRNAs correspond to the distribution of DA cells and their terminal fields in brain regions (Gall, Gold, Isackson, & Seroogy, 1992; Seroogy & Gall, 1993). The essential role of BDNF in the mesolimbic dopamine pathway has been reported in response to social stress (Berton et al., 2006; Walsh et al., 2014), survival, function and long-term adaptations of DAergic neurons (Berton et al., 2006; Gall et al., 1992; Horger et al., 1999), actions of stimulant drugs (D. L. Graham et al., 2009; Danielle L. Graham et al., 2007; Horger et al., 1999), and antidepressant treatment (Berton et al., 2006; Krishnan et al., 2007; Tsankova et al., 2006; Tsankova, Renthal, Kumar, & Nestler, 2007). In addition to CRH actions, DA release in the NAc is potentiated through BDNF-mediated TrkB activation on dopaminergic nerve terminals (Goggi, Pullar, Carney, & Bradford, 2003).

BDNF plays a role in mediating the mesolimbic reward pathway to chronic stress. Upregulation of BDNF in the NAc is observed following phasic optogenetic activation of the mesolimbic dopamine pathway in stressed animals (Walsh et al., 2014). Phasic firing of VTA DA neurons under different behavioral conditions is crucial for reward-related behaviors (Koo et al., 2012; Tsai et al., 2009), however phasic firing had no effect on NAc BDNF upregulation in stress-naïve animals (Walsh et al., 2014). In stressed animals, increased BDNF release in the NAc, following phasic stimulation, is shown to induce social avoidance behavior, an effect that is blocked by prior administration of ANA-12 (a highly potent and selective TrkB receptor antagonist that does not affect BDNF protein) or a CRH receptor antagonist (α-helical CRH), whereas in stress-naïve animals and in the absence of optical stimulation, CRH infusion had no effect on social behavior (Walsh et al., 2014). The authors reported a requirement of CRH in phasic firing-induced increase in NAc BDNF following stress, which

has important implications in understanding an organism's stress-context detecting function and response to stimuli.

2.3. Impact of stress on site-specific actions of BDNF/ TrkB signaling

2.3.1. Stress differentially regulates BDNF action in the brain's stress system

2.3.1.1. Paraventricular nucleus of the hypothalamus (PVN)

The medial parvocellular division of the PVN serves as a major site in the regulation of the HPA axis and receives afferent GABAergic projections mainly from local hypothalamic sources (Herman & Cullinan, 1997; Roland & Sawchenko, 1993) playing a prominent regulatory control of afferent CRH neurons in the PVN (Calogero, Gallucci, Chrousos, & Gold, 1988; Cullinan, 2000). Studies provide support for GABA's role in stress integration of the hypothalamus. Within the CNS, fast GABA transmission is dependent on the $GABA_A$ receptor-mediated influx of chloride (Cl^-), and stress is shown to impair the efficacy of Cl^- transport, compromising the integrity of the GABA-mediated inhibition (Hewitt, Wamsteeker, Kurz, & Bains, 2009). Within hypophysiotropic CRH neurons, $GABA_A$ receptor subunits (e.g. β1 and β2) (Cullinan, 2000) are shown downregulated following a chronic heterotypic stress protocol (Cullinan & Wolfe, 2000). In addition, manipulations that decrease GABA's tight regulation on CRH cells (Verkuyl, Karst, & Joëls, 2005) increase neural activity and levels of circulating corticosteroids, suggesting that such phenomenon can initiate HPA axis reactivity post stress (Cole & Sawchenko, 2002). Indeed, either exposure to a 30 min restraint stress or to Bicuculline disinhibition of $GABA_A$ synaptic responses on neuroendocrine cells increase circulating corticosteroid release (Hewitt et al., 2009), suggesting that $GABA_A$ inhibitory influence is significantly reduced following stress.

In contrast, glutamate signaling exerts a regulatory influence on the HPA axis response to stress (Evanson & Herman, 2015). For example, direct infusion of glutamate into the PVN is shown to increase ACTH secretion (Darlington, Miyamoto, Keil, & Dallman, 1989). Moreover, chronic variable stress increases apposition of vesicular glutamate transporter 2 (vGluT2, a marker of glutamate expression (Herzog et al., 2001)) to CRH neurons in the parvocellular PVN, indicative of a dense innervation of glutamatergic fibers to

stimulate CRH neurons, while no alterations in GABAergic innervation was reported. Changes in expression of vGluT2 are associated with changes in the quantal size (i.e. the parameter controlling the strength of synaptic transmission) of glutamate released at synapses (Daniels et al., 2004). In a previous study, chronic central stimulation of the dorsomedial parvocellular region of the PVN was reported to reduce the expression of GR and GABA receptor subunits while enhancing expression of glutamate receptors and co-secreted peptides, e.g. CRH (Herman, Flak, & Jankord, 2008). This is consistent with attenuated inhibitory influence of GABA following chronic unpredictable stress (Verkuyl et al., 2005) being achieved via the diminished expression of $GABA_A$ receptor subunits in the PVN, which increased excitation of CRH neurons following stress exposure (Cullinan & Wolfe, 2000). These findings suggest that chronic stress-induced neuroplastic changes in the parvocellular subdivision may enhance hyper-responsiveness of the HPA axis to incoming stimuli.

Notably, BDNF's mediation of the expression of GABA and glutamate, is shown through regulation of fast synaptic inhibition and excitation, BDNF reducing GABAergic and increasing glutamatergic synapses, respectively (Brünig, Penschuck, Berninger, Benson, & Fritschy, 2001). For instance, elevated BDNF levels have been shown to block surface expression of $GABA_A$ receptors, effects which can be reversed by prior administration of the TrkB receptor antagonist, K252A (Hewitt & Bains, 2006; Hewitt et al., 2009). Interestingly, BDNF potentiation of glutamate release is more persistent when the postsynaptic neuron is glutamatergic, but not when it is GABAergic (Schinder, Berninger, & Poo, 2000). This is because excitatory synapses onto excitatory pyramidal neurons elicit long term potentiation and increases the frequency but not amplitude of miniature excitatory postsynaptic currents (mEPSCs), whereas excitatory synapses onto inhibitory GABAergic interneurons are weakened and undergo long term depression in a feed forward manner (Ashwood, Lancaster, & Wheal, 1984; McMahon & Kauer, 1997). In reverse, glutamate (via NMDA and non-NMDA receptors) and acetylcholine (via muscarinic receptors) trigger the upregulation of BDNF mRNA, whereas downregulation is effected via $GABA_A$ receptors (See review (Lindholm, Castrén, Berzaghi, Blöchl, & Thoenen, 1994)). Subtle changes in the balance of both these neurotransmitters alter BDNF expression affecting synaptic transmission and neuronal development, and antidepressant properties of TrkB receptor blockade are in

part attributable to inhibition of stress-induced BDNF effects in the PVN (Schaich et al., 2016). These studies indicate that modulations by BDNF may act to enhance presynaptic transmitter release and postsynaptic responsiveness via a TrkB phosphorylation-dependent pathway, and modify synaptic strength.

BDNF is considered a stress-responsive intercellular messenger in HPA axis activity (Givalois et al., 2004; Jeanneteau et al., 2012; Murakami et al., 2005; Masashi Nibuya, Takahashi, Russell, & Duman, 1999; Smith & Cizza, 1996). Increased BDNF mRNA in the PVN and lateral part of the hypothalamus and the pituitary is reported following single or repeated restraint stress (Givalois et al., 2001; Smith, Makino, Kim, & Kvetnansky, 1995), while decreased BDNF mRNA levels are noted in extra-hypothalamic regions such as the BLA, cingulate cortex and hippocampus (Smith, Makino, Kim, et al., 1995). For example, Givalois et al., (2001) demonstrated upregulation in pituitary BDNF mRNA within 15 min post immobilization stress followed by increased BDNF protein 45 min later, and accompanied by diminished TrkB mRNA expression at 15 and 30 min time intervals. Impairment of GR function in the parvocellular portion of the PVN similarly upregulated BDNF levels, triggering a gradual increase in CRH expression, accompanied by elevated ACTH and corticosterone secretion and disinhibition of the HPA axis (Givalois et al., 2004; Jeanneteau et al., 2012). In another study, overexpression of BDNF triggered an increase in CRH expression (Cao et al., 2009). BDNF's ability to increase CRH expression is suggested mediated through TrkB-CREB signaling (Jeanneteau et al., 2012).

2.3.1.2. Hippocampus

The hippocampus expresses the highest levels of BDNF and TrkB in the mammalian brain (Murer, Yan, & Raisman-Vozari, 2001), and BDNF's role in learning, memory and synaptic plasticity has been extensively studied, including intracellular signaling cascades and neurotransmission. Following membrane depolarization, BDNF release occurs through a regulated pathway of secretion that targets the somatodendritic domain of hippocampal pyramidal neurons (Goodman et al., 1996), suggesting that BDNF is released from cell bodies and dendrites (Dugich-Djordjevic et al., 1995). Release of BDNF from the dendrites is followed by binding to TrkB receptors located on presynaptic axon terminals (Dugich-Djordjevic et al., 1995). Both BDNF and TrkB are

implicated in the structural and functional aspects of the adaptive response of hippocampal neurons to stress (Nibuya, Morinobu, & Duman, 1995; Vaidya & Duman, 2001). Elevations in BDNF expression at this site is suggested to reinstate feedback inhibition of the HPA axis in response to antidepressant treatment (Nibuya et al., 1995).

It is well established that antidepressant therapy exerts beneficial actions on the HPA axis, including blockade of corticosterone-mediated decrease in BDNF expression (Barden, 2004; Dwivedi, Rizavi, & Pandey, 2006). Increased BDNF and TrkB expression, as well as CREB protein, have been observed in patients treated with antidepressants at the time of death, compared to non-treated subjects; in contrast to reduced expression of BDNF and TrkB in the post-mortem hippocampus and prefrontal cortex of suicide victims (Chen, Dowlatshahi, MacQueen, Wang, & Young, 2001; Dwivedi et al., 2003). Treatment with the TrkB antagonist, ANA-12, is shown to attenuate the inflammatory effects of lipopolysaccharide on depression behavior by normalizing alterations in dendritic spines in the PFC, hippocampus and NAc and reducing immobility in a social defeat stress model of depression (J. Zhang, Wu, et al., 2015). Along with BDNF, NT-3 and $p75^{NTR}$ play a role in depression by influencing both the frequency and amplitude of synaptic currents in nerve muscle synapses, and through LTP in hippocampal neurons (Chao, 2003; Kang & Schuman, 1995; Levine, Dreyfus, Black, & Plummer, 1995; Lohof, Ip, & Poo, 1993), exerting effects on behavior.

The length, timing, and type of stressor can affect BDNF expression as certain exon-specific BDNF transcripts are shown upregulated or downregulated in distinct hippocampal subfields (Marmigère, Givalois, Rage, Arancibia, & Tapia-Arancibia, 2003; Nair et al., 2006). Increased BDNF signaling induces phosphorylation of TrkB receptors in its cytoplasmic domain and a later increase in exon IV-specific BDNF mRNA (Jiang et al., 2005). The activity-dependent exon IV promoter is a major target for corticosterone-induced transcriptional regulation of hippocampal BDNF (Hansson et al., 2006), of which decreases were observed following increases in GR levels (Smith, Makino, Kvetnansky, & Post, 1995). Furthermore, heightened GR levels following HPA axis hyperactivity have been linked to BDNF downregulation in the hippocampus (Fuchikami, Morinobu, Kurata, Yamamoto, & Yamawaki, 2009; Smith, Makino, Kvetnansky, et

al., 1995; Ueyama et al., 1997), resulting in atrophy of limbic structures implicated in depression (see review (J. D. Bremner, 2006)). For example, a decrease in BDNF mRNA levels following 6h of daily restraint stress was associated with dendritic atrophy/ death of hippocampal pyramidal neurons and reduction of hippocampal volume in rats (Murakami et al., 2005). However, Jeanneteau et al. (2008) showed that chronic administration of metyrapone (a glucocorticoid synthesis inhibitor) or co-administration with dexamethasone can enhance GR and TrkB phosphorylation in the rodent hippocampus and increase corticosterone levels in the absence of neurotrophin production, demonstrating independent effects of glucocorticoids to activate TrkB. This upregulation of catalytic TrkB (or TrkB.FL) mRNA is suggested to be a compensatory adaptation utilized by the hippocampus to cope with repeated stress (Nibuya et al., 1999; Shi, Shao, Yuan, Pan, & Li, 2010), suggesting bidirectional regulation of BDNF and TrkB expression in this region.

The involvement of BDNF and TrkB in LTP and synaptic plasticity, suggests that their actions may impact cognitive functions, such as hippocampus-mediated learning and memory. TrkB gene knockout in mice during postnatal development impaired learning and severely reduced threshold for LTP at CA1 hippocampal synapses, and altered coping responses in stressful and complex learning paradigms in adult homozygous mutant mice (Minichiello et al., 1999). Of note, rhythmic circadian and acute stress-induced increases in glucocorticoids are shown essential for memory acquisition and consolidation, hippocampal neuronal survival, facilitation of glutamate neurotransmission, induction of immediate early genes and dendritic spine formation (Uchoa et al., 2014). In contrast, chronic glucocorticoid elevations from prolonged stress exposure dysregulate BDNF expression in the hippocampus (Shi et al., 2010; Smith, Makino, Kvetnansky, et al., 1995; Ueyama et al., 1997), altering neuronal plasticity and impairing memory processes. For instance, repeated exposure to restraint stress for 6 h/day for 21 days resulted in impaired spatial memory performance in male rats (Luine, Villegas, Martinez, & McEwen, 1994), and in females, estrogen is proposed to foster stress resilience following 1 week of repeated restraint stress (Wei et al., 2014). Alterations in neuronal plasticity are also noted in patients with major depression disorder, effects that can be reversible with chronic antidepressant treatment. This reversal was assessed through the measurement of serum or plasma BDNF concentrations.

2.3.1.3. Prefrontal cortex (PFC)

Together with the hippocampus, the PFC is a brain region most sensitive to the deleterious impact of stress exposure. The medial PFC (mPFC) is involved in mood related disturbances induced by chronic stressors (Arnsten, 2009). In particular, depression has been associated with hyper- and hypo-activity of the ventromedial PFC (vmPFC) and the dorsomedial PFC (dmPFC), respectively (Koenigs & Grafman, 2009). Bidirectional connections between the vmPFC and subcortical structures including the amygdala, hypothalamus and NAc are involved in emotion processing (Arnsten, 2009; Quirk & Mueller, 2008), and are shown to participate in conditioned fear extinction (Milad et al., 2007; Milad & Quirk, 2002). Notably, weaker stressors tend to activate the left hemisphere of the PFC, while stimulation of the right hemisphere is observed following chronic stress exposure which affects dendritic arborization (Perez-Cruz, Simon, Czéh, Flügge, & Fuchs, 2009; Sullivan, 2004).

The PFC, along with the hippocampus, is also implicated in the feedback inhibition of glucocorticoid stress responses, this region containing a high density of corticosteroid receptors (Diorio, Viau, & Meaney, 1993; B. S. McEwen, Kloet, & Rostene, 1986) and studies have reported strong glutamatergic activation of the PFC following acute stress or glucocorticoid administration (See review (Popoli, Yan, McEwen, & Sanacora, 2011)). In a recent study, Chiba et al. (2012) demonstrated that chronic restraint stress-induced increases in glucocorticoid secretion was accompanied by downregulation of GR expression at the PFC, attenuating BDNF levels and triggering rat-specific depression phenotypes. Chronic corticosterone secretion similarly decreased TrkB protein in the PFC and hippocampus enhancing anxiety and depression behaviors (Kutiyanawalla, Jr, & Pillai, 2011). In contrast, antidepressant treatment increase expression of BDNF and pCREB in the prefrontal cortex through activation of a MAPK p38 and ERK1/2 pathway (Alboni et al., 2010). These studies support glucocorticoid and BDNF as mediators of PFC sensitivity to acute and chronic stressors, and indicate that chronic stress may enhance depression pathophysiology via excitatory glutamatergic synapses.

2.3.1.4. Amygdala

The preponderant role of the amygdala in the neural basis of emotion is well acknowledged, being involved in the emission of emotional responses and related memory formation (Cardinal, Parkinson, Hall, & Everitt, 2002). With regards to control of emotional processes, the basolateral amygdala (BLA), which projects to the PFC and portions of the ventral striatum (such as the NAc) and regulates the central nucleus of the amygdala (CeA), plays a role in emotional Pavlovian learning and complex emotional responses. The CeA rather connects to the hypothalamus, VTA and brainstem and promotes behavioral autonomic and neuroendocrine responses (Cardinal et al., 2002; Everitt et al., 1999). The amygdala also contributes to regulation of HPA axis activity (Forray & Gysling, 2004; B. S. McEwen, 2000). Under stress conditions, the amygdala potentiates activation of stress pathways in the hypothalamus and brainstem, impairing PFC regulation (Arnsten, 2009).

BDNF is differentially affected at the hippocampus and amygdala in stress conditions, leading to opposite structural changes in these regions, e.g. dendritic atrophy of the CA3 pyramidal neurons in the hippocampus and enhanced dendritic arborisation in the pyramidal and stellate neurons of the BLA (Vyas, Mitra, Shankaranarayana Rao, & Chattarji, 2002). Indeed, while chronic immobilization stress leads to dendritic atrophy in CA3 pyramidal neurons of the hippocampus, structural hypertrophy is observed at the BLA accompanied by elevated anxiety in the elevated plus maze (Vyas et al., 2002). Heightened anxiety and amygdaloid hypertrophy have been shown to persist up to 21 days following stress recovery, an effect contrasting a more rapid reinstatement of normal morphology at the hippocampal CA3 layer (Vyas, Pillai, & Chattarji, 2004). In line with these observations, BDNF expression following immobilization stress remained increased in the BLA up to 21 days, while at the CA3 BDNF had returned to baseline levels within the same period (Lakshminarasimhan & Chattarji, 2012). Behaviorally, amygdalar BDNF/TrkB signaling has adaptive function and appears necessary for acquisition of fear conditioning (Ou, Yeh, & Gean, 2010), and this effect is prevented by administration of the TrkB antagonist K252A, TrkB antibodies (TrkB-IgG) or TrkB.T1 lentiviral infection (Ou et al., 2010; Rattiner, Davis, French, & Ressler, 2004). Taken together, these findings illustrate

that chronic stress-induced structural plasticity in the amygdala follow a temporal profile and point to a role of BDNF in the behavioral modifications underlying affective states or mood disorders.

2.3.2. Increased BDNF signaling in the reward system and depression susceptibility

2.3.2.1. Nucleus accumbens (NAc) and ventral tegmental area (VTA)

Exposure to acute restraint stress stimulates the activity of the dopaminergic system, which within the medial shell of the NAc is proposed to trigger a switch in hedonic valence. The synaptic weights on NAc are suggested to be very different with respect to hedonic valence (Roitman, Wheeler, & Carelli, 2005), such as incentive salience (e.g. desire) vs. fearful salience (e.g. dread), increasing the possibility that there are different mechanisms inducing this neuroanatomical coding switch from an appetitive to aversive behavioral response towards a stimulus. Richard and Berridge (2011) pointed out that within the medial shell of the NAc, neuroanatomical coding plays an important role in determining appetitive versus some forms of aversive motivation related to fear, stress, and pain generated by glutamate disruptions, suggesting that the switch in motivational valence are dynamic to match the environmental ambience generated at the moment, and that this is a result of the roles of local D1 and D2 receptor signaling in dopamine/glutamate interaction in the NAc. Furthermore, it is suggested that GABA/glutamatergic manipulations at rostral sites in the NAc medial shell (i.e. “rostral hotspot”) typically induce appetitive behaviors, whereas similar neurochemical manipulations at caudal sites (i.e. “caudal coldspot”) produce fearful or defensive behaviors (Castro, Cole, & Berridge, 2015).

Of interest, elevated BDNF levels in the reward circuitry of the brain can predict susceptibility to stress-induced anxiety and depression (Berton et al., 2006). For example, intra-VTA infusions of BDNF resulted in a depression-like effect (Eisch et al., 2003) and infusions of BDNF into the NAc increased susceptibility to social defeat stress, although no changes were observed in TrkB and phospho-TrkB levels of susceptible versus control mice (Krishnan et al., 2007). In contrast, deletion of BDNF within the VTA-NAc pathway in adult mice has been shown to reduce susceptibility to adverse stress effects in response to aversive social experiences and to induce similar effects produced by chronic treatments with antidepressants (Berton et al., 2006; Krishnan et al., 2007). In assessing the molecular mechanisms underlying such resilience, Krishnan and colleagues (2007)

report the importance of plasticity within the brain's reward circuits in actively maintaining an emotional homeostasis.

Within the NAc, TrkB mRNA is expressed in D1+ and predominantly in D2+ medium spiny neurons (MSNs) (Smith, Lobo, Spencer, & Kalivas, 2013), of which selective TrkB deletion from D2+ MSNs attenuated cocaine reward and increased neuronal excitability (as measured by c-Fos induction, marker of neuronal activity); whereas selective TrkB deletion in D1+ MSNs enhanced behavioral responses to cocaine, but decreased c-Fos induction in the NAc shell, but not the NAc core, without a significant change in baseline neuronal excitability. This suggested that blocked BDNF/TrkB signaling differentially altered the functionality of both D1+ and D2+ NAc MSNs and may have led to an imbalance in behavioral output, through increased activity and underactivation of the D1+ and D2+ MSN pathways, respectively (Lobo et al., 2010). These studies provide further evidence for the role of the NAc in integrating and attaching valence to the excitatory and inhibitory signals downstream of mesolimbic dopamine neurons and regulating mood (Carlezon & Thomas, 2009).

BDNF is involved in the short term potentiation of dopamine release in the NAc through phosphorylation of TrkB on dopaminergic nerve terminals (Goggi et al., 2003), and may play a role in the promotion of plasticity at glutamatergic synapses onto VTA dopaminergic neurons (Pu, Liu, & Poo, 2006). It has been reported that dopamine release and dopamine-related behaviors are enhanced following increased levels of BDNF or activation of TrkB in the NAc (Narita, Aoki, Takagi, Yajima, & Suzuki, 2003). In this context, acute versus prolonged exposure to various unavoidable stressors show differential effects to increase or reduce dopamine release in the NAc shell, respectively (See review (Shirayama & Chaki, 2006)). Studies suggest that promotion of BDNF-TrkB-CREB signaling and downstream cascades of BDNF in the NAc can increase susceptibility to chronic social defeat stress. This effect is thought mediated via repressive histone methylation (G9a) in the NAc (Covington et al., 2011) and support altered BDNF signaling as a factor mediating individual predisposition to mood disorders upon stress exposure.

2.4. Sex dimorphic modulation of BDNF signaling

2.4.1. Why are male and female differences important to assess?

Sex-specific differences exist in the activity of the HPA axis, both in the basal state and during activation resulting from stress exposure (Liu et al., 2011). Recent comprehensive reviews explore the neurobiological underpinnings of sex-specific differences in neuroendocrine response to stress (including the critical point of divergence during development), emotional and arousal responses, as well as sex biases in disease vulnerability (Bangasser & Valentino, 2014; Panagiotakopoulos & Neigh, 2014). Men show increased susceptibility to substance-related disorders, whereas a 2-fold greater prevalence of depression is reported in women compared to men (Grant et al., 2004; Kendler, 1998; Kessler, 2003; Kornstein, 1997), in part due to differences in sex steroid hormone- and circulating corticosteroid- binding globulin (CBG) levels (Handa, Burgess, Kerr, & O'Keefe, 1994; Herman & Cullinan, 1997; Herman et al., 1996). In rats, CBG levels in plasma were shown increased in prenatally stressed adult females, but not in adult males (McCormick, Smythe, Sharma, & Meaney, 1995). Moreover, female rats exhibit increased ACTH response to stress, earlier onset and faster rise of corticosterone (CORT) secretion compared to males (Burgess & Handa, 1992; Handa et al., 1994), as well as prolonged HPA axis activation in response to inescapable foot shock (Heinsbroek, Van Haaren, Feenstra, Endert, & Van de Poll, 1991). Female rats also show heightened ACTH and CORT secretion following exposure to potent stressors (forced running, immobilization, and footshock) compared to males (Kant et al., 1983; McCormick et al., 1995). Other studies attributed sex-specific differences to CORT levels facilitating adaptation to acute stressors in female rodents while impairing adequate glucocorticoid secretion following repeated restraint stress (Haleem, Kennett, & Curzon, 1988) or chronic unpredictable mild stress exposure (Lu et al., 2015). In support of this contention, attenuation of glucocorticoid synthesis using metyrapone prior to exposure to 5 consecutive days of restraint stress in female rats lowered plasma CORT secretion to levels typical of restrained males not treated with metyrapone and attenuated deficits in behavioral responses (Haleem et al., 1988). These studies support the role of gender to observed differences in brain

regulation of corticosteroid release, increasing susceptibility to chronic and/or repeated stress exposure and prevalence of depression in females.

Researchers implicate levels of circulating estradiol (the primary female sex hormone) as a contributive factor to the enhanced sensitivity in females, as these levels fluctuate across the estrous and reproductive cycle events, and during chronic stress exposure (Herrera & Mather, 2015). A recent report by Wei et al. (2014) proposed that estrogen fosters stress resilience in females, showing that it protects against the detrimental effects of 1 week of repeated restraint stress on glutamatergic transmission and PFC-dependent cognition. Furthermore, sex-specific differences have been noted in spine density and dendritic morphology of pyramidal neurons in the CA1 region, attributed to increased dendritic spine density in females during the proestrus phase of the estrous cycle compared to males who lack high levels of circulating estrogen (Gould, Woolley, Frankfurt, & McEwen, 1990; Woolley, Gould, Frankfurt, & McEwen, 1990). Moreover, the expression and release of BDNF in the DG of the rat hippocampus is shown increased via actions of estrogen, inducing synaptogenesis, while administration of the TrkB antagonist, K252A, blocked this effect in hippocampal slice cultures (Sato, Akaishi, Matsuki, Ohno, & Nakazawa, 2007).

Sexual dimorphic changes in BDNF signaling have been observed in male and female rats in stress related brain regions and in the reward pathway (Franklin & Perrot-Sinal, 2006; Lin et al., 2009; Marco et al., 2013; Scharfman & MacLusky, 2014). For instance, female rats exposed to a 3 h period of restraint stress showed robust reduction in hippocampal BDNF levels and voluntary wheel-running and increased corticosterone secretion compared to males (Yamaura et al., 2013). Interestingly, using a chronic unpredictable stress paradigm (CUS), Autry and colleagues (2009) showed the increased vulnerability to stress in female, compared to male, mice to be related to sex specific differences in stress-induced depletion of BDNF in the forebrain (i.e. similar loss of BDNF in males not resulting in a greater susceptibility to depression behavior). Such findings are interesting considering that ovarian hormones directly affect BDNF mRNA and protein in the CNS (Franklin & Perrot-Sinal, 2006). In another study, BDNF alterations were suggested region specific, Greenberg and colleagues (2014) showing increased BDNF protein in the bed nucleus of the stria terminalis of

adult female mice but not in males following social defeat stress. In addition, the authors showed that ANA-12 blockade of BDNF action on TrkB receptors at this locus increased social interactions in stressed females. Thus, studies support sex dimorphism in response to stress, and the differential gender-based response profiles to antidepressant treatment. To date, adult populations have been the focus of existing studies and there is a need to investigate other developmental periods and gender-specific vulnerabilities to early life stress.

2.5. Developmental differences in HPA axis function and sensitivity to stress

2.5.1. Adolescence vs. adulthood

Early life exposure to stressful events is known to exert a major impact on immediate and long-term development of neuroendocrine, cognitive, and behavioral systems (Ilin & Richter-Levin, 2009). It can enhance stress responsiveness and cause long-term abnormalities in adult behavior (Cirulli et al., 2009; Luoni et al., 2014). When assessing the boundaries of the adolescent period in rodents, most researchers agreed that the prototypic age range for adolescence conservatively ranges from postnatal day (PND) 28 to 45 (reviewed in (O'Dell, 2009)) and PND 60 for young adulthood (reviewed in (McCormick & Green, 2013)). In this respect, the adolescence period is a critical time for functional neurodevelopment, with increased sensitivity to behavioral and psychological events that may alter the trajectory of brain development. Exposure to early life stress, depending on stressor intensity and duration, has been shown to improve HPA regulation and promote lifelong resilience to stress or to induce a hyper-reactive HPA axis that may contribute to lifelong vulnerability to stress (Maniam, Antoniadis, & Morris, 2014). For instance, adolescents are particularly vulnerable to depressive disorders due to ongoing brain development and a neural circuitry that is rich in corticosteroid receptors. In addition, male and female prepubertal rats exhibit greater HPA reactivity, notable in a more pronounced and slower recovery to ACTH and CORT response relative to adults (Hall & Romeo, 2014; Romeo, Lee, & McEwen, 2004; Vázquez & Akil, 1993). The adolescent brain may be more vulnerable to the effects of stressors due to developmental rate and differences in HPA axis functionality resulting in a prolonged release of glucocorticoids in response to a stressor (see review (Green & McCormick, 2016; Romeo, 2010)) i.e., early life

experience is one of the main determinants of HPA axis activity, alterations to this axis being linked to anxiety and depression in later life.

Importantly, adolescents differ from adults with regards to neurochemical mechanisms involved in excitatory and inhibitory influences on dopamine transmission in the brain reward circuit. During adolescence, excitatory glutamatergic systems that facilitate dopamine are overdeveloped, whereas inhibitory GABAergic systems are underdeveloped (O'Dell, 2009). Thus, the VTA cell bodies that release dopamine in the NAc show enhanced excitation and reduced inhibition, which has been linked to differential vulnerability in this population, in part related to neurotrophic factors such as NGF and BDNF being highly affected by stress (Cirulli et al., 2009; de Almeida et al., 2013). BDNF expression undergoes significant changes related to its role in the maturation of brain structures (Hofer et al., 1990), and stress-induced impairments in the signaling of this neurotrophin can lead to persistent alterations in postnatal brain function and behavior (Cirulli et al., 2009). Early life adversity has been shown to significantly attenuate BDNF expression in the adult PFC and hippocampus (Fumagalli, Bedogni, Perez, Racagni, & Riva, 2004; Luoni et al., 2014), which may increase vulnerability to psychiatric disorders (Fumagalli et al., 2004). Of note, TrkB receptor has been shown increased in the hippocampus of young and aged rats following chronic mild repeated stress, whereas levels of BDNF were decreased (Shi et al., 2010). Functionally, the lack of TrkB signaling within the hippocampus of developing mice has been shown to impair GABAergic neurotransmission and lead to age-dependent hyperexcitability of neuronal circuits (Carmona et al., 2006). In addition, TrkB knockout in the developing forebrain exacerbated learning deficits in adult mutant mice, impairments being prominent in complex/stressful paradigms and related to changes in LTP in pyramidal CA1 neurons (Minichiello et al., 1999).

The question arises as to which endogenous mechanisms are most vulnerable during this sensitive period and play a role in behavioral alterations following stress. This represents an important question considering the growing incidence of mood disorders triggered in early adolescence, and which result in a reduced capacity to subsequently mediate emotional responses. Gaining insight into the neurobiology of the adolescent brain is an

important step for understanding the increased vulnerability towards the disruptive effects of stress in adulthood.

3. Thesis study objectives

Considering that stress exposure induces alterations in a variety of brain circuits and that the site-specific actions of BDNF can be modulated by TrkB antagonism, we aimed to address the resultant behavioral and neurochemical effects of selectively targeting BDNF/TrkB signaling in the NAc shell of the reward pathway vs. other cortical regions in adult male rats, as well as the systemic blockade of TrkB receptors in the context of sex differences in adolescent rats prior to assessment in adulthood.

To investigate the mechanisms at play, this thesis (1) characterized the effects of repeated heterotypic stress exposure on the activity of BDNF and TrkB in the reward and stress circuitries; (2) determined whether blockade of TrkB receptors can enhance resilience to stress exposure in certain behaviors, such as increased exploration and social interaction, in Wistar rats; and finally (3) evaluated sex differences in stress response following ANA-12 treatment in adolescent and adult rats.

Table 1 summarizes, at a glance the three papers that are part of this thesis. Paper 1 and 2 reports complementary findings on the same animals that were directly infused with ANA-12 or vehicle in the NAc shell prior to exposure to a chronic heterotypic stress paradigm, but addresses different facets of the effects of heterotypic stress and TrkB inhibition on anxiety in adult male Wistar rats. **Paper 1** "Blockade of TrkB receptors in the nucleus accumbens prior to heterotypic stress alters corticotropin-releasing hormone (CRH), vesicular glutamate transporter 2 (vGluT2) and glucocorticoid receptor (GR) within the mesolimbic pathway" (Azogu and Plamondon, 2017): This study showed how TrkB signaling at the NAc impacted expression of stress and mood regulators in the interconnected circuitry. We utilized ELISA (blood corticosterone secretion), behavioral testing (open field test (OFT), elevated plus maze (EPM) and forced swim test (FST)) and immunohistochemical detection (including CRH- and vGluT2- ir in the PVN and BLA, and GR- and TrkB- ir in the NAc shell and core, and cingulate cortex) to determine effects of intra-accumbal treatment on anxiety and

depressive-like effects as well as on signaling pathways involved in regulation of the stress response, mood and emotionality. **Paper 2** "Inhibition of TrkB at the nucleus accumbens, using ANA-12, regulates basal and stress-induced orexin A expression within the mesolimbic system and affects anxiety, sociability and motivation" (Azogu and Plamondon, 2017): This study took a novel approach by evaluating the ability of ANA-12 to prevent stress-induced changes in Orx-A, FosB/ΔFosB and TH expression in the mesolimbic circuitry (i.e. LH, PfA, dorsal and ventral hippocampus and ventral tegmental area) and cognitive, emotional and social impairments (including social interaction/ preference test, a modified Y maze conditioned place preference test and a Y maze passive avoidance task). **Paper 3** "Sex differences in corticosterone secretion, behavioral phenotypes and expression of TrkB.T1 and TrkB.FL receptor isoforms: Impact of systemic TrkB inhibition and heterotypic stress exposure in adolescence" (Azogu, Liang, and Plamondon, submitted): This study looked at enduring effects of TrkB inhibition and repeated stress exposure in adolescence on sex differences in endocrine (corticosterone via ELISA), behavioral (OFT, EPM and FST), and plasticity signaling responses (Full-length TrkB and truncated TrkB receptor isoforms via Western blotting). Behavioral changes and TrkB receptor isoforms were assessed in young adulthood.

This thesis work will foster new knowledge on the role of important regulators of brain plasticity in regulating immediate and lasting effects of stressors experienced in adolescence and/or adulthood, and will further research on the importance of assessing sex dimorphism in stress pathology.

Table 1. Thesis studies at a glance.

Paper title	Gender	Route of administration and dosage	Housing	Measures
Blockade of TrkB receptors in the nucleus accumbens prior to heterotypic stress alters corticotropin-releasing hormone (CRH), vesicular glutamate transporter 2 (vGluT2) and glucocorticoid receptor (GR) within the mesolimbic pathway *Hormones and Behavior, 2017*	Adult male Wistar rats	Intra-NAc infusion of ANA-12 or vehicle solution (0.25µg/ 0.5µl)	Standard (1 per cage)	- Open field test (locomotion and exploration) - Elevated plus maze (anxiety) - Forced swim test (behavioral despair) - Corticosterone - CRH - TrkB - GR - vGluT2
Inhibition of TrkB at the nucleus accumbens, using ANA-12, regulates basal and stress-induced orexin A expression within the mesolimbic system and affects anxiety, sociability and motivation *Neuropharmacology, 2017*	Adult male Wistar rats	Intra-NAc infusion of ANA-12 or vehicle solution (0.25µg/ 0.5µl)	Standard (1 per cage)	- Social interaction/ preference test (social memory and novelty) - Y maze conditioned place preference test (place preference) - Y maze passive avoidance task (learning and memory retention) - Orexin A - Tyrosine hydroxylase - FosB/ΔFosB
Sex differences in corticosterone secretion, behavioral phenotypes and expression of TrkB.T1 and TrkB.FL receptor isoforms: Impact of systemic TrkB inhibition and heterotypic stress exposure in adolescence *Submitted*	Adolescent male and female Wistar rats	Intra-peritoneal injection of ANA-12 or vehicle solution (0.5mg/kg at a volume of 10ml/kg)	Standard (2 per cage)	- Open field test (locomotion and exploration) - Elevated plus maze (anxiety) - Forced swim test (active vs. passive coping responses) - Corticosterone - TrkB.FL protein - TrkB.T1 protein

Article 1

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Authorial contributions

Idu Azogu conceived and designed the study, contributed to implementation, data analyses, interpretation, and manuscript draft and revisions. She performed the surgeries for unilateral intra-NAc cannula implantation for all experimental groups and collected serum blood samples with the assistance of Sylvie Emond (Research assistant) and Charlene Habibi (Summer NSERC student co-supervised by Idu). She also prepared brain sections with the assistance of UROP students, Nivedh Patro and Michela Grazia Panarella, and performed the immunohistochemical analyses for all brain regions assessed with CRH, vGluT2, TrkB and GR. She ran ELISA to analyze blood CORT concentrations with the assistance of Jacky Liang (lab manager) and Nesma Etoubashi (Fall NSERC student co-supervised by Idu).

Dr. Hélène Plamondon supervised the research project, interpreted the data analyses, and revised the manuscript.

Abstract

Inhibition of stress-induced elevations in brain-derived neurotrophic factor (BDNF) or its primary receptor tyrosine-related kinase B (TrkB) within the reward pathway may modulate vulnerability to anxiety and mood disorders. The current study examined the role of BDNF/TrkB signaling on biochemistry and behavior under basal conditions and following exposure to a 10-day heterotypic stress paradigm in male rats. Effects of intra-accumbal administration of TrkB antagonist ANA-12 (0.25 μg/0.5 μl/min) on anxiety, and expression of Trk-B, corticotropin-releasing hormone (CRH), vesicular glutamate transporter 2 (vGluT2) and glucocorticoid receptor (GR) within the mesolimbic pathway were determined. Notably, ANA-12 attenuated anxiety-like behavior in stress rats while increasing anxiety in the non-stress group in the elevated plus maze (EPM). At the neurochemical level, ANA-12 blocked the increased vGluT2 and CRH expressions in the hypothalamic PVN and basolateral amygdala in stress rats, while it enhanced vGLuT2 and CRH expressions in non-stress rats. ANA-12 also showed state-dependent effects at the NAc core, attenuating TrkB-ir in non-stress rats while reversing reduced expression in stressed rats. At the cingulate cortex, ANA-12 normalized stress-induced increase in TrkB expression. Notably, ANA-12 showed region-specific effects on GR-ir at the NAc core and shell, with increased GR-ir in non-stress rats, although the drug attenuated stress-induced GR-ir expression only in the core portion of the NAc, while having no impact at the cingulate cortex. Elevated blood CORT levels post stress was not influenced by ANA-12 treatment. Together, these findings suggest that BDNF-mediated TrkB activation exerts differential impact in regulating emotional response under basal and stress conditions.

Keywords: Heterotypic stress; BDNF; TrkB; ANA-12; nucleus accumbens; emotionality; HPA axis.

1. Introduction

Brain-derived neurotrophic factor (BDNF) is widely expressed throughout the brain (Alcantara et al., 1997; Murakami, Imbe, Morikawa, Kubo, & Senba, 2005), and is essential for proper neuronal growth, survival and differentiation during development (Leibrock et al., 1989), and for synaptic function and plasticity of CNS neurons (long-term potentiation and long-term depression) in the mature brain (Lindsay, Wiegand, Altar, & DiStefano, 1994; Matsuda et al., 2009; McAllister, Katz, & Lo, 1999; Thoenen, 1995). Among observed properties, BDNF is reported to protect neurons against glutamate excitotoxicity (Jiang et al., 2005), modulate *N*-methyl-*D*-aspartate (NMDA) receptor phosphorylation (Suen et al., 1997) and underlie learning and memory processes. In recent years, BDNF has been shown to exert a profound influence on resilience and increased susceptibility to depression (Duman & Li, 2012), with reduced BDNF levels being implicated in altered behavioral responses induced by changes in serotonin brain levels (Autry & Monteggia, 2012; Martinowich & Lu, 2008) or trophic effects on noradrenergic neurons (Fawcett et al., 1998; Saarelainen et al., 2003). Such evidence has led to the proposed neurotrophic hypothesis of depression (Duman, 2004; Duman & Aghajanian, 2012; Duman & Li, 2012; Duman & Voleti, 2012; Hosang, Shiles, Tansey, McGuffin, & Uher, 2014), particularly suited to depressive disorders developing from stress exposure (Chao, 2003).

Notably, BDNF exerts important regulatory influences on the brain stress and reward systems (Martinowich, Manji, & Lu, 2007) as a stress and activity-dependent neurotrophin involved in the activity of the hypothalamic-pituitary-adrenal (HPA) axis (Jeanneteau et al., 2012). BDNF signaling is negatively regulated by glucocorticoids, which impair synaptic plasticity in the brain by inhibiting dendritic spine density, neurogenesis and long-term potentiation (Rothman & Mattson, 2013), particularly during stress. A decrease in BDNF mRNA levels following 6h of daily restraint stress leads to dendritic atrophy/death of pyramidal neurons and reduction of hippocampal volume in rats (Murakami et al., 2005). Repeated stress also causes changes in the prefrontal cortex (PFC) and amygdala, which are thought to subserve cognitive and emotional impairments (de Pablos et al., 2006). In the PFC, reduced BDNF-induced glutamate release is associated with downregulated glucocorticoid receptors following stress-induced glucocorticoid release (Chiba et al., 2012). The BDNF

Val66Met polymorphism is also associated with altered HPA axis reactivity (Colzato, Van der Does, Kouwenhoven, Elzinga, & Hommel, 2011; Shalev et al., 2009), increasing an individual's vulnerability to alterations in mood and depression (Stein, Daniels, Savitz, & Harvey, 2008). Due to a broad range of physiological actions, BDNF's involvement has been suggested in local responses to various types of neuronal insults or stress (Kozisek, Middlemas, & Bylund, 2008; Nitta et al., 1999).

Exogenous BDNF administration within midbrain structures or hippocampus induces antidepressant-like behavioral effects in animal models of depression, comparable to the effects of chronic treatment with pharmacological antidepressants (Chao, 2003; Shirayama, Chen, Nakagawa, Russell, & Duman, 2002). BDNF-mediated TrkB receptor activation is required for the behavioral effects induced by antidepressants, and is proposed as a key mechanism of antidepressant action (Saarelainen et al., 2003). Contrasting these effects, BDNF infusion into the nucleus accumbens (NAc), a subcortical limbic region involved in mood regulation, motor function and motivational valence, triggers pro-depressant effects, whereas inhibition of its action (e.g. via adeno-associated viral infection of dominant-negative receptor TrkB-T1 (Eisch et al., 2003) or via systemic administration of selective TrkB receptor antagonist ANA-12 (Cazorla et al., 2011) promotes antidepressant effects. In this context, exposure to chronic stress enhances NAc-BDNF expression, an effect that depends on the degree of rodents' resilience (versus susceptibility) to the stress effects (e.g. anhedonia, increased anxiety and impaired coping skills) (Feder, Nestler, & Charney, 2009; Krishnan et al., 2007). Within the mesolimbic circuitry, increased spontaneous firing activity of ventral tegmental area (VTA) dopamine (DA) neurons resulting in BDNF release within the nucleus accumbens neurons has been shown to mediate susceptibility to social defeat in mice (Krishnan et al., 2007). BDNF/TrkB signaling has also been implicated in the structural and synaptic plasticity of the dopaminergic VTA-NAc reward pathway in disorders implicating stress as a predisposing factor, including depression (Christoffel, Golden, & Russo, 2011; Eisch et al., 2003; Russo & Nestler, 2013).

Of note, the topographical organization of connections has led to proposed distinct functional attributes for the NAc core and shell portions (Deutch & Cameron, 1992; Heimer, Zahm, Churchill, Kalivas, &

Wohltmann, 1991; Kalivas & Duffy, 1995). The NAc shell receives dense excitatory projections from the BLA (Christoffel et al., 2011; Kirouac & Ganguly, 1995) via the bed nucleus of the stria terminalis and the ventral subiculum (the major output region of the hippocampal complex) (Brog, Salyapongse, Deutch, & Zahm, 1993; Kita & Kitai, 1990; McDonald, 1991b, 1991a), together with glutamatergic projections from the PFC (Sesack et al., 1989) and dopaminergic projections from the VTA (Floresco, Yang, Phillips, & Blaha, 1998). The NAc core projections from the BLA and parahippocampal regions are less dense. Within the NAc shell, anatomical coding plays an important role in the motivational valence of a stimulus (appetitive versus aversive), a phenomenon that involves dopamine/glutamate interaction in the NAc and local D1 and D2 signaling (Richard & Berridge, 2011), with selective activation of DA transmission being reported in the NAc shell, but not core portion, following 15 min of restraint stress or mild footshock (Deutch & Cameron, 1992; Kalivas & Duffy, 1995). In addition, indirect innervation of the NAc shell to the PVN is reported via efferent connections to the lateral hypothalamus (LH), which sends projections to the medial hypothalamus (Stratford, 2005).

At present, no studies have explored the anti-depressant effects of the TrkB receptor antagonist ANA-12 in an animal model of repeated heterotypic stress. Therefore, particular interest was directed towards BDNF/TrkB signaling in the brain's reward pathway and the mechanisms involved in mediating stress-induced physiological responses and neurochemical changes. The current study examined the role of BDNF/TrkB signaling in neuroendocrine responses under basal conditions and following a 10-day exposure to a heterotypic stress paradigm (alternating exposure to 30 min of restraint and 15 min of forced swim stressors), using NAc shell micro-infusions of ANA-12. In adult male Wistar rats, we determined 1) effect on endogenous glucocorticoid levels in rats, by assessing changes in blood corticosterone (CORT), 2) lasting impact on behavioral abnormalities related to anxiety, locomotor activity and behavioral despair in the open field test (OFT), elevated plus maze (EPM) and forced swim test (FST) and, 3) assessed whether alterations in BDNF/TrkB signaling triggered neurochemical changes in the post-mortem brain ~9 days following cessation of the stress paradigm via immunohistochemical detection of corticotropin-releasing hormone (CRH) and vesicular glutamate transporter 2 (vGluT2) expression in the PVN and BLA, in addition to TrkB and GR

expression in the PFC (anterior cingulate cortex) and NAc shell and core. This pharmacological study may enhance understanding of the modalities by which TrkB activation within the NAc shell regulates DA and glutamate release, and CRH-mediated stress response.

2. Methodology

2.1. Animals and housing

Adult male Wistar rats weighing between 250-275g were obtained from Charles River Laboratories (Rochefort, Quebec, Canada), housed two to a cage and handled daily to minimize stress. They were maintained on a 12h light/dark cycle (lights on at 7:00am), room temperature (21-23°C) with 60% relative humidity, and ad libitum access to water and standard Purina rat chow. Animals acclimated five days to the animal facility prior to intra-NAc guide cannula implantation surgery. All procedures were carried out in accordance with the Canadian Council of Animal care and approved by the University of Ottawa Animal Care Committee. All efforts were made to minimize animals' suffering.

2.2. Guide cannula implantation surgery

Experimentally naïve male rats were anaesthetized in a small chamber by inhalation of 2 to 3% isoflurane in oxygen and then transferred to a stereotaxic apparatus under the same anaesthesia. The head was shaved and the area disinfected prior to placement on the stereotaxic frame and skin incision. Body core temperature was kept constant at ~ 37°C throughout the surgery using a feedback regulated heating blanket (Homeothermic Blanket Control Unit, Harvard Instruments, Natick, MA). A stainless steel 22-gauge guide cannula was unilaterally implanted in the NAc shell of the right hemisphere, according to the Paxinos and Watson (1998) rat brain atlas. Stereotaxic coordinates were as follows: antero-posterior, 1.2mm from Bregma; lateral, -1.5mm from the midline (angled at 6°); and dorsoventral, 7.0mm below dura (Li et al., 2013). Intra-NAc internal injectors (28-gauge) extended 0.5mm past the end of the guide cannula, for intra-NAc injections at a final depth of 7.5mm below dura. Guide cannulas were anchored to the skull with 3 stainless steel screws (Plastics One, Roanoke, VA, USA) using dental acrylic cement and stainless steel obturators (28-gauge) secured into the guide

cannulas to prevent occlusion. Post-surgery, rats received a subcutaneous injection of the non-steroidal anti-inflammatory drug Meloxicam (1.5 mg/kg, Boehringer Ingelheim Ltd.). They were then placed in their cage resting on a heating pad in an air-controlled incubator for a few hours post-op prior to returning to their vivarium room. Rats were individually housed and allowed to recover for 10 days post-surgery, with gentle handling for at least 2 min daily after the fourth recovery day (Rodd et al., 2004). A standard operating procedure chart was used to evaluate daily the animals' health and recovery. Unilateral injections were chosen as this injection method has yielded similar effects as bilateral injections, and the former minimize stress to the animal (Olson et al., 2005); also see (Bolaños et al., 2003; Carlezon et al., 1997).

2.3. Weight assessments (food intake, water consumption and body weights)

As a primary assessment of stress response, food intake and water consumption were measured during the 10-day stress paradigm. Body weights were recorded for all rat groups as a secondary measure in the morning of each session. All measures (in grams) were obtained using a standard laboratory scale.

2.4. Drug infusion

The highly selective TrkB receptor antagonist *N*-[2-[[(Hexahydro-2-oxo-1*H*-azepin-3-yl)amino]carbonyl]phenyl]benzo[b]thiophene-2-carboxamide (ANA-12, SML0209, Sigma Aldrich) has been identified as highly potent in blocking TrkB receptor activation by BDNF without altering the activity of TrkA or TrkC receptors or neuronal viability (Cazorla et al., 2011). Intra-NAc shell micro-infusion of 0.25 μg of ANA-12 was delivered at a rate of 0.5 μl/ min in the right hemisphere. The vehicle was composed of 0.9% saline solution containing 20% (2-Hydroxypropyl)-β-Cyclodextrin (H107, Sigma-Aldrich; (Girbovan, Morin, & Plamondon, 2012)). A subthreshold dose of ANA-12 was selected from a pilot study, accounting for repeated drug administration at set intervals in our paradigm. Intra-accumbal injection was done using a stainless steel internal injector (28 gauge) attached with polyethylene tubing (PE-10, Becton Dickinson, Sparks, Maryland) to a 10 μl–capacity glass Hamilton syringe (Gastight® #1001, Hamilton Co., Reno, Nevada) mounted on a micro-infusion pump (Harvard Apparatus Canada, Saint-Laurent, Quebec). Injection needle remained in place for an

additional 60 sec post infusion to minimize backflow of the drug/vehicle. On days 1, 4, 7 and 10 of the experimental paradigm, rats either received ANA-12 or a vehicle solution 30 min prior to stress/no stress exposure. Animals were divided into 4 groups (n = 10 to 11 per group): ANA-12 + stress (**AS**), ANA-12 + no stress (**ANS**), Vehicle + stress (**VS**), and Vehicle + no stress (**VNS**). Timeline of the study is depicted in Fig. 1.

2.5. Blood corticosterone levels (collection via tail venipuncture)

Blood samples were collected between 9AM and 1PM in un-anaesthetized rats for baseline values on days 1 and 10, and for predetermined intervals on day 5 of the 10-day stress paradigm (at which point animals have already received two prior days of drug or vehicle infusion. *See Section 2.4 above*). The blood collection time intervals are: basal, and at 0, 30 and 60 min after cessation of restraint stressor. Sample collection required one small needle prick on the lateral tail vein and the rapid collection (in less than 3 min) of a drop of blood (Milot, James, Merali, & Plamondon, 2012) on Whatman blood stain cards (Sigma-Aldrich, Canada). This method provides reliable CORT values and requires a minimal amount of blood from the animal minimizing associated stress (Milot et al., 2012). Once collected, samples were allowed to dry at room temperature overnight and then stored at -80°C until ELISA procedure.

2.6. Stress paradigm

Restraint stress and forced swim stress are two widely used physical stressors due to their easy implementation and their strong psychological impact on the experimental animals (Zamora-Gonzalez, Santerre, Palomera-Avalos, & Morales-Villagran, 2013). Both induce rapid activation of the HPA axis and heightened response to stimuli (Zamora-Gonzalez et al., 2013), as well as increased DA neuron activation (Valenti, Lodge, & Grace, 2011). They have also been shown to increase phosphorylated CREB (pCREB) in various brain regions such as the hippocampus, amygdala, and nucleus accumbens (Bilang-Bleuel, Rech, De Carli, Holsboer, & Reul, 2002; Miller, Jimenez, & Mathe, 2007). A 10-day heterotypic stress paradigm was adapted from a 28-day combinatory stress paradigm alternating restraint and forced swim stressors to address the issue of habituation to repeated exposure to a single stressor (Zamora-Gonzalez et al., 2013). Briefly, on odd

numbered days up to day 9, animals were placed in a clear plastic restraining bag that prevented movement (but allowed proper breathing) for a 30 min period (between 9:30AM and 11AM). On even number days up to day 10, animals experience a 15 min forced swim session, being individually introduced to a transparent cylindrical container (20.32 cm in diameter and 43.18 cm in height) filled with tap water (23 ± 2°C) at a height of approximately 33 cm. Black cardboard pieces were used between the four swimming cylinders and at the extreme edges to obscure the rat's view of the other animals being tested at the same time. Immediately after swimming, animals were dried off and placed in a warm incubator before being returned to their home cage. Control animals were handled for up to 5 min with no additional manipulation and returned to their home cages.

2.7. Behavioral testing

Animals were tested on measures of anxiety, risk-taking, and behavioral despair. Testing occurred within 4 - 5 h after restraint stress (Valenti, Gill, & Grace, 2012), with animals being assessed in the most anxiety-sensitive tests first. More precisely, for the open field test and the elevated plus maze, testing was initiated 2 h after the last forced swim stress session. The forced swim test was administered on the last day of behavioral testing sequence (i.e. ~ 9 days after the last stress session) between 9:30AM and 2:00PM (see Fig. 1 for the experimental timeline). Animals were transported to the enclosed hallway outside the testing room and left to acclimatize to the environment for 30 min prior to testing. General room lighting was 200 lux in the animal vivarium (shelf area), 400 lux (hallway); 800-900 lux in the behavioral testing room (OFT, EPM), infusion room, and monitoring room (restraint stress and blood collection); and behavioral testing room (forced swim) was lit at 650-700 lux.

2.7.1. Open field test

The open field test is used to measure anxiety-related locomotor activity in a novel and open environment. The arena was made of gray Plexiglas (LWH: 75 cm × 75 cm × 30 cm) with a grid dividing the floor into 36 identical squares. It stood on a table 90cm above the floor and white curtains separated the arena and the experimenter's recording zones. Rats were placed in the periphery of the arena and resulting behavior

was monitored over the course of 5 min. An observer and a video-tracking system (PC computer and overhead video camera) simultaneously recorded the duration and number of entries in the center zone and in the periphery and manually scored the number of squares walked in the center and peripheral zones, as well as the latency to initially enter the center zone.

2.7.2. Elevated plus maze

The elevated plus maze (EPM) is widely used as a test for assessing anxiety-like responses of rodents (Walf & Frye, 2007). The test consisted of a plus shaped Plexiglas structure with four arms (two enclosed and two open arms, each measuring 50cm × 10cm). The enclosed arms had walls that were 40cm in height. The EPM rested on a table 60cm above the floor and was surrounded by plain white walls. The experimenter's recording zone was separated from the test by white curtains. Rats were placed in the center zone facing an open arm, and allowed to freely explore the maze for 5 min. An observer and a video-tracking system (PC computer and overhead video camera) simultaneously recorded the duration and number of entries in the open and closed arms, the number of times the rat crossed the junction of the four arms into an opposite maze arm, risk assessment behaviors (defined here as frequency of stretch-attend posture to scan the open arms from the safety of the closed arm and frequency of unprotected head dips in the open arms (Carobrez & Bertoglio, 2005; Walf & Frye, 2007)), frequency of rears in the closed arms, and freezing/immobility. An open arm entry was counted when the animal had all four paws in the open arm. Percentage of open arm time ((time in open arms / total time in arms) × 100) and index of open arm avoidance (100 – (percentage of open arm time + percentage of open arm entries) / 2) (Pellow & File, 1986; Podhorna & Brown, 2002; Trullas & Skolnick, 1993) were calculated. Animals exhibiting reduced anxiety typically spent increased time in the open arm and made more head dips, whereas animals with elevated anxiety typically show increased stretch-attend posture and open arm avoidance scores.

2.7.3. Forced swim test

The Porsolt forced swim test is used to assess depressive behavior or anhedonia (Porsolt, Le Pichon, & Jalfre, 1977; Porsolt, Anton, Blavet, & Jalfre, 1978). For the animals that initially had the forced swim as a stressor, the pretraining session was omitted while animals that did not undergo the stressor received a pre-training session (15 min) prior to a 5 min test session on the next day. Animals were allowed to swim in transparent Plexiglas cylinders (20.32 cm in diameter and 43.18 cm in height) filled with tap water (25 ± 1°C) at a height of approximately 33cm for 15 min. A video camera and computer screen captured their movements. On the next day, testing involved a 5 min period in the cylinder. Behavioral parameters included active escape-directed behaviors (diving, swimming and climbing) and immobile behavior (floating). This last was defined as the absence of all other movements for ≥ 2.0 seconds (Pliakas et al., 2001), except those needed to keep the animal's head above water. Increased immobility in the forced swim test is indicative of increased behavioral despair (Slattery & Cryan, 2012) or impaired coping responses. After the test, animals were placed on a heating pad before being returned to their home cages. The cylinders were cleaned and the water was renewed in between animals.

2.8. Corticosterone immunoassay

Sample Whatman cards were thawed from -80°C and CORT levels from blood droplets were determined in duplicates using a commercially available ELISA kit (Corticosterone EIA kit, Enzo Life Sciences, Cat. No. ADI-901-097). Briefly, a 3.0 mm diameter circle of each drop sample (n = 8 per group per blood collection interval) was punched from the blood stain cards using a Gem Hole Punch (McGill Inc., Marengo, IL), and placed in labeled glass tubes containing 280 µl of a solution consisting of assay buffer diluted in dH_20 at 1:10 concentration. The tubes were covered with parafilm and shaken on the Belly Dancer® Laboratory shaker (Structure Probe Inc., West Chester, PA) for 24 h at RT prior to the ELISA procedure. On the next day, 214.5 µl of each blood sample and 5.5 µl of steroid displacement reagent (SDR) were mixed in labeled aliquots and vortexed. Standards and samples were prepared in 96-well plates as recommended by the manufacturer. CORT concentrations were determined in a PowerWave^{TM} XS2 Microplate Spectrophotometer (BioTek, Winooski,

VT). Resulting CORT concentrations were calculated using a four-parameter equation that relied on constant values determined from the standard curve and quantified in units of pg/ml of corticosterone per punch. The analytic range of the assay was 32 – 20,000 pg/ml.

2.9. Histology

Following the last behavioral test session (9 days after last stress session), animals were anaesthetized with isoflurane and quickly decapitated. Fresh brain tissues were collected, frozen on dry ice and stored at -80˚C. Brains were sectioned into 14 μm coronal slices (i.e. 2 cell widths) on a cryostat (Leica CM1900, Leica Microsystems, Germany) at -17˚C, mounted on polarized slides (Fisherbrand Superfrost® Plus Microscope Slides) and stored at -80˚C until further histological analysis. Only rats with unilateral injection tips in NAc shell were included in the study. Site of cannula placement are depicted on representative images from the Paxinos and Watson, 1998 rat brain atlas (Fig. 2).

2.10. Immunohistochemistry (IHC)

A subset of adult male Wistar rats (n = 6 - 8 per group) were used for immunohistochemical detection of corticotropin-releasing hormone (CRH; raised against synthetic ovine CRH), vesicular glutamate transporter 2 (vGluT2; peptide corresponding to the C-terminal of rat vGluT2), tyrosine-related kinase B (TrkB; epitope corresponding to amino acids 160-340 mapping within the extracellular domain of TrkB of human origin), and glucocorticoid receptors (GR; raised against a fragment of a well conserved region of human GR). Antibody specificity information was derived from manufacturers' data sheets. Brain regions of interest included the medial prefrontal cortex (the anterior cingulate cortex) and nucleus accumbens (shell and core) – Bregma 2.70mm to 0.70mm; and the paraventricular nucleus of the hypothalamus and basolateral amygdala – Bregma -0.92mm to -2.12mm. Briefly, brain sections were post fixed with 4% paraformaldehyde containing 2% picric acid in 0.1M phosphate buffered saline (PBS, pH = 7.4) for 5 min, then rinsed 3 x 5 min in 0.1M PBS. This was followed by 30 min in blocking buffer, 1% bovine serum albumin (BSA) in PBS – Triton (0.3%). The sections were incubated for 24h at 4°C using primary antibodies mixed in 1% BSA in PBS – Triton (0.02%): polyclonal

rabbit anti-CRH (1:400, 20084, ImmunoStar), polyclonal guinea pig anti-vGluT2 (1:5000, AB2251, Millipore), polyclonal rabbit anti-TrkB (1:400, sc-8316, Santa Cruz Biotechnology), and polyclonal mouse anti-GR (1:400, sc-56851, Santa Cruz Biotechnology). Following primary antibody incubations, sections were rinsed 3 x 5 min in 0.1M PBS and subsequently incubated with the proper secondary antibody: 2 h at room temperature in biotinylated donkey anti-rabbit IgG (1:500, Invitrogen), biotinylated donkey anti-mouse IgG (1:500, Invitrogen), and biotinylated goat anti-guinea pig IgG (1:500, Invitrogen). Following another series of three rinses, the sections were incubated in 1µg/ml (Hoechst 33342, Invitrogen Canada Inc.) at room temperature for 10 minutes in the dark to label binding to AT regions of DNA. After rinsing three times, an anti-fade medium containing 0.1% *p*-phenylenediamine in phosphate buffered glycerol was applied and the slides cover-slipped and sealed with nail polish. Special controls were conducted to test for antibody specificity.

2.11. Histological quantification

Signal detection was accomplished using an Olympus DX51 microscope (Center Valley, PA, USA). Digital images for immunofluorescence (photomicrographs) were obtained using the ProgRes Capture Pro 2.7.6 software under a 20x objective lens (eyepiece 10x; numerical aperture 0.75). For all regions of interest, immunoreactivity was quantified using the ImageJ software (ImageJ, National Institutes of Health, Bethesda, Maryland, USA) and the method described by Hayes and colleagues (2005), which calculates the percent immunofluorescence reactivity of the total area of interest relative to a subthreshold background. At least four anatomically matched pictures of both hemispheres were analyzed for each brain region of interest. Data is presented as background corrected standardized image densities for each brain region. Confocal photomicrographs were produced using an Olympus BX51 microscope (Center Valley, PA, USA) with a 60x oil lens and FV1000 Fluoview software.

2.12. Statistical analysis

Two-way ANOVA was performed using SPSS software (version 22) to assess treatment (ANA-12 vs. Vehicle) and stress (Stress vs. No stress) main effects and interactions on the dependent variables of each

behavioral test output (OFT, EPM and FST), basal blood cort (for days 1 and 10) and neurochemical alterations (vGluT2, CRH, GR and TrkB). The assumptions of homogeneity of variance and Mauchly's test of sphericity were verified. When appropriate, a Huynh-Feldt ($\varepsilon > 0.75$) or Greenhouse-Geisser ($\varepsilon < 0.75$) correction for violations to the assumption of sphericity was applied and corrections were made to the degrees of freedom. Effect size was calculated and the (partial η_p^2) proportion of variance accounted throughout the respective analyses. Two-way repeated measures ANOVA was used to explore changes in food intake, water consumption and body weight across days, and differences in blood Corticosterone levels (expressed as pg/ml) for day 5. For these analyses, simple effects analysis with Bonferroni correction (adjustment at critical alpha level of 0.05) was used when significant interactions were obtained. Values are presented as mean ± SEM. Paired samples t-tests were performed for within group differences in immunohistochemical readouts for the right hemisphere, left hemisphere and the laterality index ((R - L/ R + L) x 100). Statistical significance was set at $p < 0.05$. Pearson's correlations explored relationships between behaviors of the OFT.

3. Behavioral results

3.1. ANA-12 increased bodyweight, water consumption and food intake

Two-way repeated measures ANOVA revealed main effect of group for bodyweight (g) (F (2.81,101.23) = 46.78, $p < 0.001$, $\eta_p^2 = 0.57$), change in water consumption (g) (F (4.78,176.96) = 8.81, $p < 0.001$, $\eta_p^2 = 0.19$), and change in food intake (g) (F (4.83,178.79) = 6.35, $p < 0.001$, $\eta_p^2 = 0.15$). This was related to increased weight, water and food intake in ANA-12 treated rats relative to the veh-treated groups, independent of their stress condition (data not shown). Higher bodyweight in ANA-12 treated groups reached significance on days 2 to 5, while significant between groups drug effects on water and food intake were limited to days 1 and 3, respectively.

3.2. Effect of treatment and stress in the OFT

The OFT was conducted 2 h following the last forced swim stress session. Two-way ANOVA revealed a main effect of treatment (**T**) on time spent in the center zone (F (1,37) = 5.09, $p = 0.03$, $\eta_p^2 = 0.12$), and a

significant treatment x stress (T x **S**) interaction (F (1,37) = 9.32, p = 0.004, η_p^2 = 0.20), related to increased time spent in the center in AS rats compared to non-stress counterparts and veh-treated stress group ($p < 0.01$) (Fig. 3a). Analysis of time spent in the peripheral zone (Fig. 3b) did not reveal significant group differences ($p > 0.05$). For locomotion in the center zone, two way ANOVAs revealed increased locomotion (i.e. number of squares walked) in ANA-12 compared to veh-treated groups (F (1,37) = 12.83, p = 0.001, η_p^2 = 0.26) (Fig. 3c). For the number of squares walked in the peripheral zone, there was a main effect of T (F (1,37) = 20.06, $p < 0.001$, η_p^2 = 0.35) and stress (S) (F (1,37) = 26.03, $p < 0.001$, η_p^2 = 0.41) (Fig. 3d). In addition, analysis showed a T x S interaction (F (1,37) = 9.64, p = 0.004, η_p^2 = 0.21) for locomotion in the peripheral zone due to elevated locomotion in the VS group relative to the AS and VNS groups ($p < 0.001$). The latency to enter the center zone also revealed a main effect of T (F (1,37) = 11.92, p = 0.001, η_p^2 = 0.24), S (F (1,37) = 5.04, p = 0.031, η_p^2 = 0.12) and a T x S interaction (F (1,37) = 6.42, p = 0.016, η_p^2 = 0.15) (Figure not shown). The VS group showed increased latency to enter the center zone compared to their non-stress counterpart and the AS group ($p < 0.01$). Pearson's product-moment correlation coefficient revealed that latency to enter the center zone was negatively correlated with walking in center (r = -0.41, p = 0.008) and time spent in the center (r = -0.46, p = 0.003). In contrast, it was positively correlated with time in the periphery (r = 0.42, p = 0.007) and walking in the periphery (r = 0.39, p = 0.013).

3.3. Effect of treatment and stress in the EPM

For percent open arm time, simple effect comparisons following a significant T x S interaction (F (1,37) = 26.24, $p < 0.001$, η_p^2 = 0.42) indicated that the AS ($p < 0.001$) and VNS ($p < 0.05$) groups had a higher percentage of time spent in the open arm compared to the VS and ANS groups (Fig. 4a). For time spent in the closed arms (Fig. 4b), a significant T x S interaction (F (1,37) = 6.00, p = 0.019, η_p^2 = 0.14) revealed that ANA-12 treatment led to reduced closed arm time in stressed rats when compared to their non-stress counterparts and the veh-treated stress group ($p < 0.05$). For rearing frequency (Fig. 4c), a main effect of S (F (1,37) = 16.45, $p < 0.001$, η_p^2 = 0.31) and a significant T x S interaction (F (1,37) = 4.17, p = 0.048, η_p^2 = 0.10) was observed, due to increased frequency of rearing in AS rats compared to non-stress counterparts ($p < 0.001$). Interestingly,

stressed groups showed reduced freezing/immobility behavior in both the open and closed arms (F (1,37) = 11.70, p = 0.002, η_p^2 = 0.24) than the non-stress groups, an effect paralleled by a significant increase in the frequency of crossings (F (1,37) = 16.07, p < 0.001, η_p^2 = 0.30) in stressed animals (data not shown).

For open arm avoidance (Fig. 4d), which represents an index of anxiety level, a T x S interaction (F (1,37) = 9.69, p = 0.004, η_p^2 = 0.21) was found related to reduced open arm avoidance score in AS rats (p = 0.031). In contrast, the ANS group showed increased open arm avoidance score when compared to the AS and VNS groups (p < 0.05). These findings are further supported by T x S interactions for risk assessment behaviors such as the frequency of stretch/attend posture (F (1,37) = 32.90, p < 0.001, η_p^2 = 0.47) (Fig. 4e) and the frequency of head dips (F (1,37) = 35.66, p < 0.001, η_p^2 = 0.49) (Fig. 4f). The VS (p < 0.05) and ANS (p < 0.001) groups made more stretch/attend postures from the safety of the closed arms and less head dips in the open arms (p < 0.001) compared to the AS and VNS groups. Increased open arm risk assessment behaviors in AS and VNS groups are consistent with increased time in the open arms by these groups.

3.4 Effect of ANA-12 and stressor exposure on behavior in the FST

The FST is commonly used as a measure of behavioral despair in rodents exposed to various stressors. In our case, as the forced swim was part of the heterotypic stress paradigm, and considering pre-exposure of the stressed animals to the test, only the non-stressed rat groups were subjected to a pretraining session prior to the FST. Two-way ANOVA revealed a main effect of S on time spent swimming (F (1,37) = 18.22, p < 0.001, η_p^2 = 0.33) (Fig. 5a) and on immobility (F (1,37) = 18.56, p < 0.001, η_p^2 = 0.33) (Fig. 5b). This was due to increased escape-like behavior with higher swim time and reduced immobility in the stressed compared to the non-stressed groups, regardless of drug treatment. Interestingly, the VNS group showed a drastic reduction in swimming and climbing behaviors compared to AS and VS groups and a significant increase in immobility compared to all other groups.

4. Corticosterone and immunohistochemical results

4.1 Effects of heterotypic stress on endogenous CORT levels in rats

Figure 6 basal CORT serum levels at day 1, 5 and 10 of the experimental design. On day 5, two-way repeated measures ANOVA revealed differences in CORT levels related to the collection intervals (F (1.47,41.19) = 30.07, $p < 0.001$, $\eta_p^2 = 0.52$). Specifically, high CORT levels were observed at 0 min after restraint stress when compared to all other blood collection intervals (basal and at 30 and 60 min post stress) ($p < 0.001$); while levels remained higher at the 30 compared to 60 min post restraint interval ($p < 0.001$). A significant day x stress interaction (F (1.47, 41.19) = 33.28, $p < 0.001$, $\eta_p^2 = 0.54$) was observed due to increased CORT in stressed rats immediately ($p < 0.001$, $\eta_p^2 = 0.58$) and 30 min ($p = 0.001$, $\eta_p^2 = 0.35$) post stress, regardless of drug treatment. Levels reached values comparable to that of baseline at the 60 min post stress interval. Interestingly, CORT levels (basal to 0 min post restraint stress) in AS and VS males were increased by approximately 620% and 652%, respectively, and declined thereafter over 60 min by approximately 90% to levels lower than basal CORT levels. Baseline assessments for days 1 and 10, prior to drug or vehicle infusion, were comparable across groups.

4.2 VGluT2-ir and CRH-ir in the parvocellular portion of the hypothalamic PVN

For VGluT2-ir (Fig. 7a), a T x S interaction (F (1,24) = 7.17, $p = 0.013$, $\eta_p^2 = 0.23$) was related to elevated expression in the VS compared to VNS group ($p = 0.028$, $\eta_p^2 = 0.19$). For CRH-ir (Fig. 7b), a T x S interaction (F (1,21) = 8.17, $p = 0.01$, $\eta_p^2 = 0.28$) revealed that differences were present in the non-stress groups ($p = 0.009$, $\eta_p^2 = 0.29$) and the veh-treated groups ($p = 0.036$, $\eta_p^2 = 0.19$), CRH-ir being lower in the VNS group when compared to the ANS and VS groups.

4.3 VGluT2-ir and CRH-ir in the BLA

At the BLA, T x S interaction (F (1,25) = 13.14, $p = 0.001$, $\eta_p^2 = 0.35$) was observed for vGluT2-ir (Fig. 7a), attributable to reduced expression in the AS ($p = 0.045$, $\eta_p^2 = 0.15$) and VNS ($p = 0.018$, $\eta_p^2 = 0.20$) groups when compared to the VS group. ANS showed increased vGluT2-ir relative to the AS ($p = 0.016$, $\eta_p^2 = 0.21$) and VNS ($p = 0.006$, $\eta_p^2 = 0.27$) groups. For CRH-ir (Fig. 7b), a significant T x S interaction (F (1,25) = 5.89, p

= 0.023, η_p^2 = 0.19) was related to a marked increase in CRH-ir in the ANS (p = 0.006, η_p^2 = 0.27) and VS (p = 0.046, η_p^2 = 0.15) groups compared to the VNS group.

4.4 GR-ir and TrkB-ir in the NAc shell and core

In the NAc shell, analysis of GR-ir values (Fig. 8a) revealed a main effect of T (*F* (1,21) = 7.89, p = 0.011, η_p^2 = 0.27) and S (*F* (1,21) = 17.47, p < 0.001, η_p^2 = 0.45), and a T x S interaction (*F* (1,21) = 6.44, p = 0.019, η_p^2 = 0.24). VNS group showed reduced GR-ir compared to VS and ANS groups (p < 0.001). For TrkB-ir (Fig. 8b), main effects of T (*F* (1,21) = 4.67, p = 0.042, η_p^2 = 0.18) and S (*F* (1,21) = 10.67, p = 0.004, η_p^2 = 0.34) were observed. ANA-12 led to higher TrkB expression compared to vehicle treatment. Expression was increased in the non-stress groups.

For GR-ir at the NAc core (Fig. 8a), a T x S interaction (*F* (1,20) = 25.59, p < 0.001, η_p^2 = 0.56) was found attributable to reduced GR-ir in the AS (p = 0.001, η_p^2 = 0.41) and VNS (p < 0.001, η_p^2 = 0.56) groups compared to the VS group. However, ANA-12 increased GR-ir in the non-stress group compared to the stress (p = 0.044, η_p^2 = 0.19) and VNS (p = 0.003, η_p^2 = 0.37) groups. For TrkB-ir (Fig. 8b), a T x S interaction (*F* (1,20) = 14.90, p = 0.001, η_p^2 = 0.43) was found related to reduced TrkB-ir in the ANS (p = 0.002, η_p^2 = 0.39) and VS (p = 0.033, η_p^2 = 0.21) groups compared to the AS and VNS group (relative to the ANS group only) (p = 0.005, η_p^2 = 0.34).

4.5 GR-ir and TrkB-ir in the anterior cingulate cortex (ACC)

In the ACC, analysis of GR-ir (Fig. 8a) revealed a main effect of S (*F* (1,24) = 8.86, p = 0.007, η_p^2 = 0.27), in which the stress groups had more GR expression than the non-stress groups. For TrkB-ir (Fig. 8b), a T x S interaction (*F* (1,22) = 8.82, p = 0.007, η_p^2 = 0.29) was attributed to reduced TrkB expression in the AS (p = 0.003, η_p^2 = 0.34) and VNS (p = 0.018, η_p^2 = 0.23) groups when compared to the VS group.

4.6 Lateralization of ANA-12 effects on molecular readouts

Considering unilateral drug infusions in the right hemisphere, paired sample t-tests were performed to assess within group differences in the expression of CRH, vGluT2, GR and TrkB in the right versus left hemisphere. The nucleus accumbens and amygdala showed comparable right and left brain expression patterns.

At the PVN, ANA-12 led to increased CRH expression ($t(6) = 3.034$, $p = .023$, $d = 1.15$) by reduced presynaptic vGluT2 ($t(6) = -4.160$, $p = .006$, $d = -1.57$) in the right versus left hemisphere. Considering the stimulatory role of glutamate in CRH release at the PVN; reduced presynaptic vGluT2 could thus play a role in increased CRH expression in ANA-12 treated stressed rats. ANA-12 also had a predominant effect on GR expression in the right cingulate cortex, which was observed in the stressed ($t(6) = 3.516$, $p = .013$, $d = 1.33$) and non-stressed ($t(6) = 4.304$, $p = .005$, $d = 1.63$) rats.

5. Discussion

The current study examined the role of TrkB receptor activation in the NAc shell on signaling pathways known to influence regulation of emotional behavior in a situation where daily stressors are experienced. We examined the ability of NAc shell ANA-12 infusions to regulate neuroendocrine responses and anxiety-like behavior under stress and non-stress conditions. To our knowledge, this is the first study to report beneficial effects of selectively blocking BDNF/TrkB signaling in the NAc shell to regulate biochemical and behavioral responses post stress. Notably, our findings support that TrkB signaling plays a role to modulate anxiety and/or mood within the mesolimbic circuitry that appears state-dependent.

5.1. Inhibition of TrkB receptors in the NAc shell has lasting impact on behavior following cessation of stress

Via CRH action at the NAc, stress increases BDNF expression in the brain's reward pathway (Walsh et al., 2014). In rodents, preventing BDNF production in dopaminergic regions or inhibiting BDNF/TrkB in the NAc has been shown to reduce anxiety in socially stressed animals and to induce strong anti-depressive effects (Berton et al., 2006). Herein, we show that TrkB receptor blockade in the NAc shell has persistent effects on biochemical and behavioral responses measured after cessation of drug treatment and stress exposure. Moreover, ANA-12 effects appeared state-dependent and can be opposite in stress versus non-stress rats. Thus, while blockade of TrkB receptors led to anxiolytic behavior in the OFT and EPM in stressed animals (i.e, enhanced time spent in the anxiogenic center zone), ANA-12 promoted anxiety behavior in non-stress rats, especially in the EPM as evidenced by increased index of open arm avoidance in the EPM and frequency of risk

assessments (i.e. stretch/attend posture in the closed arms). This contrasts with the diminished anxiety-related behaviors in the EPM and novelty-suppressed feeding paradigm reported in naïve mice treated acutely with a systemic 0.5 mg/kg dose of ANA-12, of which administration was shown to inhibit 20% and 10% of TrkB activity in the striatum and cortex within 2h of administration, respectively (Cazorla et al., 2011). This may indicate that action via other brain circuits may be recruited more efficiently and/or differently upon central and/or repeated drug administration. For example, endocannabinoids were shown to have different actions under normal versus challenging conditions and region-specific input to regulation of the stress response (Hill & Tasker, 2012; Knowles, de la Tremblaye, Azogu, & Plamondon, 2015). The impact of internal states and context specific action of neurochemical pathways warrants further examination.

Effects of ANA-12 treatment on antidepressant activity were examined in the forced swim test. Our findings show reduced immobility (as noted in the EPM) and increased swimming/climbing behaviors in both stress groups relative to the non-stress groups. These effects are opposite to expectations and previous observations in chronically stressed animals (Rygula et al., 2005; Strekalova, Spanagel, Bartsch, Henn, & Gass, 2004), and may illustrate development of a resilience effect from prior exposure to the same stressor, and familiarity and/or habituation to the experience. Of interest, similar to ANA-12 effects in naïve mice in the FST (Cazorla et al., 2011), ANA-12 reduced immobility time and depressive-like behavior in non-stress rats compared to the veh-treated counterparts. This supports an intrinsic role of TrkB signaling at the NAc in regulation of mood, which appears distinct from its role in regulating anxiety.

5.2. TrkB inhibition failed to affect stress-induced elevation of blood CORT levels despite blunted CRH-ir.

In the current study, increased CORT secretion was observed post restraint stress, a response that was not affected by ANA-12 pre-treatment. Animals did not appear to habituate to the heterotypic repeated stress paradigm, in line with effects on CORT secretion obtained using a similar, although longer alternating stress paradigm in BALB/c mice (Zamora-Gonzalez et al., 2013). Basal CORT measures of the non-stress groups also coincide with previously reported values (van Haarst, Oitzl, Workel, & de Kloet, 1996). CORT values of non-

stress rats did not differ from baseline values on Day 5, when ANA-12 or vehicle solutions were not administered. On that day, a steeper decline in CORT levels over the course of 60 min post stress was observed in the stress groups, and this may be attributed to glucocorticoid feedback inhibition. The extensive literature on stress effects support that chronic and repeated stress can lead to dysregulation of the HPA axis or facilitate HPA axis responses to subsequent stressors, thereby mediating active coping mechanisms. Conversely, repeated stress exposure can cause habituation of the HPA axis and/or a gradual decrease in its activity. These differential responses have a strong importance in an animal's ability to cope with stress and its ability to prevent allostatic load on the brain. Habituation of experimental animals in various models of homotypic and heterotypic stressors have been reported (e.g. in models of chronic mild stress (Azpiroz et al., 1999; Edgar, Silberman, Cremaschi, Zieher, & Genaro, 2003); see Review (Grissom & Bhatnagar, 2009)), therefore it is not unprecedented that following cessation of the stressor, progressive reduction of CORT following stress challenge toward basal concentrations was noted.

The observation that NAc shell blockade of TrkB receptor activation had no effects on stress-induced CORT secretion is intriguing considering mutual interplay between glucocorticoid and BDNF secretion. Acute or chronic immobilization stress and glucocorticoids have been reported to downregulate the expression of BDNF in the hippocampus, however leaving hippocampal expression of TrkB unaffected (Smith, Makino, Kvetnansky, & Post, 1995). In contrast, reduced GR function elevates BDNF protein and phosphorylated TrkB levels at the PVN, contributing to disinhibition of HPA axis function and heightened CRH expression (Jeanneteau et al., 2012). Notably, TrkB hypomorphic mice show reduced basal ACTH and CORT levels, and stress-induced increases in CORT levels were decreased 1 h post stress compared with wild type controls (Jeanneteau et al., 2012). Moreover, enhanced glucocorticoid stimulation (e.g. dexamethasone) has been shown to involve TrkB receptor activation, via a mechanism dependent on GR transcriptional activity, but independent of increased production of neurotrophins such as NGF and BDNF (Jeanneteau, Garabedian, & Chao, 2008). Integrative action between glucocorticoids and TrkB reported in the context of elevated glucocorticoids could also serve to minimize the impact of a site-specific blockade of TrkB in the NAc shell possibly via heightened

action in other brain regions (e.g., the hypothalamus). Site-specificity in the interplay of physiological regulators is intriguing and represents an important area for future research.

At the PVN and BLA, ANA-12 led to elevation of CRH-ir expression in non-stress rats (to comparable levels as that observed in VS rats). These observations suggest that inhibition of TrkB receptors at the NAc shell under basal conditions triggers a body response that resembles that of a stressor. However, while under stress, activation of TrkB at the NAc shell appears to be minimally involved in regulating stress-induced CRH response. Although mechanisms acting at the NAc region remains to be determined, stress exposure increases BDNF mRNA in the parvocellular region of the PVN (Castren, Thoenen, & Lindholm, 1995; M. A. Smith, Makino, Kim, & Kvetnansky, 1995) and in the BLA (Lakshminarasimhan & Chattarji, 2012), and this precedes increases in the expression of CRH and arginine-vasopressin (Givalois et al., 2004). Notably, BDNF-induced TrkB activation was recently shown to stimulate GR transcriptional activity (Lambert et al., 2013), and BDNF via TrkB activation led to a three-fold increase in CRH in primary rat hypothalamic neurons, whereas GR activation led to suppressed BDNF and CRH secretion at the PVN (Jeanneteau et al., 2012). While the NAc shell does not directly innervate the PVN, it has been shown to indirectly influence this locus via efferent connections to the lateral hypothalamus (LH), which sends projections to the medial hypothalamus (Stratford, 2005). Thus, increased Fos-like immunoreactivity has been noted in the LH and PVN following unilateral (Stratford, 2005) and bilateral (Stratford & Kelley, 1999) injections of muscimol (GABAA agonist) into the NAc shell. Our study further supports that TrkB actions at the NAc have repercussion on the regulation of HPA axis in both normal and stressful conditions.

5.3. ANA-12 treatment triggers differential responding in GR- and TrkB-ir expression

Brain areas that are rich in GR such as the hippocampal formation and the central division of the extended amygdala are reported to preferentially innervate the NAc shell (Brog et al., 1993). In our study, stress increased GR levels in the NAc shell while it lowered TrkB-ir expression, in line with reported stress- or CORT-potentiated increases in GR expression in this brain region (Barrot et al., 2000; Marinelli, Aouizerate, Barrot, Le Moal, & Piazza, 1998). ANA-12 treatment attenuated TrkB-ir observed post stress, while the drug

had a stimulating effect on TrkB-ir in non-stress rats. Reduced TrkB-ir levels in stressed animals could be related to repeated BDNF secretion in the NAc shell due to the stress. ANA-12 treatment helped re-establish TrkB levels to control values 9 days post stress. These findings complement reports of stress-induced decreases in BDNF mRNA and protein and increased TrkB mRNA expression at the hippocampus following repeated immobilization stress (Nibuya, Morinobu, & Duman, 1995; M. A. Smith, Makino, Kvetnansky, et al., 1995), indicating that BDNF and TrkB expression can be regulated in opposite directions.

In contrast, ANA-12 treatment at the NAc core reduced GR expression in the stress group while it elevated TrkB-ir expression level in the same group. Furthermore, ANA-12 acted to restore stress-induced GR and TrkB expressions to comparable levels as that of the non-stress vehicle group. Expression profile was reversed (upregulated GR; downregulated TrkB) for the ANS and VS groups, which are in line with findings at the NAc shell showing that under normal conditions blocking TrkB receptors could trigger physiological responses that resemble a stressor. The differential responses observed at the shell and core portions could be related to described regional functionality (Deutch & Cameron, 1992; Heimer et al., 1991; Kalivas & Duffy, 1995). For example, both NAc subregions were reported to respond differentially to fluctuating basal levels of glucocorticoids, with functional activity of dopaminergic responsiveness in the NAc shell being dependent on fluctuating levels of glucocorticoids relative to the core, in which suppression of glucocorticoids did not modify dopamine transmission (Barrot et al., 2000). This effect in the NAc shell was contingent on GR (Marinelli et al., 1998), which are reported to be the only corticosteroid receptors at that site (Ahima, Krozowski, & Harlan, 1991; Ahima & Harlan, 1990). The core may require supra-physiological fluctuations of circulating GRs in order to see an effect on extracellular dopamine neurotransmission (Tye, Miller, & Blaha, 2009).

Among prefrontal areas that directly innervate the hypothalamus and interact with the NAc, the anterior cingulate cortex (ACC) has been implicated in stress-induced functional and structural changes (Diorio, Viau, & Meaney, 1993; Yamashita et al., 2013), and is highly influential in linking behavioral outcomes to motivation (Bush, Luu, & Posner, 2000). At that brain locus, stress significantly increased GR-ir levels, an observation consistent with reports of enhanced glutamatergic transmission in prefrontal cortex and increased surface

expression of NMDA receptor subunits through activation of GR following acute stressor (Yuen et al., 2009). Our findings support elevated TrkB expression in the VS group when compared to their non-stress counterparts and the stress ANA-12 groups. This is surprising considering that chronic restraint stress or 3-week daily corticosterone administration trigger alterations or remodeling in dendritic arborisation in the ACC and prelimbic cortex of rats (Liston et al., 2006; Radley et al., 2004; Wellman, 2001). The reduced apical dendritic length and number of pyramidal neurons has been associated with reduced BDNF at the prefrontal cortex under stressful conditions, which could promote externalization of TrkB receptors, related to increased expression in the vehicle-stressed rats. In contrast, ANA-12 administration may have regulated BDNF secretion, bringing TrkB receptor expression to comparable levels as that observed in the control groups. Moreover, our findings support integrative/ synergistic actions of glucocorticoids and neurotrophin signaling systems in regulating TrkB signaling (Jeanneteau et al., 2008).

5.4. Altered vGluT2-ir in the PVN and BLA following chronic stress and TrkB inhibition

In response to stress, HPA axis activation leads to glutamate release associated with upregulation of BDNF mRNA expression (Tapia-Arancibia, Rage, Givalois, & Arancibia, 2004) affecting brain regions such as the anterior pituitary, PFC and hippocampus which connect to the PVN to mutually influence the neuroendocrine response (Evanson & Herman, 2015). Neurons in these areas express markers for the vesicular glutamate transporters (vGluTs) 1, 2, and 3 (Fremeau et al., 2002; Ziegler, Cullinan, & Herman, 2002), which for the most part act to facilitate glutamate release and provide high-affinity reuptake for released glutamate (Fremeau, Voglmaier, Seal, & Edwards, 2004). Of these, the vGluT2 localized on presynaptic terminals show the highest immunoreactive expression in the hypothalamus, densely innervating the medial subdivision of the parvocellular PVN (Evanson & Herman, 2015; Wittmann, Lechan, Liposits, & Fekete, 2005). Our findings indicate that ANA-12 treatment attenuated stress-induced vGluT2-ir expression in the BLA, in addition to lowering levels at the PVN comparable to those of the VNS group. Together with observations of CRH-ir, these findings further support the role of TrkB receptors at the NAc shell in maintaining activation of PVN and BLA neurons upon stress exposure. Observation of biochemical changes at a delayed interval following stress

exposure further indicates that prevention of such activation has a lasting impact on this system. Of interest, increased vGluT2-ir expression at the PVN and BLA in the VS group appears comparable to that observed after administration of the TrkB antagonist in unstressed animals, further supporting opposite changes in stress and non-stress conditions, paralleling contrasts also observed at behavioral effects (e.g. anxiety).

Concordant changes in CRH-ir and vGluT2-ir at the PVN and BLA are in line with effects reported with chronic variable stress increasing glutamatergic expression in synaptic boutons making contacts with CRH-ir somata and dendrites (Flak, Ostrander, Tasker, & Herman, 2009), likely increasing excitability of these neurons. Apposition of vGluT2-ir terminal boutons on CRH neurons within the parvocellular division of the PVN has been observed (Flak et al., 2009; Wittmann et al., 2005), and this is in line with the regulatory action of glutamatergic synaptic inputs on CRH cells, which may involve glucocorticoid-mediated fast negative feedback of the HPA axis (Levy & Tasker, 2012). Glucocorticoids activate membrane receptors on parvocellular PVN neurons, and this action is suggested to trigger endocannabinoid release acting in a retrograde fashion to suppress glutamate release at presynaptic glutamate terminals. Rapid suppression of postsynaptic glutamate release on parvocellular neurons of the PVN, including CRH neurons, has also been shown dependent on glucocorticoids and (corticosterone), an effect blocked by the CB1 receptor antagonists AM251 and AM281 (Di, Malcher-Lopes, Halmos, & Tasker, 2003).

6. Conclusion

The current study suggests a strong interplay between the brain's stress and reward systems that is in part mediated by BDNF/TrkB signaling. The nucleus accumbens, via dopamine neurons, plays a decisive role in emotional regulation. TrkB receptors are expressed in D1+ and D2+ medium spiny neuron (MSN) subtypes (Smith, Lobo, Spencer, & Kalivas, 2013), a significant enrichment of TrkB mRNA being observed in D2+ MSNs of the NAc (Lobo et al., 2010). Interestingly, TrkB deletion selectively from D1+ or D2+ neurons is reported to exert opposite effects on cocaine reward in NAc neurons (Lobo et al., 2010). This suggests that inhibition of BDNF/TrkB signaling alters the function of both D1+ and D2+ NAc MSNs in producing balanced behavioral output. Inhibition of TrkB signaling in the NAc shell may have created an imbalance of these two

MSN populations, perhaps through increased activity of the D1+ MSN pathway and underactivation of the D2+ MSN pathway (Lobo et al., 2010). It thus appears interesting for future studies to further characterize impact on dopamine release and receptor expression at the nucleus accumbens.

Importantly, our observations support that TrkB actions on physiological systems appear state-sensitive, and that blockade of TrkB receptors exert negative impact on anxiety under normal/unchallenged conditions. Our findings show that site-specific blockade of TrkB receptors led to discrete biochemical changes in interconnected structures (anterior cingulate cortex, PVN, BLA and NAc core). The reported observations further support a determinant role of BDNF/TrkB receptor signaling in stress-induced emotional disorders. The role of BDNF in the pathogenesis and facilitation of depression and anxiety in humans remains controversial. Although inhibiting BDNF signaling may not lead to depressive phenotype, inhibiting TrkB receptor signaling in individuals susceptible to stress-related anxiety and depression is suggested to have clinical significance as a therapeutic strategy for attenuating or treating these disorders. In contrast, inhibiting TrkB receptor signaling in low stress individuals may show state-dependent effects in enhancing anxiety-like response. Thus, differential concentration of TrkB receptors in stress or reward regions of the brain means that selective antagonism of BDNF actions has potential application in the attenuation of stress-induced mood disorders.

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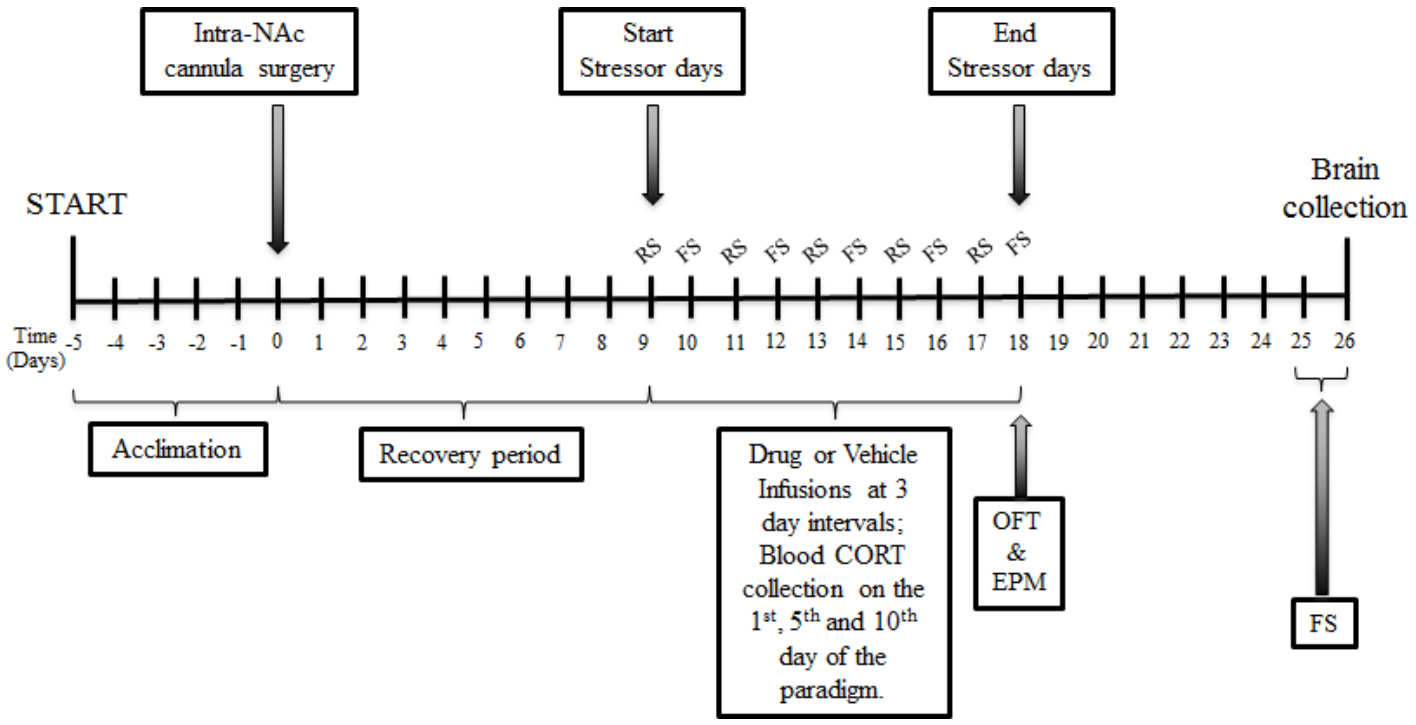

Figure 1. Experimental timeline. Animals arrive at the vivarium and are allowed 5 days to acclimate to the environment prior to surgery. Post-surgical recovery is for 10 days and then the animals begin the 10 day stress paradigm. During the stress paradigm, blood corticosterone samples are taken on the 1st, 5th and 10th days, whereas infusions occur on the 1st, 4th, 7th, and 10th days. RS: restraint stress; FS: forced swim; OFT: open field test; EPM: elevated plus maze.

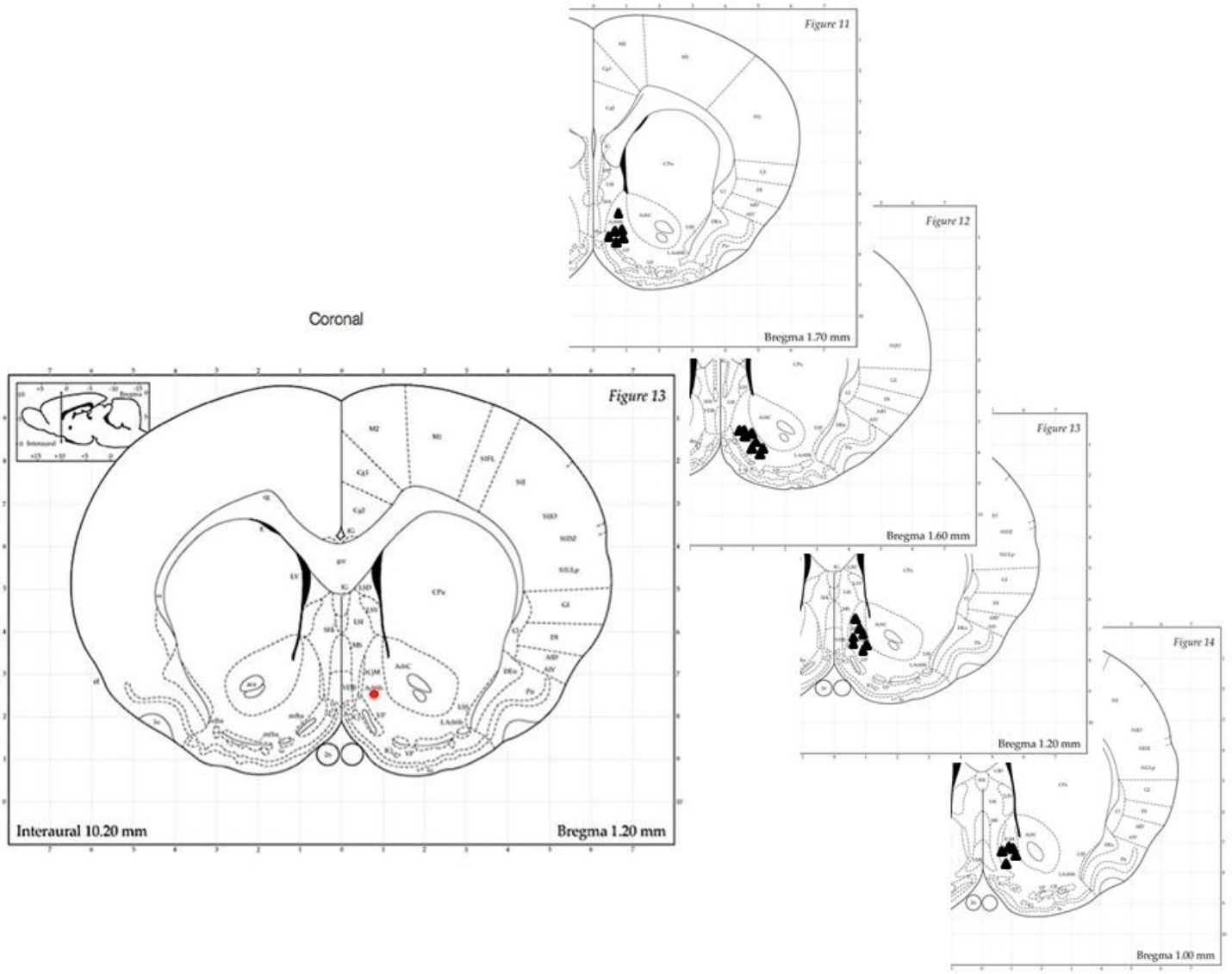

Figure 2. Cannula implantation site. Representative line drawings (Paxinos and Watson, 1998, adapted from labs.gaidi.ca/rat-brain-atlas) of coronal sections containing the intended site of cannula implantation (larger image, red dot) and the approximate implantation site locations (smaller images, black triangles).

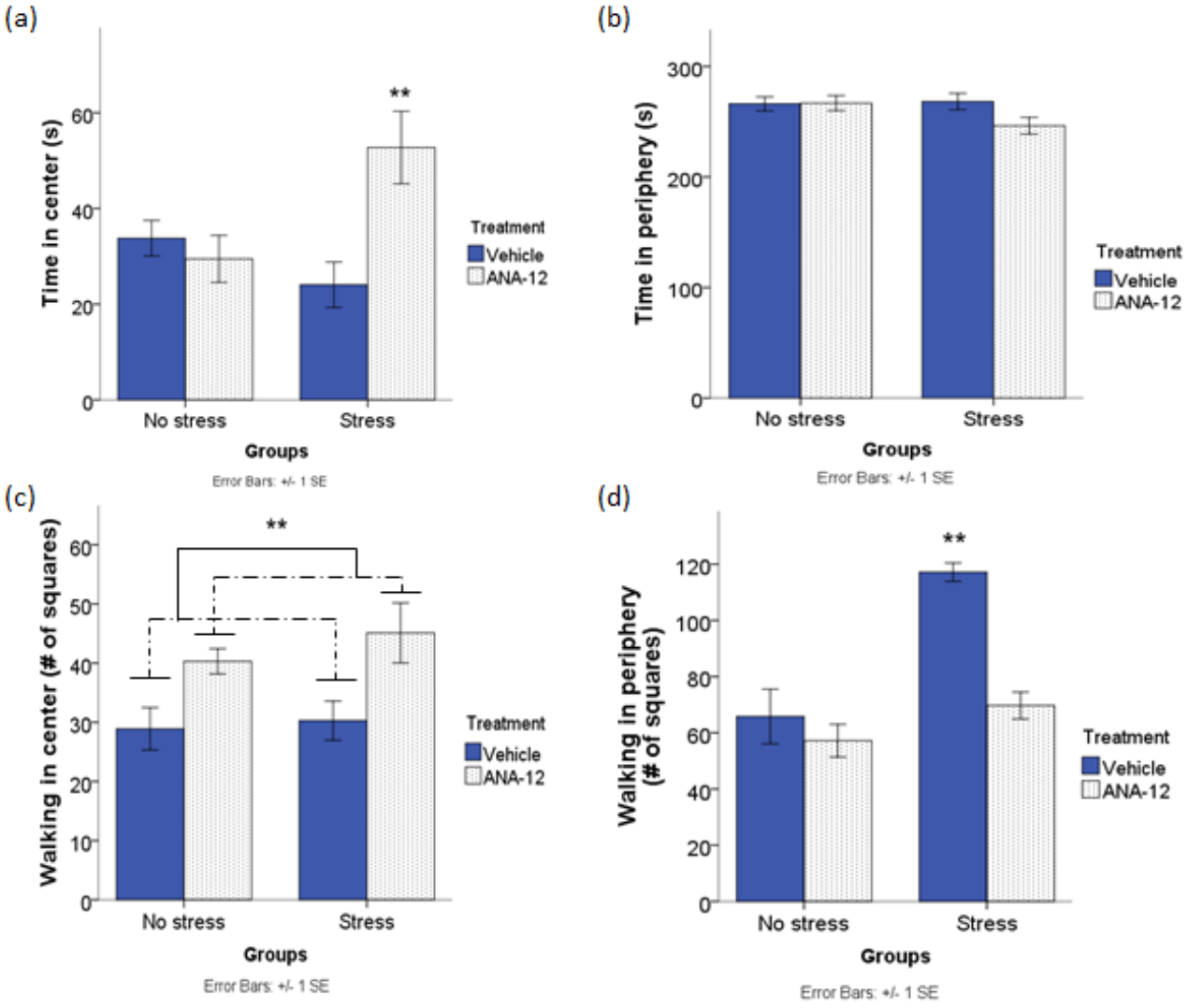

Figure 3. Inhibition of TrkB in the NAc shell of repeatedly stressed rats promotes center zone locomotion and exploration in the open field test. (a) AS group spent more time in the center when compared to the ANS and the VS groups, ANA-12 promoting anxiolytic behavior in stressed rats. (b) No significant group differences were found, however the AS group spent less time in the periphery. (c) ANA-12 injected groups walked more in the center, suggesting an effect of the treatment on locomotor activity. (d) VS group showed increased locomotion in the periphery compared to all groups. ** $p < .01$. Data are represented as mean ± SEM.

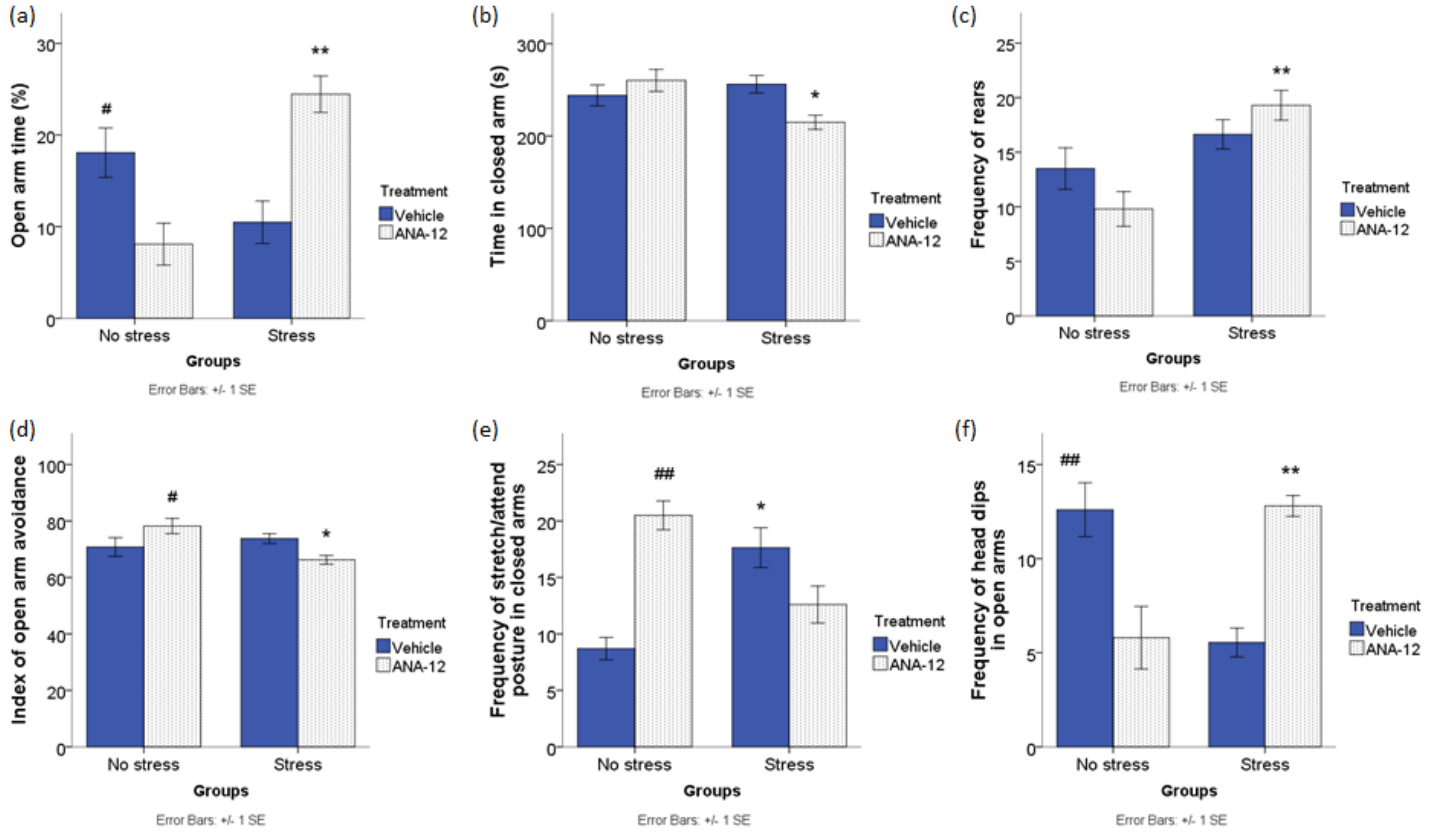

Figure 4. State-dependent effects of TrkB receptor blockade in the NAc shell in stressed and non-stressed rats in the elevated plus maze. (a) AS and VNS groups showed a higher percentage of open arm time compared to the ANS and VS groups, (b) AS group spent less time in the closed arm compared to the ANS and VS groups, (c) In terms of locomotor activity, the AS group made significantly more rears than the ANS group. The stress groups showed increased rearing behavior in the closed arms than the non-stress groups. (d) ANS group showed increased anxiety level with a high index of open arm avoidance compared to the AS and VNS groups. Conversely, the AS showed reduced open arm avoidance compared to the VS group. (e) ANS and VS showed an increased frequency of stretch/attend postures from closed arms compared to AS and VNS groups. (f) AS and VNS groups showed increased head dip frequency in the open arms compared to the ANS and VNS groups. *, # $p < .05$; **, ## $p < .01$. Data are represented as mean ± SEM.

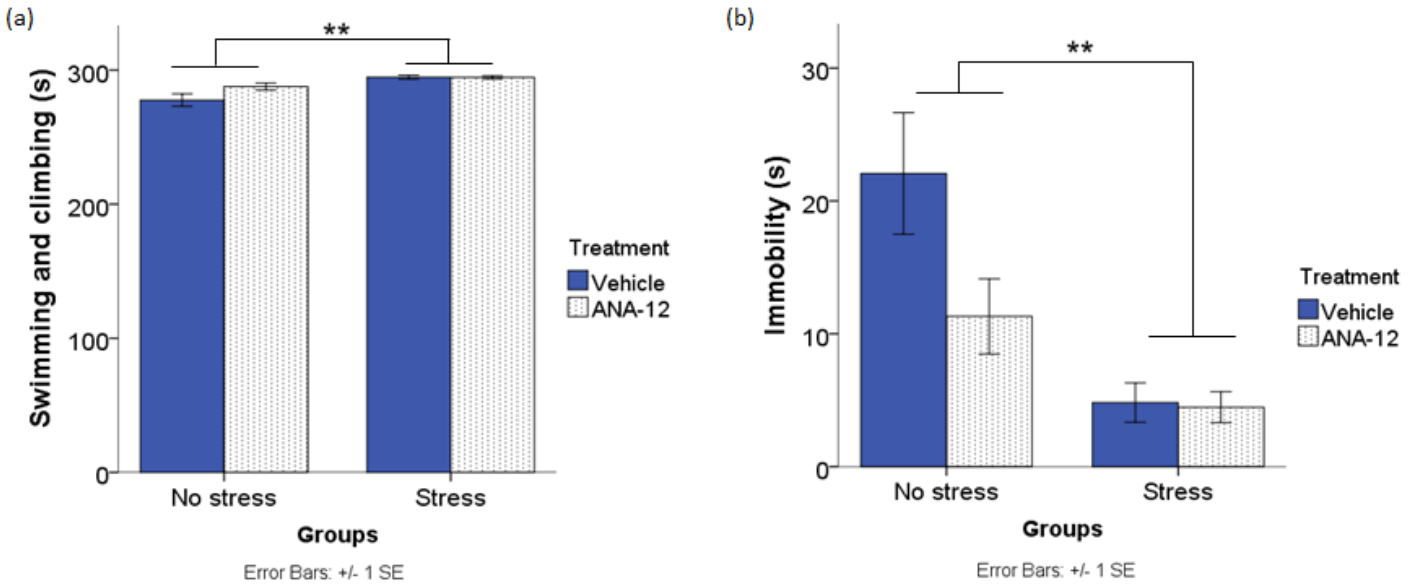

Figure 5. Active escape-like behavior and immobility in the forced swim test. On active escape-like behavior (a) and immobility (b), both stressed groups showed habituation with increased swimming and climbing and reduced immobility when compared to the non-stressed groups. Interestingly, the VNS group showed a highly significant reduction in swimming and climbing behavior compared to both the stress (AS and VS) groups, but a significant increase in immobility compared to all other groups. ANA-12 reduced immobility in non-stress rats compared to vehicle controls. ** $p < .01$. Data are represented as mean ± SEM.

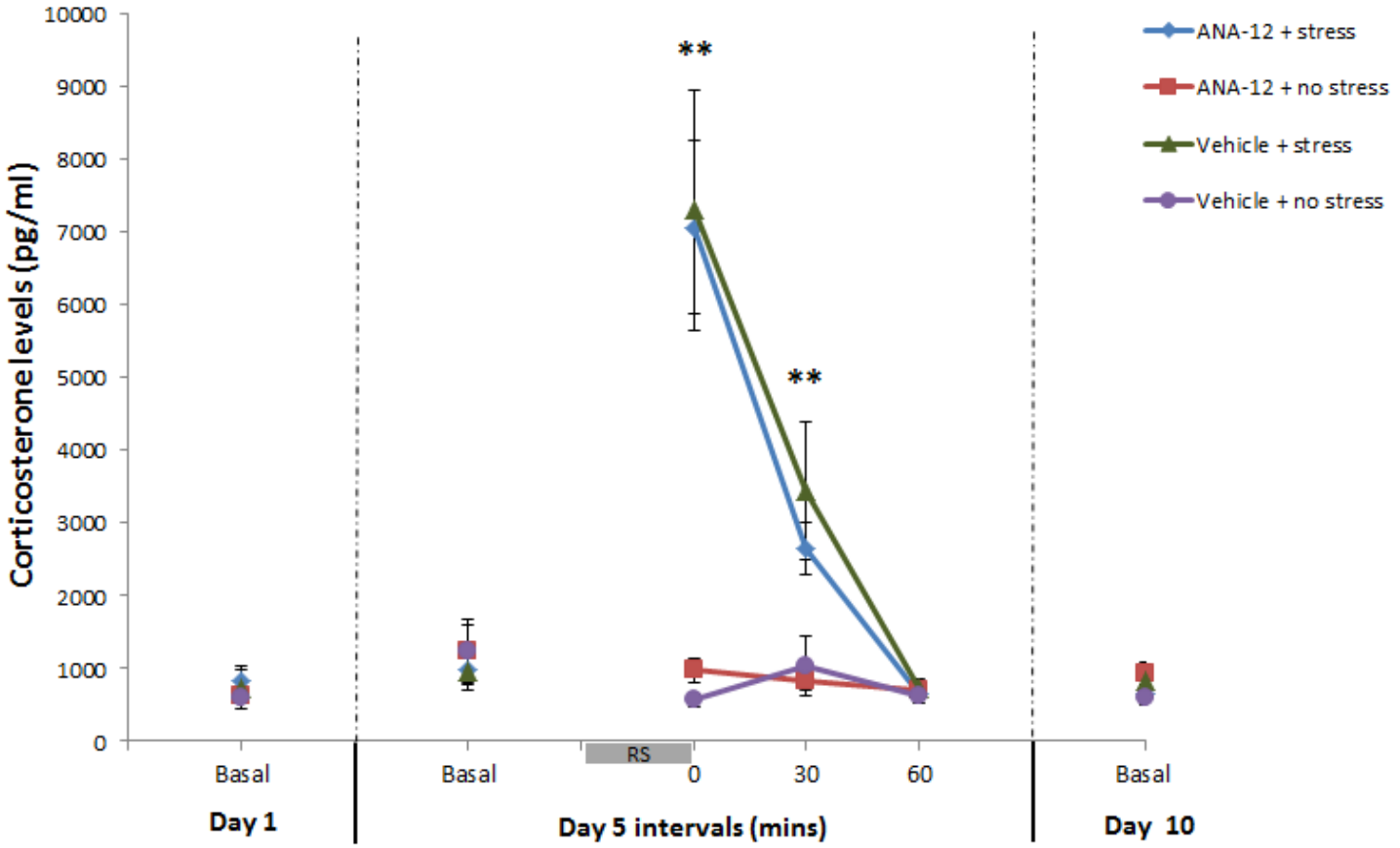

Figure 6: Effects of exposure to the heterotypic stress paradigm on CORT levels. Blood Corticosterone levels collected on days 1 and 10 (basal only) and at predetermined intervals on day 5 of a heterotypic repeated stress paradigm in animals treated with ANA-12 or vehicle (n = 8 per group) prior to stress (basal), 1 h following the first blood collection (0 min), and 30 and 60 min later. There was no drug or vehicle infusion on day 5. RS: restraint stress (30 min). No main effect of treatment on CORT levels was found. Data are represented as mean ± SEM. ** $p < 0.01$.

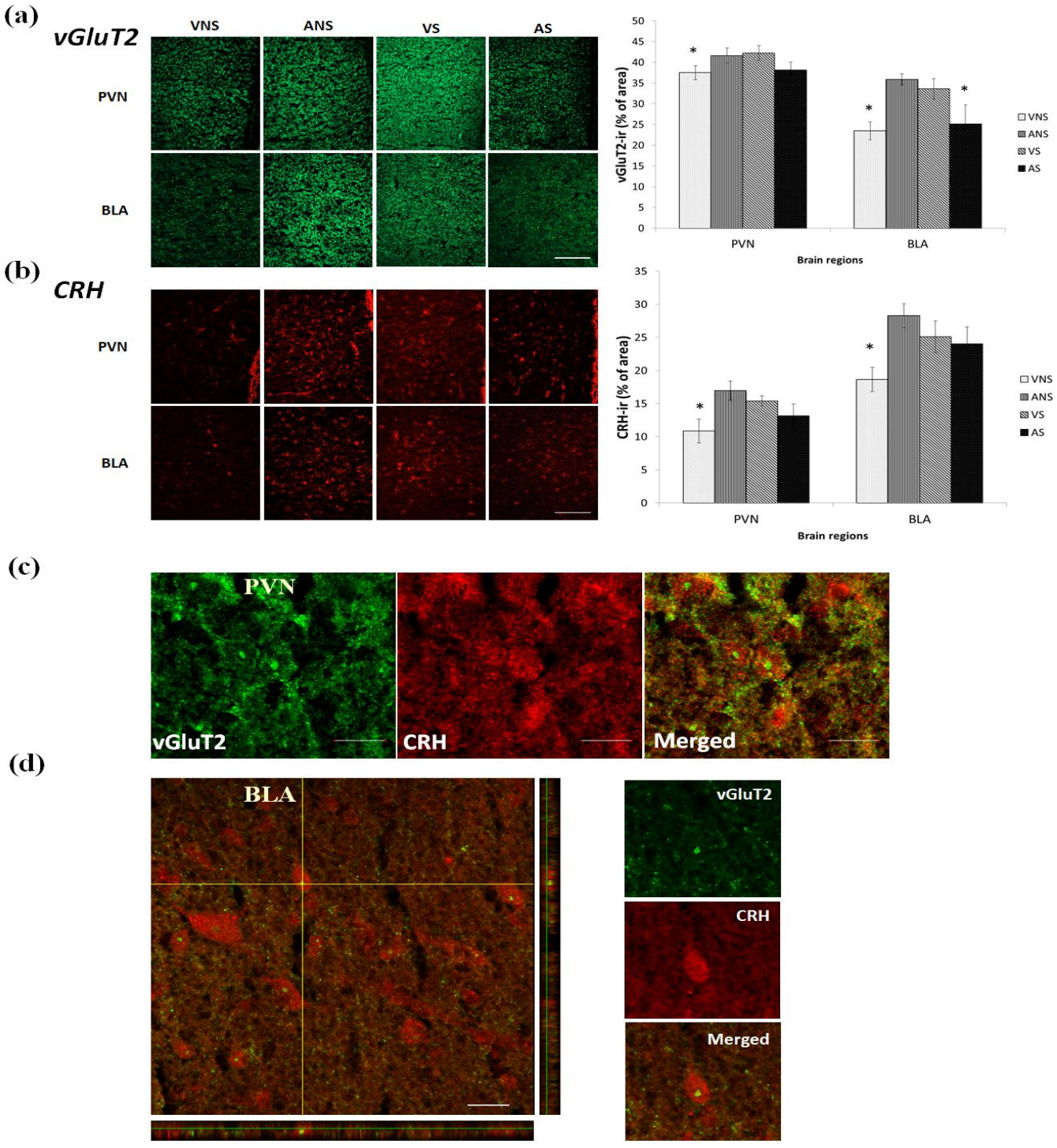

Figure 7: CRH- and vGluT2- ir in the paraventricular nucleus of the hypothalamus and the basolateral amygdala. Fluorescence photomicrographs (200x magnification) showing vGluT2-ir (green) and CRH-ir (red) in the PVN and BLA of VNS, ANS, VS and AS rats. Scale bar 100µm. (a) Graph for vGluT2-ir shows that the VNS group had significantly lower vGluT2 expression in the PVN compared to the VS group. In the BLA, lower expression of vGluT2 was observed in the VNS and AS groups compared to the ANS and the VS groups. (b) Graph for CRH-ir shows reduced PVN and BLA CRH expression in the VNS group when compared to the ANS and the VS groups. (c) Fluorescent photomicrograph taken on the confocal microscope from the PVN of an AS rat and (d) representative z-stack analysis with confocal microscopy from the BLA of a VNS rat revealed that VGluT2-ir is apposed upon CRH-ir neurons and is also found localized in the soma. Solid lines indicate the position for the z-stack images. Scale bar 20µm. Data are represented as mean ± SEM. * $p < 0.05$.

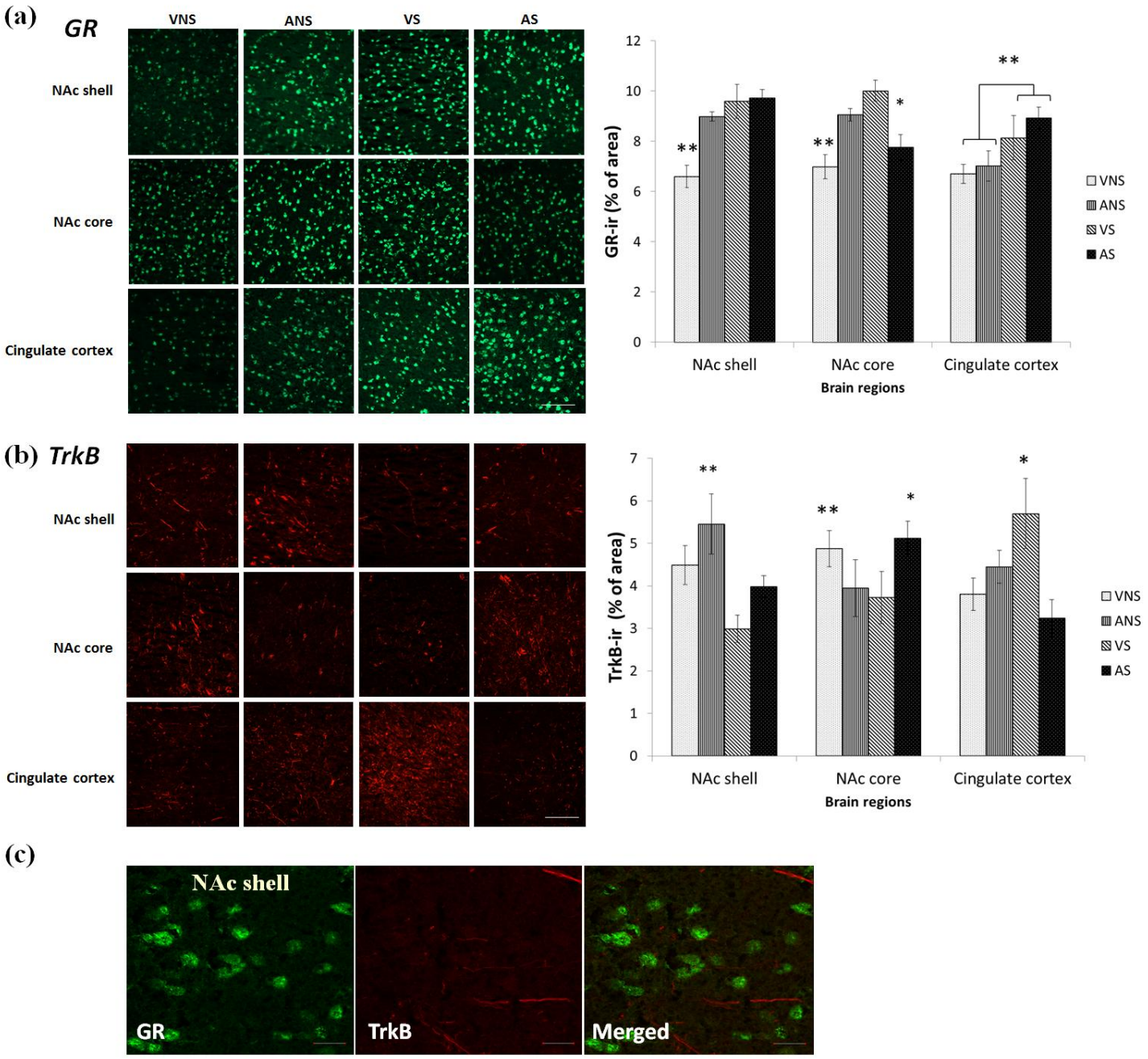

Figure 8. GR- and TrkB- ir in the nucleus accumbens shell, core and in the cingulate cortex. Fluorescence photomicrographs (200x magnification) showing GR-ir (green) and TrkB-ir (red) in the NAc shell, core and cingulate cortex. Scale bar 100µm. (a) Graph represents GR-ir levels in each group. NAc shell: VNS group showed reduced GR-ir compared to the ANS and VS groups. Stress rats showed increased GR-ir compared to non-stress animals. NAc core: Increased GR-ir in the ANS and VS animals compared to the AS and VNS groups. Cingulate cortex: Stressed groups showed higher levels of GR compared to non-stress groups. (b) Graph represents TrkB-ir levels in each group. NAc shell: ANS group showed elevated TrkB-ir levels compared to the VS groups. Of note, stress animals had lower TrkB-ir compared to non-stress animals. NAc core: Increased TrkB-ir was observed in the VNS (compared to ANS only) and AS animals compared to the ANS and VS groups. Cingulate cortex: VS animals displayed elevated TrkB-ir compared to the AS and VNS groups. (c) Confocal photomicrographs of GR-ir and TrkB-ir in the NAc shell of an AS rat indicate no colocalization. Scale bar 20µm. Data are represented as mean ± SEM. * $p < .05$, ** $p < .01$.

Article 2

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Authorial contributions

Idu Azogu conceived and designed the study, contributed to implementation, data analyses, interpretation, and manuscript draft and revisions. She performed the surgeries for unilateral intra-NAc cannula implantation for all experimental groups with the assistance of Sylvie Emond (Research assistant) and Charlene Habibi (Summer NSERC student co-supervised by Idu). She also prepared brain sections with the assistance of UROP students, Nivedh Patro and Michela Grazia Panarella, and performed the immunohistochemical analyses for all brain regions assessed with orexin A, FosB/ΔFosB and tyrosine hydroxylase.

Dr. Hélène Plamondon supervised the research project, interpreted the data analyses, and revised the manuscript.

Abstract
Repeated stress exposure can lead to the development of anxiety and mood disorders. An emerging biological substrate of depression and associated pathology is the nucleus accumbens (NAc), which through interactions with limbic, cognitive and motor circuits can regulate a variety of stress responses. Within these circuits, orexin neurons are involved in arousal and stress adaptability, effects proposed mediated via brain-derived neurotrophic factor signaling. This study tested the hypotheses that 1) repeated exposure to heterotypic stress alters social ability and preference and passive avoidant behaviors, 2) TrkB receptors at the NAc shell regulates stress-induced behavioral responses and orexin expression within the mesocorticolimbic system. Our findings indicate that ANA-12 (0.25 μg/0.5 μl) enhanced sociability during the social interaction test, although treatment had no effect on social preference. The development of conditioned place preference, and fear retention in the passive avoidance test were also facilitated by ANA-12. Biochemical assessments on brain tissues collected within 2 h of a forced swim exposure revealed that ANA-12 increased orexin A immunoreactivity (ir) in the hypothalamic perifornical area, while expression was reduced in the ventral portion of the hippocampal CA1 layer, irrespective of the stress condition. This contrasts changes at the VTA characterized by elevated versus reduced orexin A-ir in ANA-12-treated stress and non-stress rats, respectively. Colocalized orexin A- and tyrosine hydroxylase (TH)-ir at the VTA supports a different temporal expression post stress, TH-ir being unaffected 9 days post stress. These findings support a role for TrkB receptors in regulating basal and stress-induced social, cognitive and motivational behavior, and modulatory actions of BDNF, via TrkB signaling, on Orx A signaling upon stress exposure.
Keywords: Heterotypic stress; TrkB receptors; ANA-12; Nucleus accumbens; Contextual Memory; Motivation; Rats.

1. Introduction

Repeated stress exposure has been associated with prolonged activation of brain circuits (including the hypothalamic paraventricular nucleus; PVN), leading to increased sensitization and reduced capacity to adequately process subsequent stressors (Stahl, 2008). In the reward circuitry of the adult brain, stress induces region-specific changes that promote expression of the neurotrophin brain-derived neurotrophic factor (BDNF) and its primary receptor tyrosine-related kinase B (TrkB) at the nucleus accumbens (NAc) and ventral tegmental area (VTA), whereas expression is reduced in other brain regions of the stress circuitry (e.g. hippocampus). Thus, repeated exposure to social defeat stress in mice upregulates BDNF levels in the NAc (Berton et al., 2006; Krishnan et al., 2007), a phenomenon related to increased susceptibility to mood disorders, such as anxiety and depression (Lindvall et al., 1994). The development of experience-dependent social aversion also involves BDNF secretion (Berton et al., 2006), which facilitates stress- and drug-induced neuroadaptation in the mesocorticolimbic system. BDNF-containing projections to the NAc originate from the VTA and PFC. Within this pathway, adenovirus TrkB receptor knockdown in the NAc shell prevents stress-induced increase in ΔFosB expression at the NAc, as well as increased BDNF and type 1 glutamate (GluA1) receptor expression at the VTA (Wang et al., 2014). In a similar fashion, we recently demonstrated (Azogu and Plamondon, 2017) that TrkB receptor blockade at the NAc shell blocked stress-induced inhibition of TrkB-ir in the NAc core and shell. This underlines an important role of TrkB receptors within the reward circuitry in actively maintaining homeostasis.

Furthermore, recent findings have supported an involvement of orexins (also known as hypocretins (HCRT)) in motivation and stress response (Arendt et al., 2013; Yamanaka et al., 2006). Thus, orexin has been proposed to promote adaptive responses to stress (Giardino and de Lecea, 2014; Mahler et al., 2014; Srinivasan et al., 2013; Suzuki et al., 2005), in part via stimulation of BDNF secretion (Yamada et al., 2009). The HCRT signaling molecules, orexin A and orexin B, are derived from a single precursor prepro-orexin gene and mainly expressed in the lateral hypothalamus (LH), posterior hypothalamus, and perifornical area (PfA) (Cluderay et al., 2002; Hervieu et al., 2001; Peyron et al., 1998; Tsunematsu and Yamanaka, 2012). They act via two G-

protein-coupled receptors, orexin receptor-1 (OX_1R) and orexin receptor-2 (OX_2R) (Cluderay et al., 2002; Sakamoto et al., 2004; Tsunematsu and Yamanaka, 2012). OX_1R binding to orexin A is one order of magnitude higher than binding to orexin B, while OX_2R binds to both orexin A and orexin B with similar affinities (Sakurai, 2014; Sakurai et al., 1998). Hypocretinergic cells project widely to monoaminergic and cholinergic neuronal systems throughout the brain (Nambu et al., 1999; Peyron et al., 1998) and are also present within the peripheral autonomic nervous and endocrine systems (Korotkova et al., 2003; Winsky-Sommerer et al., 2004). These neuropeptides play a modulatory role in sleep and wakefulness, arousal, feeding behavior, energy homeostasis, and reward processing (see reviews (Bubser et al., 2005; Sakurai, 2014).

Several reports have supported the role of the orexin system in neuroendocrine responses related to HPA axis activation (Al-Barazanji et al., 2001). For example, anxiogenic behavior (Suzuki et al., 2005) and decreased brain reward function (Boutrel et al., 2013) in rodents following intracerebroventricular (i.c.v.) injection of orexin A have been shown to depend on activated PVN-corticotropin releasing hormone (CRH) and arginine vasopressin neurons (Al-Barazanji et al., 2001; Russell et al., 2001; Sakamoto et al., 2004; Winsky-Sommerer et al., 2004), and on elevated ACTH and glucocorticoid secretion (Al-Barazanji et al., 2001; Russell et al., 2001; Srinivasan et al., 2013; Suzuki et al., 2005). In addition, pretreatment with the CRH antagonist α-helical CRF 9-41 blocked the ability of centrally infused orexin A to activate the HPA axis (Samson et al., 2002). Endogenous activation of the orexin system has also been reported in response to different stressful stimuli. Acute immobilization or cold stress were shown to upregulate orexin mRNA expression in the LH (Ida et al., 2000; Tung et al., 2016), while chronic stress downregulated orexin at the same locus (Lutter et al., 2008) and upregulated orexin in the hippocampus, more specifically the ventral portion (Nollet et al., 2012) that is closely involved in regulating emotional responses (Fanselow and Dong, 2010). These site-specific responses to elevated stress may impair coping responses and contribute to depression phenotypes.

The NAc is a site known to regulate orexin cells (Bubser et al., 2005) and the shell subregion of the NAc subserves both negative and appetitive motivational states, such as drug-seeking, feeding behavior and arousal via dopamine (DA) receptors (Baldo et al., 2004; Qi et al., 2013). Dense projections of orexin to the NAc and

VTA influence the activity of the VTA-NAc reward circuit (Nestler and Carlezon, 2006). Production of orexin in the hypothalamus is known to innervate the VTA and remain in close apposition to DA neurons, potently exciting these neurons (Bubser and Schmidt, 1990; Fadel et al., 2002; Horvath et al., 1999; Korotkova et al., 2003), and causing DA release in VTA target regions, such as the NAc and prefrontal cortex (Narita et al., 2006; Vittoz and Berridge, 2006). A previous report found a co-distribution of orexin fibers and DA in the NAc shell (Baldo et al., 2003; Fadel and Deutch, 2002), suggesting that function of the NAc shell DA system can be regulated by orexin transmission and promote motivated behaviors, such as the reinstatement of stress-induced morphine CPP (Qi et al., 2013). Orexins are activated by FosB/ΔFosB, which is shown to accumulate after repeated stress or antidepressant treatments. In addition, Fos expression (indicative of neuronal activation) in orexin neurons of the LH/PFA have been observed following stress, such as acute footshock (Harris and Aston-Jones, 2006). The interplay between hypothalamic neurons expressing orexin and the brain's reward circuitry support a role for the peptide in the modulation of anxiety and depression states.

Considering that BDNF secretion may facilitate orexin A-mediated responses to stress and support its role in reward function, we investigated the effects of TrkB receptor blockade at the NAc shell on region-specific orexin A expression and species-specific emotional behavior following exposure to a 10-day heterotypic stress paradigm. The downstream neurobiological effects of ANA-12 were assessed with an emphasis on FosB/ΔFosB expression in orexin A-ir cell bodies and fibers of the hypothalamus and dorsal hippocampus, and the co-localized expression of orexin A and tyrosine hydroxylase (TH) in the ventral hippocampus and VTA.

2. Material and methods

2.1. Animals

Adult male Wistar rats weighing between 250-275g were obtained from Charles River Laboratories (Rochefort, Quebec, Canada) and doubly housed in Plexiglas cages with daily handling to minimize stress. They were maintained on a 12h light/dark cycle (lights on at 7:00am), room temperature (21-23°C) with 60% relative humidity, and ad libitum access to water and standard Purina rat chow. Following five days of

acclimation to the animal facility, animals underwent intra-NAc guide cannula implantation surgery. Efforts were made to minimize animals' suffering. All procedures were carried out in accordance with the Canadian Council of Animal Care and approved by the University of Ottawa Animal Care Committee. Experimentation complies with the ARRIVE guidelines and is in accordance with the National Institutes of Health guide for the care and use of laboratory animals (NIH Publications No. 8023, revised 1978).

2.2. Guide cannula implantation surgery

As previously reported (Azogu and Plamondon, 2017), the intra-accumbal cannula implantation surgeries were performed under continuous isoflurane anaesthesia by inhalation of 2 to 3% isoflurane delivered in oxygen. Body core temperature was maintained at 37°C using a feedback regulated heating blanket (Homeothermic Blanket Control Unit, Harvard Instruments, Natick, MA). A stainless steel 22-gauge guide cannula was surgically implanted in the right NAc shell (inserted 1.2mm posterior to Bregma; -1.5mm lateral from the midline (angled at 6°); and lowered 7.0mm below dura ((Paxinos and Watson, 1998) rat brain atlas) (Li et al., 2013) and secured to the skull with three stainless steel screws (Plastics One, Roanoke, VA, USA) using dental acrylic cement (Jet Liquid acrylic resin, Lang Dental Manufacturing Co., Inc.). Stainless steel 28-gauge insect pin (dummy cannula) was secured into the guide cannula to prevent occlusion. Animals received Meloxicam (1.5 mg/kg, s.c. Boehringer Ingelheim (Canada) Ltd.), a non-steroidal anti-inflammatory drug, immediately after surgery and were then placed in their cage resting on a heating pad or in an air-controlled incubator for a few hours before being returned to their vivarium room. Unilateral cannulation was selected as it involved less stress to the animal (also see (Bolaños et al., 2003; Carlezon et al., 1997)), and bilateral injections were likely to yield similar effects to those observed with unilateral injections, although of greater magnitude (Olson et al., 2005; Wang et al., 2015). Animals were housed individually and allowed 10 days for recovery, with gentle handling for at least 2 min daily after the fourth recovery day (Rodd et al., 2004).

2.3. Drug infusion

Micro-infusion of 0.25 µg of the highly selective TrkB receptor antagonist *N*-[2-[[(Hexahydro-2-oxo-1*H*-azepin-3-yl)amino]carbonyl]phenyl]benzo[b]thiophene-2-carboxamide (ANA-12, SML0209, Sigma Aldrich) was administered at a rate of 0.5 µl/min into the right hemisphere of the NAc shell. The vehicle was composed of 0.9% saline solution containing 20% (2-Hydroxypropyl)-β-Cyclodextrin (H107, Sigma-Aldrich; (Girbovan et al., 2012)). This dosage was selected considering repeated drug administration in our paradigm (Azogu and Plamondon, 2017). Dummy cannula was carefully removed from the guide cannulas in freely moving animals, and an internal injector (28-gauge), which extended 0.5mm below the end of the guide cannula, was inserted for intra-accumbal injections at a final depth of 7.5mm below dura. Injection needle remained in place for an additional 60 sec post infusion to ensure diffusion to the NAc shell tissue and minimize backflow. Rats received intra-NAc micro-infusions of either ANA-12 or vehicle 30 min prior to initiation of the stress/no stress session on days 1, 4, 7 and 10 of the stress paradigm. Animals were divided into 4 groups (n = 10 to 11 per group): ANA-12 + stress (AS), ANA-12 + no stress (ANS), Vehicle + stress (VS), and Vehicle + no stress (VNS). An overview of the experimental timeline is depicted in Fig. 1.

2.4. Heterotypic stress paradigm

The 10 day heterotypic stress paradigm is adapted from a 28-day chronic combinatory stress model based on alternating two mild physical stressors: restraint stress and forced swim stress (Zamora-Gonzalez et al., 2013). This alternative schedule is reported to steadily activate the HPA axis, while reducing habituation to the stressors. Thus, between 9:30 and 11:00 AM on odd numbered days, animals in stress groups were restrained in a clear plastic container for 30 min (with an air hole to allow easy breathing). Following the same hour schedule on even numbered days, stress rats underwent 15-min of forced swimming in a transparent cylindrical container (20.32 cm in diameter and 43.18 cm in height) filled with tap water (23 ± 2°C) at a height of approximately 33 cm. Animals were dried off post-swim and placed in a warm incubator or heating pad before being returned to their home cage. Control animals were handled for up to 5 minutes with no additional manipulation. 2 h prior to euthanasia, exposure to forced swimming was applied as previously performed in our lab (de la Tremblaye et al., 2016).

2.5. Behavioral testing

All rats were subjected to behavioral testing in the social interaction and social novelty preference test (SIT/SP), Y maze conditioned place preference (YMCPP) and Y maze passive avoidance task (YMPAT). Animals were transported to the enclosed hallway outside the testing room 30 min prior to testing. All behavioral testing were carried out between 0930 and 1600 in the order depicted in Fig. 1. Overhead lights provided 800-900 lux in the infusion, restraint stress and behavioral testing rooms (SIT/SP and Y maze) and 650-700 lux in the forced swim stress room. The behavioral tests were thoroughly cleaned with 70% ethanol in-between animals. Note: assessment of rat-specific anxious behavior will hereafter be referred to as "anxiety" rather than "anxiety-like".

2.5.1 Social interaction/ social novelty preference test (SIT/SP)

The SIT/SP was standardized to assess sociability and preference for social novelty in mice as an index of social avoidance in mouse models of autism (Moy et al., 2004). The test was conducted on the day following the last forced swim stressor. In this three chamber sociability paradigm, also known as Crawley's sociability and preference for social novelty protocol (Crawley, 2004; Moy et al., 2004), a rodent is presented with a choice between initiating direct approach with a novel conspecific rodent and spending more time in this chamber versus spending time in the chamber containing the novel inanimate object and directly interacting with the object. In the second test (SP), the experimental rodent is presented with a choice between the now-familiar conspecific and a second novel conspecific. The testing apparatus consisted of a modified open field arena (LWH: 75cm × 75cm × 30cm) that stood on a table 90cm above the floor and white curtains separated the arena and the experimenter's recording zones. Two clear Plexiglas walls with removable middle partitions divided the arena into three chambers. As adapted from Kaidanovich-Beilin and colleagues (2011), the rat was placed in the middle chamber, which represented the starting point, while the two outer chambers contained identical wire containment cups. The rat was allowed to habituate to the test for 5 min. During the first 10 min test session (social interaction), the rat was allowed to freely explore both chambers, one containing the novel rat (Stranger 1, S1) under the wire cup and the second chamber containing a similar but empty cup (EC). During session 2

(social novelty preference, 10 min), the 2nd chamber now housed a novel stranger rat (Stranger 2, S2) under the wire cup. Placement of stranger rats was interchanged between trials for the different animals. Measurements included the duration of entries in each chamber, direct interactions [i.e. the duration of contacts between S1 and the EC during session 1, and S1 (now-familiar rat) and S2 during session 2], and indirect interactions [i.e. duration of other behaviors in each chamber during session 1 and session 2 not pertaining to direct contact with the conspecifics (walking, grooming and freezing)]. The exploration ratio in session 1 was defined as [$T_{Stranger1}$ /($T_{Stranger1}$ + $T_{Empty\ cup}$)]. An exploration ratio > 0.5 indicated that contact with S1 was elevated relative to contact with the EC (i.e. a level of preference for S1 indicating normal sociability, motivation and affiliation), while an exploration ratio < 0.5 indicated that contact with S1 was reduced relative to contact with the EC. The exploration ratio in session 2 was defined as [$T_{Stranger2}$ / ($T_{Stranger2}$ + $T_{Stranger1}$)]. An exploration ratio > 0.5 indicated that contact with S2 was increased relative to contact with S1 (i.e. preference for S2 indicating social memory and predilection for novel experiences).

2.5.2. Y maze conditioned place preference (YMCPP)

The YMCPP was conducted over the course of 4 days following the SIT/SP test. Experimental protocol in the Y maze was modified to assess place preference, a classic conditioning paradigm in which an animal learns to associate the reinforcing effects of food or pharmacological drug to a particular environment. A biased place conditioning protocol was selected. The test was conducted in a Y-shaped Plexiglas structure (LWH: 35.5cm × 15cm × 30cm) with three arms and Plexiglas sliding doors (START arm – grey door, left and right arms – white doors), placed on a table (90cm above the floor) with an overhead camera, and separated by white curtains from the experimenter's area. Steel-caged grates were secured on top of each maze arm to prevent the animal from jumping out of the maze. Floor texture did not differ for all three arms, however distinct visual cues (horizontal vs. vertical black and white striped walls) were used to allow for visual discrimination. During the habituation session, a rat was placed in the enclosed tail end of the maze (START) for 2 min, after which the sliding door was raised, and the rat was allowed to freely explore the maze for 10 min. Duration of time spent in all three arms was recorded and scored by an experimenter blind to the animal groups, in order to determine

which of the two experimental arms could be labeled as the "least preferred". For the next two days, rats were subjected to four counter-balanced forced choice run sessions per day (conditioning sessions; i.e. a total of 5 min in each arm × 2 sessions × 2 days) in which they were allowed access to either the least preferred arm, which was paired with Honey Nut Cheerios®, or the other arm (non-food paired) for 5 min periods. Briefly, after being placed in the START arm for 2 min, rats entered the food-paired arm and a sliding Plexiglas door was lowered, blocking their exit. After a 5 min session, the rat was returned to the START arm for 2 min after which only the non-food paired arm was available for entry for 5 min. A 30-min interval was observed before the rat underwent additional conditioning sessions, which should aid the rat to develop a preference for the food-paired arm compared to the non-rewarded arm. During the test session on the 4^{th} day, a similar procedure as observed during the habituation session was implemented, allowing free access to all arms. An experimenter blind to the animal groups scored the time spent in each arm. Calculated measures include time spent in the non-food paired and food paired arms, the percent change in time spent in the initially least preferred arm [(test – habituation)/ habituation) x 100] (Millot et al., 2014), and the preference ratio (time in food paired arm/ (time in food paired arm + time in non-food paired arm)) during the test session were scored. A preference ratio of 0.5 indicated no preference for either arm, with ratios > 0.5 indicating a preference for the food paired arm and ratios < 0.5 indicating a preference for the non-food paired arm (Yates et al., 2013). If the time spent in the initially least preferred arm was greater during the test than during the habituation session, the animal is considered to have undergone a CPP (Huston et al., 2013; Jerlhag et al., 2010). Considering that food rewards (e.g. Honey Nut Cheerios®) act as positive reinforcement, the animals were placed on an overnight-restricted diet (with access to 4-5 food pellets) beginning on the day of the YMCPP habituation and ending on the last day of the test.

2.5.3. Y maze passive avoidance task (YMPAT)

The YMPAT assesses learning and memory in an environment paired with an aversive stimulus by measuring the latency to re-enter the aversive arm on a subsequent maze exposure in rodents (Azogu et al., 2015). This paradigm is based on previous studies validating the use of air-puffs as an alternative to foot shock

to induce aversive learning in passive avoidance paradigms (Engelmann et al., 1996; Moriarty et al., 2012). The test took place on the day after completion of the YMCPP. The Y-shaped Plexiglas structure (LWH: 35.5cm × 15cm × 30cm) consisted in a three-arm structure with steel-caged grates placed on top of each arm, and Plexiglas sliding doors (START arm – grey door, left and right arms – white doors). The apparatus resided on a table 90cm above the floor with an overhead camera, and was separated by white curtains from the experimenter's area. Briefly, after a 2 min period in the START arm, all three doors were raised and the rat was allowed to freely explore each arm. As soon as the rat entered an arm with its four paws (L1), a sliding door was gently closed behind it and the rat then received small jets of compressed air every 15 sec over a 60 sec period before removal from the maze. A 5-min interval was observed before re-exposure to the maze. Upon the second exposure (L2), the arm paired with the aversive stimuli was the only one available for re-entry, in order to assess inhibitory avoidance. Latency to re-enter this arm was measured in sec, as well as the number of risk assessments made by the rat prior to entry into the arm, and the length of time spent in the aversive arm. The level of risk assessment was scored as the number of times the rat approached the arm without entering it. Length of rat's immobility in the aversive arm was assessed as a measure of anxiety-related expectation. Maximal latency for re-entry into the aversive arm was 5 min.

2.6. Histology

Nine days following the last stress session, animals were anaesthetized with isoflurane and quickly decapitated. Brains were rapidly collected, frozen on dry ice and stored at -80°C until sectioning into 14 μm coronal slices (i.e. 2 cell widths) and three sections placed per slide using a cryostat (Leica CM1900, Leica Microsystems, Germany) at -17°C. Every 9^{th} section (i.e. at 252 μm intervals; collected a tissue and skipped the next one) was collected on polarized slides (Fisherbrand Superfrost® Plus Microscope Slides) and stored at -80°C until further histological analysis. Following histological examination, only data from rats showing exact cannula placement in the NAc shell were kept in the study (Fig. 2).

2.7. Immunohistochemistry

Sectioned brains (n = 5 to 7 per group) were processed for immunohistochemical detection of FosB/ΔFosB, orexin A, and TH. The dorsal hippocampus (CA1/CA3 pyramidal layers), lateral hypothalamus (LH) and perifornical area (PfA) (Bregma -3.14 to -4.16mm) were dual-labeled for orexin A and FosB/ΔFosB, while sections of the ventral hippocampus (CA1/CA3 pyramidal layers) and ventral tegmental area (VTA, parabrachial pigmented nucleus) (Bregma -5.60 to 6.30mm) served to detect orexin A and TH. Briefly, sections were post fixed for 5 min with 4% paraformaldehyde containing 2% picric acid in 0.1M phosphate buffered saline (PBS, pH = 7.4), rinsed 3 x 5 min in 0.1M PBS and incubated for 30 min in blocking buffer (1% bovine serum albumin (BSA) in PBS – Triton (0.3%)). The sections were incubated for 24h at 4°C using primary antibodies mixed in 1% BSA in PBS – Triton (0.02%): polyclonal rabbit anti-FosB (1:500, CAT#: sc-48, RRID: AB_631515, Santa Cruz; epitope mapping within an internal region of Fos B of mouse origin, recognizes both FosB and ΔFosB), polyclonal goat anti-Orexin A (1:300, CAT#: sc-8070, RRID: AB_653610, Santa Cruz; epitope mapping at the C-terminus of Orexin-A of human origin) or monoclonal mouse anti-TH (1:1000, CAT#: MAB318, RRID: AB_2201528, Millipore Corporation; recognizes an epitope on the outside of the regulatory N-terminus). Subsequently, sections were rinsed 3 x 5 min in 0.1M PBS and incubated for 2 h at RT with the corresponding secondary antibody: biotinylated donkey anti-mouse IgG (1:500, Invitrogen), biotinylated donkey anti-goat IgG (1:500, Invitrogen) and biotinylated donkey anti-rabbit IgG (1:500, Invitrogen). After another 3 x 5 min rinses, the sections were incubated at RT in 1µg/ml (Hoechst 33342, Invitrogen Canada Inc.) for 10 minutes to label binding to AT regions of DNA. Following a last series of rinses, the slides were applied with an anti-fade medium containing 0.1% *p*-phenylenediamine in phosphate buffered glycerol, cover-slipped and sealed with nail polish. Special controls were conducted to test for antibody specificity: secondary antibody only control, i.e. incubation of the tissue in diluent (PBS-Triton-BSA) without the primary antibody followed by incubation in the secondary antibody.

2.8. Histological quantification

Signal detection was accomplished using an Olympus DX51 fluorescence microscope (Center Valley, PA, USA), of which digital immunofluorescent images were obtained using the ProgRes Capture Pro 2.7.6 software under a 20x objective lens (eyepiece 10x; numerical aperture 0.75). Immunoreactivity was quantified with the Image J software (Image J, National Institutes of Health, Bethesda, MD) and the method described by Hayes and colleagues (Hayes et al., 2005), which calculates the percent immunofluorescence reactivity of the total area assessed relative to a subthreshold background. One of the auto-threshold methods, Moments, in Image J was employed to allow for consistency across images in assessing optical density of areas of interest. At least four anatomically matched pictures of both hemispheres were analyzed to produce an average score for each brain area. Data was presented as background corrected standardized image densities for each brain region. Bilateral counts of orexin A-ir, including FosB/ΔFosB double-labeled orexin neurons was performed for the LH (located from the lateral side of the PfA to the optic tract) and PfA (area surrounding the fornix) and averaged across each animal for each brain area. Representative confocal photomicrograph was taken using an Olympus BX51 microscope (Center Valley, PA, USA) with a 60x oil lens magnification and FV1000 Fluoview software.

2.9. Statistical analysis

Statistical analysis was performed using SPSS software (version 23). Separate two-way ANOVAs assessed the main effects and interactions of treatment (ANA-12 vs. vehicle) and stress (stress vs. no stress) on the dependent variables - behavioral test output (SIT/ SP, YMCPP and YMPAT) or neurochemical alterations (orexin A, FosB/ΔFosB and TH) - across all groups. The assumptions of normality and homogeneity of variance, along with Mauchly's test of sphericity, were verified, and the (partial η_p^2) proportion of variance accounted for throughout the respective analyses. Paired samples *t* tests assessed within group differences during the habituation and test sessions in the YMCPP and right vs. left hemispheric differences in immunohistochemical expression. In the YMCPP, Student's *t* tests were used following two-way ANOVA to determine between group differences. Finally, one sample *t* tests assessed differences from the normal population score of 0.5 (i.e. the dashed line indicating an equal exploration or preference) for individual

exploration ratios in the SIT/SP and the preference ratio in the YMCPP (Yates et al., 2013). Effect size manual calculations of Cohen's d determined the magnitude of within-group differences on two means. One-way repeated measures ANOVA served to analyze contact with stranger 1 during the SIT and SP, differences in time spent in the food paired arm during the habituation and test sessions for the YMCPP, and latencies in the YMPAT. Following these analyses, simple main effects with Bonferroni correction (alpha levels were adjusted for the number of comparisons) were used. Pearson's product-moment correlation (2-tailed) was used to explore the relationship between behavioral and neurochemical responses (Table 1A and B). Data are presented as mean ± standard error of the mean, unless otherwise stated. Statistical significance was set at $p < 0.05$.

3. Results

3.1. Effect of TrkB receptor blockade on Social interaction and Preference tests (SIT/ SP)

3.1.1. Contact with Stranger 1 during social interaction and preference tests

One-way repeated measures ANOVA revealed a main effect of time spent with stranger 1 (S1) in the SIT and SP test, attributable to all rats showing decreased interaction time with S1 in the SP test compared to initial time spent in the SIT (Mean ± SD: 158.30 ± 6.99 vs. 64.90 ± 6.38 sec) (F (1,39) = 181.52, $p < .0001$, $\eta_p^2 = .82$) (data not shown). This indicates that all rats were sensitive to reduced novelty of S1 upon the second exposure.

3.1.2. Social interaction (sociability)

3.1.2.1. Direct Interaction

Two-way ANOVA failed to show significant differences of treatment (F (1,36) = 3.51, $p = .069$, $\eta_p^2 = .09$) or stress (F (1,36) = 3.01, $p = .091$, $\eta_p^2 = .08$). Effects were marginal and related to increase contact of AS group with S1, the ANA-12 group also showing a tendency for decreased exploration of the empty cup (EC) compared to the veh-treated groups (F (1,36) = 3.42, $p = .073$, $\eta_p^2 = .09$). Paired samples t test for within group comparisons in contact with S1 vs. EC revealed a preference for S1 in all the groups (Fig. 3A): AS ($t(9) = 7.14$, $p < .0001$, $d = 2.26$), ANS ($t(9) = 5.59$, $p < .0001$, $d = 1.77$), VS ($t(9) = 2.34$, $p = .044$, $d = 0.74$) and VNS ($t(9) = 4.49$, $p = .002$, $d = 1.42$). One sample t tests revealed that all groups exhibited an exploration ratio above 0.5

($p < .01$). A main effect of treatment (F (1,36) = 6.49, p = .015, η_p^2 = .15) for the exploration ratio (Fig. 3B) was indicative of higher sociability with S1 vs. EC by the ANA-12 treated groups compared to the veh-treated groups.

3.1.2.2. Indirect interaction

Two-way ANOVA revealed main effects of treatment for increased time spent in the S1-paired chamber (Fig. 3C) (F (1,36) = 5.15, p = .029, η_p^2 = .13) and exhibition of other behaviors, e.g. grooming, (F (1,36) = 5.13, p = .030, η_p^2 = .13) by ANA-12 compared to vehicle-treated groups. This behavior was in reverse to the reduced time in the EC-paired chamber by ANA-12 treated animals (Fig. 3C) (F (1,36) = 11.54, p = .002, η_p^2 = .24) compared to their veh-treated counterparts. Paired samples t test revealed that the AS (t(9) = 5.58, p < .0001, d = 1.76) and ANS (t(9) = 4.37, p = .002, d = 1.38) groups spent significantly more time in the S1-paired chamber, while the VS and VNS groups showed a comparable reduced exploration. This finding is further supported by one sample t tests (Fig. 3D) which showed that only the AS (t(9) = 5.62, p < .0001, d = 6.39) and ANS (t(9) = 4.75, p = .001, d = 6.43) groups exhibited an exploration ratio above 0.5. A main effect of treatment (F (1,36) = 8.46, p = .006, η_p^2 = .19) for the exploration ratio (Fig. 3D) was indicative of a significant preference by the ANA-12 treated groups to explore the S1 vs. the EC chamber. Time in the middle chamber did not significantly differ between groups.

3.1.3. Social preference (social memory and predilection for social novelty)

3.1.3.1. Direct interaction

Two-way ANOVA on contact with S1 (now-familiar rat) and contact with stranger 2 (S2) (Fig. 3E) failed to show main effects or interactions ($p > .05$). Paired samples t test for within group comparisons (Figure 3E) revealed that only the VS (t(9) = 3.76, p = .005, d = 1.19) and VNS (t(9) = 2.68, p = .025, d = 0.85) groups significantly increased their contact time with S2 compared to S1. All groups showed social memory in increased inclination to spend time with S2 vs. S1 ($p < .05$), when compared to the normal exploration score of 0.5.

3.1.3.2. Indirect interaction

Two-way ANOVA revealed increased time spent in S1-paired chamber (Fig. 3G) by ANA-12 treated groups (F (1,36) = 6.82, p = .013, η_p^2 = .16) and significantly more time exhibiting other behaviors in chamber 1 (F (1,36) = 9.34, p = .004, η_p^2 = .21) compared to the veh-treated groups. Time spent in S2-paired chamber and other behaviors in this chamber did not significantly differ between groups (p > .05). Paired samples *t* test for within group comparisons in time spent in S2-paired chamber vs. S1-paired chamber (Fig. 3G) revealed significantly higher time spent in the S2-paired chamber by the VS (t(9) = 2.65, p = .026, d = 0.84) group only. For the exploration ratio, a main effect of treatment (F (1,36) = 7.24, p = .011, η_p^2 = .17) (Fig. 3H) was found related to reduced time in the chamber containing novel S2 vs. the S1-paired chamber in ANA-12 compared to the veh-treated groups. One sample *t* tests revealed that only the VS group (t(9) = 3.01, p = .015, d = 4.20) showed a significant increase in social memory by exploring S2-paired chamber more than the S1-paired chamber. Time in the middle chamber did not significantly differ between groups.

3.2. Effect of TrkB receptor blockade on Y maze conditioned place preference (YMCPP)

3.2.1. Preference

One-way repeated measures ANOVA revealed a main effect of preference (Fig. 4A) notable by increased time spent by all groups in the least preferred "food-paired" arm during the post conditioning test compared to time spent in the same arm during the preconditioning session (Mean ± SD: 136.30 ± 36.12 vs. 174.98 ± 57.69 sec) (F (1,39) = 16.42, p < .001, η_p^2 = .30). This indicates that all rats learned to associate the initially least preferred arm with food reward and exhibited conditioned place preference. Assessment of both arms during the habituation and test sessions (Fig. 4A) yielded main effects of treatment (F (1,36) = 6.13, p = .018, η_p^2 = .15) and stress (F (1,36) = 4.454, p = .042, η_p^2 = .11). Stressed animals treated with ANA-12 showed increased time spent in the non-food paired arm during the test session compared to the vehicle non-stressed animals.

3.2.2. Preference ratio

Two-way ANOVA on preference ratio did not reveal significant between group differences ($p > .05$) (Fig. 4B). Following one-sample *t* tests, individual groups did not show a significant preference above the normal preference score of 0.5 for either arm during the test session ($p > .05$), although both stress groups expressed a slightly diminished preference for the food-paired arm. The possibility that changes in preference could have been observed earlier on during the test session was assessed for the 1 min and 2 min time points, however no statistical differences were noted.

3.2.3. Time in least preferred 'food paired' arm

Two-way ANOVA on time spent in food paired arm during the test session (Fig. 4C) revealed a main effect of treatment (F (1,36) = 5.25, $p = .028$, $\eta_p^2 = .13$), attributable to increased time spent by the ANA-12 treated groups compared to their vehicle counterparts. In contrast, animals did not differ for the time spent in this arm during the habituation session ($p > .05$). Paired samples *t* test for within group comparisons of the test vs. habituation sessions revealed significant elevations of the time in food paired arm for the ANA-12 rats [AS ($t(9) = 2.39$, $p = .041$, $d = 0.76$) and ANS groups ($t(9) = 3.36$, $p = .008$, $d = 1.06$)], ANA-12 treated groups showing a mean ratio increase of the percent change in preference for the food paired arm during habituation and test sessions compared to the veh-treated groups (Fig. 4D).

3.3. Effect of TrkB receptor blockade on Y maze passive avoidance test (YMPAT)

3.3.1. Latencies

One-way repeated measures ANOVA on latencies revealed differences (F (1,39) = 89.70, $p < .001$, $\eta_p^2 = .70$) due to all animals showing increased time to re-enter the aversive arm compared to the latency to enter the arm prior to air puff application [Mean ± SD: Latency 1 - 20.65 ± 19.31 vs. Latency 2 - 68.20 ± 36.02 sec) (Fig. 5A). Two-way ANOVA exploring Latency 2 revealed a main effect of treatment (F (1,36) = 9.70, $p = .004$, $\eta_p^2 = .21$) related to ANA-12-treated rats showing increased latency to re-enter the aversive arm compared to the veh-treated groups, indicative of an effect of the drug to enhance retention of the aversive experience (Fig 5A).

No group differences were observed for L1 ($p > .05$). No main effects or interactions were observed on other measures (time in aversive arm (Fig. 5B); risk assessment and immobility – data not shown).

3.4. Effect of TrkB receptor blockade on orexin A-ir and FosB/ΔFosB-ir in the lateral hypothalamus (LH) and perifornical area (PfA)

In the LH, two-way ANOVA revealed no main effects or interactions for percent area of orexin A-ir and FosB/ΔFosB-ir (Fig. 6C and D) ($p > .05$). This contrasts observation in the PfA for percent area of orexin A-ir (Fig. 6C) that revealed a main effect of treatment (F (1,16) = 29.88, $p < .001$, $\eta_p^2 = .65$), due to elevated levels of orexin A in ANA-12 compared to the veh-treated rats. Analysis of percent area for PfA FosB/ΔFosB-ir revealed no main effects or interactions ($p > .05$) (Fig. 6D).

3.5. Effect of TrkB receptor blockade on orexin A-ir and ΔFosB-ir in the dorsal hippocampal CA1 (dCA1) and CA3 (dCA3) layers.

For orexin A-ir and FosB/ΔFosB-ir in the dCA1 and dCA3, two-way ANOVA for percent area revealed no main effects or interactions ($p > .05$) (Fig. 7B and C).

3.6. TH-ir and orexin A-ir in the ventral hippocampal CA1 (vCA1) and CA3 (vCA3) and the ventral tegmental area (VTA)

In the vCA1, no effects were found for TH-ir (Fig. 8B), however two-way ANOVA of orexin A-ir (Fig. 8C) revealed a main effect of treatment (F (1,24) = 4.94, $p = .036$, $\eta_p^2 = .17$), due to decreased expression in ANA-12- compared to veh-treated animals. In the vCA3, analysis of percent area for TH- and orexin A-ir failed to show main effects or interactions (Fig. 8B and C).

In the VTA, analysis of TH-ir revealed no main effects or interactions ($p > .05$) (Fig. 9B). Two-way ANOVA for percent area of orexin A-ir (Fig. 9B) revealed a main effect of stress (F (1,20) = 5.898, $p = .025$, $\eta_p^2 = .23$), due to elevated orexin A-ir in the stress compared to the non-stress groups. Post hoc analysis revealed that ANA-12 treatment decreased vs. increased orexin A expression in non-stress vs. stress animals ($p < .05$), showing state-dependent effects.

3.7. Effects of unilateral right NAc shell microinfusion on hemispheric differences in molecular readouts.

Paired sample t-tests were performed to assess within group differences in the expression of Orx A, FosB/ΔFosB and TH in the right vs. left hemisphere. The LH, dCA3, vCA3 and VTA showed comparable hemispheric differences. In veh-treated non-stress groups, reduced orexin A expression was observed in the right hemisphere in the PfA ($t(4) = -3.26$, $p = .031$, $d = -1.46$) and vCA1 ($t(6) = -4.79$, $p = .003$, $d = -1.81$) while veh-treated stress group showed reduced right hemispheric orexin A-ir at the dCA1 ($t(5) = -3.09$, $p = .027$, $d = -1.26$). ANA-12 treatment in the non-stress group showed a greater expression of orexin A in the right dCA1 compared to the left ($t(5) = 3.06$, $p = .028$, $d = 1.25$).

3.8. Pearson correlations

Pearson product moment correlation coefficient was conducted to assess the relationship between behavioral and neurochemical responses. Results show that orexin A-ir at the PfA was positively correlated with SIT exploration ratio ($r = .46$, $p = .041$) and YMPAT latency to re-enter the aversive arm upon second exposure ($r = .70$, $p = .001$). In contrast, it was negatively correlated to SP (i.e., time spent in S2-paired chamber vs. S1-paired chamber) ($r = -.63$, $p = .003$). In the dCA3, orexin A-ir was negatively correlated with SP (i.e. direct contact with S2 vs. S1) ($r = -.43$, $p = .038$), and with the YMPAT latency to re-enter aversive arm upon second exposure ($r = -.42$, $p = .04$). Increased direct contact with S2 during the SP test was correlated with increased expression of orexin A in the VTA ($r = .45$, $p = .028$).

Pearson product moment correlation coefficient also assessed the relationship between expressions of the different biochemical markers. Orexin A-ir was positively correlated with FosB/ΔFosB expression in the PfA and LH ($r = .43$, $p = .046$ and $r = .48$, $p = .032$, respectively), while expression was negatively correlated with orexin A-ir in the vCA1 ($r = -.45$, $p = .045$). FosB/ΔFosB-ir in the PfA and LH were positively correlated ($r = .64$, $p = .001$), while orexin A expression in the VTA was negatively correlated with that of PfA FosB/ΔFosB ($r = -.48$, $p = .018$). Our findings support contrasting biochemical expression of discrete signals at PfA versus that observed in the vCA1 and VTA. At the vCA1, orexin A-ir appeared negatively correlated to TH-ir ($r = -.40$, $p = .036$), while orexin A-ir in the vCA1 and vCA3 were positively correlated ($r = .45$, $p =$

.015). Of note, TH-ir at the vCA3 was positively correlated with TH-ir ($r = .45$, $p = .028$) and orexin A-ir ($r = .54$, $p = .007$) at the VTA, while it was negatively correlated with FosB/ΔFosB-ir in the dCA1 ($r = -.41$, $p = .029$) and dCA3 ($r = -.58$, $p = .003$). A negative correlation was also observed between the vCA3 orexin A-ir and dCA3 FosB/ΔFosB-ir ($r = -.41$, $p = .046$). These results support opposite changes in activation profile noted in dorsal and ventral hippocampal regions, which may be due to their functional distinctiveness. Summary of significant correlations are reported in Tables 1A and B.

4. Discussion

To our knowledge, this is the first study to demonstrate the role of TrkB receptor activation on the mediation of orexin A, FosB/ΔFosB, and TH expression in the mesolimbic circuitry following heterotypic stress, and to explore the impact of blocking TrkB receptor activation at the NAc shell on social cognition and conditioned preference and aversion in normal and stressful conditions.

Behaviorally, our findings show that TrkB receptors in the NAc shell mediate sociability, but not novelty-induced seeking in male Wistar rats. We show that NAc ANA-12 infusion enhanced sociability more than vehicle treatment, independently of stress exposure. These observations are consistent with increased social interaction demonstrated following infusions of ANA-12 in the NAc of male mice (Walsh et al., 2014; Wook Koo et al., 2016) and in the anterior BNST in stressed female mice (Greenberg et al., 2014). Interestingly, all groups showed reduced exploration of a familiar animal when presented with a novel conspecific during the social preference test. Despite a preference for S2 in the social preference test, ANA-12 treated rats spent increased time in the S1-paired chamber and displayed a significant reduction in exploratory behavior within the S2 paired chamber compared to the veh-treated groups as indicated by a below 0.5 exploration ratio in drug-treated rats. This could indicate reduced social cognition. Similar impairment in novelty preference in the SIT has recently been reported in BDNF heterozygous male mice (showing 50% reduction in BDNF expression) while sociability remained intact (Manning and van den Buuse, 2016). In terms of brain mechanisms, a recent optogenetic study has linked reduced social preference to elevations in the cellular balance of excitation and inhibition (E/I balance) within neural microcircuitry, involving overexcitation

of pyramidal neurons in the mPFC (Yizhar et al., 2011). A previous study from our lab also showed decreased TrkB-ir in the cingulate cortex of stressed rats treated with ANA-12 (Azogu and Plamondon, 2017). Related to our observations, reduced social preference in mutant mice has been shown associated with reduced TrkB expression at the PFC (Kaminitz et al., 2014). Our findings support that recruitment of TrkB receptors at the nucleus accumbens shell plays a role in regulating social preference/cognition.

We have previously demonstrated significant elevation of CORT secretion following stressor exposure in the 10-day stress paradigm. Stress rats exhibited increased anxiety in the EPM, which was reduced by ANA-12 treatment (Azogu and Plamondon, 2017). Therefore, the lack of stress effects in the social interaction and social preference tests are intriguing. Of note, a few factors have been suggested important when considering social behaviors. Indeed, social interactions may act as a buffer of stressful experiences that enhances resilience, as conspecifics are used in the test and experimental animals may perceive familiarity in their scents (Beery and Kaufer, 2015; Muroy et al., 2016). This may increase or decrease social behavior. Moreover, rats in our study were single housed due to their cannula surgery to avoid accidental removal of the dummy cannulas that may occur during play fighting with a cagemate, thus social interaction may be increased by social deprivation (Heinrichs and Koob, 2006; Panksepp and Beatty, 1980).

Conditioned place preference (CPP, biased protocol) was assessed using a modified Y maze and the rewarding properties of palatable food to condition the animals. The 10-day heterotypic stress paradigm failed to affect conditioned place preference, suggesting that stress exposure had no lasting impact on the hedonic valence of palatable food. All groups learned the association of the food reward in the least preferred arm, suggesting that all were able to discriminate between a neutral versus previously rewarding environment. In rats and humans, access to palatable food can buffer against the effects of stressors, a factor that could explain why stress failed to affect responses observed in a CPP paradigm using food reward (Figlewicz, 2015; MacKay et al., 2017). This is also consistent with a transient reduction observed in sucrose preference that was rapidly normalized in rats, showing lasting HPA axis dysregulation (de la Tremblaye et al., 2016). Interestingly, the time spent in the food paired environment during the test was significantly elevated in the ANA-12-treated

compared to veh-treated groups, suggesting that these animals might have been more sensitive to the hedonic properties of the palatable food. This is an interesting finding considering that bilateral infusions of ANA-12 into the NAc core and shell was reported to induce antidepressant effects in a rat model of learned helplessness (Shirayama et al., 2015) as well as following LPS-induced depression-like behavior (Zhang et al., 2015). It is also consistent with BDNF acting within the hippocampus, amygdala and prefrontal cortex to promote antidepressant phenotype (Duman and Monteggia, 2006), whereas increased levels in the VTA-NAc pathway induce susceptibility to a depression behavior (Berton et al., 2006; Eisch et al., 2003; Krishnan et al., 2007; Nestler and Carlezon, 2006). Thus, it appears possible that preventing activation of TrkB receptors at the NAc prevents activation of neuronal circuits dampening hedonic responses. This requires further investigation using other motivational paradigms and looking at possible impact on other regulators of reward mechanisms. Considering different factors shown to affect responses in this test, precautions were taken to test animals at the same hour during conditioning and test sessions. It thus appears unlikely that diurnal changes in Fos activation in PfA orexin A neurons noted to affect arousal (Estabrooke et al., 2001) or the susceptibility to time of day modulation of CPP described in Wistar rats (Cain et al., 2004) may be involved in the discussed group differences. In the YM passive avoidance test, all rats learned the association and were able to remember the arm associated with presentation of the puffed air jets, showing increased latency to re-enter this arm on the second test session. Of interest, the ANA-12 treated animals took longer to re-enter the aversive arm than vehicle-treated counterparts. However, once there, ANA-12 stress rats stayed significantly longer in the aversive arm than vehicle-treated rats. Although the precise regulators remains to be established, reduced anxiety observed in ANA-12 animals (Cazorla et al., 2011) may promote memory retention of passive avoidance, possibly via improved encoding or consolidation of the aversive experience as well as enhanced coping with a stressful experience.

Biochemically, we used double immunostaining to assess orexin A and FosB/ΔFosB activation (Furlong et al., 2009) attributable to stress and TrkB receptor blockade and possible synergistic effects. In our study, the fact that animals underwent a 5 min forced swim 2 h prior to euthanasia allowed for the examination of how

TrkB receptor blockade modified response to an acute stressor in animal groups that had not previously experienced the forced swim stress. A rapid and transient immunohistochemical expression of FosB/ΔFosB has been demonstrated following a variety of acute stressors, while accumulation has been observed in response to chronic exposure to certain forms of stress due to its unusual protein stability and a very long half-life (Chen et al., 1997). Noteworthy, Perrotti et al. (2004) observed that the induction of c-Fos, full-length FosB, and Fra-1/2 is almost completely desensitized in several brain areas after a course of chronic stress, whereas there is a selective induction of 35-37 kDa isoforms of ΔFosB following a consecutive 10-day exposure to 1h of immobilization stress or unpredictable stress. Our findings revealed comparable TH and FosB/ΔFosB expression in stress naïve animals and those exposed to repeated heterotypic stress. Considering previous reports, expression in stress-naïve rats likely represents a rapid induction of stress-activated markers 2h following stress exposure, while delayed ΔFosB expression levels from chronic stress exposure may prevail in groups that were exposed to repeated heterotypic stress exposure. A minimal contribution of FosB expression is expected, full-length FosB being desensitized in response to acute stress in chronically stressed rats (Perrotti et al., 2004). Nonetheless, due to the long interval between the heterotypic and acute stress exposure in our study [many days have elapsed compared to the 24h delay period in Perrotti et al. (2004)], it is possible that expression observed in response to acute stress exposure was partially composed from residual ΔFosB activation from prior stress exposure, or simply emerged from comparable FosB/ΔFosB activation profile in all animals following acute FS. ANA-12 treatment had no significant impact on TH and FosB/ΔFosB expression levels.

In contrast, ANA-12 treatment led to opposing effects on orexin A expression at the PfA and ventral hippocampus CA1 layer. We believe these findings to provide the first evidence that TrkB antagonism in the NAc shell stimulates orexin A-ir in the PfA, while it reduces its expression at the ventral hippocampus, independently of stress exposure. At the hippocampus, ANA-12 treatment markedly attenuated orexin A expression in the ventral portion, principally affecting the vCA1. The role of orexin at the ventral hippocampus remains largely unknown, although stimulation of orexin secretion at the basolateral amygdala (BLA) is shown

to selectively activate the PVN, NAc and ventral hippocampal neurons (Kim and Han, 2016). This is interesting considering heightened retention of fearful stimuli in the YM-PAT in ANA-12-treated animals. Furthermore, a recent study revealed the existence of a circadian oscillator in the hippocampus whose oscillation can be regulated by orexin, affecting the expression of Alzheimer's disease-risk genes (Ma et al., 2016). This supports a role for orexin in hippocampal-dependent memory functions, including memory formation and consolidation (Yang et al., 2014). Furthermore, orexin connections from the BLA to the NAc and from BLA to the ventral hippocampus have been proposed to exert important regulatory actions of sociability and mood-related behaviors (Kim and Han, 2016).

These findings are concordant with the behavioral responses that were observed in ANA-12-treated rats and with orexin's role in wakefulness and arousal (Estabrooke et al., 2001). The observation of increased time spent by ANA-12 compared to vehicle-treated rats in the food paired arm during the CPP test support a proposed role of orexin secretion in drug-seeking following extinction, and reinstatement induced by cues or context (Aston-Jones et al., 2010). Moreover, Pearson's correlations on our data indicate that orexin A-ir at the PfA positively correlates with sociability and passive avoidance behavior in the SIT and YMPAT, respectively, which responses were both positively influenced by ANA-12 treatment. Our findings however do not support a role of orexin A in regulating social cognition. With regards to hemispheric laterality of peptide expression, an effect of stress was manifest by increased right PfA orexin A-ir in stress groups and increased right dCA1 orexin A-ir in ANA-12 treated groups. This is interesting considering the acknowledged role of the right hemisphere as a main regulator of negatively valenced and sensitive emotional responses in animals and humans (Canli et al., 1998; Kensinger and Choi, 2009; Ley and Bryden, 1979; Natale et al., 1983). Carefulness may be used in interpreting correlational data as all groups were pooled together, thus findings may not be representative of individual group responses.

Expression of orexin A in the LH did not differ among the experimental groups. This brain locus being a main synthesis site, LH orexin via distinct connections has been involved in various responses, including conditioned stimuli responses associated with food and drug rewards (Aston-Jones et al., 2010; Fadel et al.,

2002; Harris et al., 2005). The absence of changes at the LH is in line with the observation that all rats, drug-treated or not, showed conditioned responses to pleasurable and aversive experiences. In this context, studies report a dichotomy in orexin neuron function in the LH and PfA regions, indicating that the regulation of responses to reward predictive stimuli occur in the LH, whereas in the PfA, orexin neurons mediate responses to stressful or arousing events (Aston-Jones et al., 2010; Harris et al., 2007, 2005; Harris and Aston-Jones, 2006; Martin-Fardon et al., 2016; Plaza-Zabala et al., 2010). Attributes differentiating the responses of the ANA-12 vs. vehicle-treated rats were linked to qualitative features of the responses (intensity, persistence in exploration of a visited site, increased latency to re-enter the aversive arm, etc). These responses may be linked to orexin secretion in structures more closely associated with stimulus salience and/or memory consolidation occurring in the context of conditioned responses. This is consistent with the demonstrated role of orexin in drug-seeking following extinction (maintenance), and reinstatement induced by cues or context (Aston-Jones et al., 2010). Contrary to earlier findings showing activation of the LH/PfA orexin neurons following acute stress (Harris and Aston-Jones, 2006), the delayed response to chronic stressor exposure in this experiment did not involve LH orexin A neuronal activation. In a similar fashion, TrkB receptor activation, but not stress, was the determinant factor affecting PfA orexin A expression.

In contrast, we observed an effect of stress and ANA-12 at the VTA, associated with increased orexin A-ir expression. This is interesting in terms of motivational effects of ANA-12 as an heightened activation of orexin neurons in the hypothalamus can induce firing of the VTA neurons via direct postsynaptic mechanisms (Korotkova et al., 2003) and increase dopamine efflux in the mPFC and NAc (Narita et al., 2003; Vittoz et al., 2008). Notably, ANA-12 treatment in stressed animals prevented attenuation of orexin A expression observed in the non-stress animals. Although mechanisms remain largely unknown, orexin synthesis in the hypothalamus is known to innervate the VTA and remain in close apposition to DA neurons, potently exciting these neuron (Bubser et al., 2005; Fadel et al., 2002; Horvath et al., 1999; Korotkova et al., 2003). Notwithstanding orexin changes at the VTA in response to stress, our study showed that TH-ir was no longer affected 9 days post stress, TH-ir being comparable at the ventral hippocampus and VTA across the groups. A contributing factor may

relate to TH expression being part of a faster acting system than changes observed with peptides, this system being no longer recruited at longer intervals post stressor exposure. However, dopaminergic transmission is impacted by other stressors at short (Anstrom and Woodward, 2005; Brischoux et al., 2009) and long intervals (Holly and Miczek, 2016; Ortiz et al., 1996). Prolonged activation may thus depend on the stressor intensity and duration.

5. Conclusion

In summary, our study provides further support for a participation of BDNF secretion to orexin A-mediated responses to stress. Our data shows evidence for the site-specific regulations of orexin neurons and fibers in the PfA and vCA1, irrespective of stress, following intra-accumbens shell TrkB receptor inhibition with ANA-12, and stress-induced increases in orexin A within the VTA. We also report that ANA-12 or stress is not sufficient to activate orexin neurons in the LH or orexin fibers in the dorsal hippocampus, and in this context, suggest that the role of orexins in functional distinctiveness of the dorsal and ventral hippocampus remains to be further defined. Lastly, our data suggest that TrkB inhibition plays a role in regulating various behaviors related to arousal and emotionality such as sociability, conditioned preference and inhibitory avoidance (memory retention for aversive stimuli), of which increased orexin A in the PfA is implicated to have an effect. Antagonism of the TrkB receptor in normal and stress states appear to represent a key mechanism in maintaining neuroendocrine responses and memory functionality.

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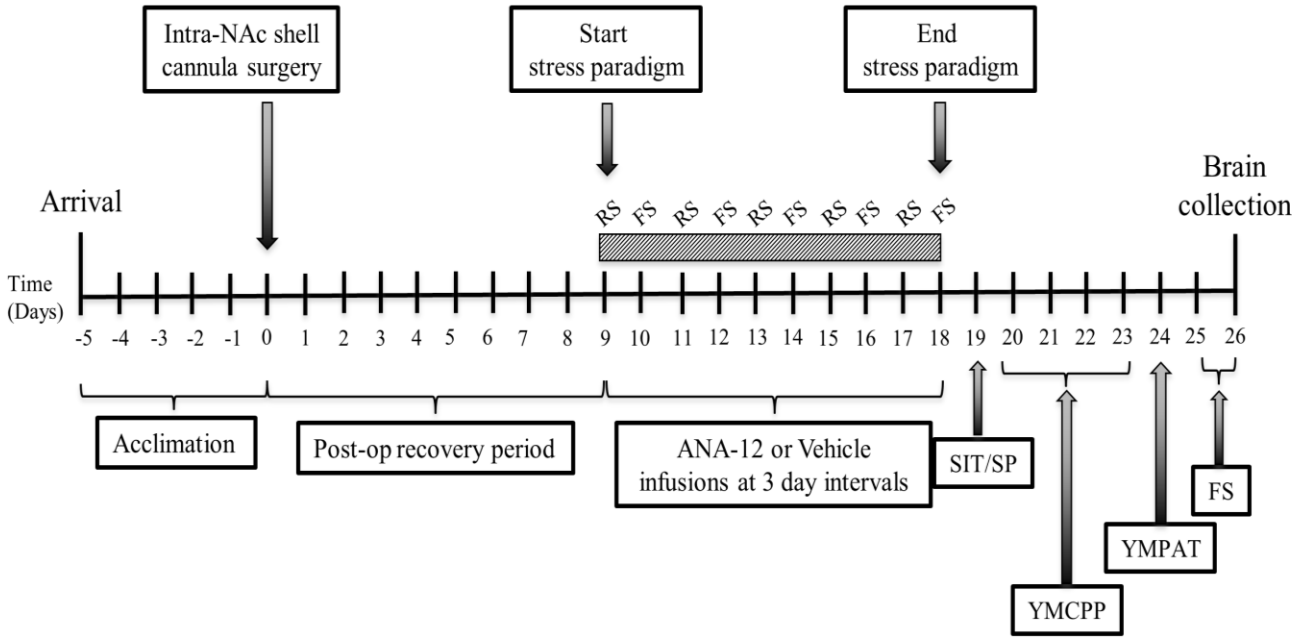

Figure 1. Experimental timeline. Animals underwent cannula implantation surgery 5 days after arriving to the facility. Following 10 days of surgical recovery, they began the 10-day heterotypic stress paradigm consisting of alternating days of 30 min of restraint stress and 15 min forced swim stress. Drug or vehicle was infused at 3 day intervals during the paradigm. Animals underwent a final acute FS 2 h prior to euthanasia and brain collection. RS: restraint stress; FS: forced swim; SIT/ SP: social interaction test/ social preference; YMCPP: Y maze conditioned place preference.

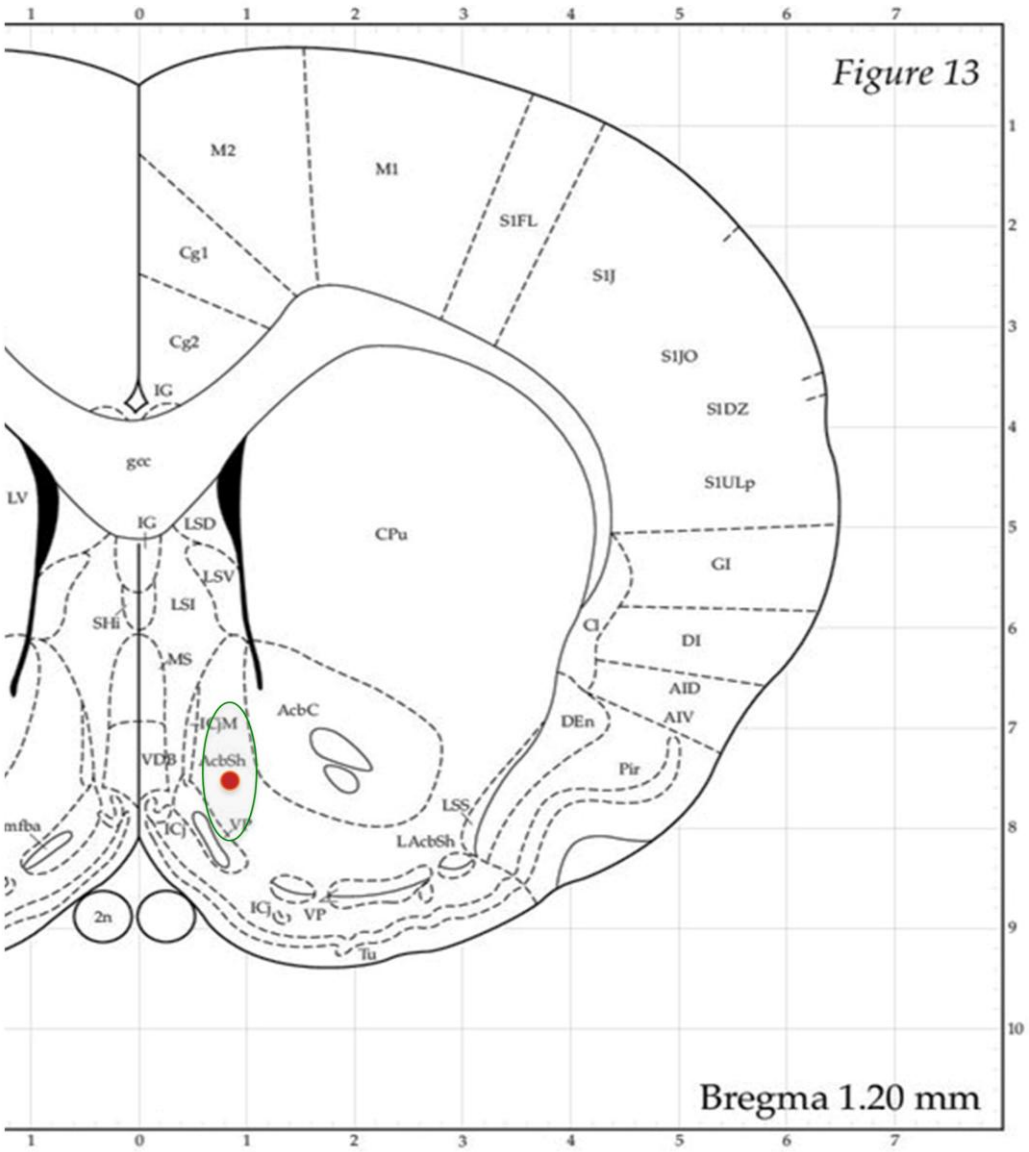

Figure 2. Unilateral cannula implantation site. Schematic diagram of the cannula implantation site on the right nucleus accumbens shell. Stereotaxic coordinates: 1.2mm posterior to Bregma; -1.5mm lateral from the midline (angled at 6°); and lowered 7.0mm below dura (Paxinos and Watson 1998 rat brain atlas). Injection needle extended 0.5mm below the tip of the cannula for a final depth of 7.5mm below dura (red dot). Green circle, possible injection sites. Image derived from labs.gaidi.ca/rat-brain-atlas/

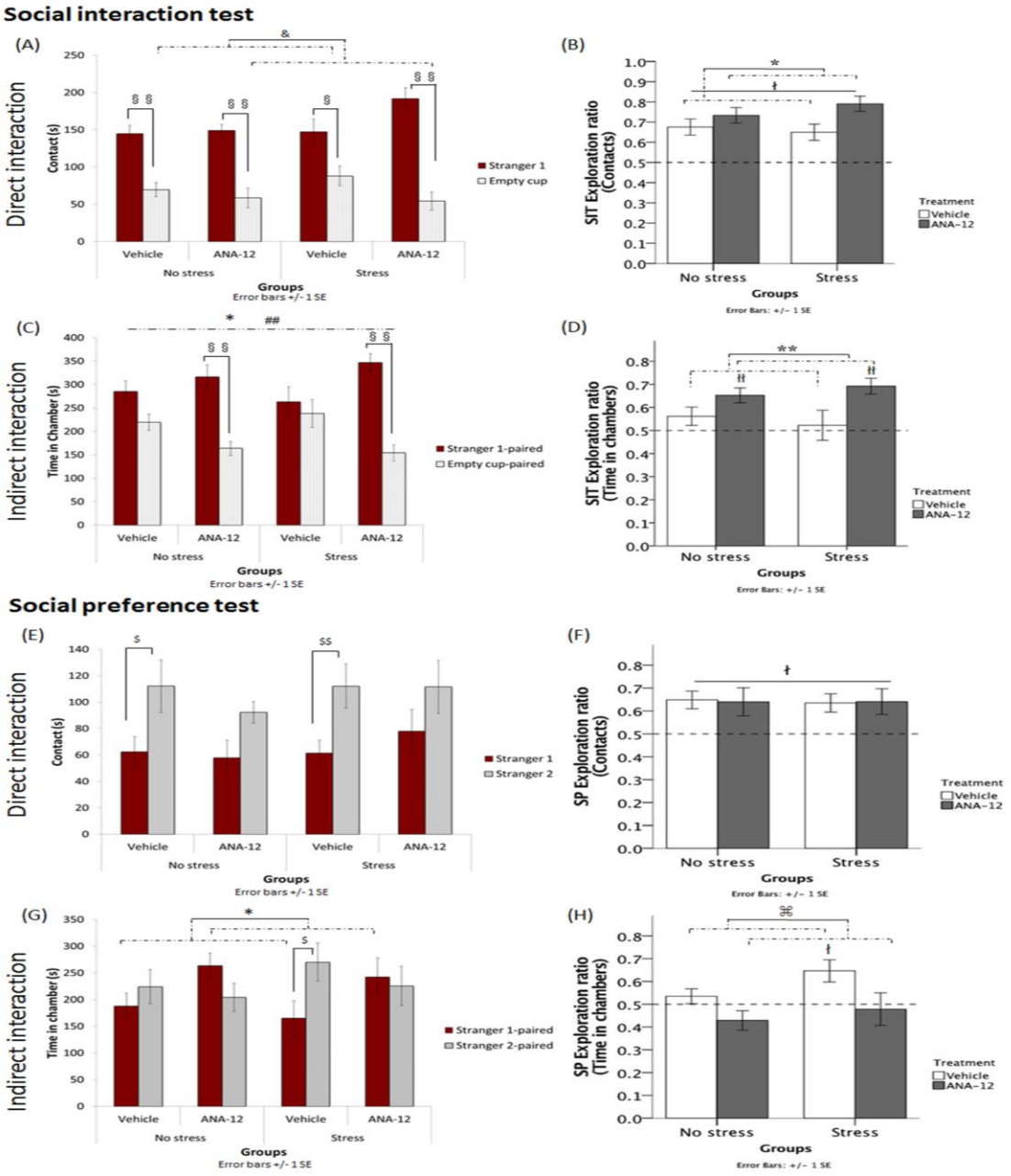

Figure 3. Social interaction and preference. (A and C) Graphs show direct and indirect interaction with stranger 1 (S1) and the empty cup (EC) by all groups during the social interaction test. (A) ANA-12 groups spent less time with the EC compared to vehicle groups (&, *marginal* p = .073). Each group spent more time with S1 compared to the EC (§ p < .05, §§ p < .01). (C) ANA-12 groups spent more time in the S1-paired chamber (* p = .029) in contrast to vehicle groups that spent more time in the EC-paired chamber (## p = .002). ANA-12 groups also spent more time in the S1 paired chamber vs. the EC –paired chamber (§§ p < .01). (B and D) Graphs show higher exploration ratio for direct and indirect interaction time by the ANA-12 groups compared to the vehicle groups (* p = .015, ** p = .006). ł and łł, indicate increased preference by all or specific groups to explore S1 directly or indirectly compared to EC (p < .05 and p < .01, respectively). (E and G) Graphs show direct and indirect interaction with S1 and novel stranger 2 (S2) by all groups in the social preference test. (E) Vehicle groups spent more time with S2 compared to S1 ($ p = .025; $$ p = .005). Interestingly, ANA-12 groups spent more time in the S1-paired chamber compared to vehicles (* p = .013). (F and H) Graphs show higher exploration ratio for direct interaction by all groups. However, vehicle groups showed increased indirect interaction time compared to ANA-12 groups (⌘ p = .011). ł, indicates increased preference to explore S2 directly or indirectly compared to S1 (p < .05). Data are expressed as mean ± SEM.

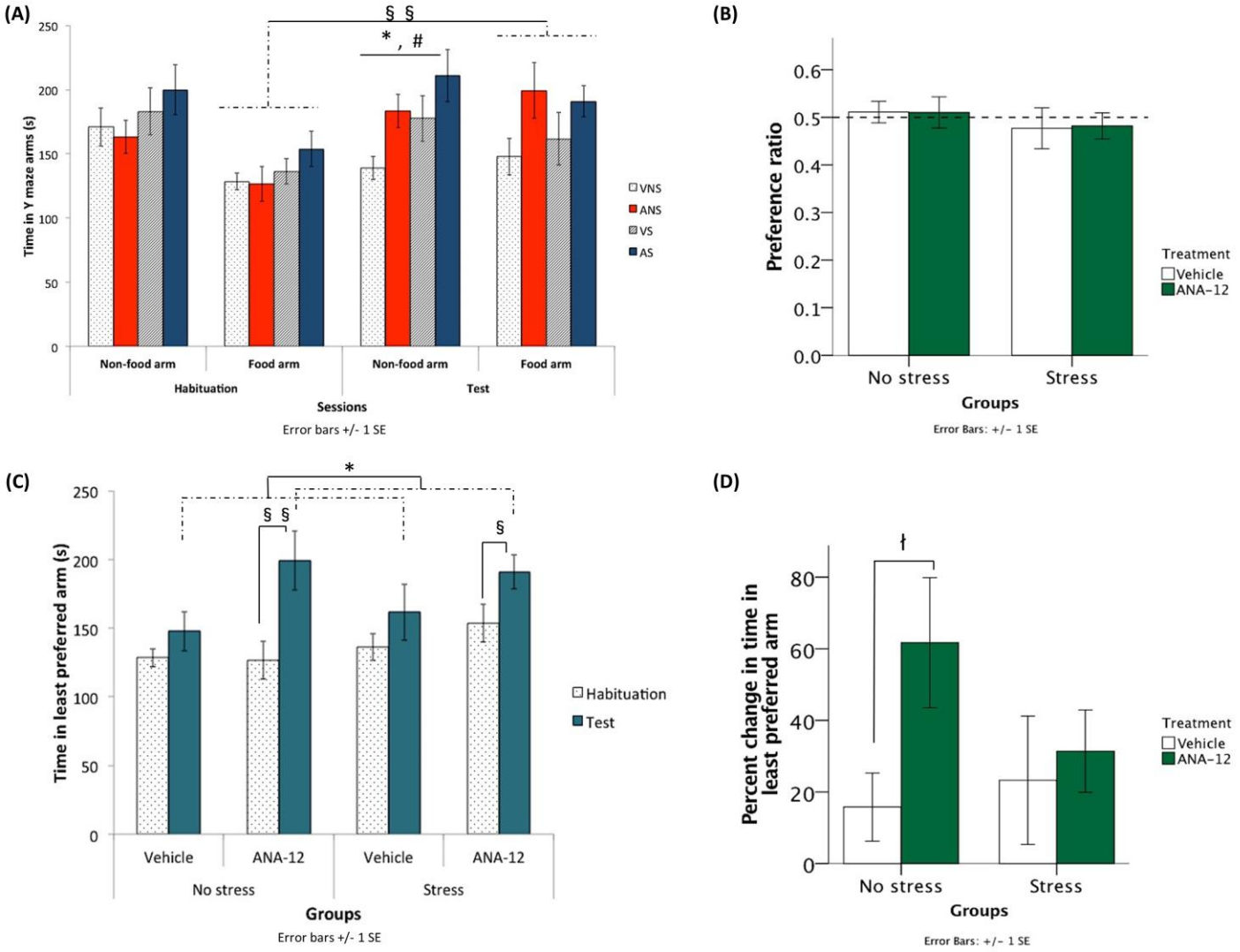

Figure 4. Conditioned place preference in the YMCPP. (A) All groups showed increased time spent in the least preferred arm during the test session vs. the habituation session (§§ $p < .01$). Drug (* $p = .018$) and stress (# $p = .042$) varied the time spent in the non-food arm during the test session only. (B) Graph shows neutral preference for either arm during the test session by all groups. (C) ANA-12 groups spent more time in the least preferred arm during the test session vs. the habituation session (§ $p = .041$, §§ $p = .008$) and compared to vehicle groups (* $p = .028$). (D) Percent change in time spent in the least preferred arm significantly differed between the non-stress groups only. VNS: vehicle + no stress; ANS: ANA-12 + no stress; VS: Vehicle + stress; AS: ANA-12 + stress. Data are expressed as mean ± SEM.

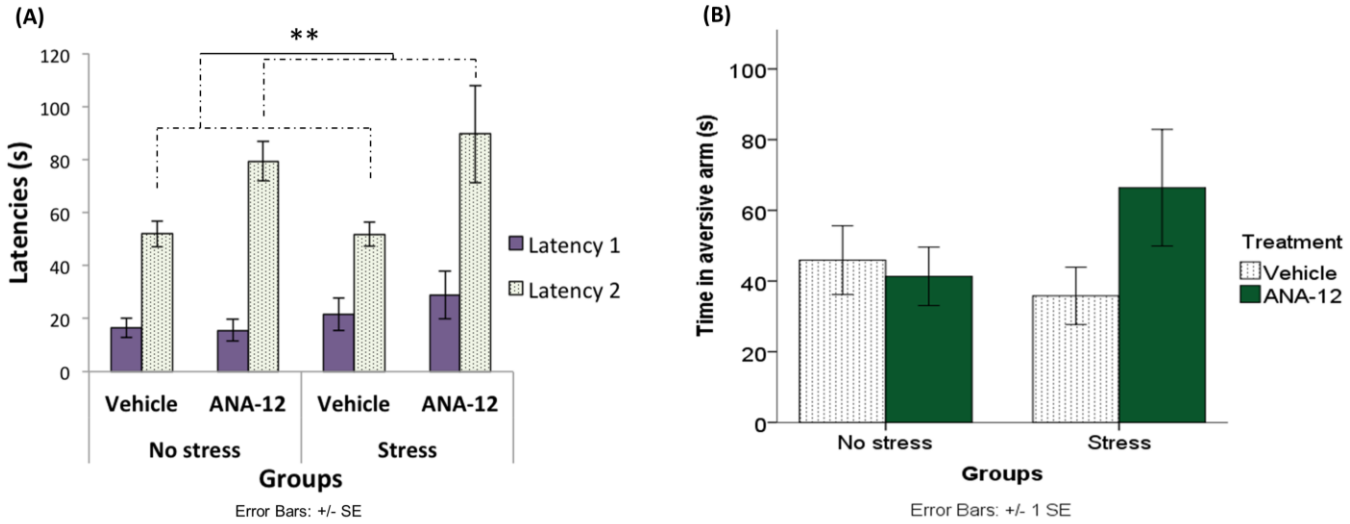

Figure 5. Fear avoidance learning in the YMPAT. (A) Graph shows increased latency to enter aversive arm upon second maze exposure by the ANA-12 groups compared to the vehicle groups (** p = .004). (B) Graph shows time spent in aversive arm during second maze exposure for the duration of the 5-minute test. Data are expressed as mean ± SEM.

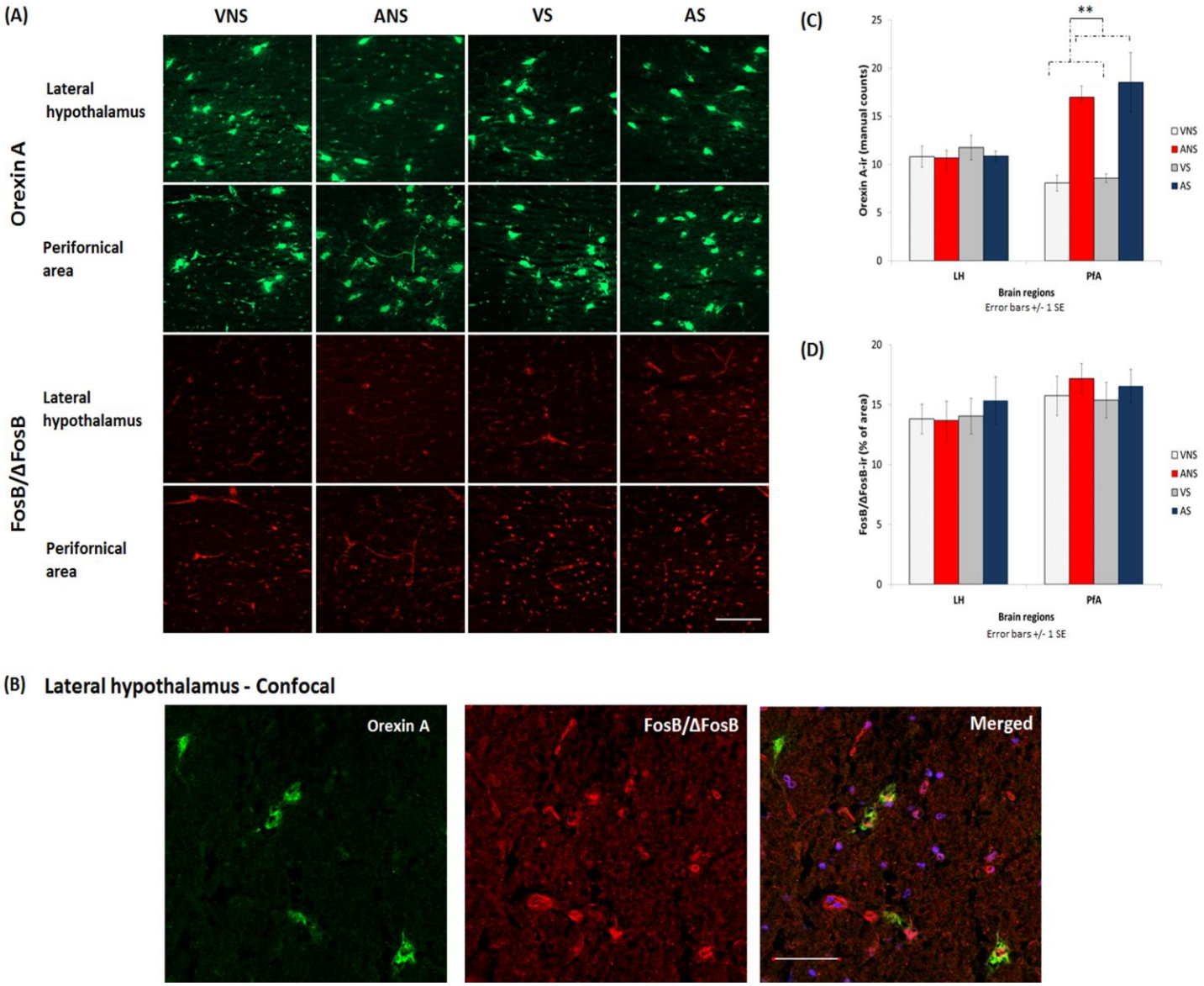

Figure 6. Orexin A and FosB/ΔFosB-ir in the lateral hypothalamus. (A) Fluorescence photomicrographs (200x magnification) showing orexin A-ir (green) and FosB/ΔFosB-ir (red) in the lateral hypothalamus (LH) and perifornical area (PfA). Scale bar 100µm. (B) Representative confocal images of orexin A- and FosB/ΔFosB- ir double immunostaining in the LH. Scale bar 50µm. (C and D) Graphs show manual counts of orexin A-ir and optical density-derived expression levels of FosB/ΔFosB in in the LH and PfA. ANA-12 treatment led to elevated orexin A release in the stress and non-stress groups compared to vehicle groups (** $p < .001$). VNS: vehicle + no stress; ANS: ANA-12 + no stress; VS: Vehicle + stress; AS: ANA-12 + stress. Data are expressed as mean ± SEM.

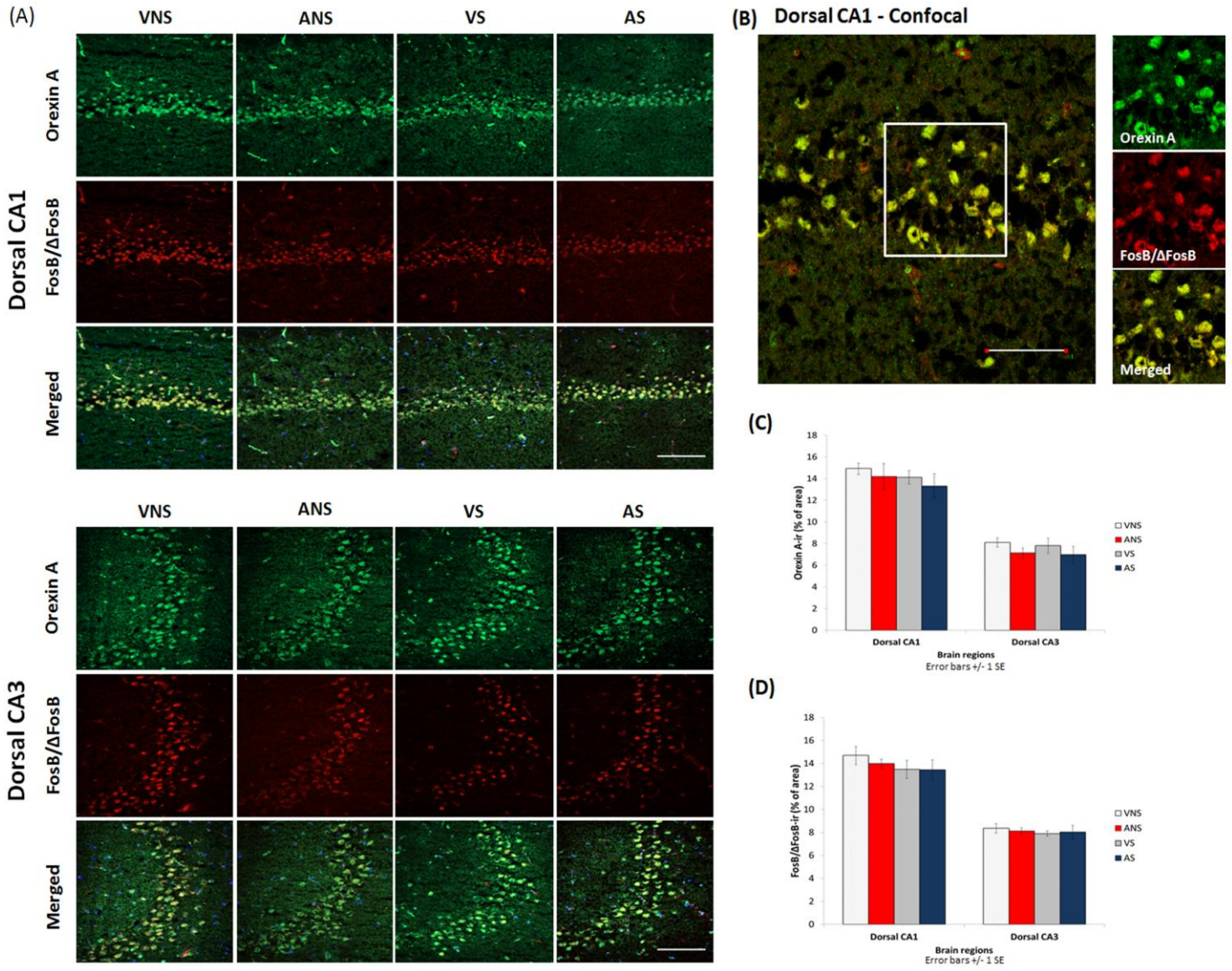

Figure 7. Orexin A and FosB/ΔFosB-ir in the CA1 and CA3 of the dorsal hippocampus. (A) Fluorescence photomicrographs (200x magnification) showing orexin A-ir (green), FosB/ΔFosB-ir (red), and Hoechst (blue, see merged images) in the dorsal hippocampus (dCA1 and dCA3). Scale bar 100µm. (B) Representative confocal images of orexin A- and FosB/ΔFosB- ir double immunostaining in the dorsal CA1. Scale bar 50µm. (C and D) Graphs show expression of orexin A- and ΔFosB- ir levels in both regions. VNS: vehicle + no stress; ANS: ANA-12 + no stress; VS: Vehicle + stress; AS: ANA-12 + stress. Data are expressed as mean ± SEM.

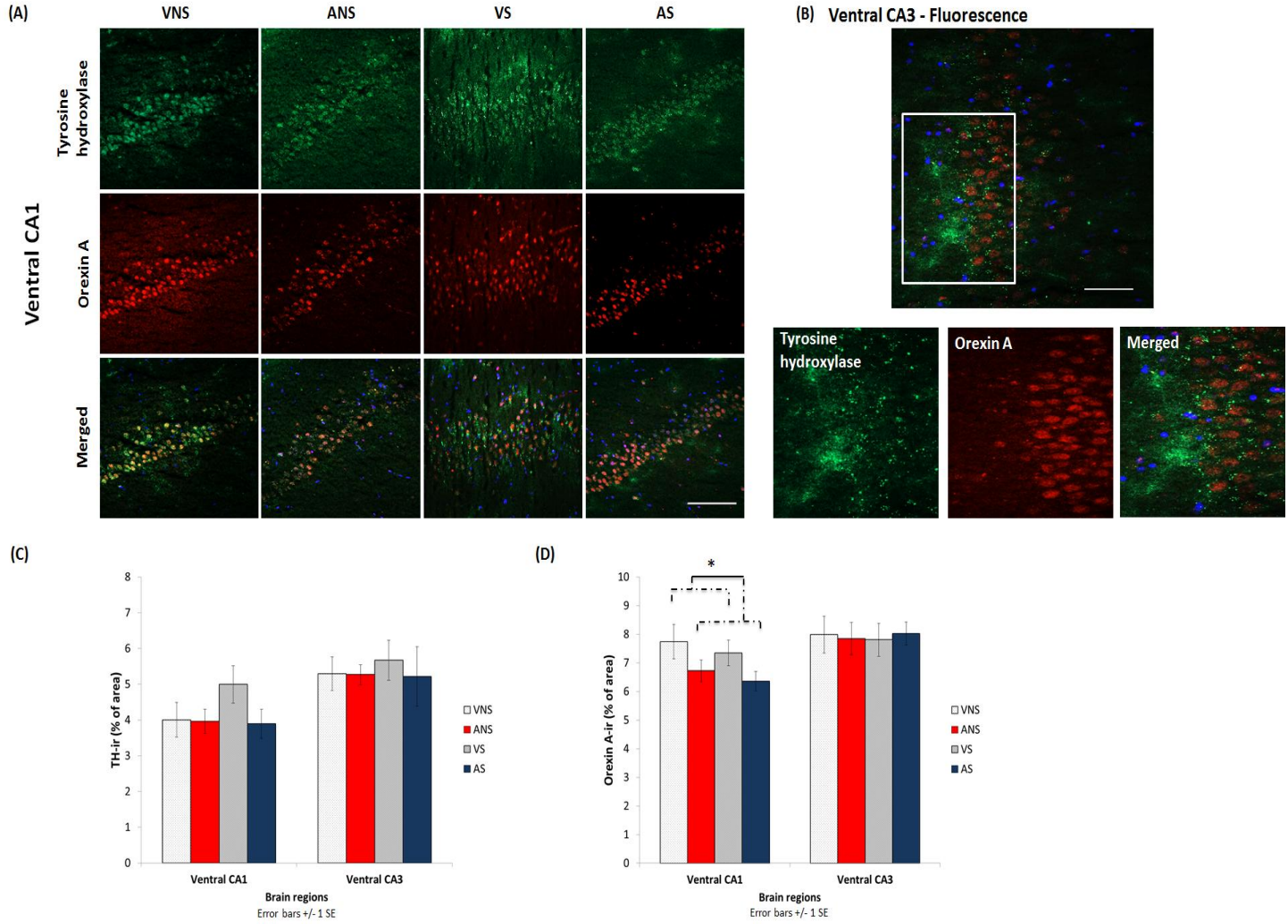

Figure 8. Orexin A and tyrosine hydroxylase-ir in the ventral hippocampus (CA1 and CA3). (A) Fluorescence photomicrographs (200x magnification) showing tyrosine hydroxylase (TH) -ir (green), Orexin A-ir (red), and Hoechst (blue, see merged images) in the ventral hippocampus (vCA1 and vCA3). Scale bar 100 µm. (B) Enlarged fluorescence images of the vCA3. (C) Graph represents TH-ir in each region. (D) Graph represents orexin A-ir levels in each region. ANA-12 treatment significantly reduced orexin A-ir in the vCA1 (* p = .036). VNS: vehicle + no stress; ANS: ANA-12 + no stress; VS: Vehicle + stress; AS: ANA-12 + stress. Data are expressed as mean ± SEM.

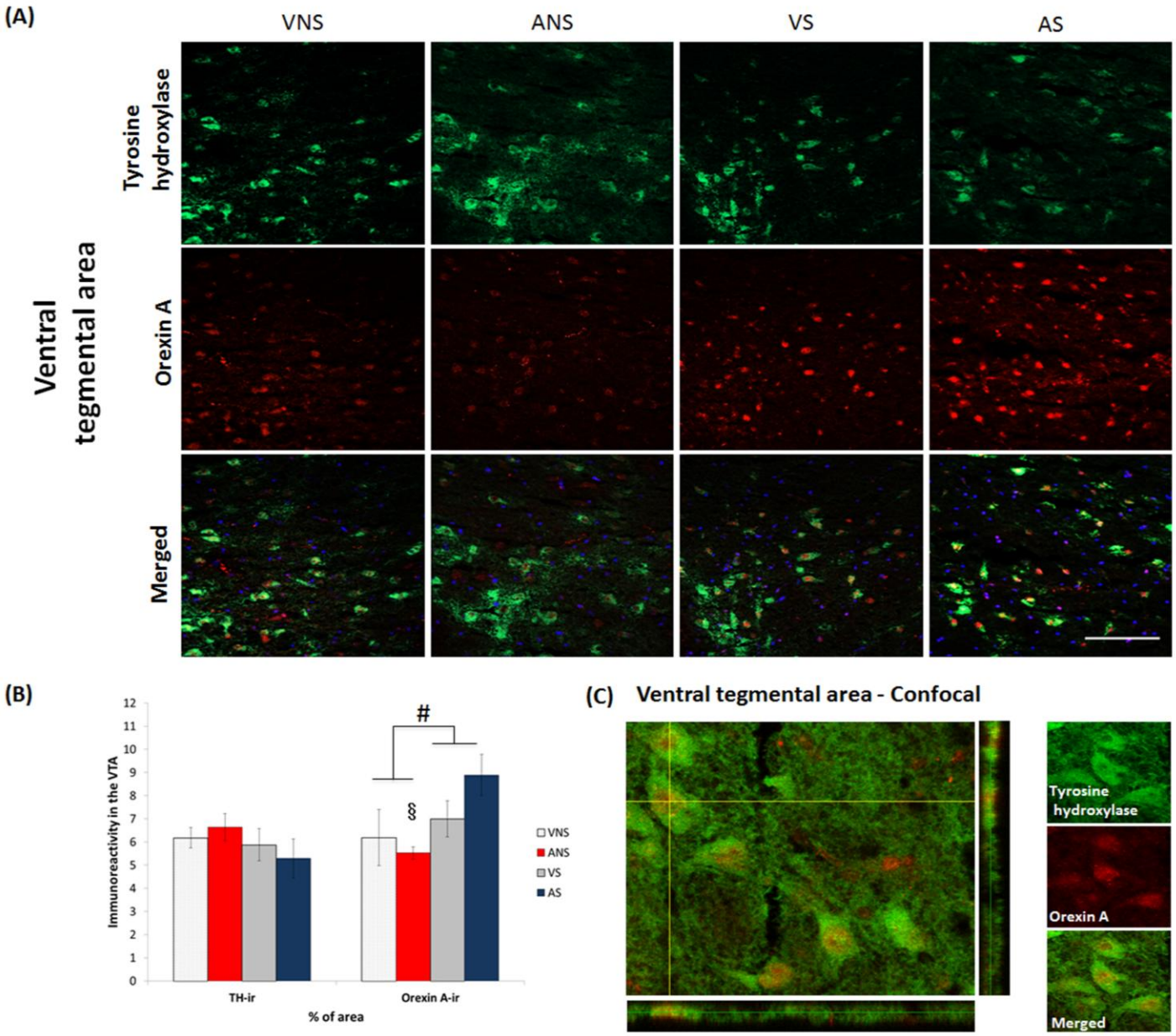

Figure 9. Orexin A and tyrosine hydroxylase-ir in the ventral tegmental area. (A) Fluorescence photomicrographs (200x magnification) showing tyrosine hydroxylase (TH)-ir (green), orexin A-ir (red), and Hoechst (blue, see merged images) in the ventral tegmental area (VTA). Scale bar 100 µm. (B) Graph represents TH- and orexin A- ir. Stress led to increased orexin A-ir in the VTA (# $p = .025$). ANA-12 treatment in stressed animals reversed the decrease in VTA orexin A expression observed in non-stress animals (§ $p < .05$). (C) Representative z-stack analysis taken on a confocal microscope reveals orexin A input to dopaminergic neurons in the VTA. Solid lines indicate the position for the z-stack images. Scale bar 50µm. VNS: vehicle + no stress; ANS: ANA-12 + no stress; VS: Vehicle + stress; AS: ANA-12 + stress. Data are expressed as mean ± SEM.

Table 1A

Pearson correlation for significant relationships between behaviors and neurochemical changes in brain areas

Behavioral test	Behavioral measure	Brain region and cell type	Correlations	n
SIT	Exploration ratio for time spent in S1-paired chamber vs. EC-paired chamber	PfA orexin A	r = .46, p = .041 *	20
SP	Direct contact with S2	VTA orexin A	r = .45, p = .028*	24
	Exploration ratio (Contact with S2 vs. S1)	dCA3 orexin A	r = -.43, p = .038*	24
	Exploration ratio for time spent in S2-paired chamber vs. S1-paired chamber	PfA orexin A	r = -.63, p = .003**	20
		LH FosB/ΔFosB	r = -.50, p = .013*	24
		vCA1 TH	r = .46, p = .013*	28
YMPAT	Latency 2 (Time to re-enter aversive arm)	PfA orexin A	r = .70, p = .001**	20
		dCA3 orexin A	r = -.42, p = .040*	24

Table 1B

Pearson correlation for significant relationships between neurochemical changes in brain areas

Brain region and cell type	Brain region and cell type	Correlations	n
PfA orexin A	PfA FosB/ΔFosB	r = .43, p = .046*	20
	LH FosB/ΔFosB	r = .48, p = .032*	20
	vCA1 orexin A	r = -.45, p = .045*	20
PfA FosB/ΔFosB	LH FosB/ΔFosB	r = .64, p = .001**	24
	VTA orexin A	r = -.48, p = .018*	24
vCA1 orexin A	vCA1 TH	r = -.40, p = .036*	28
	vCA3 orexin A	r = .45, p = .015*	28
vCA3 orexin A	dCA3 FosB/ΔFosB	r = -.41, p = .046*	24
vCA3 TH	dCA1 FosB/ΔFosB	r = -.41, p = .029*	28
	dCA3 ΔFosB	r = -.58, p = .003**	24
	VTA Orx A	r = .54, p = .007**	24
	VTA TH	r = .45, p = .028*	24

Table 1. Significant Pearson product moment correlations. Table 1A: Pearson's product moment correlations for relationships between behaviors and immunohistochemical markers in brain areas. Table 1B: Correlation between different immunohistochemical markers. PfA: Perifornical area; VTA: ventral tegmental area: dCA3: dorsal CA3 of the hippocampus, dCA1: dorsal CA1 of the hippocampus; LH: lateral hypothalamus; vCA1: ventral CA1 of the hippocampus; vCA3: ventral CA3 of the hippocampus. * $p < .05$, ** $p < .01$. Data are expressed as mean ± SEM.

Article 3

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Authorial contributions

Idu Azogu conceived and designed the study, contributed to implementation, data analyses, interpretation, and manuscript draft and revisions. She completed behavioral testing and scoring, and blood CORT collection with the assistance of Sylvie Emond (Research assistant), Dimitri De Mel, Isabelle Cossette, Oluwabukola Akindolie, and Catherine Baillargeon (Honors and UROP students co-supervised by Idu). She ran ELISA to analyze blood CORT concentrations with the assistance of Jacky Liang (lab manager) and Dimitri De Mel (Honors student co-supervised by Idu), and completed Western blotting with the help of Robin Beal (Fall NSERC student) and Mohammed Sayeem (Co-op student) that were co-supervised by Idu.

Jacky Liang assisted with blood CORT analysis via ELISA with Dimitri De Mel, as well as worked on Western blotting with Robin Beal and Mohammed Sayeem.

Dr. Hélène Plamondon supervised the research project, interpreted the data analyses, and revised the manuscript.

Abstract

Stress exposure has been implicated in the development of mood disorders, although little is known about the lasting effects of repeated stress during the adolescence period on sex differences in endocrine and plasticity signaling responses in adulthood. Using a 10-day heterotypic stress paradigm (postnatal day (PND) 26 to 35), we examined sex-specific immediate and enduring impact of early life stress and inhibition of tyrosine-related kinase B (TrkB) receptor (ANA-12; 0.5mg/kg, i.p.) on 1) adolescent blood corticosterone levels, 2) adult locomotion, anxiety and coping behavior, and 3) region-specific differences in endogenous TrkB full-length (TrkB.FL) and truncated (TrkB.T1) receptor isoforms. Blood collected on days 1, 5 and 10 revealed elevated basal and stress-induced CORT secretion in females compared to males, while ANA-12 attenuated CORT elevations post stress exposure in both genders. When tested as adults (from PND 61), females showed increased exploratory behavior in the open field and elevated plus maze compared to males, and increased passive coping in the forced swim by stress-naïve females. Biochemically, vehicle-treated males showed elevated TrkB.T1 and TrkB.FL expression compared to vehicle-treated females at the PFC, hippocampus and NAc, which levels were consistently attenuated by ANA-12 treatment in non-stress males, while ANA-12 enhanced expression in non-stress females, compared to vehicle-treated counterparts, except at the hippocampus. With regards to stress exposure, expression of both isoforms was strongly down-regulated at NAc affecting males only, associated with increased TrkB.T1 expression at the PFC. In females, stress alone had no impact, although ANA-12 treatment significantly elevated of expression TrkB.T1 and TrkB.FL in all structures, expression being increased for TrkB.T1 compared to TrkB.FL isoform and magnitude of the changes being region-specific. In contrast, ANA-12 effects in stressed males were restricted to inhibition of both isoforms' expression at the hippocampus. Together, our findings support that TrkB activation, contingent on stress exposure, affects TrkB isoform regulation during adulthood. Gender-specific biochemical responses at delayed intervals following juvenile stress exposure further support the need to include the sex variable in animal models.

Keywords: Heterotypic stress, ANA-12, adolescence, sex differences, corticosterone, TrkB.FL, TrkB.T1, behavior.

1. Introduction

Stress-induced alterations in the signaling of brain-derived neurotrophic factor (BDNF) have emerged as a major component in the vulnerability to mood disorders in adulthood (Duman & Monteggia, 2006). Notably, BDNF plays an important role as a stress and activity-dependent neurotrophin involved in the activity of the hypothalamic-pituitary-adrenal (HPA) axis. It is shown negatively regulated by downstream effectors of the HPA axis, namely glucocorticoids (corticosterone (CORT) in rodents) (Smith, 1996; Suri & Vaidya, 2013). Chronic exposure to CORT suppresses BDNF-induced synaptic plasticity in the hippocampus (Numakawa et al., 2009), a phenomenon related to downregulation of glucocorticoid receptors (GR) and subsequent inhibition of BDNF-induced glutamate release, which dampens long-term potentiation and induces cognitive impairments (Minichiello et al., 1999). In the PFC and hippocampus, significant reductions of BDNF expression is related to early life adversity (Fumagalli, Bedogni, Perez, Racagni, & Riva, 2004; Luoni et al., 2014).

Within the mesocorticolimbic dopamine system, increased susceptibility to stress-related anxiety and depression during the adolescence period has been noted (Costello et al., 2002; Ernst, Pine, & Hardin, 2006). This period (conservatively ranging from PND 28 to 49 as puberty onset varies as a function of rat strain and rearing conditions (Mayo Fiske, 1941; O'Dell, 2009; Sengupta, 2013)) is characterized by increased risk taking, impulsivity and reward/ novelty seeking behaviors (Ernst et al., 2006). Adolescence is also a critical time for ongoing brain development, regulation of neurotransmitter systems, programming of neurotrophic factor levels and promotion of lifelong resilience or vulnerability to stress (Andersen & Teicher, 2008; McCormick & Mathews, 2007; O'Dell, 2009). The adolescent brain is vulnerable to the effects of stressors due to the heightened rate of development, pubertal changes occurring in the hypothalamo-pituitary-gonadal axis (Romeo, 2003) and differences in functionality of the HPA axis resulting in an extended release of glucocorticoids in response to a stressor (Romeo, 2010). Over the course of the adolescent period, sex differences in HPA functionality in rodents have been paralleled with initiation of sex hormone regulation (McCormick & Mathews, 2007). Moreover, prolonged stress exposure is shown to decrease hippocampal BDNF expression and promote lasting elevations in CORT levels in young, but not adult rats (Taliaz et al., 2011).

Importantly, chronic stress during adolescence disrupts HPA axis-mediated glucocorticoid secretion in a sexually dimorphic manner. Sex differences have been noted in the basal state and following stress exposure (Liu et al., 2011), sometimes paralleled by abnormal levels of BDNF signaling (Marco et al., 2013; Scharfman & MacLusky, 2014), attributed to the direct effect of ovarian hormones on central BDNF mRNA and protein regulation (Franklin & Perrot-Sinal, 2006). Studies support differences in brain regulation of corticosteroid release and increased susceptibility to chronic/repeated stress exposure to contribute to prevalence of depression in females (Dalla, Pitychoutis, Kokras, & Papadopoulou-Daifoti, 2011; Handa, Burgess, Kerr, & O'Keefe, 1994; Lu et al., 2015). Female rats exhibit higher CORT levels following acute and repeated stress exposure, an increase dependent on circulating gonadal hormones (Seale, Wood, Atkinson, Harbuz, & Lightman, 2004).

Notably, mRNAs for estrogen receptors are co-localized with BDNF and TrkB in various developing forebrain regions, including the hippocampus (Solum & Handa, 2002). The presence of at least two different classes of TrkB receptors, derived by alternative splicing of the TrkB mRNA, suggest that these receptors may carry out different signaling capabilities upon BDNF binding: the catalytic, full length receptor (TrkBgp145 or TrkB.FL) and the c-terminal truncated form that lacks the cytoplasmic tyrosine kinase domain (TrkBgp95 or TrkB.T1), preferentially localized in neurons and glial cells, respectively (Barbacid, 1994; Eide, Lowenstein, & Reichardt, 1993; Haapasalo, Koponen, Hoppe, Wong, & Castrén, 2001). BDNF mediates most of its effects to induce differentiation, cell proliferation and neuronal survival, and subsequent activation of downstream signaling cascades via phosphorylation of TrkB.FL receptor (Chao, 2003; Numakawa et al., 2010). However, TrkB.T1 is reported to reversibly bind and internalize BDNF, preventing its diffusion, thereby reducing the responses normally induced by BDNF binding to TrkB.FL (Biffo, Offenhäuser, Carter, & Barde, 1995; Haapasalo et al., 2002). Indeed, responsiveness to BDNF is attenuated in cortical tissues when truncated receptors outnumber the full length TrkB by a 4:1 ratio (Knusel, Rabin, Hefti, & Kaplan, 1994).

Differential contributions of TrkB receptor isoforms have been proposed in response to pathological conditions (Manadas, Melo, Gomes, & Duarte, 2007). For instance, excitotoxicity in cultured hippocampal neurons downregulates TrkB.FL and upregulates TrkB.T1, a response shown to switch BDNF's role from a

neurotrophic to a neuroprotective dominant function (Gomes et al., 2012). Notably, upregulation of TrkB.FL in the rat hippocampus following repeated stress has been presented as a compensatory/adaptive response (Nibuya, Takahashi, Russell, & Duman, 1999). TrkB isoform expression appear site-specific, and basal TrkB.T1 is increased and TrkB.FL is decreased within the nucleus accumbens (NAc) in adult compared to juvenile rats, whereas the opposite is noted in the PFC (Perreault, Fan, O'Dowd, & George, 2013). Notably, the PFC is shown to modulate the activity of the NAc as both regions continue to mature (Davey, Yücel, & Allen, 2008). At the hippocampus and hypothalamus, TrkB.FL expression gradually decreases over the course of development, whereas TrkB.T1 is increased in the first postnatal week in these regions and remains stable during development, reducing in aged rats (Silhol et al., 2005). At present, few studies have investigated gender differences in TrkB isoform expression following stress, although sex is known to affect TrkB isoforms in the song system of zebra finches (Tang & Wade, 2012) and in mice exposed to neonatal hypoxia-ischemia (Chavez-Valdez, Martin, Razdan, Gauda, & Northington, 2014).

Therefore, the current study aimed to characterize the role of BDNF-TrkB receptor activation upon heterotypic stress exposure, induced at the onset of the adolescent period, in regulating sex specific changes in corticosterone secretion and anxiety-related behavior in adulthood. As part of this characterization, the experiment explored sex differences in the effects of ANA-12, a selective TrkB receptor antagonist, on protein expression of TrkB.FL and TrkB.T1 in dopamine projecting regions of the PFC, NAc and hippocampus of young adult rats using Western blotting.

2. Methodology

2.1. Animals and housing

Male and female Wistar rats, aged postnatal day (PND) 21 ± 2 (weighing between 50 and 60g upon arrival), were obtained from Charles River Laboratories (Rochefort, Quebec, Canada) and housed two to a cage (same sex) with daily gentle handling to minimize stress. Rats were maintained on a 12h light/dark cycle (lights on at 7:00am), room temperature (21-23°C) with 60% relative humidity, and ad libitum access to water and standard Purina rat chow. Following a 5-day acclimation period to the animal facility, exposure to the 10-day

heterotypic stress paradigm started. All procedures were carried out in accordance with the Canadian Council of Animal care and approved by the University of Ottawa Animal Care Committee. Experimentation complied with the ARRIVE guidelines and was in accordance with the National Institutes of Health guide for the care and use of laboratory animals (NIH Publications No. 8023, revised 1978).

2.2. Drug/ vehicle injection

The highly selective TrkB receptor antagonist, ANA-12, *N*-[2-[[(Hexahydro-2-oxo-1*H*-azepin-3-yl)amino]carbonyl]phenyl]benzo[b]thiophene-2-carboxamide (SML0209, Sigma-Aldrich) is shown to cross the blood-brain barrier after systemic administration, with no effects on the TrkA or TrkC receptors (Cazorla et al., 2011). Rats were randomly divided into 8 experimental groups (n = 13 per male/female groups) as follows: ANA-12 + stress (AS), ANA-12 + no stress (ANS), Vehicle + stress (VS), and Vehicle + no stress (VNS). On PND 26, experimentally naïve adolescent rats received ANA-12 (0.5mg/kg, i.p.) at a volume of 10ml/kg (Kishi, Hirooka, & Sunagawa, 2012; Leggio et al., 2014; J. Zhang et al., 2015) or vehicle (0.9% saline containing 20% (2-hydroxypropyl)-β-cyclodextrin; H107, Sigma-Aldrich) 30 min prior to beginning the stress (or control - no stress) sessions on days 1, 4, 7 and 10 of the 10-day stress paradigm. ANA-12 dosage was selected based on pharmacokinetics revealing brain active concentrations as early as 30 minutes (~400nM) and up to 6 hours after i.p. injection (~10nM) in mice (Cazorla et al., 2011). Experimental timeline is represented in Fig. 1.

2.3. Blood corticosterone collection (tail venipuncture)

Blood samples were collected from un-anaesthetized rats at predetermined intervals on days 1, 5 and 10 of the 10-day experimental paradigm. Following a small vertical incision on the lateral tail vein, two droplets of blood were collected in less than 3 min on special Whatman BFC180 bloodstain cards (Sigma-Aldrich, Canada) as previously described (Milot, James, Merali, & Plamondon, 2012). Cards were allowed to dry overnight at room temperature prior to storage in -80°C. Blood collection occurred prior to injections (basal) and at 30, 60, and 90 min time intervals after onset of stressor.

2.4. Stress paradigm

A 10 day heterotypic stress paradigm combining restraint and forced swim stress was selected to address the issue of animals habituating to repeated exposure to a single stressor (Zamora-Gonzalez, Santerre, Palomera-Avalos, & Morales-Villagran, 2013). Briefly, on odd numbered days up to day 9, animals were immobilized in a clear plastic restraining bag with air holes for 30 min (between 9:30AM and 11AM). The 15 min forced swim stressor was administered on even number days up to day 10, during which animals were introduced to a transparent cylindrical container (20.32 cm in diameter and 43.18 cm in height) filled with tap water (23 ± 2°C) at a height of approximately 33 cm. Black cardboard pieces were placed between each cylinder and at the extreme edges when multiple animals were being stressed at the same time. Animals were dried off post swim and placed on a heating pad before being returned to their home cage. Control rats were handled for 3-5 min with no additional manipulation. The stress paradigm ended at PND 35 and rats were thereafter handled daily prior to behavioral testing at PND 61.

2.5. Assessment of the estrous cycle

In order for animals to become accustomed to manipulations and reduce initial stress related to handling, estrous cycle monitoring was initiated on PND 55, six days prior to behavioral testing, and then continued on the mornings of each behavioral test up to the end of the study. A fine plastic pipette tip was used for flushing a small volume of warm 0.9% saline solution or tap water into the vaginal opening and collected samples placed on special concave slides. Fresh cells were immediately examined under a light microscope with a 10x objective. Representative bright-field images of estrous cycle stages (Fig. 2) were captured with a ProgRes MF scan camera and ProgRes Capture Pro Mac 2.7.6 software (Jenoptik, Germany) using an Olympus DX51 microscope (Center Valley, PA, USA) with a 20x objective lens. For the OFT/ EPM tests, the number of animals in each phase of the estrous cycle (proestrus, estrus, metestrus, diestrus) were as follows: AS (6, 4, 0, 4), ANS (4, 4, 5, 1), VS (1, 6, 5, 0) and VNS (5, 4, 4, 0). For the FST: AS (5, 8, 1, 0), ANS (5, 5, 2, 2), VS (3, 4, 5, 0) and VNS (5, 2, 4, 2). Thus, power was not sufficient to run this data further to address this variable.

2.6. Behavioral testing

Behavioral testing began on PND 61. Rats were transported to the enclosed hallway outside the testing room 30 min prior to testing between 0930 and 1600. Overhead room lighting in open field test (OFT) and elevated plus maze (EPM) room was 800-900 lux and 650-700 lux in the forced swim room. Testing apparatus were thoroughly cleaned with 70% ethanol in-between animals. Data for all tests were logged using the ODlog software (ODlog 2.0, MacroPod Software).

2.6.1. Open field test (OFT)

The OFT assesses exploration and locomotor activity in a novel open field environment. The arena was placed on a table 90cm above the floor and was separated from the experimenter's recording zones by white curtains. Testing apparatus was made of gray Plexiglas (LWH: 75 cm × 75 cm × 30 cm) with a grid dividing the floor into 36 identical squares. On PND 61, rats were placed on a corner of the peripheral zone within the arena and their behavior recorded for 5 min, using a PC computer and overhead video camera. Assessments included the frequency of entries in the center and peripheral zones, along with the number of squares walked in these zones and the latency to initially enter the center zone from the periphery.

2.6.2. Elevated plus maze (EPM)

The EPM test was conducted following the OFT on PND 61. The test consisted of a plus-shaped Plexiglas structure with two enclosed arms (with walls 40 cm in height) and two open arms, all measuring 50cm × 10cm, elevated 60cm above the floor and surrounded by plain white walls with white curtains on one side to separate the experimenter's recording area set up with a video-tracking system (PC computer and overhead video camera). Rats were introduced to the closed arms of the EPM facing an open arm, and allowed to freely explore the apparatus for 5 min. The duration and frequency of entries in the open and closed arms (counted only when the animal had all four paws in the specific arm), the frequency of opposite arm crossings, risk assessments (defined as stretch-attend posture to scan the open arms from the safety of the closed arm), head dips in the open arm, rears in the closed arms, and freezing/ immobility were assessed. Percentage of open arm

time (time in open arms / total time in arms) × 100) and index of open arm avoidance (100 – (percentage of open arm time + percentage of open arm entries) / 2) (Pellow & File, 1986; Podhorna & Brown, 2002; Trullas & Skolnick, 1993) were calculated. At the end of the 5 min test, the rat was placed back in its home cage. Increased open arm time and frequency of head dips were attributed to reduced anxiety, whereas increased stretch-attend posture and open arm avoidance were indicative of elevated anxiety.

2.6.3. Forced swim test (FST)

At PND 69 or 70, the Porsolt FST (Porsolt et al., 1978, 1977) was used to assess behavioral despair (Slattery and Cryan, 2012). Rats were exposed to a 15 min forced swim pretraining session prior to a 5 min test session on the next day. The stressed animals were omitted from the pretraining session due to prior exposure. Briefly, rats were allowed to swim in transparent Plexiglas cylinders (20.32 cm in diameter and 43.18 cm in height) filled with tap water (25 ± 1 °C) at a height of approximately 33 cm for 15 min on the first exposure and for a 5 min session on the following day. Post swim, rats were dried and placed in a cage resting on a heating pad. Movements pertaining to swimming/climbing and diving (i.e. active escape-like behaviors), and immobility (i.e. the absence of all other movements for ≥ 2.0 s except those needed to keep the rat's head above water (Pliakas et al., 2001)) were recorded. The water was replenished in between animals.

2.7. Blood corticosterone immunoassay

CORT levels from blood droplets were quantified in duplicates using an enzyme-linked immunosorbent assay (ELISA) kit (Corticosterone EIA kit, AD1-901-097, ENZO Life Sciences). Standards and samples were prepared as recommended by the manufacturer. Whatman bloodstain cards were allowed to thaw for 30 min at room temperature (RT) after removal from the -80°C storage. As previously reported (Azogu & Plamondon, 2017), a 3.0mm diameter circle was punched from the cards' blood samples using a Gem Hole Punch (McGill Inc., Marengo, IL) (n = 8 per group and blood collection interval, except for the baseline collection (n = 4 per group)). Punches were placed in glass tubes containing 280 µl of assay buffer covered with parafilm and shaken for 24 h at RT on a Belly Dancer® (Structure Probe Inc., West Chester, PA). Following incubation, 214.5 µl of

each sample was mixed with 5.5 µl of steroid displacement reagent (SDR) to inhibit corticosterone from binding to proteins. Samples were then diluted 1:5 with the assay buffer. CORT concentrations were determined using a PowerWave™ XS2 Microplate Spectrophotometer (BioTek, Winooski, VT), and calculated using a four-parameter equation derived from the standard curve values. The analytic range of the assay was 32–20,000 pg/ml. Intra- and inter-assay variability were determined.

2.8. Isolation and incubation of mPFC, NAc and Hippocampal tissue dissection

Protein expression levels of TrkB receptor isoforms (TrkB.FL and TrkB.T1) were determined in brain tissue samples of male and female young adult rats killed within 2 h of the FST. Following isoflurane anesthesia, the rat brains were quickly extracted. 2mm thick coronal sections were obtained using a cooled stainless steel rat brain matrix and placed on filter paper dampened with 0.1M phosphate buffered saline (PBS) resting on an ice cold aluminum plate for dissection of the PFC, NAc and hippocampus. Dissected samples were snap-frozen in liquid nitrogen and stored in -80°C until processed.

2.8.1. Protein extraction and quantification

Samples were homogenized on ice in 10x volume homogenate buffer (HB; 10mM Tris/HCl, 0.1mM EDTA, pH 7.5) mixed with protease inhibitor cocktail (PI, 1:100, P8340, Sigma-Aldrich). Aliquoted homogenous mixture was spun at 500 × g for 10 min with F-22 micro rotor placed in a Sorvall RC6 Plus Centrifuge (Thermo Fisher Scientific, Inc.). The supernatant was collected and centrifuged at 44000 × g for 10 min with Sorvall SA-512 rotor after which the supernatant was discarded and pellets dissolved with 200µl HB containing PI. Following a final 10 min centrifugation at 44000 × g, the collected pellets were dissolved in 200µl or 100µl of HB without PI if sample was greater than 80mg or less than 80mg, respectively. Extracted proteins were pipetted into labeled 1.5ml Eppendorf tubes and stored at -80°C until protein quantification.

Protein concentration was determined by the Bradford assay using bovine serum albumin (BSA) as a standard. Samples were diluted 4x, 8x or 16x in HB, and 7.5ul were loaded per well. 225µl of Coomassie Reagent (Pierce™ Coomassie Bradford protein assay kit, 23200, Thermo Fisher Scientific, Inc.) was added to

each well and mixed on the Belly Dancer® shaker (Structure Probe Inc., West Chester, PA) for 30 sec, then incubated in the dark for 10 min at RT. The absorbance of the samples and standards at 595nm were determined using a PowerWave™ XS2 Microplate Spectrophotometer (BioTek, Winooski, VT). A standard curve was derived by plotting the average blank-corrected 595nm measurement for each BSA standard vs. its concentration in µg/ml, and used for sample protein determination. Samples were run in triplicates, mean concentrations calculated and multiplied by the dilution factor for the true protein concentrations.

2.8.2. SDS-PAGE Polyacrylamide electrophoresis and Western blot protocol

Western blotting was performed on a subset of 5-7 animals per group. 20µg of total protein per well were mixed with Laemmli buffer, boiled at 100°C for 5 min to denature proteins, then loaded in triplicates (12µl per well) on 10% SDS-PAGE gel. 0.5µl of a one-color protein molecular weight marker (928-40000, Li-Cor BioSciences) was added to a specified well. SDS-PAGE was running at 80 volts for approximately 145 min. Protein was then transferred to 0.22µm nitrocellulose membrane (EP2HY00010, Fisher Scientific) at 100 volts for approximately 60 min. After transfer, total loaded protein was quantified for western blot normalization using Revert™ Total Protein Stain kit (926-11015, Li-Cor BioSciences) as per manufacturer's suggested protocol. Briefly, membrane was rinsed with dH_2O and incubated in Revert™ Total Protein Stain solution for 5 min on the orbital shaker, then rinsed twice with the Revert™ Wash Solution for 30 sec on the shaker. After a quick rinse with dH_2O, membrane was wet-scanned with Li-Cor Odyssey Infrared Imaging System at 700nm channel. Collected scans were used for loading errors control and normalization. The membrane was then blocked with filtered 5% skim milk in PBS for 1 h at RT without shaking, followed by 4°C overnight incubation with 5% skim milk in Tris-buffered saline with 0.1% Tween 20 (TBST) containing rabbit polyclonal anti-TrkB antibody (1:1000, H-181, sc-8316, Santa Cruz Biotechnology) on the orbital shaker. Next day, membrane was quickly rinsed with TBST, and then washed 3 x 10 min in TBST with gentle shaking, prior to 1 h incubation at RT with the secondary antibody in filtered 5% skim milk with TBST (1:20000, Donkey anti-rabbit IRDye 800CW, 926-32213, Li-Cor BioSciences) on the shaker. Following 3 x 10 min rinses in

TBST, membranes were wet-scanned in PBS with Li-Cor Odyssey Infrared Imaging System at 800nm channel. The TrkB antibody recognizes two protein bands: a broad band located at approximately 95 kDa (TrkB.T1) and a narrower band centered at approximately 145 kDa (TrkB.FL). Blots were analyzed with Image Studio Lite software 5.0 (Li-Cor BioSciences) and resulting data normalized against the total protein stain, as per manufacturer's instructions, and expressed as the ratio of TrkB to total protein levels.

2.9. Statistical analysis

Using SPSS (version 23), three-way ANOVAs assessed main effects of sex (male vs. female), treatment (ANA-12 vs. vehicle) and stress (stress vs. no stress) and interactions on the dependent variables of the OFT, EPM and FST, basal blood CORT (for days 1, 5 and 10) and TrkB isoforms' expression levels. Three-way repeated measures ANOVA assessed differences in CORT levels for the remaining time intervals on days 1, 5 and 10. The assumptions of homogeneity of variance and normality were verified, along with Mauchly's test of sphericity. When appropriate, a Huynh-Feldt ($\varepsilon > 0.75$) or Greenhouse-Geisser ($\varepsilon < 0.75$) correction for violations of sphericity were applied and the degrees of freedom adjusted. Estimates of effect size were calculated and the (partial η_p^2) proportion of variance accounted for throughout the analyses. Simple main effects with Bonferroni correction (adjustment at critical alpha level of 0.017) was used in significant interactions. Data are presented as mean ± SEM. Statistical significance was set at $p < 0.05$.

3. Blood serum corticosterone results

3.1. ANA-12 treatment attenuates Day 1 elevations of CORT levels in male and female rats

As shown in Fig. 3a, three-way ANOVA on day 1 CORT values revealed main effects of sex on baseline CORT levels, related to elevated levels in the female groups ($F(1,24) = 8.41$, $p = .008$). Females also showed higher CORT levels across all three post stress intervals ($F(1,56) = 184.27$, $p < .001$). Elevated CORT levels post restraint were noted in stressed animals ($F(1,56) = 72.25$, $p < .001$). CORT levels were increased 30 min post restraint stress compared to 0 and 60 min intervals ($F(2,56) = 33.37$, $p < .001$) in females, whereas in males, CORT levels were higher at 0 min relative to 30 and 60 min intervals ($F(1.808,50.636) = 8.21$, $p = .001$).

ANA-12 treatment attenuated CORT levels relative to veh-treated groups ($F(1,56) = 127.85$, $p < .001$). Significant interactions were also detected for time (**T**) × drug (**D**) ($p = .009$), **T** × stress ($p < .001$), **T** × sex ($p < .001$), **D** × sex ($p < .001$), and stress × sex ($p < .001$), which supported the main effects.

3.2. Day 5 CORT levels are elevated in animals exposed to stress and exhibit sex differences

Fig. 3b highlights basal CORT levels on day 5. Three-way ANOVA failed to reveal main effects or interactions ($p > .05$), although repeated measures ANOVA supported decreased CORT levels across time ($F(2,112) = 50.12$, $p < .001$). Stress elevated CORT secretion ($F(1, 56) = 97.44$, $p < .001$), which was significantly increased in females relative to male groups ($F(1,56) = 43.20$, $p < .001$). A **T** × stress interaction ($F(2,112) = 63.87$, $p < .001$) was due to elevated CORT in stressed rats 0 and 30 min post stress ($p < .001$).

3.3. Day 10 CORT levels show sex differences, responsivity to stress, and impact of ANA-12 treatment

Fig. 3c shows baseline CORT levels on day 10. Three-way ANOVA indicated a main effect of sex ($F(1,24) = 23.81$, $p < .001$), females showing higher basal levels compared to male groups. A **D** × stress interaction ($F(1,24) = 5.45$, $p = .028$) also supported increased CORT secretion in ANA-12 treated non-stressed groups compared to the veh-treated counterparts ($p = .043$) and ANA-12 treated stress groups ($p = .032$). Repeated measures ANOVA indicated decreased CORT levels as time elapsed post stress ($F(1.142,63.934) = 451.79$, $p < .001$) and in ANA-12 compared to veh-treated groups ($F(1,56) = 10.05$, $p = .002$), while stress-induced CORT elevations was significantly greater in female than male groups ($F(1,56) = 8.621$, $p = .005$). A **T** × stress interaction for females ($F(1.131,31.674) = 126.32$, $p < .001$) and males ($F(1.157,32.40) = 73.03$, $p < .001$) indicated increased CORT immediately following stress cessation ($p < .001$), but not at other time intervals. These effects were further supported through significant **T** × stress ($p < .001$), **T** × sex ($p < .001$), **T** × stress × sex ($p = .001$), and **T** × **D** × stress × sex ($p = .022$) interactions.

4. Behavioral results

4.1. Sex differences and effect of drug treatment and stress on exploration and locomotion in the OFT

Three-way ANOVA showed no main effects or interactions for time spent in the center and peripheral zones, or for latency to enter the center ($p > .05$). However, females showed a higher frequency of center zone

entries ($F(1,88) = 8.58$, $p = .004$) (Fig. 4a) and peripheral zone entries ($F(1,88) = 7.26$, $p = .008$) (Fig. 4b) than males. Females walked more in the center zone (Fig. 4c) compared to males ($F(1,88) = 10.09$, $p = .002$), and ANA-12 females showed increased walking in the peripheral zone (Fig. 4d) compared to vehicle counterparts ($F(1,88) = 11$, $p = .001$) and ANA-12 treated males ($F(1,88) = 5.06$, $p = .027$). Rearing frequency was reduced in stressed groups ($F(1,88) = 8.41$, $p = .005$) and in females relative to males ($F(1,88) = 16.78$, $p < .001$).

4.2. Sex differences and effect of drug treatment and stress on anxiety behavior in the EPM

Three-way ANOVA on percent open arm time (Fig. 5a) revealed a stress effect (F (1,96) = 5.73, $p = .019$), due to reduced open arm time by stress groups. Consistently, time in the closed arm (F (1,96) = 5.70, $p = .019$) (Fig. 5b) and index of open arm avoidance (F (1,96) = 7.30, $p = .008$) (Fig. 5c) was significantly elevated in stressed groups. Females made more risk assessments from the closed arms (F (1,96) = 4.40, $p = .039$) (Fig. 5d) and unprotected head dips from the open arms (F (1,96) = 5.84, $p = .018$) (Fig. 5e) compared to males. Frequency of head dips was reduced in stress groups (F (1,96) = 4.90, $p = .029$). For rearing frequency (Fig. 5f), stress (F (1,96) = 6.55, $p = .012$) and sex (F (1,96) = 6.34, $p = .013$) effects were noted, related to reduced rearing in stressed groups, and increased rearing in female groups. Simple effect tests following a significant **D** × stress interaction for crossing frequency revealed reduced crossing by VS compared to the AS ($p = .013$) and the VNS ($p = .049$) groups, irrespective of sex. Freezing/immobility was not significant ($p > .05$).

4.3. Sex differences and effect of drug treatment and stress on passive coping and stress adaptation in the FST

Three-way ANOVA revealed significant stress × sex interactions on swimming/climbing (F (1,64) = 4.22, $p = .044$) (Fig. 6a) and immobility (Fig. 6b) (F (1,64) = 4.33, $p = .041$). Swimming/climbing was decreased, whereas immobility was increased in non-stress females compared to stress female counterparts and non-stress male groups ($p < .05$).

5. Western blot analyses of region-specific changes in TrkB isoforms

5.1. Protein expression at the prefrontal cortex

Figure 7a shows representative immunoblots for all three regions. Figure 7b shows histograms of TrkB isoforms (TrkB.T1 and TrkB.FL) at the PFC for male and female rats. For TrkB.T1, three-way ANOVA showed increased expression in males relative to females (F (1,40) = 12.72, p = .001) and in stress versus non-stress groups (F (1,40) = 9.04, p = .005). A sex × **D** interaction (F (1,40) = 7.72, p = .008) was linked to reduced TrkB.T1 levels in veh-treated females compared to male counterparts (p < .001) and ANA-12 treated females (p = .002). A sex × stress interaction (F (1,40) = 15.13, p < .001) further indicated elevated TrkB.T1 expression in stress males compared to non-stress males (p < .001) and stress females (p < .001).

For TrkB.FL, three-way ANOVA revealed increased expression in males compared to females (F (1,40) = 99.52, p < .001), and in the ANA-12 treated (F (1,40) = 6.45, p = .014) and stress (F (1,40) = 15.12, p < .001) groups. Analyses also supported significant sex × **D** (p = .011), sex × stress (p < .001), **D** × stress (p = .002) and sex × **D** × stress (p < .001) interactions. In males, ANA-12 increased TrkB.FL in stress rats (F (1,40) = 16.61, p < .001) while it had the opposite effects in non-stress rats (F (1,40) = 17.46, p < .001), relative to veh-treated counterparts. A mean ratio of 1.73 characterized the TrkB.T1 to TrkB.FL protein expression at the PFC, with females achieving a higher 2.00 ratio compared to 1.58 found in males.

5.2. Protein expression at the nucleus accumbens

Figure 7c shows histograms of TrkB.T1 and TrkB.FL at the NAc for male and female rats. Three-way ANOVA showed increased TrkB.T1 expression in males compared to females (F (1,40) = 8.93, p = .005), and attenuated levels by stress (F (1,40) = 6.69, p = .013). No main effect of **D** was observed (p > .05). Analysis also revealed significant sex × D (p < .001) and D × stress (p = .008) interactions. A sex × **D** × stress interaction was associated with a **D** × stress interaction in males (F (1,40) = 22.20, p < .001), but not for females (F (1,40) = .54, p = .467) and was related to ANA-12 reducing TrkB.T1 expression in non-stress males compared to veh-treated counterparts.

For TrkB.FL, males showed increased expression than females (F (1,40) = 24.85, p < .001), and levels were attenuated in stress vs. non-stress groups (F (1,40) = 9.12, p = .004). No main effect of **D** was found (p > .05), but analyses revealed sex × **D** (F (1,40) = 77.05, p < .001) and **D** × stress (F (1,40) = 10.43, p = .002) interactions. Similar to TrkB.T1, a sex × **D** × stress interaction (p < .001) was associated with a **D** × stress interaction in males (F (1,40) = 33.40, p < .001), but not females (F (1,40) = 1.47, p = .233). ANA-12 reduced TrkB.FL levels in non-stress males compared to veh-treated counterparts (F (1,40) = 81.52, p < .001). A mean ratio of 2.24 for TrkB.T1 to TrkB.FL expression in females was observed compared to males' ratio of 1.50, the NAc showing an overall mean ratio of 1.76 for TrkB.T1 to TrkB.FL expression in the young adult.

5.3. Protein expression at the hippocampus

Figure 7d shows histograms of TrkB isoforms at the hippocampus for male and female rats. Three-way ANOVA for TrkB.T1 showed elevated expression in males (F (1,40) = 4.44, p = .043) and in stressed animals (F (1,40) = 10.93, p = .002). ANA-12 treatment attenuated TrkB.T1 expression (F (1,40) = 9.31, p = .004). A sex × **D** interaction (F (1,40) = 115.25, p < .001) was due to ANA-12 males and vehicle females showing reduced TrkB.T1 levels compared to vehicle males and ANA-12 females (p < .001). A significant **D** × stress interaction further indicated that ANA-12 treatment reduced TrkB.T1 levels in non-stressed animals relative to vehicle counterparts and ANA-12 stress groups (p < .001).

For TrkB.FL, three-way ANOVA only indicated a main effect of drug, related to reduced expression in ANA-12 treated animals (F (1,40) = 8.88, p = .005). Similar to TrkB.T1, a sex × **D** interaction (F (1,40) = 80.09, p < .001) revealed reduced TrkB.FL levels in ANA-12 males and vehicle females compared to vehicle males and ANA-12-females (p < .001). In addition, a **D** × stress interaction (F (1,40) = 11.57, p = .002) supported decreased TrkB.FL levels in ANA-12 non-stress cohorts compared to vehicle counterparts (p < .001) and ANA-12 stress groups (p = .003). Within the hippocampus, overall TrkB.T1 to TrkB.FL expression ratio was 3.41, and within each sex, females had a mean ratio of 3.36 compared to 3.46 in males. See Figure 7e for a

summary table of magnitude and direction of changes in TrkB.FL and TrkB.TI expression in males and females for the different experimental conditions (N = 6/group).

6. Discussion

To our knowledge, this study is the first to investigate effects of TrkB receptor blockade prior to stress on expression of the full length and truncated TrkB receptor isoforms, and to report sex-specific differences in TrkB expression at the PFC, NAc and hippocampus of young adult rats. We also characterized the role of TrkB receptors in regulating CORT secretion, locomotion and anxiety in adolescent male and female rodents.

6.1. TrkB receptor blockade attenuates stress-induced CORT elevations in adolescent male and female rats

ANA-12 produced a robust effect to lower post stress CORT secretion in control and stressed adolescent male and female rats on day 1 supporting a role for TrkB receptor activation in regulating CORT secretion in adolescent animals naïve to stress exposure, which appears more important in females than males. Interestingly, in the absence of drug treatment on day 5, ANA-12 non-stress females showed a more pronounced and rapid CORT secretion than the vehicle female controls suggestive that drug treatment on day 1 and 4 increased responsiveness to handling. Furthermore, ANA-12 treatment received on day 4 had protracted effects to lower post stress CORT levels in males only, and on day 10, CORT secretion levels were reduced at the 45 and 75 min intervals in stress males compared to vehicle counterparts. These findings contrast observations in adult male rats, where ANA-12 centrally infused at the NAc shell had no effect on CORT levels (Azogu & Plamondon, 2017). Although mechanisms remain unknown, our observations may be related to differences in the organization of the rat stress axis during developmental periods. Indeed, HPA axis reactivity is heightened and exhibits an immature feedback system in adolescent males (≤ PND 30) relative to adults (≥ PND 60), marked by a delayed ACTH and CORT secretion peak and return to baseline levels in response to acute 30-min restraint stress, ether vapor or intermittent foot shock (Goldman, Winget, Hollingshead, & Levine, 1973; Vázquez, 1998; Vázquez & Akil, 1993).

Ontogenic changes in TrkB receptor expression and/or activation have yet to be documented but could also be involved. Indeed, BDNF secretion is experience-dependent, affecting brain structure and function during the juvenile and adulthood periods (Branchi, Francia, & Alleva, 2004). Increased BDNF mRNA levels in the PVN and pituitary are reported following restraint stress (Givalois et al., 2001; Rage, Givalois, Marmigère, Tapia-Arancibia, & Arancibia, 2002; Smith, Makino, Kim, & Kvetnansky, 1995), and are associated with impairment of GR function and disinhibition of the HPA axis (Givalois et al., 2004; Hewitt & Bains, 2006; Jeanneteau et al., 2012). Notably, antidepressant actions of TrkB receptor blockade are in part attributable to inhibition of stress-induced effects in the PVN (Hewitt & Bains, 2006; Schaich, Wellman, Koi, & Erdos, 2016). Interestingly, non-stress females versus stress males showed delayed impact of prior day ANA-12 treatment on CORT secretion, being increased in the former and decreased in the latter groups, which our findings suggest are linked to sex dimorphism with TrkB receptor actions.

Our findings replicate robust increases in CORT secretion following exposure to restraint or forced swim stress in adolescent rodents, with females showing elevated CORT secretion compared to males. This is consistent with increased ACTH, early onset and faster rise of CORT secretion (Burgess & Handa, 1992; Handa et al., 1994), and prolonged HPA axis activation reported in female rodents in response to a variety of stressors, e.g. forced running, immobilization and inescapable foot shock (Heinsbroek, Van Haaren, Feenstra, Endert, & Van de Poll, 1991; Iwasaki-Sekino, Mano-Otagiri, Ohata, Yamauchi, & Shibasaki, 2009; Kant et al., 1983). Studies have proposed that gender differences facilitate adaptation of females to acute stressors while impairing adequate glucocorticoid secretion following repeated restraint stress (Haleem et al., 1988) or chronic unpredictable mild stress exposure (Lu et al., 2015). This is partially supported by our observations. Thus, while CORT levels gradually diminished over time for both genders, they remained significantly elevated 75 min following the 2nd restraint stress exposure in females compared to males. Interestingly however, despite a similar effect of the forced swim stress (day 10) to elevate CORT as that observed on day 5 with restraint stress, females adapted more rapidly to this stressor. This response could indicate improved adaptation of the females to repeated stress exposure and/or involvement of differential negative feedback mechanisms.

6.2. Sex dimorphism, drug and stress effects in the OFT, EPM and FST

Gender based differences were apparent in the OFT, EPM and FST. Open field exposure was characterized by increased activity levels in females relative to males and sex differences present on measures of rearing frequency, and walking and entries in the center and periphery zones. These findings are consistent with other OFT studies (Brotto, Barr, & Gorzalka, 2000; Valle & Gorzalka, 1980) and attributed to the influence of neural changes and/or ovarian hormones on the female brain (Archer, 1975; Slater & Blizard, 1976). Our findings support consideration to gender influence in this equation. ANA-12 increased walking in the periphery in stressed females but not males, along with an overall tendency of the drug to enhance other exploratory behaviors in stressed females. The effect of TrkB receptor blockade at remote intervals in the stressed females is intriguing. The role of BDNF and TrkB has been demonstrated in various aspects of motor responses although not explored in the context of novelty-induced locomotion. Thus, reduced striatal BDNF and elevated TrkB protein levels have been associated with impaired motor function in 6-hydroxydopamine-treated rats (Sampaio, Pinton, da Rocha, Gai, & Nogueira, 2017) while cocaine-induced behavioral sensitization is accompanied by increased BDNF and TrkB protein levels at the PFC and NAc (Zhang, Zhu, Huang, & Zhang, 2015). These findings support situation-specific BDNF/TrkB interplay in regulating locomotor responses.

Similar to other studies (Frye, Petralia, & Rhodes, 2000; Johnston & File, 1991), findings indicate an overall elevated activity level in females in the EPM test, particularly manifest through increase in the frequency of unprotected head dips (i.e. directed exploration) from the open arms, and rearing and risk assessments (i.e. stretch attend posture) from the closed arms. As expected, stressed animals showed attenuated open field time, females being more affected than males. ANA-12 administration was associated with increased open arm time in stressed females although effect did not reach statistical significance. Walf and Frye (2007) have consistently observed that female rodents in diestrus (low estradiol, low progestins) versus in the proestrus cycle (high estradiol, high progestins) spent less versus more time in the open arms of the EPM than their male counterparts. Although the current study monitored the stage of the estrus cycle, the number of female animals

in each stage of the cycle per group during behavioral testing was not adequate to run statistical analyses and report impact of this factor.

In the FST, male rats, stressed or not, showed comparable avoidance and immobility behaviors, supporting that stress exposure or drug treatment had no lasting impact in these cohorts. However, in females, prior stress exposure had a decisive impact on females' behavioral responses, bringing their activity level to similar levels of males rats. This suggests that stress in this group improved coping responses. Our finding is supported by a recent report showing decreased immobility time in male and female juvenile rats exposed to chronic restraint stress (Eiland, Ramroop, Hill, Manley, & McEwen, 2012). In contrast, females that were naïve to adolescent stress exposure showed reduced escape-like behavior (i.e. swimming) and increased immobility in the FST. This is consistent with a recent study showing reduced swimming behavior in stress-naïve female Sprague Dawley rats compared counterparts exposed to 30-day of social defeat stress, although differences were not found for immobility (Ver Hoeve, Kelly, Luz, Ghanshani, & Bhatnagar, 2013). A notable, albeit non-significant, effect of ANA-12 to reduce immobility was present in stress-naïve females. We suggest this response to be linked to hyperactivity of the HPA axis in the non-stress females compared to stress females in our study, considering prior exposure to the test in the stressed rats, which may have desensitized the stressed animals. In this context, findings would be concordant with elevated stress responsiveness generally observed in females compared to males (Heinsbroek et al., 1991; Iwasaki-Sekino et al., 2009; Kant et al., 1983).

6.3. Sex dimorphism in TrkB.T1 and TrkB.FL

We examined the influence of TrkB receptor blockade upon stress exposure in the adolescent rodents on expression of full-length and truncated TrkB isoforms at the PFC, NAc and hippocampus of adult male and female rats. These regions were selected due to an involvement in emotional regulation and in the pathophysiology of depression (Nestler et al., 2002; Price & Drevets, 2010). Expression was examined within 2 h after exposure to the FST, in order to determine how prior exposure to stress and TrkB receptor blockade influenced the brain response to acute stress exposure in adulthood. An upregulation of BDNF mRNA and

protein expression has been reported in the PFC 2 h after repeated social defeat stress in male Sprague-Dawley rats (Fanous, Hammer, & Nikulina, 2010), and BDNF signaling in the hippocampus and HPA axis is suggested to be a potential target for antidepressant drug effect (Castrén & Rantamäki, 2010; Wang, Dranovsky, & Hen, 2008), promoting environmental adaptability. Notably, BDNF exhibits alterations within dopaminergic regions following a variety of stressors, such that inhibiting BDNF/TrkB signaling in the NAc induces a notable antidepressant response (Berton et al., 2006; Eisch et al., 2003).

Currently, sex dimorphism in the expression of the truncated and full length TrkB isoforms remains unknown. Males relative to females had higher levels of TrkB.T1 and TrkB.FL in the PFC, NAc and hippocampus, except for TrkB.FL in the hippocampus, which did not show sex differences. Although mechanisms remain to be elucidated, neuroregulatory effects of ovarian hormones may play a role, these hormones having a profound effect on BDNF and TrkB proteins. For instance, sex differences in the expression of both TrkB isoforms in the song system of juvenile zebra finches is shown regulated by estradiol, the primary female hormone (Tang & Wade, 2012). A number of studies report that administration of estrogen increased expression of BDNF mRNA and protein in certain subregions of hippocampal and amygdaloid structures (Gibbs, 1999; Solum & Handa, 2002; Zhou, Zhang, Cohen, & Pandey, 2005), and modulated hippocampal neurogenesis and cell proliferation in adult female, but not male rodents (Galea, 2008). Furthermore, estrogen receptors colocalize to neuronal populations that express BDNF and TrkB (Sohrabji & Lewis, 2006; Solum & Handa, 2002). Furthermore, our observations are consistent with studies showing these brain regions to be sexually dimorphic, a phenomenon also mediated by gonadal hormones (Juraska, Sisk, & DonCarlos, 2013; Kolb & Gibb, 2015; Koss & Frick, 2017). In this context, the increased expression of TrkB.T1 (which promotes apoptosis) to TrkB.FL in males compared to females may serve to promote sex differences in these brain regions. Brain regions also show different TrkB.T1 to TrkB.FL expression ratio, the hippocampus and septum exhibiting the highest TrkB.T1 to TrkB.FL expression ratio, while the neostriatum and cortical areas have relatively low ratios (Fryer et al., 1996). This was evident in our study, the hippocampus showing a 3.41

TrkB.T1 to TrkB.FL ratio, while lower expression ratios of 1.73 and 1.76 were found in the PFC and NAc, respectively.

Adolescent stress exposure and ANA-12 treatment also exert intrinsic effects on regional TrkB isoform expression in adult male and females. In females, ANA-12 increased TrkB.T1 and TrkB.FL at the PFC, NAc and hippocampus, independent of stress exposure. In males, ANA-12 reduced TrkB.T1 and TrkB.FL expression at the PFC in non-stress rats, while it had the opposite effect in stress rats. Surprisingly, adolescent stress exposure alone had a more profound impact on both TrkB isoforms in males at adulthood than females. At the NAc, stress males showed a marked decrease in TrkB.FL and TrkB.T1 expression, which was not influenced by ANA-12 treatment. In contrast, at the hippocampus, blockade of TrkB receptor activation had lasting impact to decrease TrkB.T1 and TrkB.FL expression in males, effects that were opposite to ANA-12 stimulatory effects on expression in stress females. This is a fascinating finding considering the contrasting role attributed to BDNF at the hippocampus and NAc in regulating emotional responses (Autry & Monteggia, 2012). Stress males also showed increased TrkB.T1 at the PFC while stress showed no lasting impact on the truncated isoform's expression at the hippocampus. Importantly, while stress alone did not carry lasting impact on TrkB receptor expression in adult females, ANA-12 treatment consistently elevated adult expression of both TrkB isoforms in the three mesolimbic structures, although TrkB.T1 appeared most affected than TrkB.FL expression. Generally, TrkB receptor blockade by ANA-12 had a significant impact to elevate TrkB protein levels in females to those observed in males, suggesting that TrkB receptor activation in females contribute to female susceptibility to mood disorders. This effect could have a greater impact on mood at the PFC and hippocampus, where BDNF upregulation has been involved in antidepressant actions (Yu & Chen, 2011).

Studies addressing functional role of TrkB isoforms are rare, which makes functional relevance of observed changes difficult to ascertain. Mice lacking the TrkB.T1 receptor isoform exhibit increased anxiety compared to their littermate controls, associated with morphological abnormalities in neurites within the basolateral amygdala (Carim-Todd et al., 2009), suggesting that dysregulation of TrkB.T1 expression may impact ligand signaling, neuronal morphology/survival and behavior. Observations of similar changes at the

NAc and PFC in females (notwithstanding stress exposure) and the distinctive expression pattern at these loci in males are intriguing. Indeed, changes in BDNF induced by chronic stress exposure, leading to depressive-like phenotypes, have been associated with increased BDNF levels at the NAc while reduced BDNF at the PFC and hippocampus is a hallmark on mood disorders (Yu & Chen, 2011). Noteworthy, the observation that ANA-12 treatment had most profound effects on regulation of adult expression of mesolimbic TrkB receptors in the stressed females may indicate that differences in TrkB function even prior to any stress exposure represent an important basis of adult dysfunction. This contention is consistent with the notion of vulnerability and/or predisposition to emotional disorders and is coherent with female predisposition to develop depression, as well as with the fact that stress may not systematically be deleterious to men and women. These findings further support that events, including drug treatment that affects plasticity signaling in the adolescent period, have lasting impact on biochemical changes in adulthood, which in some structures go beyond those imposed by stress exposure. Our findings also demonstrate that alterations in male and female adolescents differentially affect TrkB receptor expression in structures of the mesocorticolimbic circuitry upon exposure to an acute stressor. Our findings support hypersensitivity to the blockade of TrkB receptor in females marked by lasting dysregulation.

7. Conclusion

In summary, our findings showing sex and stress specific effects of ANA-12 on endocrine responses suggest that modulating BDNF/TrkB levels could have therapeutic implications when considering depression and other mood related disorders in humans. Sex differences were noted in behavioral responding as females exhibited greater activity levels in the OFT, increased frequency of directed exploration, rearing and risk assessments in the EPM and increased passive coping in the FST (for stress-naïve groups). We provide additional evidence for TrkB.T1 receptor as an important regulator of endogenous BDNF signaling or mediator of TrkB.FL receptor responsiveness in the PFC, NAc and hippocampus, in addition to sex dimorphism in receptor isoform expression. Still much work remains to be done to improve our understanding of sex

differences in brain region-specific expression of both TrkB receptor isoforms, in addition to how these provide comparable means to understanding stress pathologies in the human brain. We suggest future studies to address sex differences in TrkB receptor isoforms bioavailability, and determine ANA-12 dose-response profile in regulating HPA axis activation in the juvenile and adult period.

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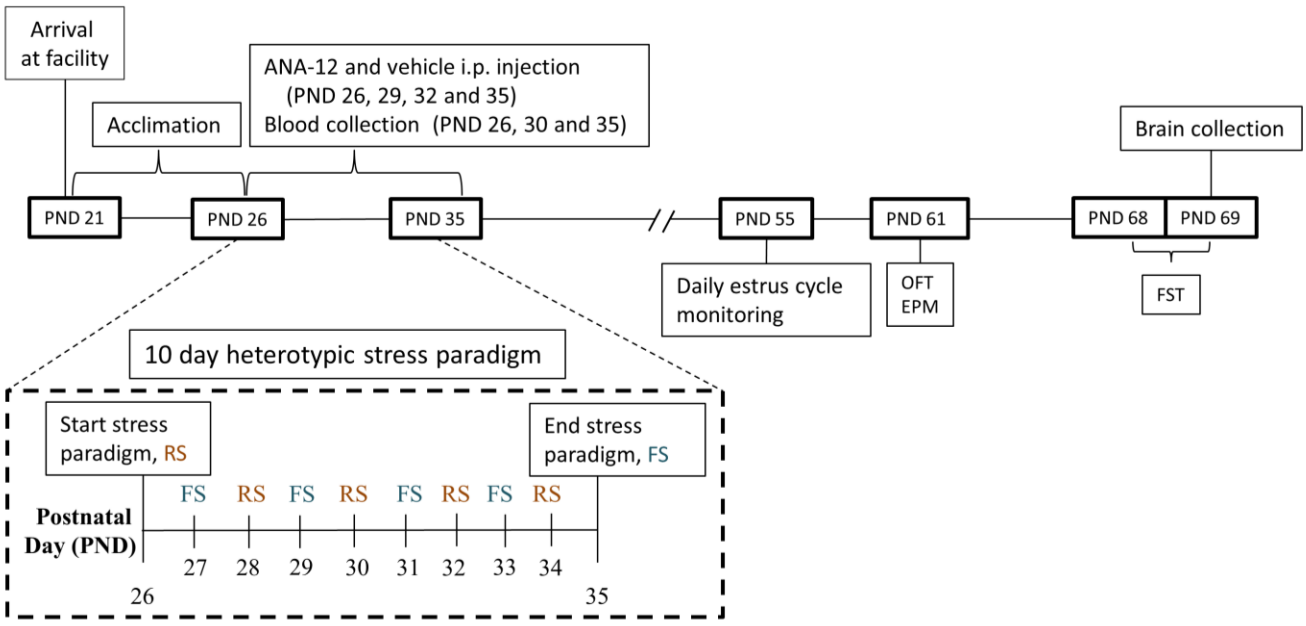


Figure 1. Experimental timeline. Animals arrived at the vivarium at ~ PND 21 and were allowed to acclimate for 5 days prior to the start of a 10 day heterotypic stress paradigm. Only stress animal groups were exposed to the repeated stressors. On days 1, 4, 7 and 10 of the paradigm (i.e. PND 26, 29, 32 and 35), all animals received either ANA-12 or vehicle (0.5 mg/kg, i.p.) before the stress or no stress sessions; whereas blood corticosterone was collected via tail venipuncture on predetermined intervals during days 1, 5 and 10 (i.e. PND 26, 30 and 35). For females, daily estrus cycle monitoring began at PND 55 for the remainder of the study. All animals were assessed in the behavioral tests from PND 61, and brains were collected at PND 68 (stress groups) or 69 (non-stress groups) within 2 hours post FST. PND: postnatal day; RS: restraint stress (30 min); FS: forced swim stress (15 min); OFT: open field test; EPM: elevated plus maze; FST: forced swim test (5 min).

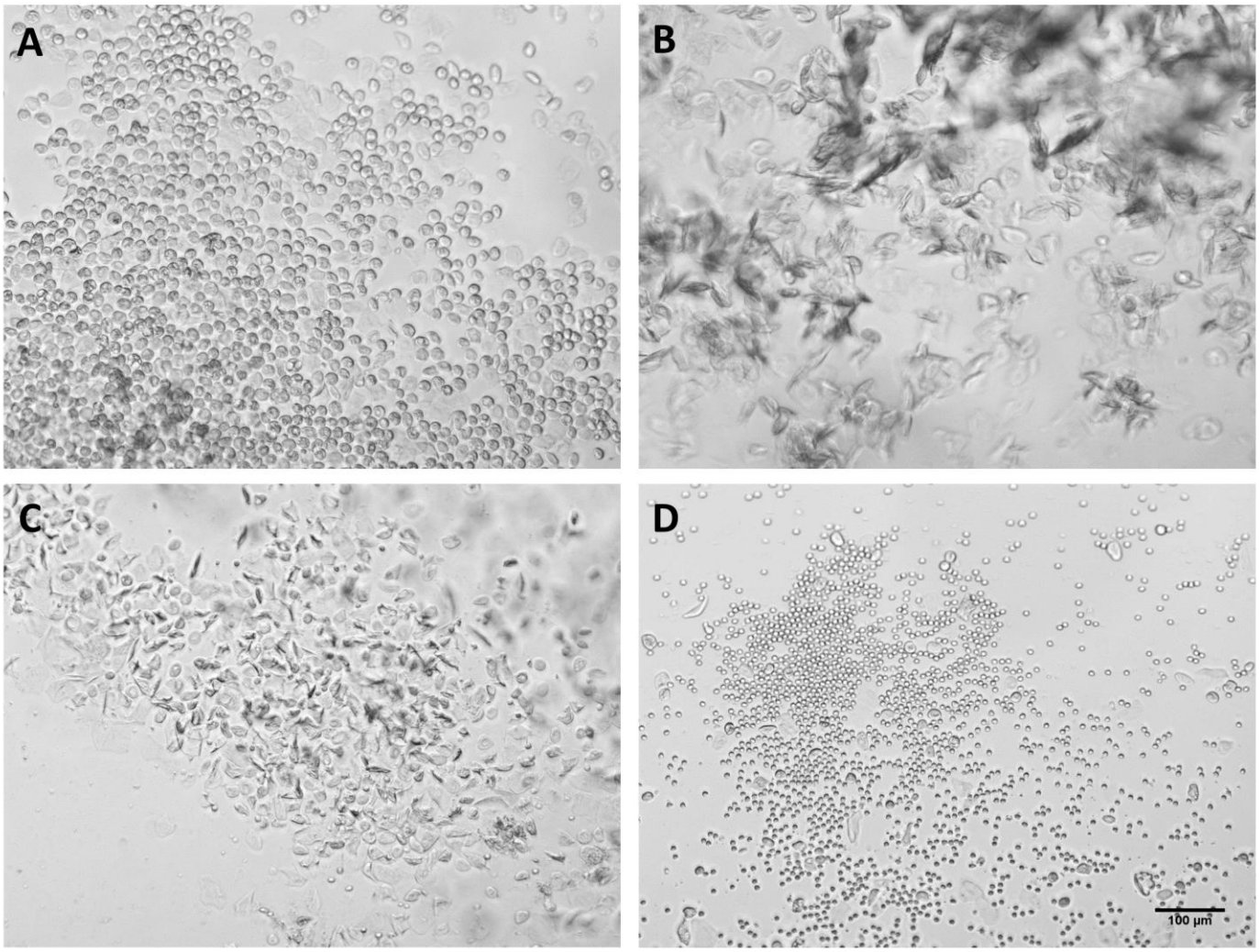


Figure 2. Representative transmitted bright field images of unstained cells resulting from vaginal lavage conducted on female rats. 200× magnification, scale bar 100 µm. Estrous cycle phases: (a) proestrus - round and nucleated epithelial cells, (b) estrus – cornified cells (irregular-shaped without nucleus), (c) metestrus – similar proportion of epithelial cells, cornified cells and leukocytes (d) diestrus – predominance of little round leukocytes. Cell description from Marcondes et al., (2002).

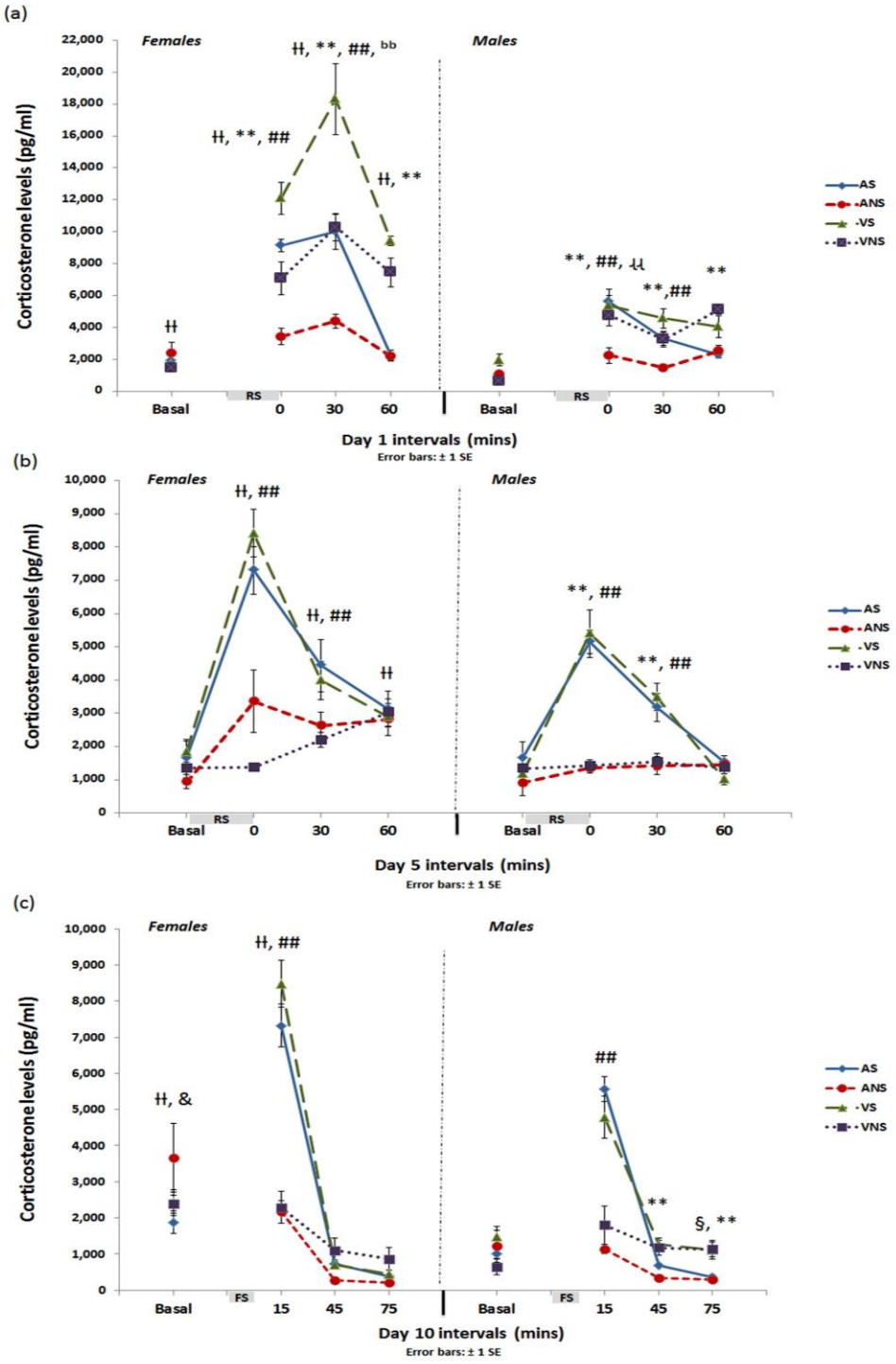


Figure 3. Sex differences and influence of ANA-12 treatment and stress exposure on blood CORT levels. CORT was collected on days 1, 5 and 10 for basal (n = 4) and predetermined collection intervals (n = 8). (a-c) females showed higher basal and interval CORT levels for days 1, 5 and 10 compared to males (ǂ $p < .01$), except on day 10, when males exhibited higher CORT levels 75 min post FS (§ $p < .05$). Stress exposure elevated CORT expression in animals (## $p < .01$), and ANA-12 treatment was efficacious in reducing CORT levels (** $p < .01$). On day 10, basal CORT was higher in ANA-12 non-stress compared to vehicle non-stress groups (& $p < .05$). In females only, Day 1 CORT level at 30 min was highest compared to 0 and 60 min post RS (^{bb} $p < .01$), whereas in males, heightened CORT level was observed at 0 min compared to 30 and 60 min post RS (ǂǂ $p < .01$). RS: restraint stress (30 min); FS: forced swim stress (15 min). Data are represented as mean ± SEM

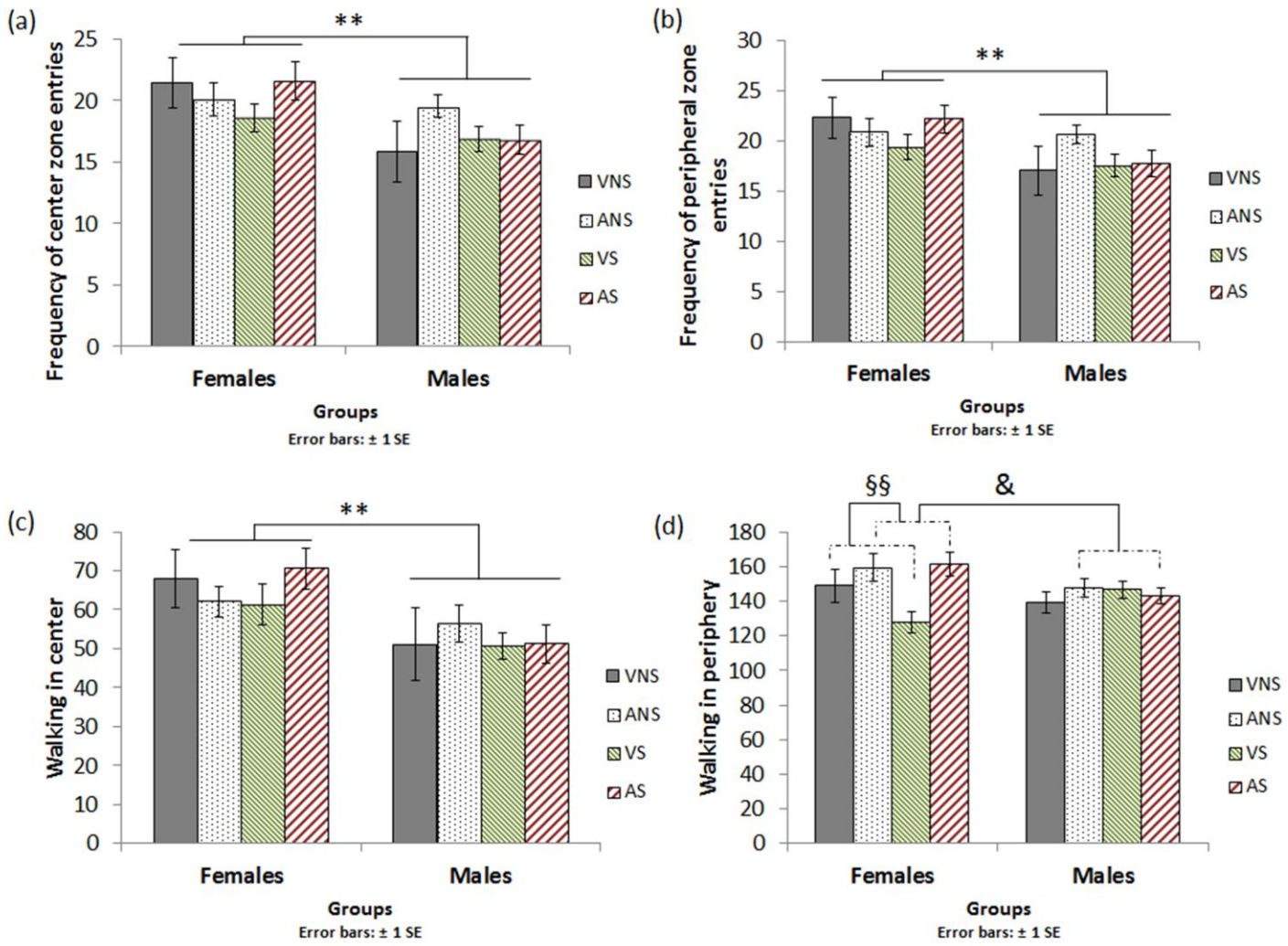


Figure 4. Sex differences and effect of treatment and stress on exploration and locomotion in the open field test (OFT) (a - c) Females showed increased frequency of center and peripheral zone entries, as well as increased walking in the center zone of the OFT compared to males (** $p < .01$). (d) ANA-12 treated females walked more in the periphery compared to ANA-12 treated males (& $p < .05$) and veh-treated females (§§ $p < .01$). Data are represented as mean ± SEM.

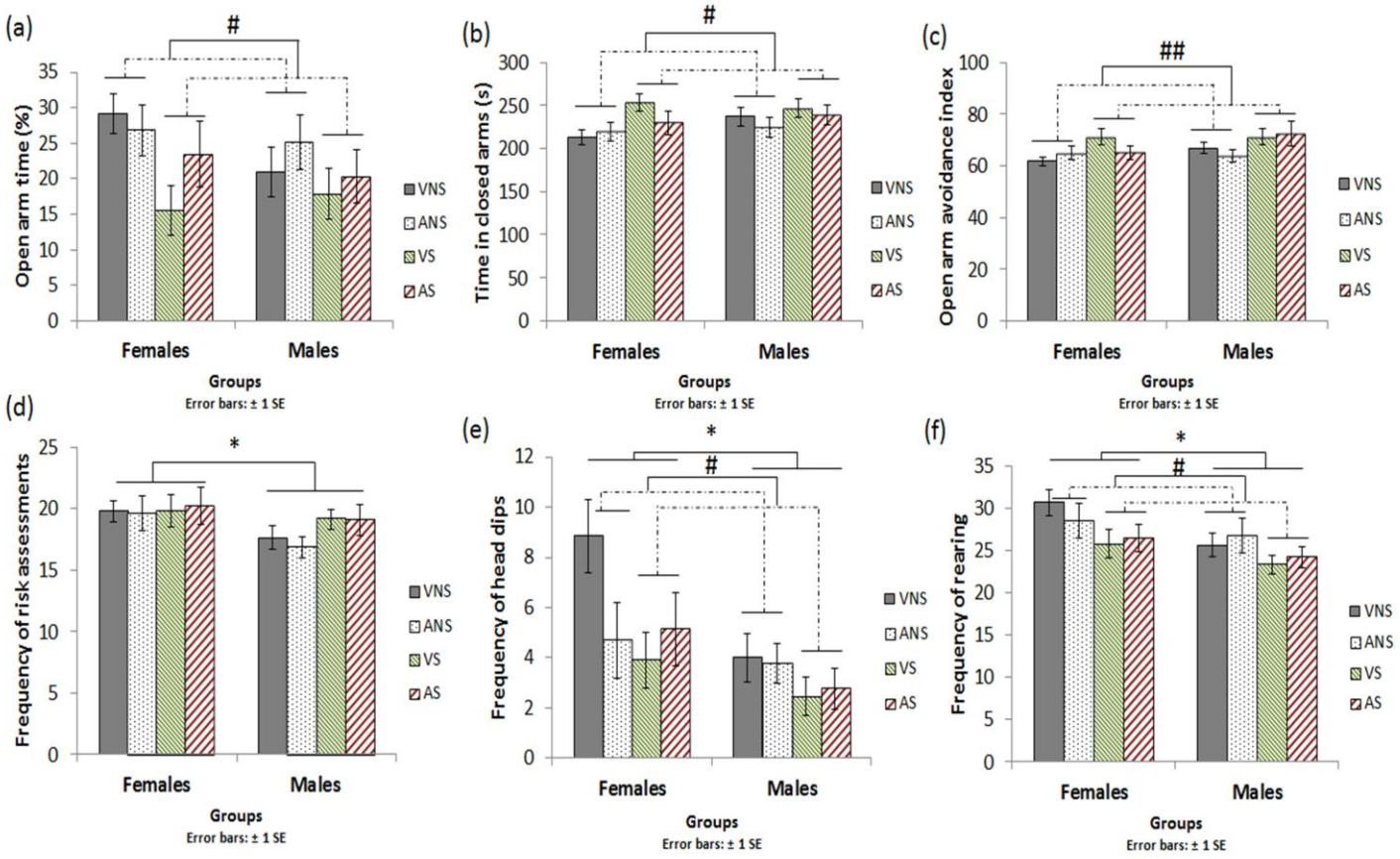


Figure 5. Sex differences and stress on anxiety behavior in the elevated plus maze (EPM). (a) Stress animals spent a smaller percent of time in the open arm compared to non-stress animals (#, $p < .05$). (b - c) Time spent in the closed arms and index of open arm avoidance were elevated in the stress groups relative to non-stress groups (# $p < .05$, ## $p < .01$). (d - e) Increased frequencies of head dips and risk assessments were exhibited by females compared to males (* $p < .05$) and frequency of head dips was higher in non-stress animals compared to stress animals (# $p < .05$). (f) Stress animals showed reduced frequency of rearing than the non-stress animals (# $p < .05$) and males also showed reduced rearing frequencies compared to females (* $p < .05$). No effects of treatment were noted. Data are represented as mean ± SEM.

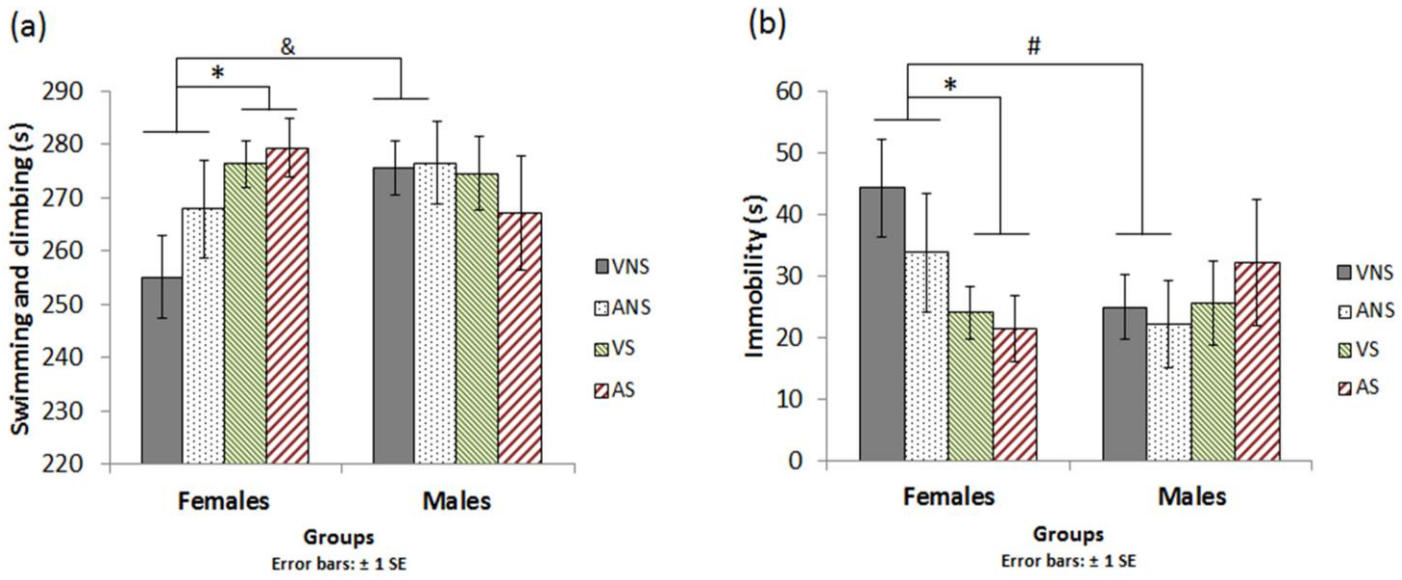
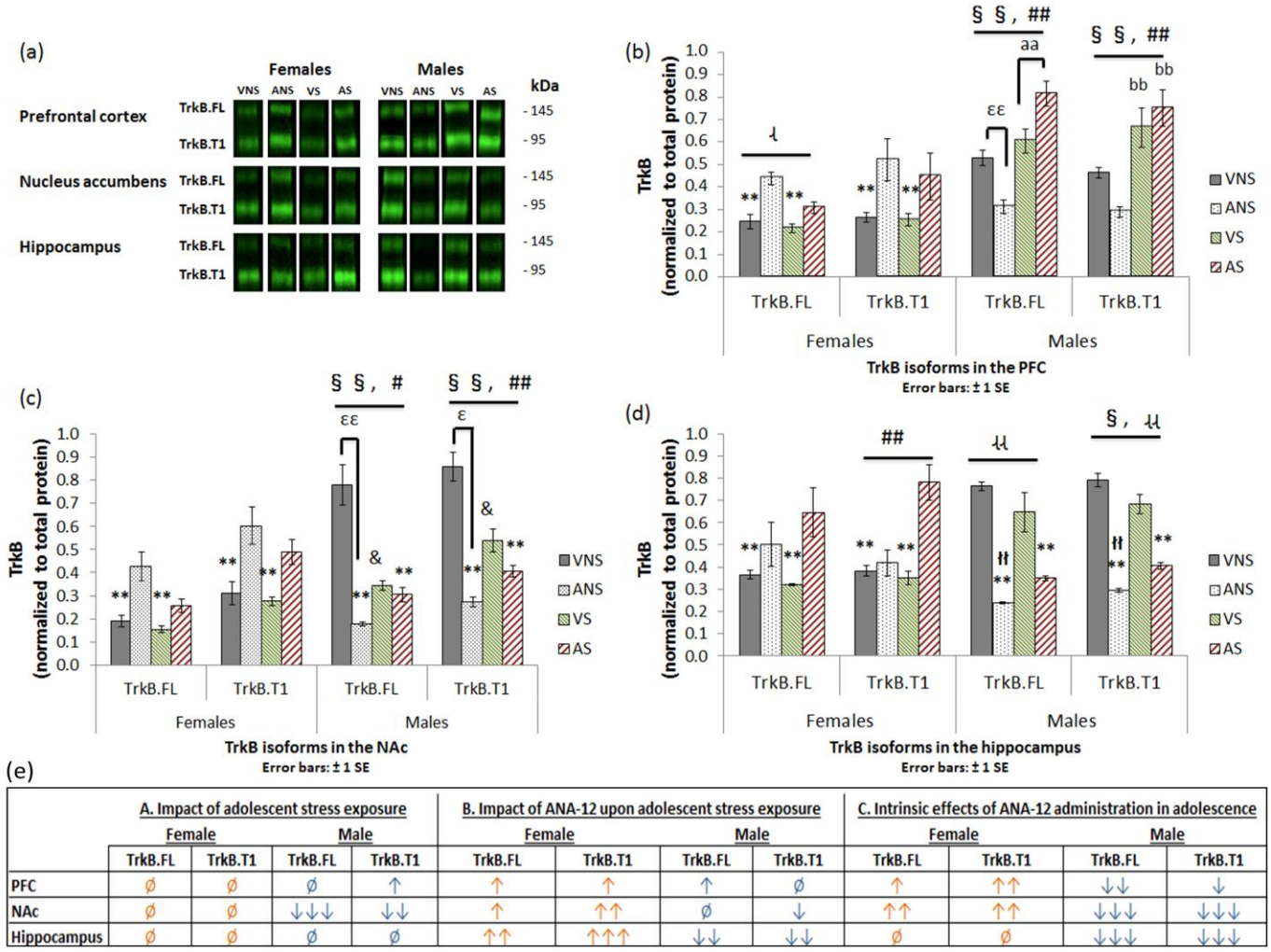


Figure 6. Sex differences and effect of stress on passive coping and stress adaptation in the forced swim test (FST). (a) Non-stress females showed reduced swimming/climbing compared to the stress females (* $p < .05$) and non-stress males (& marginal, $p = .054$). (b) Non-stress females showed increased immobility relative to the stress females (* $p < .05$) and non-stress males (# $p < .05$). Data are represented as mean ± SEM



(e)

	A. Impact of adolescent stress exposure				B. Impact of ANA-12 upon adolescent stress exposure				C. Intrinsic effects of ANA-12 administration in adolescence			
	Female		Male		Female		Male		Female		Male	
	TrkB.FL	TrkB.T1	TrkB.FL	TrkB.T1	TrkB.FL	TrkB.T1	TrkB.FL	TrkB.T1	TrkB.FL	TrkB.T1	TrkB.FL	TrkB.T1
PFC	ø	ø	ø	↑	↑	↑	↑	ø	↑	↑↑	↓↓	↓
NAc	ø	ø	↓↓↓	↓↓	↑	↑↑	ø	↓	↑↑	↑↑	↓↓↓	↓↓↓
Hippocampus	ø	ø	ø	ø	↑↑	↑↑↑	↓↓	↓↓	ø	ø	↓↓↓	↓↓↓

Figure 7. Protein expression of TrkB receptor isoforms in the PFC, NAc and hippocampus. (a) Representative immunoblots (n = 6/group) of average TrkB.FL and TrkB.T1 in male and female mice. Densitometric values were normalized to total protein. (b - d) In the PFC, NAc and hippocampus, males showed higher expression of both TrkB receptor isoforms compared to females (§ $p < .05$, §§ $p < .01$). Increased isoform expression in the PFC and hippocampus was reversed in the NAc of stressed animals (# $p < .05$, ## $p < .01$). ANA-12 treatment reduced the expression of both isoforms in the hippocampus relative to vehicle treatment, however expression of TrkB.FL in the PFC was higher in ANA-12 treated groups (ɻ $p < .05$, ɻɻ $p < .01$). Veh-treated females and ANA-12 treated males showed reduced TrkB.T1 and TrkB.FL compared to veh-treated males and ANA-12 treated females (** $p < .01$). Stress males, irrespective of treatment exhibited higher TrkB.T1 in the PFC compared to stress females and non-stress males (^{bb} $p < .01$). TrkB.FL in the PFC was higher in AS males than VS males (aa $p < .01$). ANS males showed reduced TrkB.FL in the PFC and lower expression of both isoforms in the NAc relative to VNS males (Ɛ $p < .05$, ƐƐ $p < .01$). In the NAc, expression of both isoforms was reduced in VS males compared to VNS males (& $p < .05$). Lastly, ANS groups showed a reduction of both isoforms compared with AS and VNS groups in the hippocampus (ɦ $p < .01$). (e) Schematic representation of the magnitude and direction of changes in TrkB.FL and TrkB.TI expression measured using Western blot in males and females for the different experimental conditions (N = 6/group). *(A)* Impact of adolescent stress exposure independent of drug administration. *(B)* Impact of ANA-12 upon adolescent stress exposure. *(C)* Intrinsic effects of ANA-12 administration in stress-naïve adolescent rats. Arrows designate the direction of significant effects and the number of arrows indicates the magnitude of the protein changes according to the normalized graph scale (1 - 2 fold changes: one arrow; 2 - 4: two arrows; 4 - 6: three arrows). Crossed circles indicate no significant differences in analysis of Western blot. Females are noted in orange and males in blue. PFC: prefrontal cortex; NAc: nucleus accumbens; TrkB.FL: full-length TrkB receptor; TrkB.T1: truncated TrkB receptor; AS: ANA-12 + stress; ANS: ANA-12 + no stress; VS: vehicle + stress; VNS: vehicle + no stress. Data are represented as means ± SEM.

General discussion

1. Brief overview of all chapters

Repeated exposure to different stressors has been shown to have detrimental impacts on brain health, immune activation, cognition, and emotional wellbeing in humans and animals. These disorders have been associated with heightened and prolonged activation of the hypothalamic-pituitary-adrenal (HPA) axis, of which BDNF/TrkB signaling has been implicated in the allostatic load that can severely impact the brain's reward and stress systems and impair response to environmental challenges. This thesis evaluated the endocrine, behavioral and neurochemical effects of selective TrkB inhibition in an animal model of repeated heterotypic stress. In the first two chapters, adult male Wistar rats underwent a unilateral cannula implantation in the right NAc shell to allow for specificity with the direct ANA-12 or vehicle administration. In the third chapter, male and female adolescent Wistar rats received systemic (i.p.) administration of ANA-12 or vehicle. Here, the sex-specific effects of heterotypic stress on neuroendocrine and behavioral changes, in addition to sex differences in the expression of the catalytic full-length TrkB and truncated TrkB receptor isoforms were addressed.

1.1. Summary of findings

The objective of Article 1 was to examine the effects of direct TrkB blockade in the NAc shell and repeated exposure to alternating stressors on endocrine, behavioral and neurochemical changes in adult male Wistar rats. We found that elevations in blood CORT levels post stress exposure were not impacted by ANA-12 pretreatment. However, ANA-12 led to significant attenuations versus increases in anxiety behavior observed in stress and non-stress rats, respectively. Moreover, ANA-12 elevated puncta of vGluT2 and CRH in the PVN and BLA of non-stress rats, while it diminished expression in stress rats. In the cingulate cortex, TrkB receptor blockade had no impact in the no-stress condition whereas stress-induced increase in TrkB expression was normalized by ANA-12. GR expression was influenced by stress exposure, but not by ANA-12 infusion, at this brain locus. In contrast, ANA-12 ameliorated a stress-induced decrease in TrkB-ir in the NAc core, but reduced GR expression. In the NAc shell, both stress groups exhibited increased GR-ir and decreased TrkB-ir, however ANA-12 treatment led to increased TrkB expression in non-stress rats. These findings indicated an influence of

BDNF/TrkB on signaling pathways known to differentially regulate state-dependent emotional responses. It also strengthened contributions of the NAc core and shell portions in regulating coping responses to heterotypic stress exposure. Finally, the observation of ANA-12 effects in non-stress cohorts supports intrinsic role of TrkB receptors in maintenance of homeostasis in mesocorticolimbic regions.

The objective of Article 2 was to investigate the interplay between BDNF signaling, via action of TrkB receptors, on anxiety, social memory and motivation, with a focus on orexin A colocalization with TH and FosB/ΔFosB in the mesolimbic circuitry. Results showed that ANA-12 enhanced sociability in the social interaction test, facilitated place preference, and improved fear retention in the passive avoidance test, but had no effect on social cognition in the social preference test. Noteworthy results include the findings that TrkB antagonism in the NAc shell stimulated an increase in orexin A expression in the PfA and a decrease in the vCA1, independently of stress exposure. ANA-12 also increased orexin A in the VTA of stress animals whereas expression was decreased in non-stress animals. Interestingly, FosB/ΔFosB expression did not show an increase in any of the brain areas examined after 10 days of exposure to stressful stimuli (LH, PfA and dorsal CA1 and CA3 of the hippocampus) and neither did TH-ir. As Article 2 utilized the same subset of animals from Article 1, it stands that these animals underwent an acute forced swim exposure within 2 h of euthanasia and brain collection. This may explain the non-significant differences observed across groups as neuronal activation may occur in all animals following this stimulation. Findings demonstrated a role of BDNF/TrkB signaling in regulating basal and stress-induced changes in social, motivational and anxiety-related behaviors associated with appetitive and aversive stimuli. Furthermore, the relationships between trophic and modulatory systems in the mesolimbic circuitry that maintains behavioral homeostasis and/or enhances susceptibility to affective disorders are noted to be uniquely site-specific.

Given that females are overlooked in research due to influence of hormonal fluctuations on behavior and alterations in neurochemistry, the objective of Article 3 was to examine sex differences and influence of BDNF/TrkB signaling in endocrine changes stemming from heterotypic stress exposure in adolescence, and enduring impact of the stress model on behavioral and plasticity signaling responses in adulthood. Females

showed a greater physiological stress response than males as noted by elevations in CORT levels and increased activity level in the OFT and EPM. Stress-naïve females showed enhanced passive coping in the FST, whereas immobility time was not increased or significantly different in stressed animals of both sexes. Greater focus was relegated to sex and brain regional differences in TrkB receptor isoforms. Notably, ANA-12 led to a reduction in the expression of TrkB.FL and TrkB.T1 in males whereas females showed elevations at the NAc and hippocampus. Relative to controls, ANA-12 also reduced versus increased TrkB.FL at the PFC of non-stress and stress males, respectively. With regards to stress exposure, the NAc differed from the PFC and hippocampus, in that stress down-regulated expression of both isoforms at this brain locus. Though effects of estrous cycles were absent from this study for the assessment of hormonal mediation in behavioral responses, sex dimorphism was noted in the results. Findings demonstrated acute and delayed effects of heterotypic stress exposure in male and female adolescent rats as well as biochemical and plasticity-related responses linked to sex-specific presentation of anxiety phenotype.

The general hypothesis of this thesis states that the anxiolytic and antidepressant actions of ANA-12 can be therapeutic in a preclinical animal model of heterotypic repeated stress, conferring regulation of plasticity processes, stress responses and improvements on altered behavioral impairments. Existing studies on ANA-12 support a role for the TrkB receptor antagonist in attenuating depressive behavior in rodents, reducing inflammatory response in neurons and dendrites, and affording neuroprotection in a mouse model of acute ischemic stroke (Chakravarty et al., 2015; Shirayama et al., 2015; Zhang, Wu, et al., 2015; Zhang et al., 2016).

2. Why is the study of plasticity such a big issue?

Experience-dependent plasticity, at the behavioral level, is key to the acquired learning and memory process, representing a crucial factor in the reorganization of neural activity that allows for vulnerability or resilience. Within the brain, the process of plasticity may be site-specific or extend across cortical areas, coordinating, monitoring and calibrating behavioral and physiological responses to stress (McEwen & Gianaros, 2011). Over the last decade, there has been a substantial increase in articles describing the role of trophic factors and neurotransmitters in the symptomology of depression. Recent advances in translational animal and human

research address the implications of adaptation and pathophysiology resulting from stressful experiences. Of note, allostasis is an essential process for the maintenance of homeostasis, adjustments of biological systems (including the HPA axis, the autonomic nervous system and the immune system) and promotes adaptation when faced with stressful stimuli. However, when allostatic states are prolonged, the normal protection afforded by regulatory systems is lacking, leading to the wear and tear on the body and brain (called allostatic load) (McEwen & Stellar, 1993; McEwen, 2006; McEwen & Gianaros, 2011). The hippocampus has been found in animal models to show remarkable structural plasticity, including dendritic remodelling, neurogenesis, and synaptic connectivity. Animal studies examining the effect of exposure to repeated or chronic stress have reported remodelling of the hippocampal circuitry: suppressed neurogenesis in the subgranular zone of the dentate gyrus, retracted or dendrite branching, and impaired synaptogenesis (Leuner & Gould, 2010; McEwen, 2010). This implicates the role of neurotrophin, particularly BDNF in major aspects of neuronal activity (Park & Poo, 2013). In addition, aberrant neurotrophic signaling due to a defective function of TrkB or downstream signaling cascades have shown importance in the treatment of symptoms. Lastly, the therapeutic potential of BDNF for neurological and psychiatric disorders can be affected by factors including age, sex, and time and location of administration. Notably, sex-specific effects of BDNF expression are not well represented in the female population, affecting the clinical use of preclinical research.

There is a delicate functional balance in the adaptive changes required to protect a system and the vulnerability that may lead to permanent damage. Adrenal steroids represent one of many systems that mediate this balance (McEwen et al., 1995), such that regulation of synaptic and morphological plasticity may be stressor-specific. Following stress exposure that affects systems implicated in plasticity, the question arises whether emergence of functional reorganization via structural remodelling remains possible? Short-lived stress exposure may suppress plasticity mechanisms in the hippocampus for example, leading to cognitive impairments in learning and memory and inducing dendritic atrophy in CA3 pyramidal layers (Lupien & McEwen, 1997; Mcewen et al., 1995), however these changes in morphology and function are reversible. In contrast, it is unclear whether the impact of prolonged or chronic stressors are reversible or whether the system

will adopt a new activation threshold and perform at sub-optimal levels. The latter may mount dysfunctional physiological responses, such as those noted in subjects with post-traumatic stress disorder (Bremner, 2006).

Interestingly, perpetual reorganization has been reported in the hypothalamic PVN (Herman, Flak, & Jankord, 2008). Within this brain locus, exposure to chronic stress downregulated GR, disinhibited $GABA_A$ receptor subunit, heightened glutamatergic signaling and receptor innervation of CRH neurons (Flak, Ostrander, Tasker, & Herman, 2009; Herman et al., 2008). This aggregate response points to structural remodelling occurring in the PVN, which results in a hyper-responsiveness of the HPA axis to incoming stimuli (Herman et al., 2008). Therefore, it is very important to elucidate the mechanisms by which plasticity-related systems such as the hippocampus and PVN respond to acute vs. chronic stressors, as well as which processes would aid in enhancing adaptive capabilities and attenuating damaging responses. Besides pharmaceutical interventions, many approaches to reduce the impact of chronic and repeated stress exposure and attenuate allostatic load, such as implementing changes in lifestyle, improving government policies geared towards a more health-conscious society, considering the context of experiences over the lifespan of the individual and reducing stress burden at the individual level would have a huge influence on modulating the prevalence of disease states.

3. BDNF's functional discrepancy in different sexes: crosstalk with hormones and steroid receptors

Functional heterogeneity in males and females with regards to steroid receptors may impact negative feedback of the stress circuitry, stress responses and sensitivity to hormone secretion, thereby promoting sex biases in stress pathology or coping responses. Therefore, animal studies that assess the functional differences in BDNF/TrkB signaling are crucial in understanding fundamental mechanisms at play that mediate these differential physiological responses. It is found that BDNF content within the hippocampus, amygdala, cortex and ventromedial hypothalamus is elevated in female rats relative to males after stress (Bland et al., 2005; Liu, Zhu, Kalyani, Janik, & Shi, 2014; Snigdha et al., 2011) and the opposite is detected in female mice compared to male mice (Szapacs et al., 2004), suggesting that this effect may be species- and brain-region specific, since in humans, females have significantly higher BDNF concentration in the cortex than males, but gender differences are not observed within the hippocampus (Hayley et al., 2015).

The impact of stress and external stimulus (e.g. environmental enrichment) has been shown to have an influence on BDNF expression. In particular, reduced prefrontal cortex BDNF levels were observed in the post-mortem brains of depressed females, but not males, who died by suicide; whereas within the hippocampus, post-mortem depressed male, but not female, brain samples showed signification attenuations in BDNF levels (Hayley et al., 2015). Interestingly, environmental enrichment is reported to significantly increase BDNF concentrations (Bakos et al., 2009; Vazquez-Sanroman et al., 2013), and in females, this effect was notable in the PFC, hippocampus and hypothalamus relative to males (Bakos et al., 2009; Zhu et al., 2006). Higher BDNF expression in the PFC was shown to alleviate behavioral despair and enhance hedonic capacity in ovariectomized female stress mice treated with estradiol (Karisetty, Joshi, Kumar, & Chakravarty, n.d.). Moreover, the aforementioned increase in BDNF content within the hypothalamus of females may be related to the excitatory effect of elevated BDNF levels in this region to activate and recruit PVN CRH neurons within the HPA axis (Givalois et al., 2004). For instance, dysregulated functioning of GR in the PVN resulted in elevations in the expression of CRH and increased levels of BDNF and TrkB, leading to the subsequent disinhibition of the HPA axis (Jeanneteau et al., 2012). This disinhibition is reportedly triggered by the BDNF-induced blockade of $GABA_A$ receptor surface expression on the PVN (Hewitt and Bains, 2006), suggesting that BDNF plays a role in the maintenance of HPA axis reactivity. Recently, selective deletion of forebrain GR reduced basal and stress-induced CORT secretion in females unlike males, whereas GR deletion increased depression behavior in the forced swim test (FST) and anhedonia in the sucrose preference test (SPT) in males but not in females (Solomon et al., 2012). These suggest that GR deletion has a protective effect against HPA axis hyperactivity and depression-like behaviors in females but caused impairments in GR-mediated negative feedback in males.

It has been suggested that sex hormones can also regulate activities of BDNF (Chan & Ye, 2017). This is substantiated by gender-specific differences in stress responses observed across the lifespan. This has recently been reviewed by Bale and Epperson (2015), alluding to the organizational and activational influence of gonadal hormones (e.g. androgens in males and estrogens in females) and sex chromosome genes in guiding

brain plasticity in the crucial regulatory regions of the mesolimbic circuitry (Arnold, 2009; Bale et al., 2010). Normal brain maturation occurs during the adolescent window, which may increase the vulnerability to adverse environmental stimuli and subsequent stress dysregulation. During puberty, maturity of the stress system occurs as a crosstalk between gonadal and adrenal axes (Bale & Epperson, 2015). In males, the influence of testosterone may lead to a blunted effect on the HPA axis stress reactivity, impacting the GABAergic inhibitory tone of the PVN, whereas in females, cyclicity of estrogen and progesterone led to alterations in sensitivity of glucocorticoid-mediated negative feedback mechanisms, a stimulatory influence on HPA function and modulation of dopaminergic activity. Estrogenic modulation of HPA reactivity is achieved mainly through estrogen receptors (ER) activation at the PVN, of which ERβ is the predominant isoform exerting inhibitory actions on the HPA axis, while ERα is found in stress-responsive areas (e.g. bed nucleus of the stria terminalis, medial preoptic area, and the peri-PVN) that provide indirect GABA inhibitory input to the PVN (Shughrue, Lane, & Merchenthaler, 1997; Suzuki & Handa, 2005). Through ERα-dependent mechanism, peri-PVN areas have been proposed to reduce sensitivity to glucocorticoid-mediated negative feedback, increasing stress sensitivity in female rats (Tasker & Herman, 2011). Taken together, emphasis is on the need to include sex as a variable in animal studies of anxiety and depression, as changes in hormones and neuroplasticity are sex related.

4. Mechanisms that may influence differential responding in juveniles versus adults

To date, existing studies are examining the age-dependent differences in stress pathology, i.e. mechanisms that may be at play to assess why younger rats do not respond like adults. Adversity in early life and neonatal handling can influence the long-term course of age-related cognitive impairments in animals, setting the level of responsiveness of the HPA axis and its peripheral effectors as well as the autonomic nervous system (McEwen, 2006). To start, adolescents show heightened HPA axis reactivity and exhibit an immature feedback system (Romeo, 2003, 2010, 2013; Spear, 2000). Markedly, peak levels of extracellular dopamine in the prefrontal cortex (PFC) and increased levels in the dorsal and ventral striatum have been observed during late adolescence compared to other developmental periods, central to learning and the coupling of salient events (Andersen & Teicher, 2008; Badanich, Adler, & Kirstein, 2006; Davey, Yücel, & Allen, 2008; Pascual, Boix,

Felipo, & Guerri, 2009; Philpot & Kirstein, 2004; Spear, 2000), and may enhance responsiveness to stressful stimuli. Moreover, functional age-related decline related with failure to effectively regulate the HPA axis are observed following the inability of the hippocampus to inhibit the elevated release of excitatory amino acid (EAA) post stress, and heightened post-stress-induced glutamate response in the mPFC of aged vs. young rats (Lowy, Wittenberg, & Yamamoto, 1995). EAA is involved in synaptic plasticity, LTP and memory (Magarin˜os & McEwen, 1995; Woolley & McEwen, 1993), and is also implicated in affective disorders such as schizophrenia (Ułas & Cotman, 1993). Furthermore, CORT return to baseline post stress is slower in some aged animals, and they are hypo-responsive to acute restraint (Buechel et al., 2014; Merrett, Kirkland, & Metz, 2010). In elderly humans, delayed and blunted ACTH response and attenuation of the feedback inhibition of CORT secretion as a result of reduced HPA axis sensitivity are reported (Wilkinson, Peskind, & Raskind, 1997). These findings may suggest that the capacity for resilience is higher in young relative to older rodents, although there are also increased chances of vulnerability to external and internal perturbations at this developmental stage.

5. The puzzle of TrkB isoforms, sex differences and functional impact

BDNF and activation of TrkB signaling pathways are important for the control of developmental processes. However, the presence of TrkB isoforms suggests additional mechanisms that influence a plethora of neurotrophin-induced signaling molecules (Klein et al., 1991; Middlemas, Lindberg, & Hunter, 1991). BDNF is shown to exert trophic effects via phosphorylation of TrkB.FL, effecting downstream signaling cascades. In contrast, it may also promote cell death via binding with the low affinity $P75^{NTR}$ (Chavez-Valdez, Martin, Razdan, Gauda, & Northington, 2014), especially when TrkB.FL is downregulated or not active, or when phosphorylation of TrkB.FL is combined with upregulation of truncated TrkB receptor isoforms (Biffo, Offenhäuser, Carter, & Barde, 1995; Klein et al., 1991; Miller & Kaplan, 2001). To date however, there is still a need to elucidate the function of TrkB receptor isoforms on BDNF actions in the CNS with relation to neurological disorders and stress pathology, as available reports are limited and findings can be inconsistent.

An excellent review was recently put forward by Tejeda and Diaz-Guerra (2017), discussing the relevance of combining actions geared at both BDNF and TrkB targets in order to enhance neurotrophic signaling and further characterize aberrant BDNF/TrkB signaling. Increased replication and additional studies are required, as the therapeutic potential of TrkB receptors are often undervalued. Thus far, studies on TrkB isoforms and stress are few and far between. In the post-mortem brains of suicide victims with previous history of major depression, BDNF levels and that of TrkB.FL and TrkB.T1 receptor isoforms are reduced in the prefrontal cortex (Ernst et al., 2009; Maussion et al., 2012; Pandya, Kutiyanawalla, Turecki, & Pillai, 2014) similar to the effects observed in Methylmercury-exposed wild-type mice, which also extended to increased anxiety and depression behavior (Karpova et al., 2014). Research has shown that each isoform operates on multiple enzymatic and kinase pathways (Fig. 1), which contribute to the difficulty in understanding the functional repercussion of altered expression. For instance, attenuated TrkB.FL impairs E3 ligase c-Cbl-induced stabilization by ubiquitination, whereas diminished TrkB.T1 increased Has-miR-185, a regulator of the truncated TrkB isoform (Maussion et al., 2012; Pandya et al., 2014). The isoform signature on discrete functional changes upon TrkB receptor activation has only emerged in the last decade.

Learning and memory impairments and development of anxiety have been linked to alterations in BDNF expression and/or signaling via TrkB receptor in the hippocampus (Kemppainen et al., 2012; Minichiello et al., 1999; Soliman et al., 2010). Overexpression of TrkB.FL in transgenic mice through activation of the TrkB-PLCγ pathway reduced anxiety and enhanced learning and memory (Koponen et al., 2004), as well as protected against memory deficits and depression behavior (Karpova et al., 2014). However, excess activation has been reported in the induction of epilepsy and destruction of hippocampal neurons (Liu et al., 2013), triggered by heightened and aberrant neuronal excitability. In contrast, overexpression of TrkB.T1 inhibited TrkB.FL signaling through a dominant-negative mechanism (Biffo et al., 1995; Eide et al., 1996), a phenomenon observed in the prefrontal cortex of schizophrenic subjects (Wong, Rothmond, Webster, & Shannon Weickert, 2013) and is shown to affect dendritic morphology (Yacoubian & Lo, 2000). Interestingly, TrkB.T1 deficiency in mice did not significantly impact global brain development and function, however deficiency was reported to

increase anxiety and induce morphological changes in neurite length and complexity in the BLA (Carim-Todd et al., 2009). The predominance of TrkB.T1 signaling in the adult brain highlights the need to investigate its dynamic physiological function *in vivo.*

Studies on sex differences in TrkB isoforms are scarce. Recently, Wong and colleagues (2013) reported a significant reduction in TrkB.FL protein expression in schizophrenia, which was evident in females, however sex-specific changes were not found for the TrkB.T1 receptors, suggesting that changes with this isoform may be more robust and generalized to both genders with schizophrenia. For instance, it has been suggested that increased TrkB.T1 receptors contribute to reduced overall BDNF/TrkB signaling and reduced neuronal plasticity in the DLPFC in schizophrenia, suggesting that therapies aimed at ameliorating neurotrophin deficits may need to consider blocking excessive truncated TrkB function as a means to properly regulate BDNF/TrkB signaling.

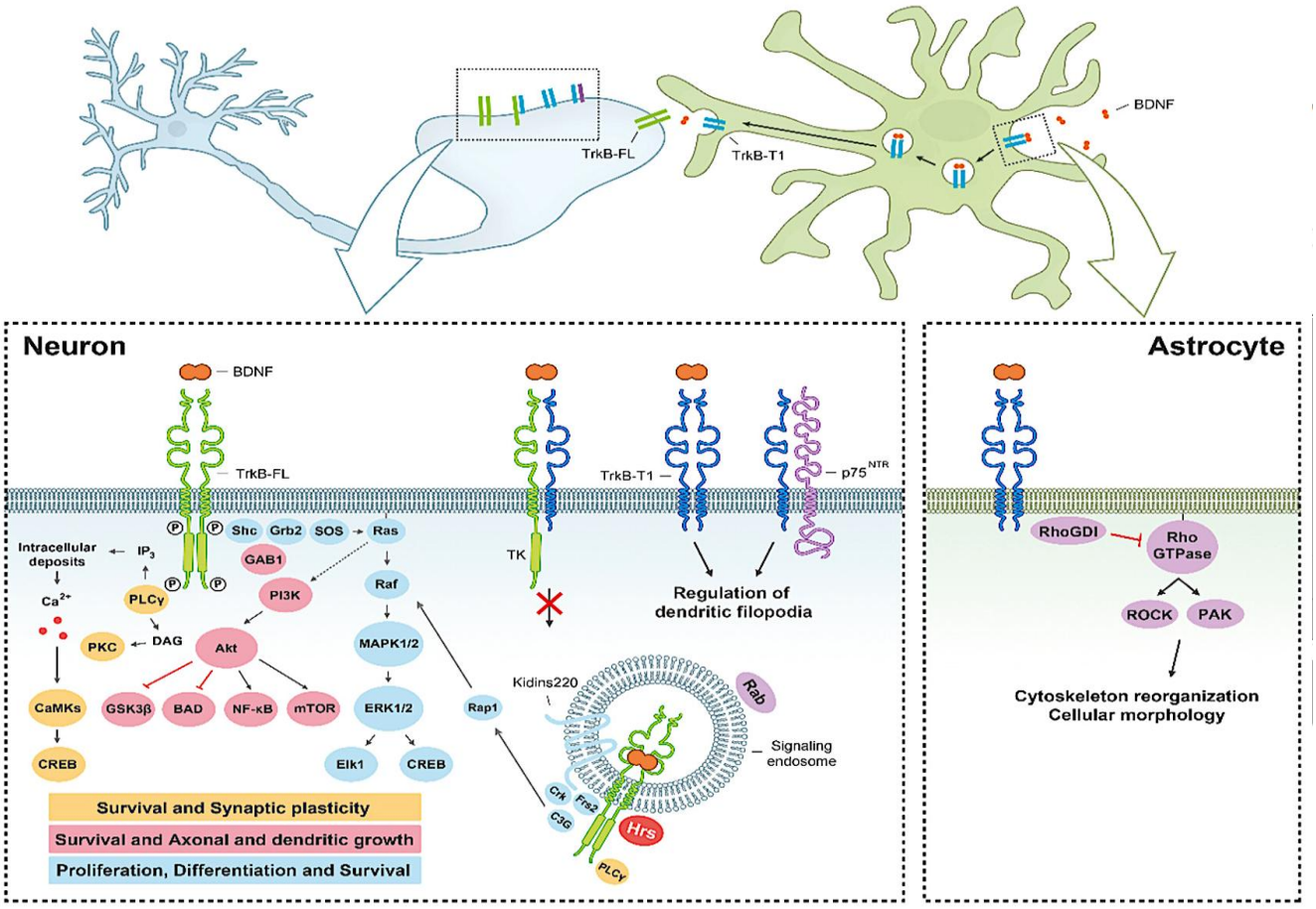


Figure 1. Function of BDNF/TrkB signalling in the CNS. BDNF binding to TrkB-FL in neurons (left diagram) induces receptor homodimerization and activation, triggering three main signalling pathways: MAPK/ERK (blue), PI3K (pink) and PLCγ (yellow), which regulate several processes central to neuronal function. The ligand-receptor complex can internalize and continue functioning in signalling endosomes. Alternatively, TrkB-T1 can form heterodimers with TrkB-FL and block its transduction cascades. TrkB-T1 is also involved in the regulation of local BDNF concentration (upper diagram) and cell morphology, both in neurons and astrocytes (respectively, left and right diagrams). P, phosphorylation sites important for receptor activation. Adapted and reprinted with permission from "Integral characterization of defective BDNF/TrkB signaling in neurological and psychiatric disorders leads the way to new therapies", by G. S. Tejeda and M. Díaz-Guerra, *Int. J. Mol. Sci. 18, 268;* doi:10.3390/ijms18020268.

6. TrkB antagonists and agonists show therapeutic efficacy to attenuate perturbations in different pathologies

At present, one of the best characterized pathologies linked with BDNF is depression, and stress is considered a major risk factor for this disease. As is often alluded, BDNF exerts antidepressant effects within the PFC and hippocampus (Autry & Monteggia, 2012; Berton et al., 2006; Eisch et al., 2003; Hashimoto, 2010; Nestler & Carlezon, 2006; Russo & Nestler, 2013). However, as much as preclinical studies have shown

prophylactic and therapeutic effects of administration of neurotrophins, clinical applications do not show improvements (Ochs et al., 2000; Tejeda & Díaz-Guerra, 2017). BDNF has a poor pharmacokinetic profile that greatly limits its therapeutic potential (Jang et al., 2010). The development of small molecule TrkB agonists (Jang et al., 2010; C. Liu, Chan, & Ye, 2016) and antagonists (Cazorla et al., 2011), or TrkB transactivation by ligands of G-protein coupled receptors, such as glucocorticoids (Mariga, Mitre, & Chao, 2017) provide an alternative therapeutic strategy to BDNF-implicated disorders. For instance, the TrkB agonist, 7,8-dihydroxyflavone (7,8-DHF) which penetrates the blood brain barrier, induces neurotrophic effects on *in vitro* excitotoxic processes (Hu, Cho, & Goldberg, 2010) and has been shown to protect neurons from apoptosis in a TrkB-dependent manner, robustly mimicking BDNF'S biochemical and physiological actions (Jang et al., 2010). Such flavonoid-based agonists are considered as promising compounds to improve neuroprotection (Tejeda & Díaz-Guerra, 2017). As for the small molecule TrkB antagonist, ANA-12, it demonstrates a 2-site mode of action, blocking activation by BDNF non-competitively in high (specific to neurons) and low (TrkB only-expressing cells) affinity sites, also showing selective TrkB receptor binding and inhibition of downstream processes without altering TrkA and TrkC functions (Cazorla et al., 2011).

Preclinical studies report that systemic administration of ANA-12 enhanced TrkB phosphorylation in the NAc of learned helplessness rats (Shirayama et al., 2015) and was shown to reduce METH-induced depression-like behavior and dopamine release as well as improve dendritic changes in the NAc shell (Ren et al., 2015), but showed no effects on BDNF amounts, being unable to increase levels of BDNF protein (Zhang, Yao, et al., 2015). Furthermore, ANA-12 bilateral infusion into the NAc shell of α7 subtype of nicotinic acetylcholine receptor knockout mice (a rat model of inflammation/depression implicated in schizophrenia) induced a rapid antidepressant effect through increased normalization of synaptogenesis and reduced anhedonia in the forced swim, tail suspension and sucrose preference tests (Zhang et al., 2016). These effects were not observed with the TrkB agonist 7,8- DHF or the selective serotonin reuptake inhibitor, fluoxetine. In another study, Shirayama et al., (2015) showed that acute bilateral infusion of 7,8- DHF, but not ANA-12, into the infralimbic part of the mPFC and hippocampus (CA3 and DG regions) promoted antidepressant effects in a rat model of learned

helplessness. Interestingly, at the NAc core and shell, antidepressant effects stemmed from ANA-12 but not 7,8-DHF bilateral injection. These findings indicate that TrkB activation in the mPFC and aforementioned hippocampal regions and TrkB antagonism at the NAc promote antidepressant effects and normalizes alterations in BDNF/TrkB signaling. Both ANA-12 and 7,8-DHF have yet to surpass pre-clinical status and be applied in clinical studies, thus more studies on their roles in various disease states that have aberrant receptor stability and function are warranted. Increasing requirements for effective therapeutic compounds targeting TrkB signaling and its downstream cascades, is also warranted in disease states that can promote excitotoxicity, especially when used in synergy with drugs having upstream actions, in order to attain balance of TrkB.FL/TrkB.T1 isoform relative levels (Tejeda & Díaz-Guerra, 2017). Moreover, possibility has been evoked that the disease process may compromise the receptors isoform properties, which could invalidate or increase difficulty in the study of these biological systems as available markers may not correctly grasp the mechanisms involved at the molecular and systems levels.

At this juncture, studies like ours are necessary, but they only touch the surface of a very complex system. This complexity is related to a great diversity of biological systems that are activated and regulated by TrkB receptors and their downstream pathways, as well as the distinct roles of cell surface signaling by BDNF-bound TrkB receptors and endosomes that bind and internalise BDNF/TrkB complexes (Tejeda & Díaz-Guerra, 2017). Further elucidation is required for signaling cascades that may be activated depending on localization of the TrkB receptor and the various functions on physiological systems. Our findings together with studies assessing receptor activation and blockade support that similar to neuromodulators, such as endocannabinoids, the system might acquire state-specific properties, which are likely to be distinct in basal, stress-related and/or neuropathological conditions.

7. Limitations

There were some limitations to this thesis work. In the first two articles, it would have been informative to measure BDNF, TrkB and phospho-TrkB expression using western blot. It is clear that western blot analyses are more sensitive from a quantitative point of view, compared to the semi-quantitative nature of

immunohistochemistry. Our choice was to investigate the expression and localization of TrkB proteins within the tissue sections, examining their basic characterization via immunohistochemistry. BDNF expression tends to be very low and detection unreliable (i.e. optical density). BDNF detection would have strengthened the conclusions as to its involvement in site-specific regulation of TrkB alterations, while phospho-TrkB could have provided an index of activity-dependent BDNF presynaptic release. In addition, it could have been interesting to assess BDNF fluctuations in the cerebrospinal fluid or in the blood, as BDNF is abundant in the CNS and PNS. BDNF levels in serum or plasma are deemed useful markers for clinical response or improvement of depressive symptoms (Gonul et al., 2005; Karege et al., 2002; Lee & Kim, 2010), and may help to further characterize the state of the neuronal network and antidepressant efficacy of ANA-12 treatment before and after administration. Although CSF measurement would be most interesting, in vivo microdialysis is a challenging technique to implement for collection in sufficient animals to enable the assessment of changes in various brain loci and the impact of stress and gender.

The route of administration (central infusion versus peripheral administration) could appear to affect CORT levels and establish sex-specific differences observed in this thesis. We also note that the testing of females at the same age as males is not representative of the age of psychological development. Females have earlier onset of puberty, so greater attention to the timing and phase of puberty in animal research may increase the comparable relevance of the work to human studies.

A posteriori, the use of the forced swim as a component of the heterotypic stress paradigm biased information concerning depressive behavior at delayed intervals as some animals were naive to the test and others had already experienced it. Although testing acute stress responses in all animals enabled the determination of possible sensitivity and/or resilience in stressed rats compared to 'stress-naive' animals, it rendered interpretation of behavioral changes more difficult (i.e. is reduced immobility and increased swimming/climbing in stressed animals indicative of despair or habituation?).

Caution may be used in interpreting correlational data as all groups were pooled together, thus results do not represent individual group differences. Furthermore, temporal resolution of behavioral testing and

neurochemical detection are low, therefore more sophisticated approaches, e.g. principal component analysis may help to target important factors driving the chemical changes.

Although the battery of tests used in this thesis were selected to measure a broad range of behaviors that are frequently assessed in animal models of mood disorders, exposure may have impacted various brain neurochemistry. Possible remedies to this issue may be to employ dynamic *in vivo* techniques to get real time measurements, such as voltammetry (spike readings), telemetry (for metabolic measurements of glucose utilization, etc.). Other possibility would be to sacrifice the animal right after behavioral testing.

8. Future directions

For future studies, it would be interesting to employ a heterotypic stress paradigm that is longer than the 10 days used in this study. This may induce a more robust effect in anxiety behaviors, and stress and plasticity markers in the hippocampus, NAc, anterior cingulate cortex, amygdala and hypothalamus. Another recommendation would be to expose animals to a reverse light-dark cycle. It is true that rodents perform better during the night (i.e. dark period) under a red light, and that testing during the light "resting" phase under white light may reduce the activity levels of the animals, increasing inhibition and cognitive disruption (Roedel, Storch, Holsboer, & Ohl, 2006). However, testing during the light phase is not uncommon. Furthermore, our behavioral tests, e.g. the SIT, is typically assessed under bright lighting conditions (File, 1980). Thus, we ensured that all animal groups in our study were exposed to similar time of day testing conditions to reduce the degree of confounding effects. Nonetheless, as scientists striving to perform comparable/ translational research, we should aim to adopt the most consistent testing environment and procedures enabling a more meaningful comparison of findings. The adoption of standardized experimental procedures recently received strong support from research reporting a distinguished influence of circadian rhythms in men and women on plasma BDNF levels, of which further assessments may reveal impairments related to disease states (Cain et al., 2017).

Both ANA-12 treated groups (Study 1) exhibited higher bodyweight compared to vehicle controls. This may be related to the well-characterized role of BDNF and TrkB in the central regulation of feeding and bodyweight, such that inhibition of TrkB signaling may lead to alterations in the homeostatic regulatory

circuitry in the hypothalamus and enhance bodyweight (such as is seen with the use of antidepressant treatments in humans). More studies are therefore required to elucidate the mechanisms involved in metabolic issues and hormonal control.

A detailed examination of male- and female- specific differences, particularly in the function of TrkB receptor isoforms linked to physiological stress response, could provide further evidence of hormone-related effects on plasticity and behavior. Furthermore, sex-specific regional differences in the interplay of physiological regulators are intriguing and represent an important area for future research. To our knowledge, there is no data available concerning diffusion profile of ANA-12 or any other molecules of similar size and injection volume, therefore this would be interesting to investigate. Lastly, a detailed comparison of the metabolism rate in brain and periphery following systemic and direct infusion, of not just ANA-12, but other small molecule TrkB antagonists and agonists, would be a valuable addition to the existing literature and would enhance replication of studies to further aid in comparable studies aiming for a move from preclinical to clinical applications.

9. Last word

In conclusion, the set of studies in this thesis provided novel findings helping to characterize BDNF/TrkB plasticity signaling in dopaminoceptive fields. We assessed how blockade of TrkB receptors affects delayed expression of multiple biochemical signals related to stress, anxiety and depression. Importantly, our research revealed that TrkB isoform expression is differentially impacted in male and females, and that changes are not solely defined by stress exposure. Indeed, effects of TrkB receptor blockade in naïve rats support intrinsic effects of its activation under physiologically stable conditions. The findings of this thesis' studies provide evidence of a mechanism of TrkB blockade, using ANA-12, to counteract anxiety and exert changes in molecular correlates 9 days after repeated stress exposure in adult male Wistar rats and 26 days after the stress paradigm in adolescent male and female rats. These observations are novel, since few studies, animal or human, have utilized females to study stress reactivity, and even fewer, have assessed sex differences in TrkB isoform. Despite an overwhelming amount of research on BDNF/TrkB signaling in stress and treatment

strategies for protecting the brain against impaired neuroprotective and neurotrophic function and altered cognitive processes, more research geared towards a clinical approach is required in order to elucidate aberrant neurotrophic signaling in mood disorders. This is especially important, as plasticity-related processes are shown more relevant in the efficacy of antidepressant therapeutic means and in improving cognitive function.

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