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Long-Term Neurobehavioral Consequences of Adolescent Stress Hormone Exposure

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Long-Term Neurobehavioral Consequences of Adolescent Stress Hormone Exposure

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B.S., Neuroscience, Furman University, 2012

Advisor: Shannon L. Gourley, Ph.D.

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Abstract

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By Elizabeth Barfield

Chronic stress during adolescence is associated with negative psychiatric outcomes in adulthood, including increased risk for, and greater severity of, depression and drug use disorders. The neurobehavioral processes that translate developmental stressor exposure into psychiatric vulnerabilities later in life are incompletely understood, but may involve the prefrontal cortex (PFC) and hippocampus, which mature during adolescence. Dysfunction of these cortico-limbic regions can impair the ability to flexibly adjust behavior when environmental contingencies change, resulting in maladaptive habits symptomatic of several psychiatric diseases. In excess, and with prolonged exposure, glucocorticoids released during stress can perturb neuronal morphology and disrupt tyrosine receptor kinase B (trkB) signaling in the PFC and hippocampus. We hypothesize that prolonged exposure to high levels of glucocorticoids (as occurs with chronic stress) during adolescence may produce enduring changes in trkB signaling and cortico-limbic neuronal morphology that underlie persistent impairments in behavioral flexibility. This dissertation first reports that exposure to elevated levels of the glucocorticoid, corticosterone (CORT), during adolescence in mice biases behavior towards inflexible habits in adulthood. These habit biases are associated with disrupted trkB signaling in the ventral hippocampus (vHC), and can be blocked by administration of a putative trkB agonist, as well as recapitulated by viral-mediated disruption of trkB in the vHC of naïve mice. Next, we show that CORT and trkB manipulations during adolescence have sex-dependent long-term effects on behavioral flexibility in an instrumental reversal task that depends on the orbital PFC (oPFC). Lastly, we demonstrate that adolescent CORT-induced impairments in the ability to use outcome-predictive associations to guide behavior extends to aversive circumstances, and are associated with enduring loss of dendritic spines – the primary sites of excitatory synapses – in the oPFC. Adolescent CORT exposure also degrades anatomical connectivity between the vHC and oPFC, necessary for the retention of associative memories that guide flexible, adaptive behavior. Together, these findings implicate a neurotrophin-regulated vHC-oPFC pathway in the enduring behavioral flexibility deficits following prolonged elevation of stress hormones during adolescence. Cortico-limbic dysfunction and associated impairments in outcome-based learning and memory may constitute a pathophysiological mechanism by which stressors during adolescence increase vulnerability to an array of psychiatric diseases.

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CHAPTER 1: PREFRONTAL TRKB AND GLUCOCORTICOID SYSTEMS IN STRESS AND DEVELOPMENTAL CONTEXTS

1.1 CONTEXT, AUTHOR'S CONTRIBUTION, AND ACKNOWLEDGEMENT OF REPRODUCTION

The following chapter reviews the mechanisms by which tyrosine receptor kinase B (trkB) and glucocorticoid receptor (GR) signaling events regulate the density and morphology of dendritic spines, and how the levels and activities of trkB and GR in the prefrontal cortex change across postnatal development, are modulated by stress, and are impacted in depression. This work was conceptualized, researched, organized, and written by the dissertation author, with editorial feedback from Dr. Shannon Gourley. This chapter is reproduced with minor edits from Barfield ET, Gourley SL (in revision) Prefrontal trkB and glucocorticoid systems in stress and developmental contexts. *Neuroscience & Biobehavioral Reviews*.

1.2 ABSTRACT

The tyrosine receptor kinase B (trkB) and glucocorticoid receptor (GR) regulate neuron structure and function and the hormonal stress response. Meanwhile, disruption of trkB and GR activity (*e.g.*, by chronic stress) can perturb neuronal morphology in key cortico-limbic regions implicated in stressor-related illnesses like depression. Further, several of the short- and long-term neurobehavioral consequences of stress depend on the developmental timing of stressor exposure. Here we review how the levels and activities of trkB and GR in the prefrontal cortex (PFC) change during development, are modulated by stress, and are implicated in depression. We review evidence that trkB- and GR-mediated signaling events impact the density and morphology of dendritic spines, the primary sites of excitatory synapses in the brain, highlighting effects in adolescents when possible. Finally, we review the role of neurotrophin and glucocorticoid systems, and their interaction, in stress-related metaplasticity. We argue that a better understanding of the long-term

effects of developmental stressors on PFC trkB, GR, and related factors, may yield insights into risk for chronic, remitting depression.

1.3 INTRODUCTION

Human cortical brain development is a non-linear process, with individual subregions maturing according to temporally distinct trajectories. The prefrontal cortex (PFC) is among the last brain regions to structurally mature (Giedd et al., 1999; Sowell et al., 2001; Gogtay et al., 2004) and undergoes significant synaptic remodeling during adolescence. Following an initial phase of spinogenesis and synaptogenesis during childhood and early adolescence is a protracted period of dendritic and synaptic pruning in the PFC that persists until young adulthood (Rakic et al., 1994; Huttenlocher, 1979; Anderson et al., 1995). Such dramatic structural modification may open a window of vulnerability to insults like stressor exposure or drugs of abuse. Indeed, some evidence suggests that stressor exposure during adolescence can impact long-term behavioral outcomes and psychiatric disease risk. For example, a stress-induced depressive episode in adolescence increases the risk of depression recurrence and treatment resistance throughout life (Thapar et al., 2012).

Adolescence is also a period of vulnerability to the development of neuropsychiatric illness; for example, up to 50% of "adult" psychiatric disorders, such as depression or schizophrenia, initially present during adolescence (Kessler et al., 2005, 2007), and many are triggered by stress. Understanding why adolescence is a period of vulnerability to the development of stress-induced psychopathology may be advanced by examining molecular mechanisms that control dendritic spine dynamics and neuroplasticity. This review examines the literature regarding developmental and stress-induced changes in receptors for two potent modulators of dendritic spines and synaptic plasticity – Brain-derived Neurotrophic Factor (BNDF) and glucocorticoids.

BDNF and signaling through its high-affinity tyrosine receptor kinase B (trkB) are involved in neural development and synaptic plasticity, are implicated in the etiology of major depression, and are differentially expressed and regulated throughout development and between brain regions (Bartkowska et al., 2010; Luberg et al., 2010). Cortisol, which binds to glucocorticoid receptors (GRs) and mineralocorticoid receptors (MRs), is a principal mediator of the stress response, and also regulates brain development and neuronal plasticity (McEwen, 2000; Liston & Gan, 2011). Prolonged exposure to high levels of glucocorticoids (as occurs with chronic stress) can have effects on neuronal architecture that are typically perceived as deleterious (*e.g.*, Wellman, 2001; Radley et al., 2004, 2006; Liston & Gan, 2011).

In the following sections, we review the structural maturation of the PFC during postnatal development (section 1.4) and the impact of stressor exposure on PFC neuronal morphology (section 1.5). Then we review the mechanisms by which trkB- and GR-mediated signaling events regulate dendritic spine densities and morphologies (sections 1.6 & 1.7), and how the expression and activity of trkB and GR in the PFC: change across postnatal development (section 1.8), may be impaired in depression (section 1.9), and are modulated by stress (section 1.10). Finally, the role of BDNF/trkB and GR systems, and their interaction, in stress-related metaplasticity is reviewed in section 1.11. We conclude by discussing the implications of these findings for understanding the pathogenesis of depression (section 1.12).

1.4 STRUCTURAL MATURATION OF THE PFC

Considerable evidence from longitudinal imaging studies indicates that the PFC is among the last brain regions to structurally mature (Giedd et al., 1999; Sowell et al., 2001; Gogtay et al., 2004). In the frontal cortex, gray matter volume increases during childhood and into adolescence, peaking at approximately age 11-12 (Lenroot & Giedd, 2006), then declines steadily until young adulthood

(Giedd et al., 1999; Sowell et al., 1999; Gogtay et al., 2004). Moreover, this cortical gray matter loss progresses in a back-to-front fashion in the frontal lobe, ending with the PFC (Gogtay et al., 2004). Region-specific trajectories of gray matter maturation may be associated with synaptic pruning, increased myelination of intra-cortical axons, or changes in programmed cell death (Huttenlocher, 1979; Juraska & Markham, 2004; Markham et al., 2007). Postmortem histological findings indicate that the general pattern of synaptic changes in the human cortex is similar across regions, with an initial overproduction of synapses peaking in early childhood and synapse elimination continuing throughout adolescence (Huttenlocher, 1997). Nevertheless, the PFC shows the highest degree of dendritic spine proliferation, reaching spine densities in childhood that are twice as high as in adults (Petanjek et al., 2011). Rates of dendritic spine elimination are also slowest in the PFC, with dendritic spine pruning continuing until the third decade of life (Petanjek et al., 2011).

This process of dendritic spine and synapse overproduction, followed by a prolonged period of pruning during adolescence, is conserved across mammalian species (*e.g.*, Gourley et al., 2012a; Bourgeois et al., 1994; reviewed Shapiro et al., 2017b) and is thought to support species-typical adolescent behavior. These changes refine synaptic connections between subregions of the PFC and with other limbic and subcortical structures such as the hippocampus, amygdala, and basal ganglia (by way of thalamic relays). Additionally, axons in the frontal cortex continue to be myelinated during adolescence (Barnea-Goraly et al., 2005; Sowell et al., 2003; Giedd et al., 1999). Synaptic overproduction, pruning, and myelination in the adolescent PFC are thought to enhance the efficiency of communication between regions, optimizing executive functions in the transition to adulthood. "Executive function" refers to a cluster of cognitive processes necessary for goal-directed decision making, working memory, attention, planning, and reasoning. Not surprisingly – considering the protracted structural maturation of the PFC – these cognitive skills continue to

develop throughout adolescence (Blakemore & Choudhury, 2006; Conklin et al., 2007; Best & Miller, 2010).

1.5 STRESSOR EXPOSURE IMPACTS NEURONAL MORPHOLOGY IN THE PFC

Abundant evidence indicates that chronic stress remodels PFC neuronal structure and induces deficits in PFC function. Rodent studies have focused almost exclusively on the medial prefrontal cortex (mPFC) – typically referring to the anterior cingulate, prelimbic (PL), and infralimbic (IL) cortices. These investigations report dendritic retraction on pyramidal neurons (Liston et al., 2006; Dias-Ferreira et al., 2009; Radley et al., 2004) and reduced spine densities following chronic stress (Radley et al., 2006). Stress-induced structural remodeling of mPFC neurons is associated with behavioral impairments in attentional set-shifting (Liston et al., 2006). Chronic exposure to the primary stress hormone corticosterone (CORT; cortisol in humans) in adult rodents also decreases spine densities on mPFC pyramidal neurons (Radley et al., 2008; Liu & Aghajanian, 2008) and leads to apical dendrite atrophy on pyramidal neurons in the mPFC (Wellman, 2001; Cerqueira et al., 2007; Liu & Aghajanian, 2008). Simplification of apical dendrites in the PL cortex has also been reported in adolescent male and female rats exposed to chronic stress beginning in pre-adolescence (Eiland et al., 2012).

Although fewer studies have examined whether morphologic effects persist after stressor exposure ends, many of those that *have* examined long-term effects in the mPFC (PL and/or anterior cingulate cortex) report that, surprisingly, neurons largely recover following a rest period of about 3 weeks (Radley et al., 2005; Bloss et al., 2011). In at least one report, stress-induced alterations in dendritic morphology and spine density in the PL cortex normalized by 10 days post-stress in male rats, and by 7 days in females (Moench & Wellman, 2017). Dendritic spine densities on pyramidal neurons in the IL cortex in mice exposed to chronic CORT in the drinking water also

recovered after a 1-week washout period without CORT (Gourley et al., 2013b). In the PL cortex, dendritic spines on pyramidal neurons were eliminated on the last day of repeated CORT exposure, but were increased (relative to control mice) 3 weeks after CORT in adult mice (Swanson et al., 2013) and normalized in density but dysmorphic in shape long after adolescent CORT exposure (Barfield et al., 2017a). These findings contrast somewhat with those of Anderson et al. (2016), who reported that chronic exposure to CORT (via subcutaneous pellets) induced dendritic spine loss and shrinkage in the PL cortex in rats, and this persisted for 3 weeks. This discrepancy may be attributable to the fact that subcutaneous CORT pellets disrupt diurnal glucocorticoid rhythmicity by clamping CORT at the peak circadian levels, while oral CORT exposure (via the drinking water) leaves circulating CORT at normal or even low levels during the daytime (when mice are sleeping and thus not consuming CORT) (Gourley et al., 2008a; Barfield et al., 2017a). Differences in visualization and quantification methods also likely contribute to discrepancies between studies. For example, Moench & Wellman (2017) visualized layer II/III pyramidal neurons by Golgi-Cox staining. Swanson et al. (2013) utilized a transgenic mouse line expressing GFP in layer V cortical pyramidal neurons (Feng et al., 2000) and characterized dendritic spines on pyramidal neurons in layer V. Meanwhile, Anderson et al. (2016) used intracellular dye injections and characterized dendritic spines in layers II, III and V.

In another subregion of the PFC, the orbital PFC (oPFC), chronic stress induces hypertrophy of apical dendrites on layer II/III pyramidal neurons (Liston et al., 2006; Dias-Ferreira et al., 2009), but dendritic spine loss (Xu et al., 2016a) and depressive-like behaviors and decreased post-synaptic density-95 (PSD-95) and kalirin-7, proteins essential for spine function, stabilization, and maturation (Ehrlich et al., 2007; Ma et al., 2003). Gourley et al. (2013b) also reported that chronic CORT reduces dendritic spine counts on neurons in deep-layer oPFC and suggested that stress-induced dendrite elaboration in the oPFC may be a protective response to stress.

The PFC may be especially vulnerable to long-term structural alterations due to stress experienced during postnatal development. For example, male rats exposed to 5 days of footshock stress in pre-adolescence (postnatal day (P) 21-25) display despair-like behavior in the forced swim test (FST) in adulthood, as well as decreased cortical thickness in the PL and IL cortices and reduced dendritic spine densities and dendrite lengths on pyramidal neurons in the IL cortex (Lyttle et al., 2015). Notably, these morphological alterations are evident >11 weeks after the last footshock stressor, and are blocked by the selective serotonin reuptake inhibitor (SSRI), fluvoxamine. Fluvoxamine also blocked stress-induced depressive-like behavior. In line with these findings, social isolation during early adolescence (P30-35) reduces synaptophysin, a pre-synaptic marker, in the IL cortex of adult rats (Leussis et al., 2008b). Synaptophysin loss was blocked by MK-801 (an NMDA receptor antagonist) or adinazolam (a benzodiazepine derivative) in adolescence (P40-55), suggesting that dampening glutamatergic activity may be an effective strategy in mitigating some of the enduring neurobehavioral effects of social adversity.

The oPFC also appears to be vulnerable to long-term effects of stress or glucocorticoid exposure in adolescence. For example, chronic exposure to CORT in rodents' drinking water during adolescence (P35-56) induces anhedonic-like behavior in adulthood and loss of dendritic spines in hippocampal CA1 and on deep-layer pyramidal neurons in the oPFC and IL cortex. Spine densities in the basal amygdala also increase (Gourley et al., 2013b). However, following a 1-week washout period (when rodents are returned to regular drinking water following the cessation of CORT), dendritic spine densities in all regions normalized, except in the oPFC. Thus, stress-related structural modifications of the oPFC can persist beyond the period of stressor or glucocorticoid exposure, and may be associated with some of the long-lasting behavioral consequences of adverse experiences in adolescence.

Consistent with this notion, the oPFC is implicated in adolescent-emergent Major Depressive Disorder (MDD) and vulnerability to psychopathology following early-life adversity. For example, adolescents with MDD exhibit hypoactivity of the oPFC during reward-related decision-making tasks (Shad et al., 2011), and early-life adversity is associated with reduced gray matter volume (Hanson et al., 2010; De Brito et al., 2013; Dannlowski et al., 2012) and cortical thickness (McLaughlin et al., 2014; Lim et al., 2017) in the oPFC. And, decrements in oPFC gray matter following childhood maltreatment may mediate vulnerability to depression (Edmiston et al., 2011). Ansell et al. (2012) found that an interaction between cumulative adverse events over the lifetime and greater subjective experience of chronic stress is associated with less gray matter volume in the oPFC. Thus, stress-related changes in oPFC volume may increase risk for developing psychopathology in the face of adverse life events.

Glucocorticoids and neurotrophins are key regulators of dendritic spine plasticity, and their *dysregulation* is associated with synapse loss and stress-related psychopathology. The actin cytoskeleton forms the structural lattice of dendritic spines. Because dendritic spines are the primary sites of excitatory synaptic transmission, their structure and function are intimately related, and disrupting cytoskeletal dynamics can have profound effects on spine structure, communication between neurons, and ultimately, on behavior (Licznerski & Duman, 2013; Wong et al., 2013). The filamentous (F-) actin meshwork within dendritic spines is constantly being polymerized and depolymerized by an array of regulatory proteins. The long-term maintenance of spines requires a balance of actin polymerization and depolymerization. Synaptic scaffolding proteins and pre- and post-synaptic adhesion systems also help provide structural support for spines. Activity-dependent neurotrophin signaling strengthens and helps maintain active synapses by activating spine stabilization pathways. These pathways are also targeted by glucocorticoids, which are necessary for spine development and maintenance. However, chronic stress or excess glucocorticoid levels lead to

dendritic spine loss, at least in part, by GR-mediated disruption of the molecular mechanisms that stabilize spines. These topics will be discussed in further detail in the next sections.

1.6 BDNF-trkB REGULATION OF DENDRITIC SPINES

BDNF signaling through trkB is involved in the development, maintenance, and plasticity of synapses throughout life. The trkB receptor exists in two forms – a full-length form (designated trkB.FL) and a truncated form (designated trkB.T1). TrkB.FL is expressed on neurons, while TrkB.T1 is expressed on both neurons and glia (Ohira & Hayashi, 2009). The truncated receptor contains the same extracellular and transmembrane domains and initial 12 intracellular amino acid sequences as the full-length receptor, but lacks the tyrosine kinase intracellular domain (Middlemas et al., 1991). Upon ligand binding, the full-length trkB receptor dimerizes and is autophosphorylated at tyrosine residues in the intracellular domains, initiating mitogen-activated protein/extracellular signal-regulated kinase (MAPK/ERK), phosphatidylinositol-3-kinase (PI3K), and phospholipase C gamma (PLCγ) signaling cascades (Reichardt, 2006; Carvalho et al., 2008). Lacking the tyrosine kinase-containing domain, TrkB.T1 is unable to activate these signaling cascades; however, emerging evidence suggests that this isoform may have distinct functions via alternative signaling pathways (see for further discussion, Ohira & Hayashi, 2009).

TrkB.FL-mediated signaling regulates the structural and functional plasticity of dendritic spines through effects on gene expression, neurotransmitter release, trafficking of synaptic proteins, and activities of membrane receptors and actin cytoskeleton regulatory proteins. These effects are mediated by the MAPK/ERK, PI3K, and PLCγ signaling cascades, which regulate overlapping but also distinct molecular processes. These pathways have been extensively reviewed elsewhere and are beyond the scope of this review (Reichardt, 2006), but here we briefly discuss the pertinent findings.

Phosphorylation at Tyr785 on trkB.FL leads to the recruitment and activation of PLCγ, which hydrolyses phosphatidyl inositides to generate diacylglyercerol (DAG) and inositol 1,4,5-trisphosphate (IP3). DAG activates protein kinase C (PKC), while IP3 stimulates the release of Ca2+ from intracellular stores, increasing the activation of Ca2+/calmodulin-dependent protein kinases and other Ca2+-regulated targets, such as adenylyl cyclase (AC) and the transcription factor cyclic adenosine monophosphate (cAMP) response element binding protein (CREB) (Minichiello, 2009; Yoshii & Constantine-Paton, 2007). TrkB signaling through PLCγ also activates the plasma membrane transient receptor potential canonical subfamily 3 (TRPC3) channel that generates sustained cationic currents at synapses (Amaral & Pozzo-Miller, 2007).

TrkB.FL phosphorylation at Tyr490 in humans (Tyr515 in mice) recruits and phosphorylates the adaptor protein, Src homologous and collagen-like protein (Shc). Activated Shc recruits a complex consisting of the adaptor Growth factor receptor-bound protein 2 (Grb2) and the Ras exchange factor son of sevenless (SOS), which activates Ras. Upon activation by Ras, the protein kinase Raf phosphorylates mitogen-activated/extracellular signal-regulated kinase 1/2 (MEK1/2), which subsequently phosphorylates ERK1/2 (English et al., 1999). ERK1/2 influences protein synthesis-dependent plasticity by activating CREB (Ying et al., 2002; Shaywitz & Greenberg, 1999) and enhancing signaling through mammalian target of rapamycin (mTOR), a kinase that regulates protein translation and long-term synaptic changes. Ras homologue enriched in brain (Rheb), an activator of mTOR, is normally inhibited by the tuberous sclerosis complex 1/2 (TSC1/2) of proteins (Dwyer & Duman, 2013). Activated ERK1/2 phosphorylates and inhibits TSC1/2, thereby activating mTOR.

Phosphorylation of Shc at Tyr515 of trkB.FL also leads to PI3K activation through Ras-dependent and -independent pathways. Ras-dependent activation of PI3K occurs via Shc/Grb2/SOS/Ras. Ras-independent activation involves Shc/Grb2 and subsequent recruitment of

Grb2-associated binding protein 1 (Gab1), which binds and activates PI3K (Holgado-Madruga et al., 1997). Phosphoinositides generated by PI3K and phosphoinositide-dependent kinases cooperatively activate the protein kinase Akt (also called Protein kinase B). Akt enhances mTOR-mediated protein translation by phosphorylating TSC1/2. Signaling through PI3K/Akt also regulates the trafficking of synaptic proteins (Yoshii & Constantine-Paton, 2007).

BDNF and trkB.FL are expressed in both pre- and post-synaptic compartments of synapses. BDNF is rapidly released from both compartments in an activity-dependent manner (Hartmann et al., 2001; Kohara et al., 2001; Kojima et al., 2001). The activation of trkB.FL on pre-synaptic sites by BDNF released from post-synaptic sites leads to stabilization of the pre-synaptic site (Lin & Koleske, 2010). Likewise, activation of trkB.FL on post-synaptic sites by pre-synaptic BDNF release contributes to stabilization of the post-synaptic site. Activity-dependent secretion of BDNF is one mechanism by which active synapses are selectively modulated (Snider & Lichtman, 1996; Boulanger & Poo, 1999). However, because BDNF is a diffusible molecule, additional mechanisms to achieve synaptic specificity of activity-dependent plasticity likely exist. Indeed, the second messenger, cAMP, regulates synaptic responses to BDNF. Specifically, Ji and colleagues (2005) report that cAMP gates BDNF-induced trkB phosphorylation and enhances the mobilization of trkB into synapses by facilitating the association of trkB with the glutamate receptor scaffolding protein PSD-95. Enhanced localization of trkB at the post-synaptic density is thought to potentiate the response to BDNF (Meyer-Franke et al., 1998). Synaptic activity increases intracellular cAMP levels – by enhancing Ca2+ influx and activation of Ca2+-stimulated AC (Wang et al., 2003) or through activation of AC by neurotransmitter-stimulated G protein-coupled receptor (GPCR) signaling. Thus, activity/cAMP-dependent recruitment of trkB to stimulated synapses and activity-dependent release of BDNF selectively potentiates BDNF-trkB signaling at active synapses.

BDNF-trkB-mediated signaling is involved in the induction, maintenance, and consolidation phases of synaptic long-term potentiation (LTP; Park & Poo, 2013; Panja & Bramham, 2014), a critical component of activity-dependent synaptic strengthening. For example, BDNF activation of trkB enhances pre-synaptic glutamate release (Gottschalk et al., 1998; Pereira et al., 2006; Matsumoto et al., 2006) by trkB-MAPK/ERK-mediated phosphorylation of the synaptic vesicle protein synapsin I, which modifies the interaction of synaptic vesicles with the actin cytoskeleton to facilitate exocytosis and neurotransmitter release (Jovanovic et al., 1996, 2000). Enhancement of glutamate release by BDNF also occurs via trkB-PLCγ-induced release of Ca2+ from pre-synaptic intracellular stores, which increases the number of docked synaptic vesicles for exocytosis (Numakawa et al., 2002, 2009).

BDNF-trkB is also involved in post-synaptic mechanisms of LTP. Several studies have shown that BDNF modulates the activity and trafficking of ionotropic glutamate receptors (Caldeira et al., 2007a,b). For example, BDNF induces translocation of the GluR1 subunit of AMPA receptors (Nakata & Nakamura, 2007) and GluA1-containing AMPA receptors (Fortin et al., 2012) to the postsynaptic membrane, and this depends on trkB-PLCγ-induced Ca2+ signaling. Furthermore, BDNF enhances NMDA-mediated synaptic currents in a trkB-dependent manner (Kolb et al., 2005) and augments the probability of NMDA receptor channel opening (Levine et al., 1998). This effect is mediated by the protein tyrosine kinase Fyn, which, when activated by trkB (Iwasaki, 1998), phosphorylates the NR2B subunit of the NMDA receptor (Xu et al, 2006). BDNF-trkB signaling also increases AMPA and NMDA receptor components (Caldeira et al., 2007b) and synaptic proteins needed to support increased synaptic activity (Kumamaru et al., 2008; Matsumoto et al., 2006) through the MAPK/ERK and PI3K-Akt-mTOR pathways.

BDNF signaling through trkB is also linked with the structural alterations associated with LTP, including the formation of nascent spines and the enlargement of existing spines. BDNF

stimulation of trkB increases dendritic spine density in the hippocampus (Tyler & Pozzo-Miller, 2001; Amaral & Pozzo-Miller, 2007), and this process is dependent on MAPK/ERK1/2 activation (Alonso et al., 2004) and activation of TRPC3 channels by the trkB-PLCγ pathway (Yoshii & Constantine-Paton, 2010). BDNF also modulates spine morphogenesis in the presence of synaptic activity (Tanaka et al., 2008) through trkB-mediated signaling events, which promote the transformation of immature spines into stabile mushroom spines. For example, activity-dependent secretion of BDNF and subsequent stimulation of trkB-PI3K-Akt signaling facilitates the trafficking of PSD-95 from the soma to dendrites and synapses (Yoshii & Constantine-Paton, 2007). Subsequent work suggested that BDNF increases synaptic PSD-95 by enhancing microtubule invasion into spines (Hu et al., 2011), since microtubules can serve as highways for delivery of synaptic proteins. PSD-95 functions as a scaffold protein at the post-synaptic membrane, binding glutamate receptors, adhesion molecules, cytoplasmic signaling enzymes, and cytoskeletal regulatory elements required to support the structural and functional properties of active, stabile spines. For example, PSD-95 binds kalirin, a Rho guanine nucleotide exchange factor (GEF) that activates Rac1 to promote actin polymerization and spine stability (Ma et al., 2003). Thus, enhanced trafficking of PSD-95 to spines following BDNF stimulation of trkB likely facilitates stabilization of nascent spines or activity-dependent growth or existing spines.

Additional mechanisms by which BDNF-trkB impact activity-dependent spine remodeling involve regulation of proteins that control actin cytoskeleton dynamics. These include the Rho family of small GTPases, such as RhoA, Rac1, and Cdc42 and other small GTPases. For example, trkB-mediated activation of the Rac-GEF, Tiam1, can act synergistically with pathways downstream of NMDA receptor activation to stabilize spines (Rex et al., 2007). Phosphorylation of Tiam1 by trkB following stimulation by BDNF (Miyamoto et al., 2006) or by CAMKII following NMDA receptor activation (Tolias et al., 2005) activates Rac1, which promotes F-actin dynamics through

activation of p21-activated protein kinase (PAK). PAK phosphorylates LIM motif-containing protein kinase 1 (LIMK1), which phosphorylates and thereby inactivates the actin depolymerizing factor, cofilin (Lai & Ip, 2013). Inactivation of cofilin allows for actin polymerization and spine growth. Moreover, the interaction of trkB and Tiam1 requires the phosphorylation of trkB at S478 by cyclin-dependent kinase 5 (Cdk5) (Cheung et al., 2007). And, Cdk5-mediated phosphorylation of trkB is required for activity-dependent spine remodeling (Lai et al., 2012). TrkB activation also increases cortactin localization to spines (Koleske, 2013). Cortactin, an F-actin binding protein, stabilizes and promotes branching of actin filaments.

1.6.1 Structural studies using mutant *Bdnf/Trkb* mice

Forebrain-specific loss of *Bdnf* and *Trkb* leads to similar outcomes in the mouse brain, including dendritic spine loss and simplification of dendrite arbors (Gorski et al., 2003; Xu et al., 2000), suggesting that BDNF acts via trkB in the healthy brain to promote spine and dendrite stability. (Here, "stability" refers to the process by which dendritic spines and dendrites *not* destined for pruning are retained.) Mice with early-onset forebrain-specific knockdown of *Trkb* in cortical pyramidal neurons (trkB loss occurs between P14 and P28) exhibit dendritic retraction as early as P28, followed by neuronal loss between P42 and P72 (Xu et al., 2000). TrkB-deficient neocortical neurons transplanted into the cerebral cortices of wild-type mice similarly display impaired dendritic growth and reduced neuronal survival (Gates et al., 2000). Furthermore, blocking trkB-mediated signaling with 1NaPP1 from P21-31 in mice expressing a *Trkb* mutant that is sensitive to chemical inactivation reduces dendritic spine densities on apical dendrites of layer II/III excitatory neurons in primary sensory cortex at P31 (Arango-Lievano et al., 2015).

In mice with early-onset forebrain-specific knockdown of *Bdnf*, pyramidal neurons develop normally until about P21, when neurons begin to shrink and dendritic arbors atrophy (Gorski et al.,

2003). Because the structural atrophy of neurons in *Bdnf* knockdown mice occurs during the period when BDNF in the cortex normally increases (P21-35, reported by Gorski et al., 2003; but see discussion in section 1.8.1), these findings hint at an essential role for developmentally-typical elevations in BDNF in the maintenance of neuronal morphology. Moreover, adult-onset forebrain-specific knockdown of *Bdnf* in mice (with progressive knockdown beginning at P21 and peaking by P56) does not impact dendritic spine density at P35, but results in a 30% loss of layer II/III dendritic spines in visual cortex at P84 (Vigers et al., 2012). This reduction in spine density following progressive loss of BDNF in adolescence and adulthood suggests that continued BDNF signaling is required for the ongoing stabilization of cortical dendritic spines.

1.7 GLUCOCORTICOID REGULATION OF DENDRITIC SPINES

Activation of the hypothalamic-pituitary-adrenal (HPA) axis by stressor exposure stimulates the synthesis and release of glucocorticoids, which bind to steroid hormone receptors throughout the body and brain to initiate a cascade of behavioral and physiological changes that enable an organism to effectively cope with stress. Glucocorticoids regulate the termination of the stress response through negative feedback at the level of the hypothalamus and pituitary, as well as other brain regions such as the PFC, hippocampus, and amygdala. Glucocorticoids act by binding to type I mineralocorticoid receptors (MR) and type II glucocorticoid receptors (GR). GRs are present on neuronal cell bodies, dendrites, and dendritic spines, and they regulate gene transcription upon activation by ligand binding. GRs also localize to plasma membranes, where they mediate intracellular signaling via non-genomic mechanisms.

Glucocorticoids exert biphasic effects on dendritic spine density and morphology; the dose and duration of exposure critically determine the degree and type of neuronal remodeling. In healthy animals. For example, acute GR blockade reduces dendritic spine head diameters on pyramidal

neurons in deep-layer mPFC, suggesting that GR tone is important for the maintenance of large, synapse-containing spines (Swanson et al., 2013). But, insufficient or excessively high levels of GR activity can have detrimental effects on neuronal morphology and behavior, in part by regulating trkB expression and activity.

Although the mechanisms underlying the modulation of spine structure by glucocorticoids remain incompletely understood, rapid effects are thought to involve glucocorticoid-induced regulation of the actin cytoskeleton. For example, acute CORT application rapidly (within 1 hr) increases spine density and spine head diameter in adult rat hippocampal organotypic slice cultures in a GR- and NMDA receptor-dependent manner (Komatsuzaki et al., 2012). Moreover, CORT-induced spinogenesis is abolished by inhibiting MEK1/2, protein kinase A (PKA), PKC, or PI3K. Phosphorylation of actin-binding proteins by these kinases can regulate spine morphology by influencing actin cytoskeleton dynamics (Lin & Koleske, 2010). For example, ERK1/2 phosphorylates cortactin, which stabilizes and promotes branching of actin filaments. CORT rapidly increases phospho-ERK1/2 (p-ERK1/2; *i.e.*, active ERK1/2) in PC12 cells (Qiu et al., 2001), and activation of GR by the synthetic glucocorticoid dexamethasone (DEX) has the same effect in hippocampal slice cultures (Jafari et al., 2012). Jafari et al. (2012) also report that acute DEX rapidly increases levels of phosphorylated cofilin in whole hippocampal homogenates, but reduces phosphorylated cofilin in hippocampal CA1 dendritic spines. Cofilin severs filamentous actin in its active, de-phosphorylated state, allowing for actin polymerization and depolymerization. Thus, a spine-selective *loss* in p-cofilin (as following DEX treatment) may permit spine remodeling.

GRs can modulate glutamatergic and BDNF-mediated neurotransmission in a ligand-dependent and -independent fashion, respectively, which would be expected to impact dendritic spine morphology and plasticity. Numakawa et al. (2009) suggest that the GR functions as an adaptor protein, forming a complex with trkB at the membrane. This trkB-GR interaction is critical

for BDNF-stimulated glutamate release via activation of trkB/PLCγ signaling. Notably, in cortical cultures, BDNF/trkB/PLCγ-stimulated glutamate release was enhanced by overexpression of GR and reduced by GR down-regulation. GRs also modulate synaptic transmission when bound by acute stress-elicited or exogenous glucocorticoids (Popoli et al., 2012). For example, acute stress rapidly enhances stimulus-evoked glutamate release from pre-synaptic terminals in the PFC through a GR-dependent mechanism that involves an increase in the readily releasable pool of synaptic vesicles (Musazzi et al., 2010). Additionally, acute stress or CORT increases the insertion of AMPA and NMDA receptors into the post-synaptic membrane in the rat PFC, potentiating the response to glutamate (Yuen et al., 2009, 2011). Glucocorticoids can also enhance glutamate neurotransmission indirectly by recruiting endocannabinoid signaling (Hill et al., 2011). Thus, acute stressor exposure, through GR-mediated mechanisms, enhances synaptic transmission, an initial step in LTP induction.

Recently, Liston and Gan (2011) reported that a low dose of CORT enhances dendritic spine turnover within several hours of application in the cortex of adolescent and adult mice, increasing spine formation and elimination to a similar degree. In addition, circadian glucocorticoid oscillations regulate spine plasticity, and these modifications are associated with learning and memory (Liston et al., 2013). Specifically, learning-associated changes in spine formation and elimination were assessed using transcranial two-photon imaging of pyramidal neurons in the motor cortex of mice before and after rotarod training. Circadian glucocorticoid peaks enhanced spine formation after motor skill learning through a non-genomic, GR-dependent mechanism involving phosphorylation of LIMK1 and cofilin. Circadian glucocorticoid troughs promoted the stabilization of a subset of newly formed learning-associated spines and the elimination of a subset of pre-existing spines through an MR-dependent transcriptional mechanism.

Glucocorticoids are also necessary for normative dendritic spine maturation during adolescence. Administration of an MR antagonist at P30 in mice reduces rates of spine formation

and elimination in the developing adolescent barrel cortex, suggesting that MR binding is critical for spine remodeling during adolescent development (Liston & Gan, 2011). Further, GR antagonism reduces spine formation rates only, revealing an important role for GR binding in adolescent spine formation. Similarly, shRNA-mediated knockdown of *Gr* throughout development decreased the density of immature thin spines on apical dendrites in primary sensory cortex of mice at P30 (Arango-Lievano et al., 2015).

Prolonged exposure to elevated glucocorticoids and excessive GR binding is associated with synaptic deficits in the cortex. In contrast to the rapid enhancement of spine turnover in the somatosensory cortex by acute CORT exposure, where rates of spine formation and elimination were roughly equivalent, repeated CORT exposure during adolescence (P30-40 in mice) does not affect spine formation, but significantly increases spine elimination (Liston & Gan, 2011). Notably, acute CORT exposure increases the elimination of recently formed spines, while chronic CORT results in the loss of both recently formed spines and stable spines established early in development (Liston & Gan, 2011, Liston et al., 2013). This loss of stably maintained spines suggests that prolonged exposure to excess glucocorticoids may disrupt mechanisms of spine stability.

Chronic glucocorticoid exposure down-regulates BDNF and trkB, critical for dendritic spine stabilization and maintenance. Chronic exposure to elevated CORT in rodents decreases BDNF protein in mPFC (Gourley et al., 2012b), *Bdnf* mRNA in oPFC (Gourley et al., 2009a), *Bdnf* exon IV mRNA (associated with activity-dependent transcription) in PFC (Dwivedi et al., 2006b), trkB protein in frontal cortex (Kutiyanawalla et al., 2011) and mPFC (Gourley et al., 2012b), and *Trkb* mRNA in PFC (Kutiyanawalla et al., 2011). Prolonged CORT exposure also reduces signaling proteins downstream of trkB, including components of the PI3K-Akt-mTOR pathway, in frontal cortex (Howell et al., 2011). Additionally, NMDA (GluN2B) and AMPA (GluR2/3) receptor subunits are decreased in PFC following chronic CORT exposure (Gourley et al., 2009a). The

effects of chronic glucocorticoid exposure on BDNF-trkB are discussed in more detail in section 1.10.1.

Notably, trkB modulates GR function via phosphorylation of GR at BDNF-sensitive sites, and this process is critical for cortical spine maturation and neuroplasticity in response to stress and antidepressant treatment. Arango-Lievano et al. (2015) report that BDNF stimulation of trkB and downstream activation of ERK1/2 and JNK induces the phosphorylation of GR at 3 sites. Mutation of these sites or blockade of trkB-mediated signaling from P21-31 in mice decreases spine densities on apical dendrites of excitatory neurons in primary sensory (S1) cortex. Further, chronic unpredictable stress from P21-31 reduces BDNF protein, GR phosphorylation, and spine densities. Fluoxetine treatment in pre-adolescence increases GR phosphorylation and ameliorates spine loss following chronic stress, but not in mice expressing GR with mutated BDNF-sensitive phosphorylation sites, indicating that the ability of fluoxetine to reverse stress-induced spine loss depends on trkB-mediated GR phosphorylation. Collectively, these findings suggest that trkB-mediated signaling regulates cortical dendritic spine formation, in part by modulating GR function. Importantly, disruption of this trkB-GR interaction (for example, by chronic stress) leads to dendritic spine loss.

1.8 DEVELOPMENTAL TRAJECTORIES OF trkB AND GR

1.8.1 TrkB in the PFC across postnatal development

Several studies have characterized trkB (full-length and truncated isoforms) and BDNF levels in the rodent, macaque, and human PFC at various developmental time points, but findings are not always congruent. Fryer and colleagues (1996) report that levels of *Trkb.fl* mRNA in the rat frontal cortex increase dramatically following birth, and remain relatively static into adulthood. Meanwhile, *Trkb.t1* mRNA levels increase sharply around P10-15 and remain static into adulthood,

except for another slight increase in mRNA levels at P20. The temporal pattern of trkB protein in the frontal cortex mirrors that of mRNA levels, with trkB.FL predominating at earlier stages of development, and trkB.T1 increasing in late postnatal development. However, mRNA levels were assessed in this report at embryonic day 15 (E15), E18, P0, P5, P10, P15, P20, P30, and adulthood, and protein levels at P0, P10, P20, P30, and adulthood, thus precluding any conclusions about changes in trkB throughout adolescent development in rats (a period from P28-56; see Spear, 2000).

In the mouse visual cortex, BDNF increases dramatically from P21-35 (Gorski et al., 2003). In a different study, Andersen and Sonntag (2014) report that *Bdnf* (total) mRNA in the PFC of rats does not change significantly across adolescent development (P20, P35, P40, P60). However, this study did find that some *Bdnf* transcripts changed throughout adolescence. At least 8 promoters within the *Bdnf* gene in rodents drive the transcription of alternative mRNA transcripts (Aid et al., 2007), allowing for temporal and spatial control of *Bdnf* (Tao et al., 1998; Timmusk et al., 1993; Baj et al., 2011). Activity-dependent expression of *Bdnf* in the cortex is primarily mediated by promoter IV (originally referred to as promoter III) (Tao et al., 1998, 2002; Lu, 2003; Sakata et al., 2009). In rat PFC, the *Bdnf* transcript containing exon IV decreases modestly from pre-adolescence to early adolescence (P20-35), then steadily increases thereafter until adulthood (P35-60) (Andersen & Sonntag, 2014). By contrast, exon IIc modestly increases in early adolescence, decreases sharply from P35 to P40, then slightly increases from P40 to adulthood. In this study, PFC dopamine D3 receptor (*Drd3*) mRNA was positively correlated with PFC *Bdnf* (total) mRNA, but negatively correlated with *Bdnf* exon IIc mRNA. Given that expression of dopamine receptors in the PFC peaks during late adolescence in rodents and humans (Naneix et al., 2012; Weickert et al., 2007), these findings suggest that *Bdnf* alternative transcripts may have different functional roles in the maturation of the PFC across adolescence.

In macaques, trkB.FL protein levels in the PFC are similar at E140, birth, and adulthood, but increased at P60 (Ohira et al., 1999). TrkB.T1 increases dramatically between birth and P60, and is modestly higher at adulthood. The late postnatal increase in trkB.T1 is in agreement with prior work in rat frontal cortex (Fryer et al., 1996) and mouse prefrontal cortex (Shapiro et al., 2017b). Hayashi and colleagues (2000) assessed trkB.FL immunoreactivity in the PFC of macaques at E140, P7, P6 months (P6m), and adulthood, and found that immunoreactivity was highest at P6m. This is also the age at which synapse density peaks in the PFC (Bourgeois et al., 1994); thus, Hayashi and colleagues (2000) speculated that trkB.FL signaling may be important for synaptogenesis in the PFC. However, few reports examine developmental changes in trkB in the PFC of non-human primates. Because adolescence in macaques typically occurs from 2 to 5 years of age, expression of the trkB isoforms throughout adolescent development of the non-human primate PFC remains to be determined.

Generally speaking, *BDNF* mRNA and protein in the macaque PFC also varies across development, and the pattern of change resembles that reported for trkB.FL. Mori and colleagues (2004) assessed BDNF protein levels in the PFC of macaques at E120, E140, birth, P2m, and adulthood. BDNF levels increase across development, peaking at P2m, then decline in adulthood. Subsequent work by this group more extensively characterized the developmental changes in BDNF (Mori et al., 2006). Specifically, *BDNF* mRNA and protein levels were measured at E120, E140, birth, P3 weeks (P3w), P2m, P3m, P6m, P2 years (P2y), P4y, and adulthood in the PFC and several other brain regions. In the PFC, BDNF protein increases steadily throughout development, reaching peak levels at P2m, which remains stable until P6m, then declines steadily until adulthood. Moreover, the decline in BDNF protein levels in primary sensory and motor cortices begins earlier in development than in the PFC, mirroring region-specific trajectories of synaptic remodeling during childhood and adolescence. This pattern suggests that BDNF signaling may be an important

regulator of spine plasticity and activity-dependent refinement of synaptic connections during cortical development.

Although the studies discussed thus far have examined developmental changes in BDNF or trkB in the PFC of rats and macaques, sub-regions within the PFC may exhibit distinct patterns of BDNF/trkB. Developmental trajectories of structural maturation vary between sub-regions of the PFC (van Eden & Uylings, 1985), suggesting that the temporal profile of trkB may also vary between PFC sub-regions. Indeed, recent work in female mice has shown that trkB.FL in the oPFC and mPFC increases across adolescent development (timepoints include P35, P42, and P56) but levels are modestly higher at P42 (mid-adolescence) in the oPFC relative to the mPFC (Shapiro et al., 2017b). Furthermore, trkB.T1 increases across adolescent development in the oPFC, but does not change in the mPFC. These findings suggest that characterizing trkB within distinct PFC sub-regions may improve the ability to detect changes across development.

At least four studies in humans report age-related expression patterns of trkB and BDNF in the dorsolateral PFC (DLPFC), including in adolescents and young adults (Fig. 1). Romanczyk and colleagues (2002) examined the developmental profile of *TRKB.FL* and *TRKB.T1* mRNA levels using in-situ hybridization. They report that *TRKB.FL* mRNA levels increase from infancy to adolescence and young adulthood, drop in adulthood, and decrease further in the aged brain. Notably, *TRKB.FL* mRNA peaks in young adulthood, and this is most prominent in superficial layers (II and III), the site of cortico-cortical projections. Further, they find that levels of *TRKB.T1* mRNA modestly increase from infancy to adulthood. Webster and colleagues (2002) report that, like *TRKB.FL*, *BDNF* mRNA increases in young adulthood. Specifically, *BDNF* mRNA is relatively low in infants and adolescents, but approximately 1/3 higher in young adults and adults. Furthermore, cortical layers III and V show the greatest age-dependent increase in *BDNF* mRNA.

In the PFC, synapses are overproduced in childhood, then eliminated throughout adolescence (Huttenlocher & Dabholkar, 1997). Thus, the peak in *TRKB.FL* and *BDNF* mRNA in young adulthood reported by Romanczyk et al. (2002) and Webster et al. (2002) occurs at the tail-end of a protracted period of major synaptic remodeling and refinement of cortical circuits. The presumed increase in BDNF-trkB signaling at this time may be critically involved in the stabilization and long-term maintenance of the synapses that are not subject to pruning. Moreover, the prominent increase in *TRKB.FL* and *BDNF* mRNA in layer III in young adulthood may reflect an important role for BDNF-trkB signaling in the refinement or stabilization of cortico-cortical connections. Layer V, which projects to subcortical areas including the striatum, also experiences a prominent age-dependent increase in *BDNF* mRNA (Webster et al., 2002). Structural MRI suggests that in addition to the PFC, striatal grey matter density also decreases throughout late adolescent development (Sowell et al., 1999). Because BDNF is anterogradely transported and released onto striatal neurons (Altar et al., 1997; Kokaia et al., 1998), an increase in BDNF within layer V of DLPFC during late adolescence/young adulthood may contribute to the maturation of cortico-striatal synapses.

The pattern of developmental changes in trkB reported by Romanczyk et al. (2002) somewhat differs from that reported by Luberg and colleagues (2010), who examined *TRKB* mRNA and protein in neonates, infants, toddlers, school-age children, teenagers, young adults, and adults. *TRKB.FL* mRNA is relatively similar across all age groups except for toddlers and adults, with expression significantly higher in toddlers compared to adults. However, there does seem to be a trend for increased *TRKB.FL* mRNA in teenagers and young adults relative to school-age children and adults, which is more in line with the two reports discussed above. Luberg et al. (2010) also found that trkB.FL protein increases from the neonatal period to infancy, then decreases with age. Further, *TRKB.T1* mRNA levels are similar in neonates, infants, young adults, and adults, but

decreased in toddlers, school-age children, and teenagers. Protein levels of trkB.T1 steadily increase from neonates to teenagers, then decrease slightly in young adults and adults.

Importantly, as in rodents, several alternative BDNF transcripts are identified in humans (Pruunsild et al., 2007; Cattaneo et al., 2016a), and their expression varies across postnatal development in distinct regional and temporal patterns (Timmusk et al., 1993, 1994; Sathanoori et al., 2004; Wong et al., 2009). The generation of alternative transcripts appears to regulate the laminar and subcellular localization of *BDNF* mRNA (Pattabiraman et al., 2005). Thus, quantification of total levels of *BDNF* mRNA may mask region- and layer-specific patterns of alternative transcript expression across postnatal development.

Wong and colleagues (2009) characterized the laminar distribution of four major BDNF alternative transcripts across development in DLPFC. Transcripts I, IV and VI are highest in infants and decline with age. Transcript II peaks in toddlers, then drops in school-age children and does not change thereafter. BDNF protein peaks in infants and decreases across development. Wong et al. (2009) also used *in situ* hybridization to examine laminar differences in *BDNF* mRNA. They report that *BDNF* mRNA hybridization is elevated in layer IV in young adults. However, the authors suggest that this pattern likely reflects increased *BDNF* mRNA in the apical dendrites of layer V pyramidal neurons, which extend into layer IV. Additionally, with increasing age, the distribution of *BDNF* mRNA in layer V and VI becomes more diffused around pyramidal neurons, suggesting that *BDNF* mRNA may be more targeted to dendritic processes as the cortex matures.

In summary, in the human DLPFC, some evidence suggests that *TRKB.FL* mRNA is highest in young adulthood (Romanczyk et al., 2002), and *BDNF* mRNA increases between adolescence and young adulthood (Webster et al., 2002). However, the *TRKB.FL* mRNA and protein levels reported by Luberg et al. (2010) were not significantly increased in young adulthood, and BDNF protein declined from infancy to adulthood in another report (Wong et al., 2009). Nevertheless, *TRKB.FL*

mRNA levels are somewhat higher in teenagers and young adults relative to infants, school-age children, and adults (Luberg et al., 2010), and dendritic *BDNF* mRNA content in layers V and VI increases with age (Wong et al., 2009).

1.8.1.1 Sex differences

One reason why findings reported by Luberg et al. (2010) and Wong et al. (2009) may not be consistent with those of Romanczyk et al. (2002) and Webster et al. (2002) is that the cohort examined by Romancyzk et al. (2002) and Webster et al. (2002) consisted primarily of males, except for the infant age group. The cohort examined by Luberg et al. (2010) and Wong et al. (2009) contained both sexes in all age groups. Given the modulatory effects of sex steroid hormones on trkB-mediated signaling (Hill, 2012a), it is possible that BDNF or trkB is developmentally regulated in a sex-dependent manner. Indeed, at least one study in mice suggests that expression of BDNF and trkB from early adolescence to adulthood may be sexually dimorphic (Hill et al., 2012b).

Hill and colleagues (2012b) report a trend for increased trkB.FL protein levels in the frontal cortex of male mice during early- to mid-adolescence (postnatal weeks 3-7), but levels of phosphorylated trkB (p-trkB) are unchanged across adolescence and adulthood. BDNF protein levels peak in young adulthood (weeks 8-10). Moreover, changes in trkB.FL and BDNF levels from adolescence to adulthood in the striatum are similar to those in the frontal cortex. This pattern of changes is different for females; there are no significant changes in trkB.FL (but see subregion-specific analyses in Shapiro et al., 2017b) and BDNF across adolescence and adulthood in the frontal cortex and striatum. However, levels of p-trkB in the frontal cortex and striatum increase starting in early adolescence, peak at mid-adolescence, then drop in young adulthood. These findings suggest that changes in the activities and levels of BDNF and trkB across development differ

between males and females. Grouping sexes together in experimental analyses may hinder the ability to detect these temporal changes.

1.8.2 GR in the PFC and HPA axis activity across postnatal development

Stress-induced activation of the HPA axis and subsequent release of glucocorticoids varies throughout postnatal development (McCormick & Mathews, 2007). In rodents, adolescents exhibit a more prolonged CORT response following acute stress (Romeo et al., 2004, 2006a; McCormick & Mathews, 2007; Foilb et al., 2011; Lui et al., 2012). An early study in rats revealed that adults exhibit a greater reduction in stress-induced CORT levels following pre-treatment with DEX than adolescents (Goldman et al., 1973), suggesting that glucocorticoid-mediated negative feedback is weaker in adolescents. Furthermore, following repeated exposure to a stressor, adult rodents show habituation of CORT responses, but adolescents exhibit a sensitized response; peak CORT levels are higher immediately following the stressor, but return to baseline faster (Doremus-Fitzwater et al., 2009; Romeo et al., 2006b). Together, these findings suggest that negative feedback on the HPA axis may be attenuated in adolescent animals relative to adults.

Given differential stress responses between rodents of differing ages, one might hypothesize that limbic and cortical regions modulating stress reactivity exhibit decreased GR during adolescence. However, multiple studies examining the developmental regulation of GR protein and mRNA in the hippocampus, paraventricular nucleus (PVN) of the hypothalamus, pituitary, and mPFC report similar levels in pre-, mid- and post-adolescent animals (Dziedzic et al., 2014; Romeo et al., 2008, 2013; Vazquez, 1998). Further, Perlman and colleagues (2007) report a significant effect of age on *GR* mRNA levels in the human DLPFC, with *peak,* rather than low, expression during adolescence. Moreover, they find that *GR* mRNA in various hippocampal subfields does not vary with age. Sinclair and colleagues (2011) report that protein and mRNA expression of GR-α, the

predominant GR isoform that translocates to the nucleus upon ligand binding to alter gene transcription (Oakley et al., 1999), increases threefold during postnatal development of the human DLPFC to peak in teenagers, then decrease in young adults and adults. Consistent with these findings, *NR3C1,* the gene that encodes the GR, in the human PFC peaks between ages 15 and 25 (Harris et al., 2009). Thus, it appears that reduced negative feedback regulation of the HPA axis in adolescents is not related to age-dependent changes in GR protein levels. Rather, GR *function* may differ between adolescents and adults.

1.9 ALTERED PFC trkB AND GR IN DEPRESSION

Depressed patients often exhibit disrupted circadian glucocorticoid cycling, resting hypercortisolemia (Yehuda et al., 1996), and impaired negative feedback control of the HPA axis (Holsboer, 2000; Barden, 2004). Furthermore, the Stress Hypothesis posits that HPA axis dysregulation may be an etiologic factor in depression (De Kloet et al., 1998; Holsboer, 2000). Indeed, successful antidepressant treatment is associated with normalization of HPA activity in depressed patients (Barden, 2004), and risk of relapse is higher in patients who do not show normalization of baseline plasma cortisol levels and feedback inhibition of the HPA axis with antidepressant treatment (O'Toole et al., 1997; Holsboer & Ising, 2010). Research in humans and animal models indicates that disruption of forebrain GR-mediated signaling contributes to dysregulation of the HPA axis and possibly, the pathophysiology of depression (Calfa et al., 2003; Boyle et al., 2005; Cattaneo & Riva, 2016b). Specifically, post-mortem studies report decreased *GR* mRNA in the frontal cortex of depressed patients (Webster et al., 2002) and PFC of teenage suicide victims (Pandey et al., 2013), potentially reflecting a compensatory downregulation of GRs in response to elevated glucocorticoids. By contrast, antidepressant treatments increase the expression and function of GR (Calfa et al., 2003; Pariante & Miller, 2001). Furthermore, polymorphisms in the

human *GR* gene (*NR3C1*) are associated with altered basal cortisol levels, negative feedback inhibition, and stress-induced cortisol responses (Wüst et al., 2004; DeRijk et al., 2008), as well as increased risk for depression (Van West et al., 2006).

Desensitization of GRs and impaired negative feedback of the HPA axis can elevate glucocorticoids, which could impact neuronal morphology via neurotrophic factors including BDNF. According to the Neurotrophic Hypothesis of Depression and Antidepressant Efficacy (Duman et al., 1997), down-regulation of BDNF by prolonged exposure to elevated glucocorticoids contributes to structural and functional alterations in cortico-limbic regions associated with depression, and chemically distinct antidepressants act by stimulating BDNF. In line with this perspective, mRNA and protein expression of BDNF (Karege et al., 2005; Dwivedi et al., 2003a; Qi et al., 2015) and trkB (Dwivedi et al., 2003a; Qi et al., 2015), as well as p-trkB levels (Dwivedi et al., 2009a), are reduced in the PFC of postmortem MDD/suicide subjects. Several studies also report alterations in signaling proteins downstream of trkB in postmortem MDD/suicide subjects, including decreased ERK1/2 signaling (Dwivedi et al., 2009b, 2006a, 2001) and increased MAPK phosphatase 2 (MKP-2) (Dwivedi et al., 2001) in the PFC; decreased PI3K-Akt signaling and increased PTEN levels (phosphatase and tensin homolog deleted on chromosome 10, which inhibits phosphorylation of Akt) in the ventral PFC (Karege et al., 2007, 2011); decreased mTOR and its downstream signaling targets in the PFC (Jernigan et al., 2011); decreased protein and mRNA levels of CREB (a transcription factor downstream of trkB) in the oPFC (Yamada et al., 2003) and Brodmann's area 9 (consisting of DLPFC and mPFC) (Dwivedi et al., 2003b); and decreased p-CREB levels in the oPFC (Yamada et al., 2003). Interestingly, CREB is decreased in PFC, but not hippocampus, of teenage suicide victims (Pandey et al., 2007). Furthermore, BDNF-trkB signaling is likely involved in the therapeutic efficacy of a variety of chemically-distinct antidepressant treatments (see Saarelainen et al., 2003; Duman & Monteggia, 2006; Rantamäki et al., 2007).

1.10 STRESS-INDUCED ALTERATIONS IN PFC trkB AND GR

Chronic stress-induced disruption of trkB-mediated signaling and GR function in key cortico-limbic regions is postulated to increase vulnerability to depression, as discussed above. Also as discussed, experiments using animal models with reduced trkB starting in early adolescence suggest that BDNF/trkB are critical for normative dendritic spine maturation and the maintenance of stabile spines throughout life. GR is also involved in adolescent spine maturation, promotes spine plasticity across the lifespan, and regulates the activity of the HPA axis. Thus, loss of neurotrophic support and impaired GR function may impact risk for psychopathology by increasing basal and stress-induced release of glucocorticoids and altering neuronal morphology and/or structural plasticity. During adolescence, disruption of trkB or GR may be particularly impactful for the still-maturing PFC. In this section, we review evidence that chronic stressors and glucocorticoid exposure modulate trkB and GR activity. We also discuss evidence from rodent models implicating dysregulation of both BDNF-trkB and GR systems in the development of depressive-like phenotypes, and restoration of these systems by antidepressants.

1.10.1 Effects of acute/chronic stressors or glucocorticoid exposure on trkB

Transient activation of the HPA axis by acute stress enhances neurotransmission and synaptic plasticity in the PFC, adaptive effects thought to enhance emotional memory processes and promote resilience to future stressors. By contrast, chronic stress impairs neuroplasticity in the PFC and dysregulates the HPA axis, maladaptive effects that may contribute to allostatic load and increase risk for the development of psychopathology. Likewise, BDNF-trkB signaling is differentially regulated by acute and chronic stressors. Acute stress increases p-ERK2 levels in the frontal cortex and PFC of mice and rats (Galeotti & Ghelardini, 2012; Meller et al., 2003; Shen et al.,

2004). In cultured cortical neurons, acute CORT or DEX increases phosphorylation of trkB, PLCγ, Akt, and ERK1/2, with peak levels occurring after 2-4 hrs (Numakawa et al., 2009). Consistent with these patterns, Jeanneteau and colleagues (2008) report that glucocorticoids can stimulate p-trkB through the genomic actions of GR, independent of effects on BDNF synthesis and release. What GR-responsive gene products are responsible for the phosphorylation of trkB remains to be determined.

1.10.1.1 Association with depressive-like phenotypes

In contrast to the stimulatory effects of acute stressor or glucocorticoid exposure on trkB-mediated signaling, chronic stressors and prolonged exposure to glucocorticoids reduce levels and activity of trkB in the PFC. These biochemical changes are associated with depressive-like behaviors, and can be blocked or reversed with antidepressant treatment (table 1). For instance, chronic forced swim stress in male rats induces anhedonic-like behavior and decreases p-ERK2 and p-CREB in the PFC, and these changes are blocked by fluoxetine treatment (Qi et al., 2006, 2008). Moreover, p-ERK2 in the PFC is positively correlated with body weight in stressed animals during stressor exposure, suggesting that the degree of disruption in PFC trkB-ERK2 signaling correlates with the severity of stress-induced physiological impairments (Qi et al., 2006).

Five weeks of chronic unpredictable stress in male mice also induces anhedonic-like and depressive-like behavior, with concomitant reductions in BDNF and p-trkB/trkB in the PFC (Zhou et al., 2017). Meanwhile, fluoxetine during the last week of stress exposure blocks these stress-induced changes. Decreased levles of p-ERK/ERK, p-Akt/Akt and p-CREB/CREB in the PFC following 8 weeks of chronic unpredictable stress has also been reported (Wang et al., 2015). In this study, Alarin (a member of the galanin family of neuropeptides) in stressed mice reverses depressive-like behavior and restores trkB signaling proteins in the PFC; furthermore, this effect depends on

trkB activation. Similarly, Zhang et al. (2016b) report that the putative trkB agonist, 7,8-dihydroxyflavone (7,8-DHF), blocks anhedonic-like behavior and loss of BDNF, p-trkB/trkB, PSD95, and synaptophysin in the PFC of mice exposed to 8 weeks of unpredictable stress. The authors of the present review also find that 7,8-DHF has antidepressant-like effects, in this case in adolescent mice (Barfield et al., 2017a).

Studies utilizing other chronic stress models, including chronic restraint, repeated social defeat, and inescapable electric shock (learned helplessness model) report largely consistent findings of reduced BDNF (protein and mRNA), trkB (protein and mRNA), p-trkB/trkB and downstream signaling proteins in the PFC when brain tissue is collected within a week after the last stress exposure (table 1) (Wang et al., 2016, 2018; Pesarico et al., 2017; Ma et al., 2016; Shirayama et al., 2015). Moreover, the restoration of BDNF-trkB levels by antidepressant treatments in stressed animals is concurrent with the reversal of stress-induced behavioral alterations. At least one study also reports concomitant stress-induced alterations in neuronal morphology in the mPFC (Ma et al., 2016). For example, in the repeated social defeat stress model used by Ma et al. (2016), 'susceptible' mice (i.e., those displaying social avoidance after the stress exposure) show increased anhedonic-like and despair behavior, reduced mature BDNF and p-trkB/trkB in the PFC, and lower dendritic spine densities in the PL cortex compared to non-stressed mice. A combination of the atypical antipsychotic brexpiprazole and a sub-threshold dose of fluoxetine produces rapid antidepressant-like effects and increases BDNF, p-trkB/trkB, and spine densities in stress-susceptible mice; moreover, pre-treatment with a trkB antagonist blocks the effects of fluoxetine + brexpiprazole.

Exposure to elevated CORT levels during early adolescence in male mice decreases the ratio of trkB.FL/trkB.T1 in the mPFC, but does not alter p-ERK1/2/ERK1/2 levels (Barfield et al., 2017a). Adolescent CORT-exposed mice also show long-term deficits in goal-directed decision making and decreased motivation, which can be blocked by the trkB agonist 7,8-DHF. Additionally,

adolescent CORT eliminates dendritic spines the PL cortex, and remaining spines are larger in volume, but not head size, suggesting aberrantly large necks. This spine dysmorphia persists for several weeks after CORT.

Despite evidence for sex differences in the prevalence of stress-related psychopathologies (Kessler, 2003; Holbrook et al., 2002), relatively few animal studies examining neurotrophic mechanisms of stress vulnerability have used females. Nevertheless, de Sousa et al. (2015) report that chronic CORT exposure in female mice decreases BDNF protein levels in the PFC and induces anhedonic-like and depressive-like behavior. In another report, chronic unpredictable stress decreases PFC BDNF in adult female, but not male, mice. And while both stress-exposed males and females develop increased immobility in the FST, only females also develop decreased sucrose preference (Karisetty et al., 2017). This pattern is consistent with our own findings that the trkB agonist 7,8-DHF can correct PFC-dependent decision-making abnormalities in CORT-exposed female adolescent mice (Barfield et al., 2017a; Barfield & Gourley, 2017b). Considering the increased incidence of depression in women (Kessler, 2003), additional studies exploring sex differences in the vulnerability to, and persistence of, stress-induced changes in PFC BDNF-trkB are certainly warranted.

Chronic stress alters levels of the precursor form of BDNF, proBDNF, which binds to the $p75^{NTR}$ and sortilin receptors, and is cleaved to yield mature BDNF (mBDNF, which binds to trkB). In contrast to mBDNF, proBDNF enhances apoptosis, inhibits neurite outgrowth, and promotes spine pruning (Teng et al., 2005; Koshimizu et al., 2009; Sun et al., 2012; Orefice et al., 2016). Shirayama et al. (2015) report that inescapable electric shock increases proBDNF in the mPFC of male rats. Repeated social defeat stress in mice also increases proBDNF in the PFC (Pesarico et al., 2017). However, in this study, $p75^{NTR}$ was reduced, which the authors suggest may be a compensatory response to increased proBDNF. Nevertheless, an isoquinoline compound (FDPI, a

putative modulator of monoaminergic activity) prevents stress-induced changes in proBDNF and $p75^{NTR}$, as well as stress-induced social avoidance (Pesarico et al., 2017).

proBDNF may contribute to the behavioral and morphological consequences of chronic stress. Male rats exposed to chronic unpredictable stress have higher levels of proBDNF (protein), $p75^{NTR}$ (protein and *Ngfr* mRNA) and sortilin receptor (protein and *Sort1* mRNA), and lower levels of *Bdnf* mRNA and trkB (protein and mRNA) in the neocortex (Bai et al., 2016). Stressed rats also exhibit anhedonic- and depressive-like behavior and decreased dendritic spine lengths in the neocortex. Furthermore, administration (*i.c.v.* or *i.p.*) of an anti-proBDNF antibody increases sucrose preference, decreases immobility in the FST, and restores spine lengths. Thus, chronic stress may "shift the balance" between two opposing pathways [proBDNF and its receptors ($p75^{NTR}$ and sortilin) *vs.* BDNF and trkB] towards proBDNF-mediated signaling, favoring neurodegeneration and spine loss. Of note, because stress reduced *Bdnf* mRNA, the stress-induced increase in proBDNF protein is likely due to inhibition of proteolytic cleavage of proBDNF to mBDNF, and not increased transcription of *Bdnf*. Interestingly, proBDNF is elevated in the serum and plasma of patients with depression (Yoshida et al., 2012; Zhou et al., 2013), and antidepressant treatments increase the enzymes that cleave proBDNF (Sartori et al., 2011; Segawa et al., 2013). In sum, chronic stress impacts the balance of proBDNF *vs.* mBDNF, which may mediate the neurobehavioral response to adversity.

In the absence of chronic stress- or CORT-induced changes in BDNF or trkB levels, BDNF-trkB function can nevertheless be impacted. Chiba et al. (2012) report that chronic restraint stress in rats does not alter trkB or BDNF levels in the PFC, but decreases BDNF-stimulated glutamate release in PFC slices, down-regulates GR, induces immobility in the FST, and decreases sucrose preference. Numakawa et al. (2009) suggest that this impairment in BDNF-trkB function may be due to decreased trkB-bound GR levels following chronic stress. As discussed above

(section 1.7), the interaction of GR with trkB is critical for BDNF-trkB activation of PLCγ signaling that stimulates glutamate release. In cultured neurons, prolonged CORT exposure decreases GR, resulting in reduced trkB-GR interaction and attenuated BDNF-induced glutamate release (Numakawa et al., 2009). Together, these patterns suggest that stress-induced glucocorticoids can impair BDNF-trkB function, at least in part, by reducing the interaction of trkB and GR in the PFC, which may contribute to stress-induced behavioral alterations. The effects of chronic stress on GR are discussed in detail in section 1.10.2.

Numerous reports indicate that the antidepressant-like efficacy of a variety of drugs can be blocked by co-administering a trkB antagonist (Wang et al., 2015; Zhang et al., 2016b; Ma et al., 2016; Zhou et al., 2017). Interestingly, multiple studies also report that administration of the trkB antagonist ANA-12 alone has antidepressant-like effects (Cazorla et al., 2011; Zhang et al., 2014, 2015). This phenomenon is thought to be mediated by antagonism of trkB receptors in the nucleus accumbens (NAc) – a brain region in which stress-induced changes in BDNF-trkB are, generally speaking, opposite those in the PFC. For example, several days after the end of a 10-day social defeat period in mice, mBDNF is decreased in the PFC and increased in the NAc (Zhang et al., 2015). Increased mBDNF and p-trkB/trkB, as well as decreased proBDNF, are also observable in the NAc of rats exposed to inescapable electric shock (Shirayama et al., 2015). In this study, infusion of the trkB agonist 7,8-DHF (but not ANA-12) in the PFC reduces escape failures and latency to escape in a new situation with a controllable shock (conditioned avoidance test) – considered antidepressant-like effects. The same behavioral patterns are evident with infusion of ANA-12 (but not 7,8-DHF) into the NAc. Antidepressant-like effects of BDNF blockade in the NAc have also been reported (discussed in Nestler and Carlezon, 2006).

Some evidence suggests that antidepressants correct stress-induced alterations in BDNF-trkB and neuronal morphology simultaneously in both the PFC and NAc. For example, Ma et al.

(2016) report that fluoxetine + brexpiprazole blocks depressive- and anhedonic-like behaviors in mice susceptible to chronic social defeat. This treatment also boosts BDNF, p-trkB/trkB and dendritic spine density in the PFC, while normalizing (reducing) BDNF, p-trkB/trkB and dendritic spine density in the NAc. These findings suggest that the therapeutic-like efficacy of antidepressants may depend on a nuanced modification of BDNF-trkB systems throughout the brain.

1.10.1.2 Persistence of stress-related changes on prefrontal cortical BDNF-trkB

The studies discussed thus far assessed short-term effects of stress on trkB-mediated signaling (determined ≤1 week after stressor cessation). Identifying and characterizing persistent alterations may yield further insight into the chronic nature of depression. Far fewer studies have examined long-term changes in BDNF or trkB following chronic stress exposure, especially in the PFC, but initial findings are summarized in table 2.

Chronic mild stress in adult male rats decreases p-ERK1/2/ERK2 in the frontal cortex, detectable 2 weeks following stressor exposure, and daily fluoxetine throughout the duration of stress normalizes p-ERK1/2/ERK2 levels (First et al., 2011). However, BDNF and trkB in the frontal cortex, as well as spatial learning and memory in the Morris water maze test, are apparently not affected by stress exposure in the same report. In another study, chronic unpredictable stress reduces *Bdnf* in the cortex of adult mice, and *Bdnf* deficiency is detectable 2 weeks following stress, in tandem with decreased sucrose preference and despair-like behavior (Yang et al., 2017). Infusion of an anti-proBDNF antibody into the anterior cingulate cortex reverses all stress-induced behavioral and molecular alterations (for discussion of proBDNF, see section 1.10.1.1).

While the long-term effects reported by Yang et al. (2017) are generally consistent with the short-term effects of chronic stress discussed earlier, studies that have directly compared short- and long-term changes in BDNF-trkB reveal less consistent results. For example, 2 hr following the last

intermittent social defeat stressor, adult male rats have either increased or unchanged levels of BDNF and *Bdnf* mRNA in the IL, PL, and anterior cingulate cortex (summarized table 2) (Fanous et al., 2010). Meanwhile, no changes in BDNF or *Bdnf* mRNA are evident 4 weeks following the last stressor. Similarly, adult male and female rats experience sex-dependent changes in p-CREB and BDNF in the PL and anterior cingulate cortex (but not IL) 1 day following chronic footshock stress, but no changes are evident 3 weeks after stress (Lin et al., 2009). Depressive-like behaviors were not assessed in these studies, precluding the ability to draw conclusions about the molecular mechanisms of stress vulnerability or resilience.

Mice susceptible to social defeat stress (*i.e.*, show social avoidance) have decreased levels of BDNF, p-trkB/trkB, and PSD-95 in the PFC 8-10 days after the last stressor, along with increased immobility and decreased sucrose preference (Yang et al., 2015, 2016; Dong et al., 2017). These stress-related behavioral and molecular changes can be reversed by a single dose of ketamine or one of its stereoisomers (R-ketamine and S-ketamine) (Dong et al., 2017; Yang et al., 2015). A trkB antagonist blocks the antidepressant-like effects of R- or S-ketamine on depressive-like behaviors, suggesting that ketamine's sustained effects are mediated by modulation of trkB. Further, ketamine, R-ketamine, and S-ketamine restore decreased dendritic spine densities in the PL cortex of stress-susceptible mice (Dong et al., 2017; Yang et al., 2015).

In line with the hypothesis that a chronic stress-related depressive-like phenotype may be characterized by synaptic deficits in cortico-limbic regions and alterations in both BDNF-trkB signaling and GR function, chronic glucocorticoid exposure in rodents produces an antidepressant-sensitive persistent depressive-like state (Gourley et al., 2008a,b), desensitizes GRs (Chiba et al., 2012), decreases BDNF and trkB in mPFC (Gourley et al., 2012b), and decreases dendritic spine density in PFC (Gourley et al., 2013b; Swanson et al., 2013). Recent work by our group has shown synergistic effects of chronically diminished mPFC BDNF and GR signaling on goal-directed

decision-making (Gourley et al., 2012b), which is disrupted in depression (Dickson & Moberly, 2013; Griffiths et al., 2014). Specifically, knockdown of *Bdnf* in the PL cortex (mimicking the effects of chronic CORT exposure) does not by itself impair goal-directed action selection, but co-administration of a subthreshold dose of the GR antagonist RU38486 impairs the ability of mice to select actions based on the likelihood that they will be rewarded. Thus, deficient BDNF in the PL cortex increases vulnerability to the effects of reduced GR binding on goal-directed decision-making processes.

The vast majority of studies examining persistent stress-induced changes in prefrontal BDNF/trkB systems have focused on the mPFC, but emerging evidence suggests that the oPFC may also be vulnerable to long-term change. For example, adult male mice exposed to chronic restraint stress have decreased p-ERK1/2 in the medial oPFC, cingulate cortex and mPFC, and decreased p-MEK1/2 in the medial oPFC 2.5 weeks following the last restraint stressor (Leem et al., 2014). The antidepressant imipramine blocks stress-induced depressive-like behavior [social avoidance, immobility in the tail suspension test (TST)] and reductions in MEK/ERK1/2 signaling, but only in the medial oPFC, strongly suggesting that trkB-ERK1/2 signaling in this region influences social interaction and stress coping.

In adult male mice, chronic CORT exposure does not alter p-ERK1/2 in the mPFC when measured 3 weeks after CORT, but does impact depressive-like behavior, which is sensitive to antidepressant treatment (Gourley et al., 2008b,c). While these findings suggest that CORT-induced depressive-like behavior may not be accompanied by gross changes in mPFC p-ERK1/2, Gourley et al. (2008c) also report that reward-related amotivation covaries with BDNF and p-ERK1/2 levels in the mPFC of CORT-exposed mice, notable because tissue extracts may be expected to include medial oPFC tissue. Additional work by this group indicated that CORT reduces *Bdnf* mRNA in the lateral oPFC, but not in the IL, detectable 3 weeks following CORT, and concomitant with

impairments in fear extinction and sucrose preference (Gourley et al., 2009a). These findings are interesting, considering CORT also decreases dendritic spine densities on pyramidal neurons in the lateral oPFC, and unlike in some other brain regions, spine densities fail to recover after a washout period (Gourley et al., 2013b). Hence, the oPFC may be particularly susceptible to long-lasting stress-related alterations in BDNF-trkB and neuronal morphology, but further studies are warranted.

Adolescent rodents may be particularly vulnerable to developing stress-related depressive-like behavior, behavioral inflexibility, and PFC BDNF-trkB deficiencies in adulthood. Three weeks following chronic mild stress exposure in early adolescent (P28-41) male rats, BDNF, p-ERK1/2/ERK1/2, and p-CREB in the mPFC is decreased, immobility in the FST increases, and behavioral flexibility in the (mPFC-dependent) attentional set-shifting task is impaired (Zhang et al., 2017). Deficits correlated with p-ERK1/ERK1. Interestingly, when tested in adolescence, rats also display anhedonic-like sucrose neglect, but this phenomenon does not persist into adulthood. A separate study exposed male mice to social defeat in early adolescence (P28-37) then singly housed them. Subsequent testing suggested that certain deficits in cognitive flexibility and mPFC BDNF may be delayed, in this case, evident 6 weeks, but not 1 week, after the last social defeat (Xu et al., 2016b). Duloxetine (serotonin and norepinephrine reuptake inhibitor) in adulthood (P65-79) reverses both behavioral and molecular alterations. The authors also note that no stress-induced changes in BDNF within the oPFC are evident at the 6-week time point. A follow-up study by this group revealed that total *Bdnf* mRNA and *Bdnf* IV (but not transcripts I and VI) mRNA are lower in the mPFC of adult male mice with a history of adolescent social defeat stress (P28-37) (Xu et al., 2017). Adolescent stress also increases histone 3 dimethylation at a region downstream of the *Bdnf* IV promoter – an epigenetic modification that represses *Bdnf* IV gene expression through chromatin remodeling. The monoamine oxidase inhibitor, tranylcypromine, in adulthood (P65-78), reverses the stress-induced changes in *Bdnf* IV mRNA and epigenetic modifications.

In contrast to the findings of Zhang et al. (2017) and Xu et al. (2016b, 2017), Desbonnet et al. (2012) report that social defeat stress in male mice from P35-45 (and individual housing from P31-end of experiment) does not alter *Bdnf* mRNA levels in the PFC when measured nearly 6 weeks following the last defeat. Desbonnet et al. (2012) exposed mice to social defeat stress during a slightly later period of adolescence, which may account for this apparent resilience to stress. Consistent with this notion, Zhang et al. (2016a) found that mice exposed to social defeat (then singly housed) from P28-37, but not P38-47 or P70-79, develop deficits in cognitive flexibility and reversal learning 6 weeks later. The authors of the present review have similarly reported that oral CORT exposure in mice during early adolescence (P31-42), but not adulthood (P70-81), disrupts goal-directed decision making several weeks after the cessation of CORT (Barfield et al., 2017a). Additionally, CORT, repeated forced swimming, or trkB antagonism during the same early adolescent period (P31-42) impairs oPFC-dependent behavioral flexibility in adulthood (Barfield & Gourley, 2017b). Although studies directly comparing the long-term effects of stress or CORT during different periods of adolescence/adulthood on PFC BDNF-trkB and function are somewhat lacking, current evidence suggests that stressors during early, but not late, adolescence may be more impactful than those in adulthood. Indeed, chronic stressors in adulthood often need to be twice as long in duration as stressors in adolescence to produce comparable long-term behavioral and molecular consequences (table 2).

1.10.2 Effects of chronic stressor exposure on GR

A well-documented consequence of chronic stressor exposure is the desensitization of GRs in the PFC and hippocampus, leading to impaired negative feedback of the HPA axis. Upon glucocorticoid binding, the GR dissociates from a chaperone protein complex, dimerizes, and translocates from the cytosol to the nucleus, where it regulates gene transcription by interacting with

glucocorticoid response elements on DNA or by modulating the activity of other transcription factors (Vandevyver et al., 2012; Oakley & Cidlowski, 2013). The genomic actions of GRs facilitate the termination of the stress response, but under conditions of chronic stress, alterations in the expression, trafficking, and transcriptional activity of GRs may contribute to dysregulation and improper termination of the HPA axis (Pariante, 2006; Pariante & Lightman, 2008).

Chronic stress in rodents decreases GR levels in the PFC (Mizoguchi et al., 2003; Chiba et al., 2012; Chen et al., 2016; Zhuang et al., 2016). Furthermore, in chronically stressed rats, DEX infusion into the PFC fails to suppress plasma CORT levels, indicating that stress-induced changes in GRs in the PFC contribute to the disrupted negative feedback on the HPA axis (Mizoguchi et al., 2003). In addition to regulating GR levels, chronic stress alters GR function by modulating the expression of GR co-chaperones. Guidotti and colleagues (2013) report that chronic stress increases cytosolic GR and the co-chaperone FKBP5 (FK506 binding protein 51) in the PFC. Because FKBP5 reduces the affinity of the GR for its ligand and restrains the translocation of GRs to the nucleus (Wochnik et al., 2005), its up-regulation by chronic stress may contribute to dysregulation of the HPA axis by impairing nuclear translocation of the GR and GR-mediated transcription. In line with this perspective, polymorphisms in the human *FKBP5* gene that are associated with higher protein/mRNA expression decrease negative feedback inhibition of the HPA axis (Binder et al., 2008).

Impaired GR function following chronic stress may also be mediated by changes in receptor phosphorylation. GR phosphorylation regulates GR transcriptional activity through modification of protein-protein interactions, which can affect stability of the receptor and the association and recruitment of co-factors (Ismaili & Garabedian, 2004). GR phosphorylation also modifies the subcellular localization of the receptor (Rogatsky et al., 1998) and plays a role in the non-genomic

actions of the GR by modulating GR-activated cytoplasmic signaling pathways (Ismaili & Garabedian, 2004).

Chronic stress in adult rats alters phosphorylation of GRs at specific residues (Adzic et al., 2009; Papadopoulou et al., 2015), and patterns of stress-induced GR phosphorylation are sexually dimorphic (Mitic et al., 2013). Several specific phosphorylation sites on the rat GR are now associated with effects of sex, stress, and antidepressants on GR function (Ismaili and Garabedian, 2004; Arango-Lievano et al., 2015). Differential phosphorylation at these sites can positively and negatively regulate GR transcriptional activation. Phosphorylation at S232 by various cyclin-dependent kinase (Cdk) complexes stimulates GR translocation to the nucleus (Adzic et al., 2009; Davies et al., 2008) and promotes transcriptional activation in a gene-specific manner (Rogatsky et al., 1998; Chen et al., 2008). Phosphorylation at S246 by c-Jun N-terminal kinase (JNK) inhibits GR transcriptional activity (Rogatsky et al., 1998) by promoting nuclear export of the receptor (Adzic et al., 2009; Davies et al., 2008). Following trkB activation by BDNF, GR is phosphorylated at S155, S287, and to a lesser extent, S246, by ERK and JNK, thus providing one avenue for cross-talk between trkB and GR signaling (see section 1.11 for further discussion).

Chronic unpredictable stress in mice decreases p-GR at S232 in the PFC but not hippocampus (Papadopoulou et al., 2015). Other adversities, like chronic isolation, decrease the ratio of p-GR at S232 *vs.* S246 in the PFC and hippocampus of female, but not male, rats (Mitic et al., 2013). Loss of p-GR at S232 and/or increase at S246 may stimulate GR export from the nucleus and decrease GR transcriptional activation, since GR transcriptional activity is highest when levels of p-GR at S232 exceed that of p-GR at S246 (Chen et al., 2008). These changes may contribute to impaired negative feedback control of the HPA axis. In humans, the ratio of p-GR at S211 (human S211, rat S232) *vs.* S226 (human S226, rat S246) is low in MDD patients (Simic et al., 2013b). Interestingly, in women but not men, this ratio negatively correlates with current reports of

depression, anxiety and stress (Simic et al., 2013a). Thus, differential patterns of GR phosphorylation between males and females may contribute to sex differences in the effects of chronic stress on GR function and the HPA axis.

Some of the effects of chronic stress in impairing negative feedback of the HPA axis may be associated with ovarian steroids, regulators of the GR and its co-regulators (Bourke et al., 2012, 2013; Malviya et al., 2013). Certain forms of chronic stress during adolescence can disrupt behavioral, HPA axis, and GR functioning in female, but not male, rats (Bourke et al., 2013; Bourke & Neigh, 2011), and behavioral vulnerabilities are also reported in adolescent female mice (Barfield & Gourley, 2017b). Greater susceptibility to stress-induced GR dysfunction in females may have implications for understanding increased incidence of depression in women (Kessler, 2003).

1.10.3 Restoration of both BDNF-trkB and HPA systems by antidepressants

Deficits in neurotrophin signaling and hormonal stress responses are implicated in the pathophysiology of depression, yet historically, studies utilizing animal models have focused on either BDNF-trkB *or* HPA axis systems. In this section, we discuss investigations that concurrently assessed the sensitivity of both to stress and importantly, behaviorally-efficacious antidepressant treatments.

Several studies employing the unpredictable chronic mild stress model in adult male rats and mice report largely consistent effects – anhedonic- and despair-like behavior, reduced BDNF in the PFC, increased blood serum CORT levels, and adrenal hypertrophy (indicative of an overactive HPA axis) (Jin et al., 2015; Abdul Shukkoor et al., 2016; Pytka et al., 2017; Zu et al., 2017) (table 3). These patterns are also seen in female mice (Filho et al., 2015). At least one study additionally reports decreased p-ERK, p-CREB, and GR (protein and mRNA) in the PFC following chronic stress (Zu et al., 2017). In all of these investigations, chronic administration of traditional, novel, or

experimental antidepressants reversed stress-induced molecular, neuroendocrine, and behavioral alterations.

Interestingly, Réus et al. (2012) found that while chronic unpredictable stress induces anhedonic-like behavior and increases circulating CORT and adrenal gland weights in adult male rats, BDNF in the PFC is not altered. Memantine (an NMDA receptor antagonist) reverses all stress-induced changes and increases BDNF in the PFC; thus, enhancing BDNF was associated with antidepressant-like efficacy, even though BDNF was not affected by stress in this particular report. The lack of an effect of stress on BDNF may be due to more mild stress conditions relative to other reports. For example, in the experiments by Abdul Shukkoor et al. (2016) and Zu et al. (2017) (see table 3 for comparisons), animals were exposed to 6 hr of restraint, while animals in Réus et al. (2012) were exposed to 1-3 hr of restraint or 1.5-2 hr of restraint at 4°C. Additionally, in experiments by Abdul Shukkoor et al. (2016) and Zu et al. (2017), animals undergoing chronic stress were singly housed. By contrast, animals were group housed for 30 days out of a 40-day stress exposure period by Réus et al. (2012), which could buffer some stress effects.

The studies discussed in this section thus far focused on short-term (≤1 week) effects of chronic stress experienced during adulthood. We identified one investigation that examined neurobiological consequences in adult male rats 2 weeks after repeated (10 days) immobilization stress (Shilpa et al., 2017). BDNF and GR are not significantly affected in the frontal cortex, but are decreased in the hippocampus. Environmental enrichment for 2 weeks reverses anhedonic- and despair-like behaviors, ameliorates spatial learning and memory impairments, normalizes hippocampal BDNF, and partially restores hippocampal GR. Although the authors report no significant stress-induced changes in BDNF and GR in the frontal cortex, different PFC subregions may be variably affected by immobilization stress. Thus, lumping all subregions together as the "frontal cortex" may mask subregion-specific changes in BDNF and GR.

Yan et al. (2016) assessed PFC BDNF-trkB, blood serum CORT levels, and depressive-like behavior in male mice exposed to CORT during adolescence (P35-56), though these variables were measured within a few days following the cessation of CORT injections. CORT decreases BDNF, p-trkB/trkB, and p-CREB/CREB in the PFC, as well as sucrose preference, and increases immobility in the FST. Blood serum CORT levels are also elevated in mice given exogenous CORT, as expected. Treatment with fluoxetine during the CORT exposure period blocks the effects of CORT on all measures, including serum CORT levels, and a trkB antagonist abolishes these effects of fluoxetine. The ability of fluoxetine to normalize serum CORT levels, in a trkB-dependent manner, suggests that fluoxetine facilitates negative feedback on the HPA axis, which may be mediated by effects on GR (Barden, 2004).

Collectively, these studies demonstrate that putative core features of depression in humans – dysregulated BDNF-trkB and HPA systems – can be recapitulated in chronic stress or chronic glucocorticoid exposure rodent models. Disruption of both systems is associated with the development of depressive-like behavior, while restoration of both systems by antidepressants is associated with the reversal of depressive-like behavior. Taken together, these findings suggest that antidepressants may treat depressive-like behaviors by modulating GR function and activating BDNF-trkB signaling in the PFC. However, in depressed humans, cognitive/behavioral symptoms, mood changes, putative deficits in neurotrophin signaling and hormonal stress response abnormalities persist well beyond the period of chronic stress exposure. Thus, it is important for future work using rodent models to determine whether stress-induced dysregulation of BDNF-trkB and HPA systems and depressive-like behavior persists in the weeks and months following stressor exposure. This is especially pertinent to studies involving prolonged stressor or glucocorticoid exposure during adolescence, since initial evidence suggests that some depressive-like behaviors,

cognitive deficits, and biochemical changes may not emerge until adulthood (Xu et al., 2016b; Zhang et al., 2017).

1.11 BDNF-trkB AND GR SYSTEMS IN STRESS-RELATED METAPLASTICITY

A key factor in understanding the long-term effects of stressor exposure is the likelihood that stressful life events will alter the molecular, cellular, or behavioral response to stressors later in life (Schmidt et al., 2013). According to this view, chronic stressors may increase susceptibility to developing psychopathology in response to subsequent stressor exposure. This priming of neurobiological systems by stressful experiences constitutes a form of stress-induced metaplasticity. Furthermore, the concept of metaplasticity may be particularly relevant for understanding the impact of adverse experiences during adolescence on behavior and stressor vulnerability and resilience later in life. In this section, we discuss evidence implicating BDNF-trkB and GR systems in stress-induced metaplastic modifications.

1.11.1 HPA axis reactivity to adolescent experience

Considerable evidence indicates that chronic stress during key developmental periods can alter subsequent function of the HPA axis, with effects potentially persisting throughout the lifespan (Meaney et al., 1996). Thus, the concept of stress-induced metaplasticity applies to the effects of early environmental stressors on the HPA response to subsequent stressors. Furthermore, stress-induced programming of the HPA axis may occur beyond prenatal, neonatal, and perinatal development (McCormick et al., 2010). Chronic stress during adolescence can also have long-term effects on subsequent stressor reactivity. For example, adult male rats with a history of chronic variable stress exposure during adolescence (P28-56) exhibit a greater and more prolonged acute stress-induced CORT response, as well as decreased levels of GR protein in hippocampus, than

control counterparts (Isgor et al., 2004). Additionally, rats exposed to a triple stressor on P28 and re-exposed to swim stress on P35 and P60 show higher basal CORT levels and reduced GR in the hippocampus at P61 (Uys et al., 2006), suggesting that trauma exposure in early adolescence may impair HPA axis function through down-regulation of GR.

Several studies suggest that the impact of adolescent experience on HPA axis activity in adulthood may be sex-specific. Chronic mild stress in rats during early adolescence results in an exaggerated stress-induced CORT response in adulthood in females, but not males (Pohl et al., 2007). Similarly, social subjugation stress in rats from P28-38 potentiates stress-induced CORT levels and increases adrenal gland weight in adult females relative to controls and adult males (Weathington et al., 2012). Chronic social stress (daily 1 hr social isolation, then housing with a new cagemate) of rats during adolescence (P33-48) exaggerates the CORT response to acute restraint stress 3 weeks later in females, but not males (McCormick et al., 2005). Collectively, these findings suggest that stressful life events during adolescence can alter the hormonal response to stressors later in life, and females may be particularly vulnerable to such long-term changes.

The cellular and molecular mechanisms responsible for long-term changes in HPA axis function following adverse experiences in adolescence are not well known. Nevertheless, decreased hippocampal GR expression and an exaggerated CORT response to acute stress in adult animals with a history of early-life stress may be mediated by hyper-methylation of the glucocorticoid receptor gene (*Nr3c1*) early in life, which persists into adulthood (Weaver et al., 2004). Suicide completers with a history of childhood abuse exhibit increased methylation of *NR3C1* at the exon 1_F promoter (human homologue of the rat exon 1_7 promoter) and reduced GR in hippocampus (McGowan et al., 2009). Methylation of *NR3C1* at exon 1_F is also associated with impaired recovery of the cortisol stress response in adolescents exposed to a social stress task (van der Knaap et al., 2015a). Furthermore, exposure to stressful life events in adolescence, but not childhood or the

perinatal period, is associated with higher methylation of *NR3C1* at exon 1_H in adolescents (van der Knaap et al., 2014). Methylation at the exon 1_H promoter at age 16 is also associated with an increased risk of an internalizing disorder diagnosis at a 3-year follow up (van der Knaap et al., 2015b). However, it remains to be determined whether methylation at exon 1_H in adolescents alters GR and/or HPA function. Nevertheless, it is possible that epigenetic modifications induced by major life stressors in adolescence may contribute, at least in part, to long-term reduction of GR, exaggerated stress-induced glucocorticoid release, and impaired negative feedback control of the HPA axis.

The majority of studies examining the effects of chronic adolescent stress on HPA axis function in adulthood have focused on the hippocampus, but the PFC also regulates HPA reactivity. For instance, mPFC (anterior cingulate and IL cortex) lesions prolong the CORT response to acute restraint stress (Diorio et al., 1993), suggesting that the mPFC participates in inhibition of the HPA axis. However, the role of GRs appears to vary between PFC subregions. In naïve male rats, viral-mediated (sh-RNA) *Gr* knockdown in both the IL and PL cortex enhances the CORT response to acute stress (McKlveen et al., 2013). Only knockdown of *Gr* in the IL cortex potentiates the acute stress-induced CORT response in chronically stressed animals, however, and increases immobility in the FST. By contrast, only knockdown of *Gr* in the PL cortex increases baseline CORT levels in chronically stressed animals. Thus, disruption of GR function in the PFC may contribute to reported alterations in HPA axis reactivity following chronic stressor exposure in adolescence – namely, increased basal glucocorticoid levels, impaired negative feedback inhibition of the HPA axis, and a heightened CORT response to acute stress.

Notably, GR levels in the PFC, but not hippocampus, drop following re-exposure to a restraint stressor in rats previously subjected to chronic restraint stress (Gadek-Michalska et al., 2013). Thus, PFC GRs may be especially sensitive to stress-induced metaplasticity. Future work

examining the effects of chronic stressor exposure during adolescence on PFC GR function in adulthood may provide new insight into mechanisms by which adverse experiences alter stress responsivity later in life.

1.11.2 GR and BDNF-trkB interactions in stress contexts

Patients with MDD can exhibit dysregulation of the HPA axis, decreased GR in the PFC, and decreased trkB and downstream signaling proteins in the PFC, suggestive of a relationship between these alterations and the pathophysiology of depression. Empirical support for this perspective comes from studies utilizing mutant mice with altered *Gr*, *Bdnf*, or *Trkb* expression. Moreover, trkB and GR signaling pathways intersect, and we will discuss evidence that their coordinated actions regulate neurobehavioral responses to stress.

1.11.2.1 Mutant models of GR disruption

Using a Cre/LoxP system, Boyle and colleagues (2005) generated mice with forebrain-specific knockout of *Gr* (FBGRKO). In this case, progressive loss of GR does not begin until ~P21, with complete deficit by ~P120-180. Consistent with a role for forebrain GR in the regulation of HPA axis activity, FBGRKO mice exhibit higher basal and peak CORT levels and impaired negative feedback inhibition of the HPA axis. FBGRKO mice also develop despair-like behavior in the FST and TST and anhedonic-like sucrose neglect. Interestingly, depressive-like behavior and altered CORT release in FBGRKO mice are observed at P180 (when GR is nearly absent) but not at P60 (when GR levels are reduced by ~50%). Furthermore, the tricyclic antidepressant, imipramine, normalizes alterations in circadian CORT levels and depression-like behavior, suggesting that imipramine may act on systems impacted by forebrain GR loss (such as BDNF-trkB, discussed below). However, imipramine does not restore negative feedback on the HPA axis, suggesting that

the up-regulation of GRs by antidepressants may be required for their ability to restore negative feedback inhibition of the HPA axis (Barden, 2004).

In contrast to mice lacking forebrain GR, mutant mice expressing 50% less GR protein than typical mice (*i.e.*, *Gr*+/- mice) do not exhibit alterations in basal or peak CORT levels, depressive-like behavior in the FST, abnormal context- and cue-dependent fear conditioning, or anxiety-like behavior (Ridder et al., 2005). However, *Gr*+/- mice exhibit a higher and more prolonged CORT response to acute restraint stress and impaired suppression of CORT in the DEX suppression test, consistent with impaired negative feedback inhibition of the HPA axis. *Gr*+/- mice also display increased "helplessness" following 2 days of inescapable and uncontrollable foot shocks. Thus, GR levels may influence gradients of HPA axis dysregulation and susceptibility to stress-induced depression; *i.e.*, as GR levels decrease, the intensity or duration of stressors sufficient to induce depressive-like behaviors and HPA axis disturbances also decreases.

Ridder et al. (2005) also report that hippocampal BDNF protein is diminished in *Gr*+/- mice, suggesting that vulnerability to depressive-like behavior in GR-deficient mice may be related to impaired BDNF-trkB signaling. Molteni and colleagues (2010) find that acute stressor exposure up-regulates *Bdnf* mRNA in the PFC of wild-type mice (an adaptive neuronal response), but not *Gr*+/- mice, suggesting that GR dysfunction may impair the adaptive neurochemical response to acute stressors involving increased BDNF-trkB signaling. In support of this idea, acute stress enhances the post-translational processing of proBDNF to mBDNF (which activates trkB) in the hippocampus of wild-type, but not *Gr*+/- mice (Molteni et al., 2010). These findings indicate that GR signaling interacts with BDNF/trkB systems to modulate neurobehavioral responses to stress.

1.11.2.2 Mutant models of BDNF disruption

The investigations using GR mutant mice discussed above suggest that defective GR expression or function impacts BDNF. Likewise, studies utilizing mutant *Bdnf* mice suggest that BDNF influences HPA axis reactivity and susceptibility to stress-related depressive-like behavior.

Mice with a knock-in of the human Val66Met single nucleotide polymorphism (SNP) in the *BDNF* gene, which decreases the activity-dependent secretion of BDNF (Egan et al., 2003; Chen et al., 2004), do not differ from wild-type (WT) mice in baseline plasma CORT levels (Yu et al., 2012). After exposure to repeated bouts of restraint stress for 7 days, however, $BDNF^{Val/Met}$ mice exhibit a greater stress-induced elevation in CORT than WT mice. PFC BDNF protein levels are lower in $BDNF^{Val/Met}$ mice, and restraint stress causes a greater loss of *Bdnf* mRNA in the PFC of $BDNF^{Val/Met}$ mice compared to WT mice. Furthermore, $BDNF^{Val/Met}$ potentiates the stress-induced development of depression-like behaviors and working memory impairments and dendritic spine loss on apical dendrites in the mPFC. Interestingly, Yu et al. (2012) also report a positive correlation between *Bdnf* mRNA in the mPFC and apical dendritic spine density in the PFC with working memory. At least one study in male rats reports that chronic CORT produces long-term impairments in conditioned fear extinction, a PFC-dependent process, in Val/Met, but not WT, rats (Gururajan et al., 2015). Thus, impairments in the activity-dependent secretion of BDNF may increase vulnerability to HPA axis hyperactivity and stress-related disruption in PFC *Bdnf* expression, neuronal morphology, and function.

These conclusions differ from those of Notaras et al. (2017), who compared the effects of late-adolescent CORT exposure (P42-56; in the drinking water) in $BDNF^{Val/Val}$, $BDNF^{Val/Met}$ and $BDNF^{Met/Met}$ mice (male and female) on depressive-like behavior and mPFC BDNF-trkB 2 weeks after the cessation of CORT. Control Met/Met mice (expressing the least amount of BDNF) are more immobile in the FST than Val/Val mice (expressing the most BDNF), and CORT exposure increases immobility in Val/Val mice. By contrast, neither genotype nor CORT exposure impacts

BDNF or trkB.FL protein levels in the mPFC in adulthood. CORT decreases trkB.T1 in Val/Val, but not Met/Met, mice, and *increases* phosphorylated trkB in both genotypes. Thus, it appears that reduced activity-dependent BDNF secretion does not increase vulnerability to CORT-induced down-regulation of mPFC BDNF-trkB in late adolescence, but may, rather, *increase* trkB-mediated signaling. Interestingly, in late-adolescent mice, CORT exposure for longer than 2 weeks (*i.e.*, extending into adulthood) induces different long-term behavioral and molecular changes (*e.g.*, see table 2), and in early-adolescent mice, CORT induces long-term depression-like behaviors and deficiencies in trkB throughout cortico-limbic regions (Barfield et al., 2017a). Determining whether late adolescence in mice is a period of stressor resilience is important because it could reveal mechanisms of stressor resilience.

Other studies utilizing different methods of reducing BDNF report sex-dependent vulnerability to stress. For example, chronic unpredictable stress exposure induces anhedonic-like sucrose neglect and increases stress-induced CORT release in female, but not male mice with forebrain-specific inducible knockdown of *Bdnf* (beginning in adulthood) (Autry et al., 2009). Monteggia et al. (2007) report that, even without stress exposure, *Bdnf* knockdown beginning in adolescence induces anhedonic-like behavior and behavioral despair in female, but not male, mice in adulthood (Monteggia et al., 2007). Whether female rodents carrying the *Bdnf* Met allele would be more vulnerable than males to HPA axis disturbance, alterations in PFC neuronal morphology, and behavioral/cognitive impairments following stress exposure in adolescence *vs.* adulthood would be an interesting focus for future investigations.

1.11.2.3 Importance of individual differences

Blugeot et al. (2011) suggest that *stress-induced* disruption of BDNF alters hippocampal neuronal morphology and increases vulnerability to depressive-like behavior upon re-exposure to

stress later in life. While this study focuses on molecular and morphological alterations in the hippocampus, it is likely that many of the findings could apply to the PFC as well and are worthy of discussion here. After a stress sensitization paradigm consisting of 4 days of social defeat, male rats exhibited HPA axis hyperactivity, reduced hippocampal BDNF, and morphological changes in the hippocampus, such as dendritic retraction and reduced spine density. Following a 4-week recovery period, two subpopulations of rats were identified: "vulnerable" rats that continued to exhibit diminished BDNF and morphological alterations in hippocampus, and "non-vulnerable" rats that recovered. Additionally, "vulnerable" but not "non-vulnerable" rats developed chronic mild stress-induced depressive-like and anhedonic-like behavior and adrenal hypertrophy. Intracerebroventricular administration of the trkB agonist, 7,8-DHF, during chronic mild stress blocked all neurochemical, morphological and behavioral alterations in "vulnerable" and "non-vulnerable" rats, including increased CORT levels in both groups. Long-lasting impairments in BDNF-trkB signaling following a priming stressful event may increase vulnerability to depressive-like behavior when an organism is re-stressed later in life, but organisms can differ significantly from one to another.

A noteworthy point regarding the study of Blugeot et al. (2011), discussed in the prior paragraph, is that persistent hippocampal BDNF deficits and morphological alterations resulting from the priming social defeat stress were related to *vulnerability* to depressive-like behavior, and not to a depressive-like state, *per se*. Furthermore, in "vulnerable" rats, HPA axis activity normalized following a recovery period after the priming stressor, but not following the subsequent chronic mild stress exposure protocol that ultimately induced depression-like behavior. Together, this pattern suggests that simultaneous dysregulation of both BDNF-trkB signaling and HPA axis activity (or GR function) may trigger depression-like phenotypes in some rodent models.

While the work of Blugeot et al. (2011) does not reveal whether the persistent BDNF deficits and chronic mild stress-related disruption of HPA axis activity in "vulnerable" rats are causally related or simply co-occurring, Taliaz and colleagues (2011) indicate that neurotrophin signaling in adolescence can alter HPA axis activity in adulthood. Specifically, viral-mediated *Bdnf* knockdown in the dorsal dentate gyrus of young rats (surgery at P21, with expected maximum gene knockdown by ~P35) induces anhedonic-like behavior when rats are tested in adulthood and elevates both baseline and acute stress-induced CORT. When control rats are exposed to chronic mild stress during adolescence (from P31-59), only those that exhibit anhedonic-like behavior in adulthood (behaviorally "non-resilient") also develop deficient hippocampal BDNF and elevated baseline and acute stress-induced CORT levels. Furthermore, unlike in young rats, *Bdnf* knockdown in adult rats does not cause long-lasting elevations in CORT levels. These findings indicate that stress-related anhedonic-like behavior is associated with reduced hippocampal BDNF and persistently elevated CORT levels, and further suggest that chronic stress-induced disruption of BDNF-trkB-mediated signaling during adolescence may contribute to dysregulation of HPA axis activity.

1.12 CONCLUSIONS

Chronic stress is a well-known risk factor for several psychiatric diseases, including depression. Abundant evidence from animal studies indicates that exposure to chronic stress recapitulates many of the core behavioral symptoms of depression in humans, as well as key structural and neuroendocrine alterations, including neuronal remodeling and synaptic loss in the PFC and impaired negative feedback inhibition of the HPA axis. Glucocorticoids and neurotrophins are critical regulators of dendritic spine structure and function, and their dysregulation is implicated in synaptic loss associated with stress-related psychopathology. As reviewed here, prefrontal trkB

and GR activities are modulated by stressor exposure. Disruption of trkB- and GR-mediated signaling events by chronic stressor exposure may set the stage for the development of psychopathology by impairing dendritic spine stability in the PFC and altering HPA activity.

Neurobiological consequences of, and behavioral outcomes following, chronic stress vary depending on the developmental timing of stressor exposure. This may be due to temporal and regional differences in the trajectory of synaptic maturation and neurotrophin and glucocorticoid receptors across postnatal development, which, together, may contribute to windows of vulnerability to adverse experiences. Indeed, trkB and GR signaling are critical for normal trajectories of brain development, and abnormal adolescent PFC development may contribute to psychiatric disease onset.

Studies using mutant *Bdnf*/*Trkb* mice indicate that decreased BDNF-trkB signaling during adolescence results in decreased cortical dendritic spine densities in adulthood, indicating that BDNF-trkB signaling is required for the ongoing stabilization of cortical dendritic spines throughout life. Additionally, some studies report that trkB and BDNF levels in the PFC markedly increase in adolescence or young adulthood, suggesting that BDNF-trkB-mediated signaling may be especially important for activity-dependent refinement of synaptic connections during adolescence. Moreover, adolescents exhibit greater and more prolonged glucocorticoid release following exposure to acute stressors, and HPA reactivity does not habituate with repeated exposure to the same stressor, unlike in adults. Increased expression of GR in the PFC in late adolescence/young adulthood may also render the PFC more susceptible to down-regulation of BDNF or trkB by excessive GR activation during this developmental period. Thus, disruption of trkB-mediated signaling by chronic stressor exposure may be particularly impactful in the adolescent PFC, potentially disrupting the trajectory of dendritic spine maturation, resulting in long-lasting structural alterations that may underlie persistent behavioral impairments and increase risk for the onset of psychiatric disease.

A key step in understanding mechanisms of susceptibility to stress-related psychopathology may be to identify long-term effects of stressor exposure, particularly stressor exposure during putative vulnerability periods like early adolescence. Initial findings in rodents, as reviewed here, suggest that persistently reduced BDNF-trkB activity following stressor or CORT exposure contributes to long-lasting structural alterations in cortico-limbic regions that underlie vulnerability to depressive-like behavior (Blugeot et al., 2011; Barfield et al., 2017a). Future work examining persistent alterations in molecular regulators of neuronal morphology following stressor exposure may yield critical insight into factors impacting risk for depression.

Importantly, adverse experiences can also impact the neurobehavioral consequences of *subsequent* stressor exposure, an example of metaplasticity. TrkB and GR systems, and their interaction, may be involved in the effects of stressful life events on the molecular, cellular, neuroendocrine, and behavioral response to stressors later in life. The coordinated actions of trkB and GR regulate neurobehavioral responses to stress, and disruption of one system may increase susceptibility to stress-induced disruption of the other system. A depressive-like state may be characterized by structural deficits in cortico-limbic brain regions and both impaired BDNF-trkB-mediated signaling and dysregulated HPA activity. Thus, treatment strategies that target both neurotrophin and glucocorticoid systems may be most effective in reversing structural deficits and associated cognitive/behavioral impairments in depression. Although stress-related psychopathologies, such as depression, are complex and multi-faceted, characterization of the long-term effects of chronic stress on trkB-glucocorticoid interactions in the PFC may facilitate the identification of risk factors and biomarkers, and may critically inform the development of novel treatments and early intervention strategies.

1.13 A BRIEF OVERVIEW AND RESEARCH STRATEGY OF THE DISSERTATION

This dissertation attempts to better understand why adverse experiences during adolescence increase vulnerability to an array of psychiatric diseases throughout the lifespan. We identify long-term behavioral, molecular, cellular, and circuit-level consequences of prolonged exposure to the GR ligand, CORT, during adolescence in mice. We focus our behavioral assessments on maladaptive, inflexible behaviors implicated in depression, substance use disorder, and post-traumatic stress disorder (among others) because the cortico-limbic regions that support behavioral flexibility are sensitive to stress and continue to mature during adolescence. We isolate specific cortico-limbic regions that show long-term changes in trkB-mediated signaling (the ventral hippocampus (vHC)), neuronal morphology (oPFC), and anatomical connectivity (vHC-oPFC) following adolescent CORT exposure. Using pharmacological and viral-mediated manipulations of trkB and circuit-specific chemogenetic inactivation techniques, we demonstrate that these CORT-induced changes are sufficient to impair flexible behaviors requiring outcome-based associative learning and memory.

Flexible, adaptive behavior in ambiguous environments requires that organisms use previously learned associations between actions or stimuli and contingent outcomes (rewarding or aversive), together with current contextual cues, to select appropriate responses based on anticipated outcomes. Failure to update or retain these outcome-predictive associations in memory when no longer valid can result in maladaptive behaviors or thought patterns. Goal-directed behavior is flexibly modulated when response-outcome contingencies change, while habit-based behavior is not. The vHC and oPFC, as well as projections *from* the vHC *to* the oPFC are necessary for guiding behaviors based on expected outcomes, and modifying behaviors when actual outcomes differ from expected outcomes (Yu & Frank, 2015; Wikenheiser & Schoenbaum, 2016).

We report in Chapter 2 that exposing mice to CORT during adolescence, but not adulthood, impairs goal-directed decision making in a food-reinforced operant conditioning task in adulthood, biasing behavior instead towards context-elicited habits. Further, we find that enduring disruption of

trkB signaling in the vHC is associated with vulnerability to inflexible habit-based behavior following CORT exposure. Chapter 3 explores the long-term effects of adolescent CORT and trkB manipulations on another index of behavioral flexibility, instrumental reversal learning, which is oPFC-dependent. We find sex-dependent effects of both manipulations on dissociable components of the task that depend on distinct oPFC sub-regions, suggestive of sexually dimorphic stress-related vulnerabilities in the medial vs. lateral oPFC. In Chapter 4, we report that adolescent CORT exposure also impairs the ability to discriminate contexts based on anticipated outcomes in aversive circumstances, resulting in maladaptive context-elicited fear responses. These deficits are long-lasting and coincide with the loss of dendritic spines in the lateral oPFC (loPFC) and vHC projections to the loPFC. Our findings indicate that a vHC-oPFC pathway that regulates outcome-based behavioral flexibility is sensitive to excess glucocorticoids during adolescence. These data also implicate neurotrophin signaling within this pathway in the enduring effects of adolescent glucocorticoid exposure on maladaptive, inflexible behaviors. Lastly, Chapter 5 synthesizes the findings of the previous chapters and integrates them with other relevant findings from the literature. We discuss the implications of this work for understanding the pathophysiological mechanisms by which early adverse experiences increase vulnerability to psychiatric disease, and for identifying novel targets of pharmacotherapies for adolescent populations exposed to chronic stress.

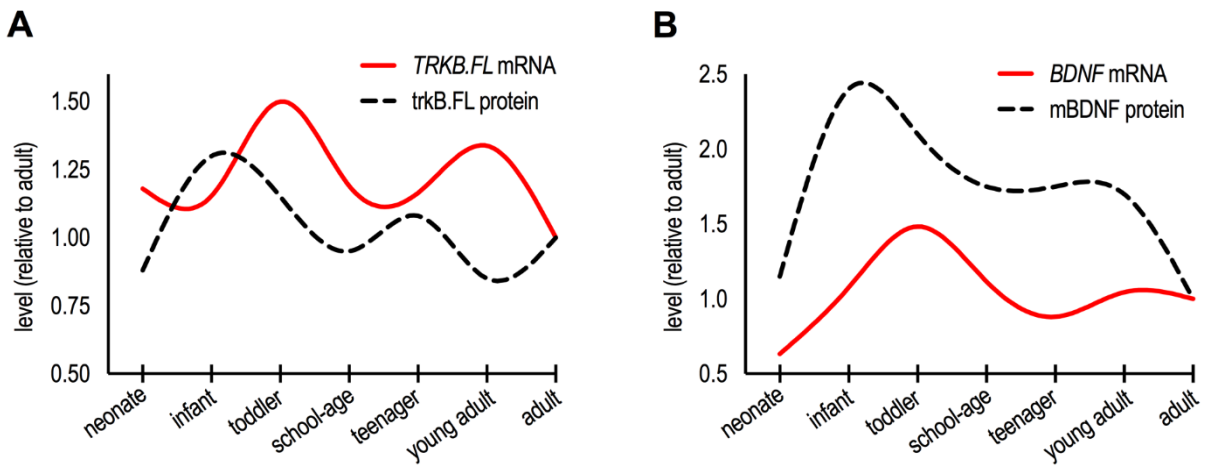

Figure 1-1. Developmental changes in trkB.FL and BDNF in human DLPFC. (A) Averages of levels of full-length trkB (*TRKB.FL)* mRNA in layers III and V reported by Romanczyk et al. (2002) and *TRKB.FL* mRNA reported by Luberg et al. (2010). trkB.FL protein levels reported by Luberg et al. (2010). **(B)** Averages of levels of *BDNF* mRNA alternative transcripts containing exons II and IV reported by Wong et al. (2009) and total *BDNF* mRNA in layers III and V reported by Webster et al. (2002). Mature BDNF (mBDNF) protein levels reported by Wong et al. (2009). Abbreviations: dorsolateral prefrontal cortex, DLPFC.

Reference	Species, age	Stress/CORT	Duration	Endpoint (timing relative to end of stressor/CORT)	Brain region	Expression changes	Behavioral changes	ADT-like effects if applicable
Qi et al., 2006	adult male rats	chronic forced swim	14 days	behavior: <1 day euth.: 1 day	PFC	↓ p-ERK2/ERK2	↓ saccharin preference	
Qi et al., 2008	adult male rats	chronic forced swim	21 days	behavior: <1 day euth.: ~4 days	PFC	↓ p-ERK2, p-CREB	↓ saccharin preference	Fluoxetine (concurrent with stressor exposure) blocked all
Zhou et al., 2017	adult male mice	chronic unpredictable stress	35 days	behavior: 4-7 days euth.: 7 days	PFC	↓ BDNF ↓ p-trkB/trkB	↓ sucrose preference ↑ immobility	Fluoxetine or biperiden (days 28-37 of the stress period) blocked all; Biperiden actions blocked by trkB antagonist
Wang et al., 2015	adult male mice	chronic unpredictable stress	56 days	behavior: 2-3 days euth.: 3 days	PFC	↓ p-ERK1/2/ERK1/2 ↓ p-Akt/Akt ↓ p-CREB/CREB	↓ sucrose preference ↑ immobility	Acute alarin (1 and 3 days after stressor) reversed all changes except sucrose preference; alarin actions blocked by trkB antagonist
Zhang et al., 2016b	adult male mice	chronic unpredictable stress	56 days	behavior: 1 day euth.: 3 days	PFC	↓ BDNF ↓ p-trkB/trkB ↓ PSD95 ↓ synaptophysin	↓ sucrose preference	7,8-DHF (days 29-56 of the stress period) blocked all (trkB antagonist blocked the effects of 7,8-DHF)
Wang et al., 2016	adult male rats	chronic restraint stress	21 days	behavior: 2 days euth.: 1 day	PFC	slight ↓ BDNF ↓ *Bdnf* mRNA ↓ p-ERK1/2/ERK1/2	↓ sucrose preference ↑ immobility	Fluoxetine or resveratrol (concurrent with stressor exposure) blocked all changes
Pesarico et al., 2017	adult male mice	social defeat	10 days	behavior: 1 day euth.: 1 day	PFC	↓ p-ERK1/2/ERK1/2 ↓ p75 receptor ↑ proBDNF ↑ p-CREB/CREB	↓ social interaction	FDPI (synthetic isoquinoline compound) (concurrent with stressor exposure) blocked all changes
Ma et al., 2016	adult male mice	social defeat (susceptible mice)	10 days	behavior: 1-3 days euth.: 4 days	PFC	↓ mBDNF ↓ p-trkB/trkB --- proBDNF	↓ sucrose preference ↑ immobility	Acute fluoxetine + brexpiprazole (2 days after stress) reversed all (trkB antagonist blocked)
					PL	↓ spine density		Fluoxetine + brexpiprazole reversed
					IL	--- spine density		
Wang et al., 2018	adult male rats	social defeat	14 days	behavior: <1 day-5 days euth.: ~6 days	mPFC	↓ BDNF --- p-ERK1/ERK1 ↓ p-ERK2/ERK2 ↓ p-CREB	↓ sucrose preference (BDNF in mPFC correlated with preference) ↓ cognitive flexibility ↓ reversal learning (BDNF in OFC correlated with better reversal learning)	
					OFC	↓ BDNF --- p-ERK1/2/ERK1/2 ↓ p-CREB		
Barfield et al., 2017a	adolescent male mice	subchronic oral CORT	11 days	behavior: >14 days euth.: <1 day	mPFC	↓ trkB.FL/trkB.T1 --- p-ERK1/2/ERK1/2	↓ goal-directed decision making	7,8-DHF (P39-47) blocked decision-making impairments and motivational

					PL	↓ spine density ↑ spine volume	↑ immobility (after acute stressor) ↓ reward-related motivation	deficits
				83 days	PL	↑ spine volume		
de Sousa et al., 2015	adult female mice	chronic CORT injections	21 days	behavior: <1 day euth.: 1 day	PFC	↓ BDNF	↓ sucrose preference ↑ immobility	Desvenlafaxine (DVS) or DVS+Alpha-lipoic acid (during last week of CORT) blocked all changes
Karisetty et al., 2017	adult male, female mice	chronic unpredictable stress	21 days	behavior: 2 days euth.: 3 days	PFC	--- BDNF (males) ↓ BDNF (females)	↓ sucrose preference (females) ↑ immobility (both sexes)	
Shirayama et al., 2015	adult male rats	inescapable electric shock	2 days	behavior: 6 days euth.: 2 or 6 days	mPFC	↑ proBDNF ↓ mBDNF ↓ p-trkB/trkB	↑ "helplessness" (failure in conditioned avoidance test)	7,8-DHF (2 days after shock) restored p-trkB (trkB antagonist blocked); 7,8-DHF infusion in the IL (but not PL) reduced "helplessness"
Bai et al., 2016	adult male rats	chronic unpredictable stress	21 days	behavior: 2-4 days euth.: 5 days	neocortex	↑ proBDNF ↑ p75 receptor (protein & mRNA) ↑ sortilin (protein & mRNA) ↓ trkB (protein & mRNA) ↓ *Bdnf* mRNA ↓ spine length	↓ sucrose preference ↑ immobility	Anti-proBDNF antibody (1 day after stress) reversed stress-induced behavioral changes and normalized spine lengths
Chiba et al., 2012	adult male rats	chronic restraint stress	28 days	behavior: <1 day-6 days euth.: 1 day	PFC	--- BDNF --- trkB ↓ BDNF-stimulated glutamate release	↓ sucrose preference ↑ immobility	

Table 1-1. Summary of studies examining short-term (immediate) effects of chronic stressors or CORT exposure on prefrontal cortical BDNF-trkB. If depressive-like behaviors or other longer-term neurobiological measures were collected, they are noted. "Immobility" refers to immobility in the forced swim test or tail suspension test. Abbreviations: antidepressant, ADT; corticosterone, CORT; euthanasia, euth.; infralimbic cortex, IL; medial prefrontal cortex, mPFC; orbital prefrontal cortex, OFC; prefrontal cortex, PFC; prelimbic cortex, PL; full-length trkB, trkB.FL; truncated trkB, trkB.T1.

Reference	Species, age	Stress/CORT	Duration	Endpoint (timing relative to end of stressor/CORT)	Brain region	Expression changes	Behavioral changes	ADT-like effects if applicable
First et al., 2011	adult male rats	chronic unpredictable stress	35 days	behavior: 1-13 days euth.: 14 days	frontal cortex	--- BDNF --- trkB ↓ p-ERK1/2/ERK2	--- spatial learning	Fluoxetine (concurrent with stressors) blocked changes in p-ERK
Yang et al., 2017	adult male mice	chronic unpredictable stress	28 days	behavior: 14 days euth.: 15 days	cortex	↓ *Bdnf* mRNA	↓ sucrose preference ↑ immobility	Anti-proBDNF injection in AC (on last day of stressor exposure) reversed all changes
Fanous et al., 2010	adult male rats	intermittent social defeat	every 3rd day for 10 days	2 hours	PL	↑ BDNF --- *Bdnf* mRNA		
					IL	--- BDNF ↑ *Bdnf* mRNA		
					AC	↑ BDNF ↑ *Bdnf* mRNA		
				28 days	PL, IL, AC	--- BDNF --- *Bdnf* mRNA		
Lin et al., 2009	adult male, female rats	chronic footshock	21 days + every other day for 21 days	1 day	PL	--- BDNF - M ↓ BDNF - F		
					IL	--- p-CREB - M, F		
					AC	↓ p-CREB - M --- p-CREB - F		
			21 days	22 days	PL, IL, AC	--- p-CREB - M, F --- BDNF - M, F		
Dong et al., 2017	adult male mice	social defeat (susceptible mice)	10 days	behavior: 2-9 days euth.: 10 days	PFC	↓ BDNF ↓ p-trkB/trkB ↓ PSD-95	↓ sucrose preference ↑ immobility	Ketamine (2 days after stress) reversed all changes
					PL	↓ spine density		
					IL	--- spine density		
Yang et al., 2015	adult male mice	social defeat (susceptible)	10 days	behavior: 2-8 days euth. 8 days	PFC	↓ BDNF ↓ p-trkB/trkB --- trkB	↓ sucrose preference ↑ immobility	R-Ketamine and S-Ketamine (1 day after stress) reversed all (trkB antagonist blocked ADT behavioral effects)
					PL	↓ spine density		
					IL	--- spine density		
Yang et al., 2016	adult male mice	social defeat (susceptible)	10 days	behavior: 2-9 days euth.: 10 days	PFC	↓ BDNF --- proBDNF ↓ p-trkB/trkB --- trkB ↓ PSD-95	↓ sucrose preference ↑ immobility	R-Ketamine (2 days after stress) reversed all changes

Leem et al., 2014	adult male mice	chronic restraint stress	21 days	behavior: 14 days euth.: 18 days	moPFC	↓ p-ERK1/2 ↓ p-MEK1/2	↓ social interaction ↑ immobility	Imipramine (concurrent with stressors) blocked behavioral changes and modifications in the moPFC
					cingulate cortex	↓ p-ERK1/2		
					mPFC	↓ p-ERK1/2		
Gourley et al., 2008b	adult male mice	chronic oral CORT	20 days	behavior: 11 days euth.: 20 days	mPFC	--- p-ERK1/2/ERK1/2	↓ reward-related motivation ↑ immobility	Amitriptyline (for 1 week following CORT) restored motivation; amitriptyline or fluoxetine (for 2 weeks following CORT) blocked immobility
Gourley et al., 2008c	adult male mice	chronic oral CORT	20 days	21 days			↓ sucrose intake	Amitriptyline (for 2 weeks following CORT) or fluoxetine (for 3 weeks following CORT) restored sucrose intake
				behavior: 11 days euth.: 20 days	mPFC	--- p-ERK1/2/ERK1/2	↓ reward-related motivation (scores covaried with BDNF and p-ERK1/2)	Amitriptyline (for 1 week following CORT) restored motivation
Gourley et al., 2012b	adult male rats	chronic oral CORT	20 days	>14 days			↓ goal-directed decision making	Amitriptyline (for 10 days following CORT) restored decision-making
	adult male mice			7 days	mPFC	↓ trkB		
				behavior: >14 days euth.: following behavioral testing	mPFC	↓ BDNF	↓ reward-related motivation (scores covaried with BDNF in CORT mice)	Riluzole (for 3 weeks following CORT) blocked effects of CORT on motivation and BDNF. Local BDNF infusion restored motivation
Gourley et al., 2009b	adult male rats	chronic oral CORT	20 days	behavior: 14 days euth.: 21 days	loPFC	↓ *Bdnf* mRNA	↓ conditioned fear extinction ↓ sucrose preference	
					IL	--- *Bdnf* mRNA		
Zhang et al., 2017	adolescent male rats	individual housing, P21-end + chronic unpredictable stress, P28-41	14 days	1 day			↓ sucrose preference	
				behavior: 21 days euth.: 32 days	mPFC	↓ BDNF ↓ p-ERK1/2/ERK1/2 ↓ p-CREB	--- sucrose preference ↑ immobility ↓ cognitive flexibility	
Xu et al., 2016b	adolescent male mice	social defeat, P28-37, then individual housing until euth.	10 days	behavior: 7 days euth.: 8 days	mPFC	↑ *Bdnf* mRNA	--- cognitive flexibility	
				behavior: 42 days euth.: 49 days	mPFC	↓ BDNF ↓ *Bdnf* mRNA	↓ cognitive flexibility	Duloxetine (P65-79) reversed all changes
					oPFC	--- BDNF --- *Bdnf* mRNA		
Xu et al., 2017	adolescent male mice	social defeat, P28-37, then individual housing until euth.	10 days	behavior: 42 days euth.: 49 days	mPFC	↓ *Bdnf* total mRNA ↓ *Bdnf IV* mRNA	↓ cognitive flexibility	Tranylcypromine (P65-78) reversed changes in behavioral measures and *Bdnf IV*

Desbonnet et al., 2012	adolescent male mice	individual housing, P31-end + social defeat, P35-45	10 days	5 days behavior: 25 days euth.: 40 days	PFC	--- *Bdnf* mRNA	↓ sucrose preference ↑ agonistic behavior --- sociability

Table 1-2. Summary of studies examining long-term effects of chronic stressors or CORT exposure on prefrontal cortical BDNF-trkB and depressive-like behaviors. In the case of stressor/CORT exposure during adolescence, ages are indicated, with "P" referring to postnatal day. "Immobility" refers to immobility in the forced swim test or tail suspension test. Note that, relative to the immediate effects of stressor/CORT exposure, effects are variable and some compensation in BDNF may be detectable. Abbreviations: anterior cingulate, AC; antidepressant, ADT; corticosterone, CORT; euthanasia, euth.; female, F; infralimbic cortex, IL; lateral orbital prefrontal cortex, loPFC; male, M; medial orbital prefrontal cortex, moPFC; medial prefrontal cortex, mPFC; orbital prefrontal cortex, oPFC; prefrontal cortex, PFC; prelimbic cortex, PL.

Reference	Species	Stress/ CORT	Duration	Assay endpoint	Brain region	Expression changes	HPA changes	Behavioral changes	ADT-like effects if applicable
Chang et al., 2016	adult male rats	chronic unpredictable stress	56 days	euth.: 2 days			↑ CORT	↓ sucrose intake at day 28 of stress exposure period	7,8-DHF (concurrent with last 4 weeks of stress) dose-dependently blocked changes in CORT
Jin et al., 2015	adult male mice	chronic unpredictable stress	28 days	<1 day	PFC	↓ BDNF	↑ CORT ↑ adrenal weight	↓ sucrose preference	Fluoxetine or oleoylethanolamide (concurrent with last 3 weeks of stress) blocked all
Zu et al., 2017	adult male mice	chronic unpredictable stress	35 days	behavior: 3-6 days euth.: 7 days	PFC	↓ BDNF protein, mRNA ↓ p-ERK1/2/ERK1/2 ↓ p-CREB/CREB	↑ CORT ↓ GR protein, mRNA	↓ sucrose preference ↑ immobility	Fluoxetine or higher doses of bacopaside I (concurrent with last 2 weeks of stress) blocked all
Pytka et al., 2017	adult male mice	chronic unpredictable stress	28 days	1 day	PFC	↓ BDNF	↑ CORT ↑ adrenal weight	↓ sucrose preference ↑ immobility	Fluoxetine or higher doses of HBK-15 (5-HT receptor antagonist) (concurrent with stressors) blocked all
Réus et al., 2012	adult male rats	chronic unpredictable stress	40 days	behavior: 1-7 days euth.: 7 days	PFC	-- BDNF	↑ CORT ↑ adrenal weight	↓ sucrose intake	Memantine (for 1 week following stress) reversed all and increased BDNF in PFC
Shukkoor et al., 2016	adult male rats	chronic unpredictable stress	42 days	<1 day	PFC	↓ BDNF	↑ CORT	↓ sucrose preference ↑ immobility	Fluoxetine (concurrent with last 4 weeks of stress) blocked all
Filho et al., 2015	adult female mice	chronic unpredictable stress	28 days	behavior: <1 day euth.: 2 days	PFC	↓ BDNF	↑ CORT	↓ sucrose preference, ↑ immobility	Fluoxetine or chrysin (concurrent with stressors) blocked all
Shilpa et al., 2017	adult male rats	chronic immobil-ization stress	10 days (2hr/day)	14 days	Frontal cortex	--- BDNF	--- GR	↓ spatial learning & memory ↓ sucrose preference ↑ immobility	Environmental enrichment (6hr/day for 2 weeks following stress) reversed all
					Hipp	↓ BDNF	↓ GR		
Yan et al., 2016	adolescent male mice	CORT injections, P35-56	21 days	behavior: 1 day euth.: 2 days	PFC	↓ BDNF ↓ p-trkB/trkB ↓ p-CREB/CREB	↑ CORT	↓ sucrose preference, ↑ immobility	Fluoxetine (concurrent with stressors) blocked all (trkB antagonist blocked effects of fluoxetine)

Table 1-3. Summary of studies examining effects of chronic stressors or CORT exposure on both prefrontal cortical BDNF-trkB and HPA systems. "Immobility" refers to immobility in the forced swim test or tail suspension test. Abbreviations: antidepressant, ADT; corticosterone, CORT; euthanasia, euth.; glucocorticoid receptor, GR; hippocampus, Hipp; hypothalamic pituitary adrenal, HPA; postnatal day, P; prefrontal cortex, PFC; serotonin, 5-HT.

CHAPTER 2: REGULATION OF ACTIONS AND HABITS BY VENTRAL HIPPOCAMPAL TRKB AND ADOLESCENT CORTICOSTEROID EXPOSURE

2.1 CONTEXT, AUTHOR'S CONTRIBUTION, AND ACKNOWLEDGEMENT OF REPRODUCTION

The following chapter describes the long-term consequences of adolescent corticosteroid exposure on goal-directed decision making and ventral hippocampal (vHC) tyrosine receptor kinase B (trkB) signaling, as well as the regulation of actions and habits by vHC trkB. The dissertation author contributed to the paper by designing and running experiments, analyzing data, and was a main contributor to the writing of the manuscript, under the guidance of Dr. Shannon Gourley. Dr. Kelsey Zimmermann and Kyle Gerber contributed to running experiments, and Dr. Ryan Parsons performed dendritic spine imaging. Dr. Kerry Ressler provided viral vectors. This chapter is reproduced from Barfield ET, Gerber KJ, Zimmermann KS, Ressler KJ, Parsons RG, Gourley SL (2017a) Regulation of actions and habits by ventral hippocampal trkB and adolescent corticosteroid exposure. *PLoS Biology* 15:1-27.

2.2 ABSTRACT

In humans and rodents, stress promotes habit-based behaviors that can interfere with action–outcome decision-making. Further, developmental stressor exposure confers long- term habit biases across rodent–primate species. Despite these homologies, mechanisms remain unclear. We first report that exposure to the primary glucocorticoid corticosterone (CORT) in adolescent mice recapitulates multiple neurobehavioral consequences of stressor exposure, including long-lasting biases towards habit-based responding in a food- reinforced operant conditioning task. In both adolescents and adults, CORT also caused a shift in the balance between full-length tyrosine kinase receptor B (trkB) and a truncated form of this neurotrophin receptor, favoring the inactive form throughout multiple cortico-limbic brain regions. In adolescents, phosphorylation of the trkB

substrate extracellular signal-regulated kinase 42/44 (ERK42/44) in the ventral hippocampus was also diminished, a long-term effect that persisted for at least 12 wk. Administration of the trkB agonist 7,8-dihydroxyflavone (7,8-DHF) during adolescence at doses that stimulated ERK42/44 corrected long-lasting corticosterone-induced behavioral abnormalities. Meanwhile, viral-mediated overexpression of truncated trkB in the ventral hippocampus reduced local ERK42/44 phosphorylation and was sufficient to induce habit-based and depression-like behaviors. Together, our findings indicate that ventral hippocampal trkB is essential to goal-directed action selection, countering habit-based behavior otherwise facilitated by developmental stress hormone exposure. They also reveal an early-life sensitive period during which trkB–ERK42/44 tone determines long-term behavioral outcomes.

2.3 INTRODUCTION

Goal-directed actions are defined as behaviors directed towards achieving a specific outcome. By contrast, habits are stimulus-elicited and insensitive to action-outcome relationships. Individuals who experience early-life stress have an increased incidence of behaviors that can lead to addiction and obesity as adults, and Patterson et al. (2013) provided evidence that these behaviors may result from an overreliance on outcome-insensitive habits. In rats, chronic stressor exposure similarly biases behavioral response strategies towards habits (Dias-Ferreira et al., 2009), and the primary glucocorticoid corticosterone (CORT) is sufficient to induce habit biases in both rats and mice (Gourley et al., 2012b). Exogenous glucocorticoids similarly enhance habit-based learning and memory in humans (Guenzel et al., 2014). And like early-life stress (Patterson et al., 2013), prenatal stress in humans and maternal separation in neonatal rats also induce inflexible habit behavior (Schwabe et al., 2012; Grissom et al., 2012).

Despite these convergences across species, how elevated glucocorticoids, particularly during specific developmental periods, cause long-term biases towards habit-based behavior remains unclear. To address this issue, we elevated CORT in mice during a timespan equivalent to early adolescence in humans, which induced habit biases in adulthood. We hypothesized that adolescent CORT exposure may have long-term behavioral consequences by impacting tyrosine kinase receptor B (trkB), the high-affinity receptor for Brain-derived Neurotrophic Factor (BDNF), in cortico-limbic regions. We were motivated by evidence that cortico-hippocampal trkB levels increase during early postnatal development and adolescence (Shapiro et al., 2017b) and are stress-sensitive (Begni et al., 2017).

Our investigations focused on the ventral hippocampus (vHC), medial prefrontal cortex (mPFC), striatum, and amygdala, brain regions implicated in action-outcome decision making – that is, in selecting behaviors based on expected consequences, rather than familiar habit-based strategies (Balleine & Dickinson, 1998; Corbit & Balleine, 2003; Yin et al., 2008; Hart et al., 2014; Smith & Graybiel, 2016). Specifically, inactivation of the mPFC or connected regions of the striatum causes failures in selecting actions based on their outcomes or on outcome value (Balleine & Dickinson, 1998; Corbit & Balleine, 2003; Yin et al., 2008; Hart et al., 2014; Smith & Graybiel, 2016). Similar behavioral impairments follow amygdala inactivation, in particular, inactivation of the basolateral compartment (Hart et al., 2014). Meanwhile, vHC inactivation disrupts goal encoding in the mPFC (Burton et al., 2009; Spellman et al., 2015), and the vHC appears to route contextual and task-relevant information to the mPFC and amygdala in order to influence reward-related decision making and response selection (Gruber et al., 2012; Ciocchi et al., 2015).

Habit biases that occur due to disruptions in cortico-limbic networks may be associated with depression-like behavior. Depressive rumination in humans can be habit-like – stimulus-elicited, resistant to change, and precipitated by stressor exposure (Watkins & Nolen-Hoeksema, 2014). A

sense of helplessness in depression has also been conceptualized as a habit-based weakness in awareness of action-outcome contingency (Griffiths et al., 2014). To investigate habit biases following adolescent CORT exposure, we used an instrumental contingency degradation procedure in which a familiar behavior was uncoupled from reward. To investigate depression-like behavior, we turned to the progressive ratio task, a classical assay of reward-related motivation. Using these separable strategies, our findings suggest that vHC trkB is necessary for goal-directed action (occluding habits), and that compromised trkB signaling *induces* habit-based and depression-like behavior. They also reveal a sensitive period during which enhancing ERK42/44 activity during adolescence can interfere with CORT-induced habit-based and depression-like behavior later in life.

2.4 MATERIALS AND METHODS

2.4.1 Subjects

Group-housed wildtype C57BL/6 mice (Jackson Labs) were used, except for dendritic spine imaging experiments, in which case, mice were *thy1*-YFP-expressing (C57BL/6 background) (Feng et al., 2000). Mice were provided a 12-hour light cycle (0800 on) and food and water *ad libitum* except during instrumental conditioning when body weights of all mice were reduced to 90-93% of baseline to motivate food-reinforced responding. Mice were males unless otherwise explicitly noted. The timing of experimental events is provided in Table 2-1, and timelines are also provided in the figures.

2.4.2 Ethics statement

Procedures were approved by the Emory University Institutional Animal Care and Use Committee, licenses 2000973, 2002802 and 4000010, and the *Guide for the Care and Use of Laboratory*

Animals in Research. In cases of euthanasia, mice were deeply anesthetized with isoflurane prior to rapid decapitation.

2.4.3 CORT exposure

CORT hemisuccinate (4-pregnen-11β 21-DIOL-3 20-DIONE 21-hemisuccinate; Steraloids) was dissolved in tap water (25μg/ml free base) according to an established protocol (Gourley et al., 2008a,b, 2012b). Mice were given CORT in place of normal drinking water. Water bottles were weighed daily, and mice were weighed every other day (Table 2-2). Average doses (mg/kg) of CORT were calculated by normalizing daily consumption values per cage to the total body weight of the animals in the same cage. Every 3 days, water bottles were emptied and refilled with fresh water or newly-prepared CORT solution.

Mice were exposed to CORT from postnatal days (P) 31-42 or 56-67 (in one cohort, P68 due to experimental error), resulting in ~5-9mg/kg/day. These periods correspond to early adolescence and early adulthood in rodents (Spear, 2000). Mice were euthanized at the end of the CORT exposure period, or they experienced a 2-, 4-, or 12-week washout period as indicated.

2.4.4 Forced swim stress

To compare blood serum CORT levels between CORT-exposed *vs.* stressor-exposed mice, naive mice were exposed to forced swim stress at P31 or daily from P31-42. Mice were placed in a glass cylinder (24cm x 15.5cm diameter) filled to 10 cm with 22-25ºC water in a dimly lit room. After 6 min, mice were allowed to dry in a warm cage lined with paper towels before being returned to the home cage. Water was changed between mice. Control mice were handled but not exposed to swim stress. Groups were also housed separately. Mice were weighed every other day (Table 2-2).

2.4.5 Blood serum CORT

We collected trunk blood at P31 or P42. Mice were briefly anaesthetized with isoflurane and then decapitated either early in the active, dark cycle (2000 hours) or late in the active cycle (0600 hours). In the case of swim stress, mice were euthanized 30 min following swimming (Bourke et al., 2013). Blood was centrifuged in chilled Eppendorph tubes at 4°C for 30 min, and serum extracted. CORT levels were analyzed in duplicate by ELISA (Assay Designs) in accordance with manufacturer's instructions with the exception of the extraction step which was excluded.

2.4.6 Gland harvesting

Adrenal and thymus glands were extracted following euthanasia by midline dissection and weighed in pairs.

2.4.7 Dendritic spine imaging, reconstruction

A widely-documented consequence of repeated stressor exposure is the elimination of dendritic spines in the mPFC. As part of our efforts to validate that our CORT exposure procedure recapitulated aspects of stressor exposure, brains from YFP-expressing mice were collected at the end of CORT exposure at P42 and submerged in chilled 4% paraformaldehyde for 48 hours, then transferred to 30% w/v sucrose. Brains were sectioned into 40 μm-thick sections at -15ºC. Dendrites on deep-layer mPFC neurons, prelimbic/medial orbital compartments, were imaged using fluorescence confocal microscopy and reconstructed in 3-D using Imaris software. Methods are described elsewhere (Gourley et al., 2013b), the only modification being that a Leica TSC SP8 microscope was used.

Eight dendrites/mouse, 16-25 μm in length and located between Bregma +1.98-+1.70 were imaged and reconstructed by a single blinded rater/experiment. In our adolescent population,

dendritic spine densities were more variable than expected based on our prior investigations of prelimbic cortical neurons (Gourley et al., 2012b; Swanson et al., 2013; Shapiro et al., 2017b), and un-blinding revealed considerable variance based on rostro-caudal positioning. Most dendrites (73%) were imaged at roughly Bregma +1.94 or +1.78. Thus, we next compared dendritic spine densities and morphological metrics by 2-factor (CORT x anatomical position) analysis of variance (ANOVA), total=4-8 dendrites/mouse, considering each dendrite an independent sample. Values +/-2 standard deviations from the mean were considered outliers and excluded, and the results of these comparisons are reported here.

To investigate long-term consequences of adolescent CORT exposure, dendritic spines were imaged and classified from mice exposed to CORT during adolescence then behaviorally tested in adulthood (methods described immediately below).

2.4.8 Instrumental response training

Mice were food-restricted and trained to nose poke for 20mg grain-based food reinforcers (Bio-Serv Precision Pellets) using Med-Associates conditioning chambers equipped with 2 nose poke recesses and a food magazine. Training was initiated with a fixed ratio 1 (FR1; also called "continuous reinforcement") schedule of reinforcement; 30 reinforcers were available for responding on each aperture (60 reinforcers/session). Sessions ended when mice acquired all reinforcers, or at 70 min. 5-7 training sessions were conducted (1/day). Unless specified, response acquisition curves represent both responses/min; there were no response biases throughout.

To assess decision-making strategies, a modified version of classical instrumental contingency degradation was used, as in our prior reports (*e.g.*, Gourley et al., 2012b; Swanson et al., 2013, 2015). In a 25-min "non-degraded" session, one nose poke recess was occluded, and responding on the other was reinforced using a variable ratio 2 schedule of reinforcement. In a 25-

min "degraded" session, the opposite aperture was occluded, and responding on the available aperture produced no programmed consequences. Instead, reinforcers were delivered into the magazine at a rate matched to each animal's reinforcement rate on the previous day. That is, food pellets were delivered independently of the animal's actions. Thus, responding on one aperture became significantly less likely to be reinforced than the other. The order of the sessions and which response-outcome contingency was "degraded" were counter-balanced.

The following day, a 5 min probe test was conducted in extinction. Both apertures were available. Mice that are sensitive to instrumental contingencies preferentially generate the response that is likely to be reinforced, a goal-directed response strategy; meanwhile, mice that have developed habits are insensitive to instrumental contingency degradation and generate both familiar responses equally, habitually (for further discussion of this task, see Balleine & O'Doherty, 2010).

Following test 1, responding was reinstated using an RI30-sec schedule of reinforcement for 4 days to promote the formation of stimulus-response habits (Dickinson et al., 1983). 30 reinforcers were again available (60 reinforcers/session, 1 session/day). Sessions ended when mice acquired all reinforcers, or at 70 min. Then, the 3-day contingency degradation and probe test protocol was repeated ("test 2").

In a separate experiment, mice were trained to nose poke using FR1 and then RI30 schedules of reinforcement. Then, mice were tested in the contingency degradation procedure 3 times (1 session/day) to quantify the development of response inhibition.

2.4.9 Context shift

To determine whether insensitivity to instrumental contingency degradation was context-dependent, we utilized a "context shift" (Swanson et al., 2015): The "non-degraded" and "degraded" training sessions and probe test 2 occurred in unique chambers located in a separate room in the

laboratory relative to training and test 1. The chambers were contextually distinct (containing a recessed lever and distinct odors) and configured differently (nose poke ports and house light located on different walls).

2.4.10 Reinforcer devaluation

Following instrumental contingency degradation, nose poking was reinstated using an RI30 schedule of reinforcement during 3 daily training sessions. Then, prefeeding devaluation was used to assess value-based response selection. Mice were placed individually in empty shoebox-style cages for a 1-hr habituation period. Then, mice were allowed access for 30 min to either standard chow or the food pellets used during instrumental conditioning. Immediately following this prefeeding, mice were placed in the conditioning chambers, and responding in a probe test conducted in extinction was measured for 10 min. This procedure was repeated the following day with the opposite food item. Prefeeding with the reinforcer pellets, but not standard chow, reduces response rates if mice select actions based on outcome value.

Mice consumed more reinforcer pellets than chow during the prefeeding period; thus, we tested mice in a third condition in which the amount of pellets available to each mouse was matched to the amount of chow that the group consumed during the prior prefeeding period. Subsequent response rates during the probe test did not differ (*i.e.*, when the pellets were restricted or not), and those following restricted access – controlling the amount of food ingested – are shown. All intake data are provided in Table S2-3.

2.4.11 Progressive ratio

In separate mice tested in the instrumental contingency degradation procedure, nose poke responding on one recess was reinstated using an FR1 schedule for 2 50-min sessions (1/day). Then,

responding on a progressive ratio schedule, in which the response requirement increased by 4 with each reinforcer delivery, was measured. Sessions ended after 180 min or when mice executed no responses for 5 min. The "break point ratio" refers to the highest number of responses:reinforcers generated.

2.4.12 Forced swim test

Following instrumental conditioning, mice were fed *ad libitum*. Within one week, mice were placed in a glass cylinder (24cm x 15.5cm diameter) filled to 10 cm with 25ºC water, as previously used to detect an *increase* in immobility following CORT (Gourley et al., 2008a). Ten-minute sessions were videotaped under dim light, and time spent immobile, defined as only movements necessary to keep the head above water, was scored by a single, blinded rater. In one experiment, an acute stressor (19 hr water deprivation) preceded the forced swim test in half of the group; "unstressed" mice in this experiment were left undisturbed.

It is important to note that while mice in this report had *ad libitum* food access at the time of forced swim testing, they had all experienced modest food restriction during instrumental conditioning experiments; this could conceivably influence mobility scores (for review, Bogdanova et al., 2013).

2.4.13 Locomotor monitoring

Following forced swim testing, locomotor activity was monitored for 24 hr using a custom-built Med-Associates locomotor monitoring system equipped with 16 photocells. Locomotor activity was quantified in photobeam breaks across the 24 hr period, which were summed into 6 hr bins (Table S2-2).

2.4.14 7,8-DHF

7,8-DHF (Sigma; dissolved in 17% dimethylsulfoxide (DMSO) and saline; 3-10mg/kg (Zhang et al., 2014)) was administered *i.p.* daily from P39-47. This period overlapped with the end of the adolescent CORT exposure period and was determined based on pilot studies. Control mice received 17% DMSO and saline.

2.4.15 Immunoblotting

Mice were euthanized at: the end of the CORT exposure procedure; 12 weeks following CORT (and following instrumental contingency degradation testing); or 30 min following the last of 8 daily injections of 7,8-DHF. Mice were briefly anaesthetized by isoflurane and euthanized by rapid decapitation. Brains were extracted and frozen at -80ºC. Brains were sectioned into 1-mm sections using a chilled brain matrix, and the mPFC, vHC, amygdala, dorsomedial striatum, and ventral striatum were extracted using a 1-mm tissue core by a single experimenter. Tissues were homogenized by sonication in lysis buffer (200 µl: 137 mM NaCl, 20 mM tris-Hcl (pH=8), 1% igepal, 10% glycerol, 1:100 Phosphatase Inhibitor Cocktails 2 and 3 (Sigma), 1:1000 Protease Inhibitor Cocktail (Sigma)), and stored at -80ºC. Protein concentrations were determined using a Bradford colorimetric assay (Pierce).

Equal amounts of protein were separated by SDS-PAGE on 7.5% gradient tris-glycine gels (Bio-rad). Following transfer to PVDF membrane, blots were blocked with 5% nonfat milk for 1 hr. Membranes were incubated with primary antibodies at 4ºC overnight and then in horseradish peroxidase secondary antibodies for 1 hr. Immunoreactivity was assessed using a chemiluminescence substrate (Pierce) and measured using a ChemiDoc MP Imaging System (Bio-rad). Densitometry values were normalized to the control sample mean from the same membrane in order to control

for fluorescence variance between gels. vHC and amygdala samples were loaded on the same gels to allow for comparisons within and between brain regions and tested at least twice.

Primary antibodies were anti-trkB (Rb, Cell Signaling, 4603s, lot 3; 1:375), anti-ERK42/44 (Rb, Cell Signaling, 9102s, lot 26; 1:2000), anti-p-ERK42/44 (Ms, Cell Signaling, 9106s, lot 43; 1:1000), and anti-PSD95 (Rb, Cell Signaling, 3450s, lot 2; 1:1000). In our initial comparisons of vHC-amygdala ERK42/44, a loading control (GAPDH; Ms, Sigma, G8795, lot 044M4808V; 1:5000) was additionally applied to further confirm equivalent loading.

2.4.16 Surgery

Naïve mice were infused with a lentivirus expressing a CMV promoter and truncated trkB receptor isoform, *Trkb.t1*, with an HA tag (titer=$5.8x10^8$ iu/ml; virus described in Rattiner et al., 2004; Heldt et al., 2014). Control mice were infused with lenti-GFP, also bearing a CMV promoter. Mice were anaesthetized with ketamine/xylazine and placed in a digitized stereotaxic frame (Stoelting). The scalp was incised, skin retracted, bregma and lambda identified, the head leveled, and stereotaxic coordinates corresponding to the vHC or CeA were located (-3.0 AP/-4.0 DV/±2.75 ML and -1.5 AP/-4.9 DV/±3.0 ML, respectively). Viral vectors were infused over 5 min, with 0.5 µl/side. Needles were left in place for 5 additional min prior to withdrawal and suturing. Three weeks later, instrumental conditioning began. Following testing, fixed brain tissue was imaged for GFP or immunostained for HA as described (Heldt et al., 2014). Mice were infused at P31 or P56 at the same coordinates; timing did not impact behavioral outcomes.

2.4.17 p-ERK42/44 immunostaining

Tissue sections from mice expressing viral vectors were blocked in a PBS solution containing 2% normal goat serum, 1% bovine serum albumin (BSA), and 0.3% Triton X-100

(Sigma) for 1 hr at room temperature. Sections were then incubated in a primary antibody solution containing 0.3% normal goat serum, 1% BSA, and 0.3% Triton X-100 at 4 °C for 48 hr. p-ERK42/44 (Rb, Cell Signaling, 9102s, lot 26; 1:400) served as the primary antibody. Sections were incubated in secondary antibody solution containing 0.5% normal goat serum and 0.3% Triton X-100, with Alexa Fluor 633 (1:200; Life Technologies) serving as the secondary antibody.

Sections were imaged on a Nikon 4550s SMZ18 microscope with settings held constant. Integrated intensity (normalized to the size of the sampling area) was measured where HA or GFP staining was also detected. Sections were compared in 2 cohorts, and fluorescence values were normalized to the control mean from each respective cohort. We analyzed 1-10 sections from each mouse, with each animal contributing a single mean value to statistical analysis.

2.4.18 Statistical analyses

Body weights, blood serum CORT, gland weights, response rates, response counts, break point ratios, and densitometry values were compared by 2-tailed ANOVA or *t*-test using SPSS with $p<0.05$ considered significant. Following interactions, post-hoc comparisons utilized Tukey's HSD tests, and results are indicated graphically. In mice bearing *Trkb.t1*-expressing viral vectors, exclusions due to mis-targeted infusions and the combination of control groups resulted in considerably uneven sample sizes (reported in the captions); thus, we compared these groups by type III ANOVA. Statistical approaches to comparing dendritic spine densities and morphologies are outlined in the corresponding section above. Values lying >2 standard deviations above the means were considered outliers and excluded.

2.5 RESULTS

2.5.1 Exogenous CORT exposure recapitulates several effects of stress: validation of the method

In both humans and rodents, glucocorticoid exposure can induce biases towards habit-based behavior, at the expense of goal-directed action (Gourley et al., 2012b; Guenzel et al., 2014). Further, stressor exposure during early developmental periods appears to confer *long-term* habit biases across rodent-primate species (Schwabe et al., 2012; Grissom et al., 2012; Patterson et al., 2013). Despite these homologies, mechanisms remain unclear. To address these issues, we exposed mice to CORT in the drinking water from postnatal day (P) 31-42, equivalent to early adolescence in humans (Spear, 2000). The timing of experimental events is provided in Table 2-1, and timelines are also provided in the figures.

We first confirmed that exogenous CORT exposure elevated blood serum CORT late in the active period (*i.e.*, nighttime) when mice had been active and ingesting CORT for several hours. Notably, levels did not differ between CORT-exposed and control groups during the early active period when mice were just waking [interaction $F_{(1,27)}=22.3, p<0.001$] (Fig 2-1a). This pattern indicates that exogenous CORT disrupts typical diurnal changes in blood serum CORT levels. Correspondingly, adrenal and thymus glands atrophied during the CORT exposure period, as expected [$t_8=5.7, p<0.001$; $t_8=4.24, p=0.003$] (Fig 2-1b). Also as expected, gland weights recovered when exogenous CORT was removed [$t_{10}=-0.25, p=0.8$; $t_{10}=0.099, p=0.9$] (Fig 2-1b). Despite this recovery, break point ratios in a progressive ratio test, an assay of reward-related motivation, were reduced in mice with a history of CORT exposure [$t_9=2.39, p=0.04$] (Fig 2-1c). This pattern is consistent with amotivation in depression and provides evidence of long-term behavioral consequences of adolescent CORT exposure.

We next compared our oral CORT exposure procedure to daily forced swim stress. Forced swimming increased blood serum CORT as expected, although this effect appeared to habituate

with repeated exposure [$F_{(2,20)}$=4.0,p=0.04] (Fig 2-1d). Nonetheless, adolescent stressor exposure decreased progressive ratio break points, as with the oral CORT procedure [t_{13}=3.56,p=0.003] (Fig 2-1e).

Another well-characterized consequence of repeated stressor exposure is the elimination of dendritic spines on pyramidal mPFC neurons (Holmes & Wellman, 2009). Thus, as another experiment validating our CORT exposure method, we enumerated dendritic spines on excitatory deep-layer pyramidal neurons in the mPFC using *thy1*-YFP expressing transgenic mice. Spines were eliminated in the anterior-most sections [interaction $F_{(1,73)}$=4.9,p=0.03] (Fig 2-1f). The effect size (Cohen's *d*) was 0.92, signaling that ~80% of dendrites in CORT-exposed mice had fewer dendritic spines than the control mean.

Dendritic spines were also reconstructed in 3-D, revealing increased volume following CORT [main effect $F_{(1,72)}$=6.2,p=0.02] (Fig 2-1g). This phenomenon could not be accounted for by an increase in the head size [Fs<1] (Fig 2-1h), suggesting that CORT induced dysmorphic spines with aberrantly large necks. This pattern was detected even several weeks following CORT exposure (Fig S2-1).

Lastly, we exposed adult mice with a history of adolescent CORT treatment to the forced swim test. In this test, attempting to escape has been termed "active coping," while immobility has been termed "passive coping" (de Kloet et al., 2016). Prior CORT exposure did not impact baseline immobility scores, however an acute stressor challenge prompted an "active" coping style in control mice, reducing immobility. CORT-exposed mice were, by comparison, more immobile, favoring a "passive" response [CORT x stress $F_{(1,21)}$=6.3,p=0.02] (Fig 2-1i). Thus, exposure to exogenous CORT in adolescence modified stressor reactivity in adulthood.

2.5.2 Subchronic CORT exposure in adolescence, but not adulthood, induces habit biases

Having characterized our model, we next determined whether the same subchronic CORT exposure procedure would impact habit biases. We first trained control and CORT-exposed mice to nose poke two separate recesses for food reinforcers. We detected no side biases, nor group differences in instrumental response acquisition rates [$F_{(1,15)}$=2.7,p=0.12; interaction $F_{(9,135)}$=1.6,p=0.12] (Fig 2-2a).

We next decreased the likelihood that one response would be reinforced. A "goal-directed" response strategy is to then preferentially engage the remaining behavior, which remains likely to be reinforced, while habit-based responding is insensitive to action-outcome contingency (Balleine & O'Doherty, 2010). In this case, mice engage both responses (both "non-degraded" and "degraded") equivalently. Following an initial test, both groups inhibited the response that was unlikely to be reinforced in a goal-directed (non-habitual) fashion [main effect $F_{(1,15)}$=11.6,p=0.004; no interaction] (test 1, Fig 2-2b). Response rates were also lower overall in the CORT-exposed mice, consistent with diminished break point ratios in the progressive ratio test (Fig 2-1c).

With additional training using random interval (RI) schedules of reinforcement that can bias responding towards habits, mice with a history of CORT exposure indeed assumed habit-based strategies, failing to differentiate between the behaviors that were more, or less, likely to be reinforced. Meanwhile, control mice differentiated between the responses, retaining goal-oriented response strategies [interaction $F_{(1,13)}$=6.0,p=0.03] (test 2, Fig 2-2b). Thus, subchronic CORT exposure *in adolescence* causes a bias towards habit formation *in adulthood*. Notably, we discovered the same patterns when we tested female, rather than male, mice (Fig S2-2).

In separate mice, test #2 occurred in a distinct context. In this case, both groups generated the response that was likely to be reinforced [main effect $F_{(1,12)}$=16.7,p=0.001; no interaction] (Fig 2-2c). Thus, adolescent CORT-induced habits were context-dependent.

Next, we assessed whether subchronic CORT exposure similarly impacted *adult* mice. CORT exposure decreased body weights as expected (Table 2-2). While instrumental response rates during training were generally lower than in our younger cohorts, control *vs.* CORT-exposed groups did not differ [*F*s<1] (Fig 2-2d). And unlike with subchronic CORT exposure in adolescence, both groups consistently generated the response most likely to be reinforced in a goal-directed fashion [main effect test 1: $F_{(1,12)}$=8.4,p=0.01; main effect test 2: $F_{(1,12)}$=36,p<0.001; no interactions] (Fig 2-2e). Thus, adolescents were more vulnerable to the habit-inducing influence of elevated glucocorticoids.

Insensitivity to instrumental contingencies is commonly associated with insensitivity to reinforcer value. However, when we tested the same mice for sensitivity to reinforcer devaluation, we found no impairment in response inhibition following *ad libitum* access to the reinforcer pellets prior to test ("devalued" condition), relative to prefeeding with regular chow ("non-devalued" condition) [main effect $F_{(1,26)}$=28,p<0.001; no interaction] (Fig 2-2f). Thus, subchronic CORT exposure during adolescence, but not adulthood, induced failures in selecting actions based on their consequences. This failure was context-dependent, while value-based action selection was intact.

In a final experiment, we exposed adolescent mice to CORT, then trained them to respond for food reinforcers following a prolonged, 4-week washout period. This procedure doubled the "recovery" period following CORT and matched the age of testing in our adult CORT-exposed population. Mice acquired the nose poke responses, without group differences [$F_{(1,21)}$=2.7,p=0.12; interaction *F*s<1.2] (Fig 2-2g). Mice were sensitive to action-outcome contingency degradation at test 1, as in all other groups [main effect $F_{(1,21)}$=5.8,p=0.03; no interaction] (Fig 2-2h). In a second test, CORT-exposed mice again preferentially generated the response most likely to be reinforced, however this preference decayed [group x response x time interaction $F_{(1,20)}$=4.2,p=0.05] (Fig 2-2h). Thus, adolescent CORT-exposed mice recovered some function with a prolonged washout period

(as opposed to our shorter washout period tested above), but habitual response biases remained detectable nearly 1 month following exposure.

2.5.3 CORT shifts cortico-limbic trkB/trkB.t1 ratios and reduces p-ERK42/44 in the vHC

The transition from goal-directed to habit-based modes of response has been characterized as a decline in behavioral control by specific PFC-limbic structures (*e.g.*, Fig 2-3a), in favor of sensorimotor circuits (Yin et al., 2008; Balleine & O'Doherty, 2010; Schwabe, 2013; Hart et al., 2014; Smith & Graybiel, 2016; Gourley & Taylor, 2016). Within the hippocampus, the ventral compartment provides the primary inputs to the PFC (Hoover et al., 2007). For these reasons, we assessed levels of the stress-sensitive neurotrophin receptor trkB and phosphorylation of its substrate ERK42/44 in a mPFC-vHC-amygdala-striatal network. Results are reported in Table 2-3. Key findings are also displayed graphically: Specifically, adolescent CORT exposure decreased the ratio of full-length trkB/trkB.t1 in the mPFC [t_{12}=4.3,p<0.001] (Fig 2-3b). This pattern was not anatomically-selective – in both the vHC and amygdala, developmental CORT also decreased trkB/trkB.t1 ratios [main effect of CORT $F_{(1,23)}$=10.7,p=0.003] (Fig 2-3c, top), as was also observed in the ventral striatum [t_{10}=3.9,p=0.003] (Table 2-3). CORT elevated overall levels of trkB.t1 in the vHC [interaction $F_{(1,21)}$=5.8,p=0.025] (Fig 2-3c, bottom) and ventral striatum [t_{10}=-2.3,p=0.046] (Table 2-3), but only in the vHC were levels of phosphorylated (active) ERK42/44 also decreased by CORT (Fig 2-3d; Table 2-3). Of note, phospho-ERK levels were also generally higher in the vHC than the amygdala [pERK42 $F_{(1,18)}$=16.4,p<0.001; pERK44 $F_{(1,18)}$=8.5,p=0.009] (Fig 2-3d).

To determine whether this effect was long-lasting, we immunoblotted for p-ERK42/44 in the vHC 12 weeks following adolescent CORT exposure, again revealing lower phosphorylated levels of both ERK isoforms [p-ERK42: t_{18}=2.7,p=0.01; p-ERK44: t_{18}=3,p=0.008] (Fig 2-3e).

We next tested adult mice exposed to CORT. CORT again decreased amygdalo-hippocampal ratios of full-length trkB/trkB.t1 [main effect $F_{(1,18)}$=9.4,p=0.007] and also elevated trkB.t1 [main effect $F_{(1,20)}$=21.2,p<0.001] (Fig 2-3f), however we identified no effect of CORT on p-ERK42/44 [main effects and interactions p>0.18] (Fig 2-3g). *Thus, a unique consequence of subchronic CORT exposure in adolescence appears to be the elevation of trkB.t1 and concurrent suppression of p-ERK42/44 within the vHC.*

2.5.4 Blockade of CORT-induced habits and amotivation by trkB stimulation

Next, we attempted to block adolescent CORT-induced habits with the trkB agonist 7,8-dihydroxyflavone (7,8-DHF). We first aimed to identify a dose range that stimulated p-ERK42/44 in the vHC. Three and 10 mg/kg, *i.p.,* induced ERK42 phosphorylation [$F_{(2,38)}$=3.9,p=0.03] (Fig 2-4a), though p-ERK44 levels were not affected [$F_{(2,38)}$=1.7,p=0.2] (Fig 2-4b). 7,8-DHF also blocked the chronic p-ERK42 deficit due to CORT exposure (Table 2-4), while having no long-term consequences for adrenal and thymus gland weights (Table S2-1), and the 10 mg/kg dose increased levels of the postsynaptic marker PSD95 [$F_{(2,24)}$=4.8,p=0.02] (Fig 2-4c).

Next, separate mice were exposed to CORT during early adolescence, and half were treated with 7,8-DHF (3 mg/kg) from P39-47, overlapping with the end of the CORT exposure period and extending into late adolescence (Spear, 2000) (Fig 2-4d). As adults, mice acquired the nose poke responses with no group differences [main effect CORT and 7,8-DHF Fs<1; CORT x 7,8-DHF interaction $F_{(1,19)}$=3.8,p=0.065; all other interactions Fs≤1.1] (Fig 2-4e). Following an initial instrumental contingency degradation test, all groups preferentially generated the response most likely to be reinforced in a goal-directed fashion as in our experiments described above [main effect $F_{(1,20)}$=24.6,p<0.001; no interaction] (test 1, not shown). With more training, CORT-exposed mice developed habit-based behavior, also as expected, but critically, *7,8-DHF blocked CORT-induced habit*

biases [CORT x response interaction $F_{(1,19)}$=7.2,p=0.015; 7,8-DHF x response interaction $F_{(1,19)}$=5.3,p=0.03] (test 2, Fig 2-4f). This effect of 7,8-DHF was also detectable in female mice (Fig S2-2).

We next tested these mice in the forced swim test. As in Fig 2-1, prior CORT did not impact immobility in the absence of stressor exposure in adulthood. However, a history of 7,8-DHF treatment reduced time spent immobile, an antidepressant-like effect that was notably detectable multiple weeks following treatment [main effect of 7,8-DHF $F_{(1,20)}$=8.3,p=0.008; no interaction] (Fig 2-4g). The reduction in time spent immobile could not obviously be attributable to general hyper-activity following 7,8-DHF treatment (Table S2-2).

We also quantified responding on a progressive ratio schedule of reinforcement. 7,8-DHF dose-dependently blocked CORT-induced deficits in break point ratios, and this blockade was detectable when either total responses or break points were compared [interactions $F_{(2,56)}$=3.9,p=0.03; $F_{(2,54)}$=3.4,p=0.04]. Break points are shown (Fig 2-4h).

2.5.5 Recapitulating the long-term effects of adolescent CORT exposure with *Trkb.t1* overexpression

To summarize, adolescent CORT exposure increases levels of trkB.t1 and decreases p-ERK42/44 in the vHC and also induces biases towards habit-based behaviors. These biases are blocked by the putative trkB agonist 7,8-DHF at doses that increase p-ERK42 in vHC. To determine whether selectively elevating trkB.t1 and decreasing p-ERK42/44 in the vHC is *sufficient* to recapitulate the behavioral effects of CORT exposure, we over-expressed *Trkb.t1* in the vHC and a major projection target, the central nucleus of the amygdala (CeA) (Fig 2-5a). vHC-targeted infusions were mostly restricted to the ventral CA1 region, with some spread into the intermediate hippocampus (Fig 2-5a; see also Fig S2-3). Amygdala-targeted infusions were largely contained

within the CeA as intended (Fig 2-5a; see also Fig S2-3). Seven mice were excluded due to mis-targeted infusions infecting white matter tracts, and control GFP-expressing mice did not differ and were combined.

At the infusion site, p-ERK42/44 levels were reduced by 15% in *Trkb.t1*-exressing mice relative to mice expressing GFP [t_{13}=2.6,p=0.02] (Fig 2-5b), mimicking the long-term consequences of adolescent CORT exposure (*cf.*, Fig 2-3d). Mice acquired the food-reinforced instrumental responses without group differences [Fs≤1] (Fig 2-5c). GFP-expressing control mice were sensitive to instrumental contingency degradation, preferentially engaging the response most likely to be reinforced. By contrast, *Trkb.t1* overexpression induced inflexible habits, indicated by a failure to respond in a selective fashion following instrumental contingency degradation [interaction $F_{(2,34)}$=5.6,p=0.008] (Fig 2-5d). Thus, *Trkb.t1* overexpression recapitulated the long-term effects of adolescent CORT exposure.

Next, we expanded these studies, deviating from our protocol used thus far, to assess whether *Trkb.t1* overexpression in the vHC also interfered with the ability to "break" habits. We accordingly trained separate mice using an RI schedule of reinforcement. Instrumental response acquisition curves are segregated according to whether the action-outcome contingency associated with each response would ultimately remain intact or be "degraded," highlighting equivalent response rates throughout [Fs<1] (Fig 2-5e).

We modified our typical instrumental contingency degradation procedure to determine whether responses could be inhibited once habits formed. Specifically, we exposed mice to alternating training sessions in which one response was reinforced or the contingency between the other response and its outcome was degraded. Initially, mice responded equivalently during these two types of training sessions, habit-based behavior. With repeated testing, control mice were ultimately able to inhibit the response that was unlikely to be reinforced. By contrast, response

strategies in *Trkb.t1*-overexpressing mice were unchanged, habit-based [group by contingency interaction $F_{(1,32)}$=6.6,p=0.02] (Fig 2-5f). Thus, *Trkb.t1* overexpression caused significant behavioral inflexibility.

Finally, we also confirmed that vHC *Trkb.t1* overexpression decreased break point ratios in a progressive ratio test [t_{16}=2.8,p=0.01] (Fig 2-5g), as with adolescent CORT exposure. This finding is consistent with evidence that vHC-targeted knockdown of the trkB ligand BDNF also induces depression-like behavior (Taliaz et al., 2010).

2.6 DISCUSSION

Early-life stress is associated with inflexible habit-based behavior in humans (Schwabe et al., 2012; Patterson et al., 2013), and in both humans and rodents, glucocorticoid exposure induces habit biases (Gourley et al., 2012b; Guenzel et al., 2014). Despite these homologies, mechanisms are largely uncharacterized. We report that CORT exposure in mice during a period equivalent to early adolescence in humans induces: a bias towards habit-based behaviors; a shift in the balance between full-length, active trkB and an inactive, truncated form of the receptor throughout multiple cortico-limbic brain regions; and p-ERK42/44 deficits selectively in the vHC (summarized in Table 2-3). Viral-mediated overexpression of *Trkb.t1* selectively in the vHC decreases local p-ERK42/44 and causes habit-based behavior. Meanwhile, *stimulating* trkB-ERK42/44 during adolescence blocks CORT-induced habits and has antidepressant-like effects that are detectable well after the treatment period, revealing an early-life sensitive period when trkB-ERK42/44 tone has long-term behavioral consequences.

We first validated our CORT exposure procedure, revealing elevated blood serum levels during the active dark cycle that were comparable to CORT levels following forced swim stress. Meanwhile, blood serum CORT during the inactive light cycle (when mice are sleeping and not

consuming CORT) was unaffected. Adolescent CORT exposure additionally reduced progressive ratio break points, as also occurs in cases of adult CORT exposure (Gourley et al., 2008a,b, 2012b; Olausson et al., 2013). This behavioral phenotype has been likened to amotivation in depression and is consistent with considerable evidence that a history of stressor exposure is a primary predictor of depression (Heim et al., 2004).

Unlike chronic CORT (*e.g.*, Gourley et al., 2008a), subchronic CORT (here) did not induce immobility in the forced swim test, but reactivity to an acute stressor was impacted. Specifically, water deprivation induced mobility in control mice (see also Yamamoto et al., 2009), a so-called "active coping" response (de Kloet & Molendijk, 2016)). Meanwhile, CORT-exposed mice maintained high levels of immobility, a "passive coping" response that has also been interpreted as depression-like (Porsolt et al., 1977). Dendritic spines were also eliminated in the mPFC, a common reaction to stressor and CORT exposure in mature rodents (Holmes & Wellman, 2009; Swanson et al., 2013). These outcomes may be related, given that switching between "active" and "passive" swimming phenotypes is dependent upon stimulation of mPFC projections to brainstem targets (Warden et al., 2012).

2.6.1 Adolescent CORT exposure has persistent neurobehavioral consequences

Adolescent CORT exposure also induced a long-term bias towards habit-based response strategies. Specifically, mice were initially able to select actions (left/right nose poke) based on their consequences (food), but with repetition, these behaviors assumed habitual qualities such that they were insensitive to response-outcome contingency. Subchronic CORT exposure did not impact adult mice, indicating that adolescents are more vulnerable to developing CORT-induced habits. Moreover, when we doubled the "recovery" period duration following adolescent CORT exposure, all mice could initially select actions based on their consequences, but response preferences faded in

CORT-exposed mice. We interpret this as uncertainty in response selection, resulting in a deferral to familiar, habit-based behaviors that are insensitive to response-outcome associations.

Behavioral insensitivity to response-outcome contingency is often associated with insensitivity to reinforcer value (Balleine & O'Doherty, 2010). This was not the case here, however, in that all mice reduced response rates following prefeeding with the reinforcer pellets, which decreases their value. Why might this be? One possibility is that subchronic CORT exposure particularly impacted hippocampal function. Lesions of the entorhinal cortex, a primary input to the hippocampus, reduce sensitivity to response-outcome contingencies but not reinforcer value, as with CORT here (Corbit et al., 2002; Lex & Hauber, 2010). This may be because organisms form an association between the context and response-outcome contingency during training. When that contingency is later violated, the hippocampus detects the discrepancy between "the context where I typically work for reward" and noncontingent pellet delivery and facilitates response inhibition. This model predicts that response strategies should be intact if instrumental contingency degradation occurs in a contextually distinct environment relative to the training environment, which was indeed the case here. In contrast, behavioral sensitivity to reinforcer devaluation is context-*independent* because it relies on an animal's ability to prospectively calculate reinforcer value. Accordingly, it is unaffected by entorhinal cortex lesions (Corbit et al., 2002; Lex & Hauber, 2010), while lesions/inactivation of other structures, such as the dorsal mPFC, basolateral amygdala, and dorsomedial striatum impair sensitivity to both response-outcome contingency and reinforcer value (Balleine & O'Doherty, 2010).

Based on these findings, we next quantified levels of the stress-sensitive neurotrophin receptor trkB in the vHC and other regions that counter habit-based behavior (dorsal mPFC, amygdala, dorsomedial striatum, and ventral striatum; see Yin et al., 2008; Balleine & O'Doherty, 2010; Hart et al., 2014; Smith & Graybiel, 2016). CORT caused widespread modifications in the

ratio of full-length:truncated isoforms, favoring the inactive isoform. (Indeed, of the brain regions tested, only the dorsomedial striatum was spared.) This is significant because trkB.t1 dimerization with full-length trkB reduces the receptor's ability to stimulate ERK/MAP kinase, PI3-kinase, and PLCγ. trkB.t1 is also linked to neurodegeneration (Danelon et al., 2016) and excitotoxicity (Gomes et al., 2012). Additionally, increasing trkB.t1 decreases cell-surface levels of full-length trkB, further reducing opportunities for trkB-mediated signaling (Haapasalo et al., 2002).

TrkB.t1 levels remained particularly robust in the vHC even following the CORT exposure period. These findings are in general agreement with prior investigations using social (Tsai et al., 2014) (though not physical (Nibuya et al., 1999)) stress. Also, transgenic mice over-expressing *Trkb.t1* are insensitive to classical antidepressants (Lindholm & Castren, 2014), further motivating us to investigate whether direct trkB stimulation could have antidepressant-like properties following CORT. Indeed, 7,8-DHF given during adolescence (P39-47) corrected CORT-induced habit behavior and amotivation and increased mobility in the forced swim test, an antidepressant-like effect. 7,8-DHF derivatives also have antidepressant-like consequences in adult mice (Liu et al., 2012; Zhang et al., 2014) and restore the reinforcing properties of cocaine following stress (Tzeng et al., 2013), but ours is the first evidence, to our knowledge, of antidepressant-like efficacy in adolescents. And importantly, effects were detectable well beyond the treatment period.

How might adolescent 7,8-DHF treatment confer long-term benefits? Enhancing trkB-mediated signaling could correct the suppressive effects of stressor or CORT exposure on neurogenesis in the vHC, or restore BDNF-trkB interactions in the hippocampus, both of which would be associated with antidepressant-like efficacy (Gourley et al., 2008a; David et al., 2009; Tanti et al., 2013). Also of note, vHC innervation of the anterior mPFC develops during adolescence (Thomases et al., 2014). We found that adolescent CORT exposure caused dendritic spine elimination in the anterior mPFC. Additionally, remaining spines were irregularly enlarged, as also

occurs following *Trkb* ablation (Danzer et al., 2008). Future studies could determine whether these modifications result from aberrant vHC input, and whether 7,8-DHF corrects abnormalities. Finally, while we find a robust p-ERK42/44 response to repeated 7,8-DHF injections, the ability of acute application to stimulate trkB, ERK42/44, and other factors has been questioned (Boltaev et al., 2017). Identifying off-target actions of 7,8-DHF could reveal novel mechanisms by which acute application benefits animal models of depression (*e.g.*, Zhang et al., 2014) and Alzheimer's Disease (discussed Boltaev et al., 2017).

2.6.2 vHC *Trkb.t1* overexpression induces habits

Despite broad-spread changes in trkB:trkB.t1 ratios following subchronic CORT exposure, p-ERK42/44 was detectably reduced only in the vHC of adolescent CORT-exposed mice. These findings led us to overexpress *Trkb.t1* in the vHC, reducing local p-ERK42/44 and causing habit-based behavior. *Trkb.t1* over-expression impairs both LTP and LTD (Li et al., 1998; Michaelsen et al., 2010; but see Saarelainen et al., 2000) and reduces BDNF (Razzoli et al., 2011), which could potentially account for a transition away from hippocampal-dependent action selection to habit-based behaviors, which are instead associated with dorsolateral striatal and cortical sensorimotor systems (Yin et al., 2008; Balleine & O'Doherty, 2010; Schwabe, 2013; Hart et al., 2014; Smith & Graybiel, 2016).

The vHC innervates the CeA (Pitkanen et al., 2000), and likely provides BDNF, given that the CeA expresses little *Bdnf* mRNA, but abundant BDNF from non-cortical sources, and among the highest levels of amygdalar trkB (Krause et al., 2008). Axonal BDNF transport can be trkB-dependent (Butowt & von Bartheld, 2005); thus, we hypothesized that vHC *Trkb.t1* overexpression may render the CeA BDNF-deficient, and that *Trkb.t1* overexpression in the CeA may similarly influence decision-making strategies. Indeed, *Trkb.t1* overexpression in the posterior CeA caused a

deferral to habit-based strategies. This finding provides novel evidence that the healthy posterior CeA is involved in selecting behaviors according to their consequences. This may occur via regulation of appetitive arousal, rather than encoding specific action-value information *per se* (Corbit & Balleine, 2005). By contrast, the anterior (and *not* posterior) CeA is essential to habit-based behavior, potentially due to interactions with the dorsolateral striatum (Lingawi & Balleine, 2012).

Importantly, trkB.t1 is expressed in neurons (Armanini et al., 1995; Kryl et al., 1999; Gomes et al., 2012) and glia (Klein et al., 1990; Beck et al., 1993; Silhol et al., 2005). Lentiviruses, as used here, preferentially infect excitatory neurons, but moderate glial infection would be anticipated (Ehrengruber et al., 2001). Future investigations could elucidate cell-type-specific effects of *Trkb.t1* overexpression. Also, it is notable that we did not overexpress *Trkb.t1* in the mPFC. We were motivated by evidence that selective reduction of its ligand BDNF in this region fails to induce habit-based behavior (Gourley et al., 2012b; Hinton et al., 2014). mPFC-selective *Bdnf* knockdown does cause depression-like amotivation, however, and targeted BDNF infusions provide partial recovery from CORT-induced amotivation (Gourley et al., 2012b). Thus, systemic 7,8-DHF treatment here may have ameliorated CORT-induced depression-like amotivation by acting in multiple brain regions, not strictly the vHC.

2.6.3 Conclusions

To summarize, subchronic CORT exposure in adolescence imbalances trkB/trkB.t1 throughout several brain regions and selectively decreases vHC p-ERK42/44. A *Trkb.t1* overexpression procedure that reduces p-ERK42/44 recapitulates CORT-induced behavioral abnormalities. Interestingly, the "pro-habit" effects of vHC *Trkb.t1* overexpression were not age-dependent, given that viral vector infusion at P31 *and* P56 induced behavioral inflexibility. We argue that adolescents are not necessarily uniquely vulnerable to *Trkb.t1* overexpression, but are rather

more vulnerable to a corticosteroid-induced triggering of neurobiological factors associated with depression-like and habit-based behaviors, in this case, the concomitant elevation of trkB.t1 and reduction in p-ERK42/44 in the vHC.

2.7 ACKNOWLEDGEMENTS

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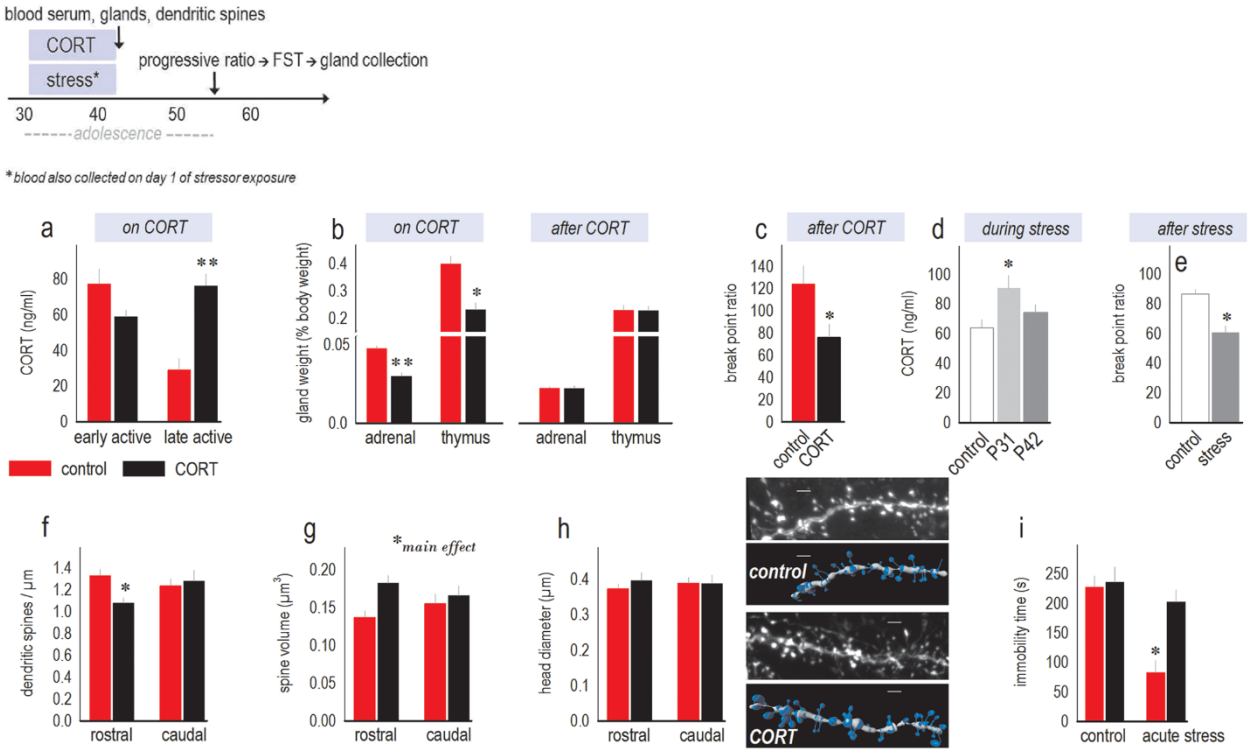

Figure 2-1. Effects of CORT exposure during adolescence: validation of the procedure. Top: Timeline of experimental events. See also Table 2-1. (a) Blood serum CORT levels at the end of an 11-day CORT exposure period (from P31-42) did not differ between groups at the beginning of the active cycle (following sleep), but were elevated in CORT-drinking mice (relative to control mice) at the end of the awake, active cycle. *n*=5-10/group. (b) Adrenal and thymus gland weights also atrophied following exogenous CORT exposure (left), but glands recovered with a washout period (right). *n*=5-6/group. (c) In a progressive ratio test, a history of CORT exposure reduced break point ratios. *n*=5-6/group. (d) Forced swim stress in adolescence also increased blood serum CORT (though this effect appeared to habituate with repeated exposure). *n*=5-11/group. (e) Further, break point ratios were also reduced, as with CORT exposure. *n*=5-10/group. (f) CORT exposure from P31-42 also eliminated dendritic spines on excitatory neurons in the anterior mPFC (prelimbic subregion) of *thy1*-YFP expressing transgenic mice. *n*=7 mice/group. (g) CORT increased overall

spine volume. (h) Dendritic spine head diameter did not, however, change. Representative dendrites are adjacent (unprocessed image at top, followed by reconstruction). (i) Finally, in the forced swim test, a history of subchronic CORT exposure did not impact mobility, but modified reactivity to an acute stressor: Acute stress induced mobility in control mice, but mice with a history of CORT exposure remained immobile. n=5-7/group. *$p \leq 0.05$, **$p < 0.001$ compared to control (red or white bars). Scale bar=2 μm. CORT, corticosterone; FST, forced swim test; mPFC, medial prefrontal cortex; P, postnatal day; YFP, yellow fluorescent protein.

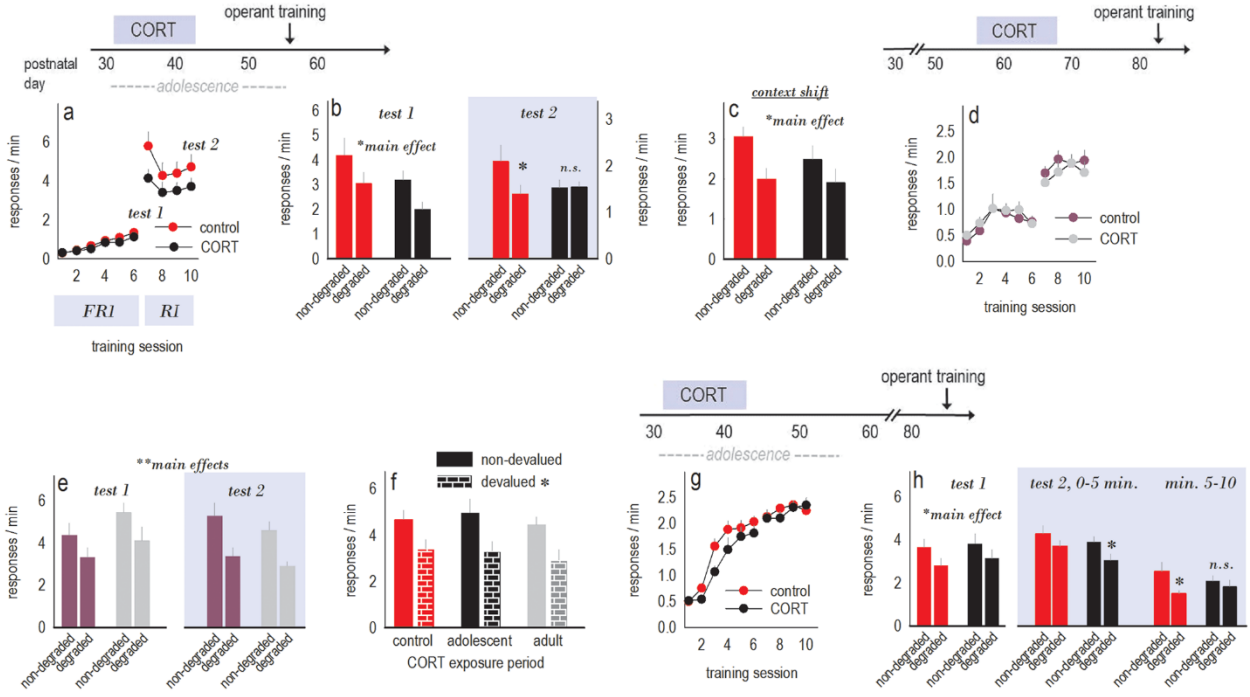

Figure 2-2. Greater vulnerability to CORT-induced habits in adolescents than adults.

Experimental timelines are positioned above the response acquisition curves associated with each experiment. Response acquisition curves represent both responses/min, and breaks in the curves represent tests for sensitivity to instrumental contingency degradation. (a) Instrumental response acquisition was intact 2 weeks following adolescent CORT exposure. Text below the x axis notes the schedules of reinforcement used throughout (FR1 before test 1, followed by an RI schedule). (b) Sensitivity to instrumental contingency degradation was also initially intact (test 1), in that mice inhibited a response that was unlikely to be reinforced ("degraded" condition), but CORT-exposed mice then developed habit-based response strategies, failing to differentiate between responses (test 2). *n*=8-9/group. (c) In a separate group, test 2 was conducted in a distinct environment ("context shift"). In this case, all mice preferentially engaged the response most likely to be reinforced in a goal-directed manner, indicating that CORT-induced habits (in b) are context-dependent.

n=7/group. (d) A history of subchronic CORT exposure *in adulthood* also did not impact instrumental response acquisition. (e) Unlike with adolescent CORT exposure, however, both groups inhibited a response that was unlikely to be reinforced in a goal-directed fashion. *n*=7/group. (f) Additionally, all mice inhibited responding following prefeeding with the reinforcer pellets ("devalued"), relative to prefeeding with chow ("non-devalued"), regardless of age of CORT exposure. (g) Another group of adolescent CORT-exposed mice was allowed a longer (4-wk) washout period in order to match the timing of testing in adult CORT-exposed mice. Mice acquired the responses without group differences. (h) Response preferences were intact in test 1, as above. During test 2, mice were initially able to differentiate between the responses that were likely vs. unlikely to be reinforced, but response preference decayed in the CORT-exposed mice. *n*=11-12/group. Bars/symbols=means+SEMs, **p*<0.05, ***p*<0.001 vs. non-degraded or main effects, as indicated. CORT, corticosterone; FR1, fixed ratio 1; RI, random interval.

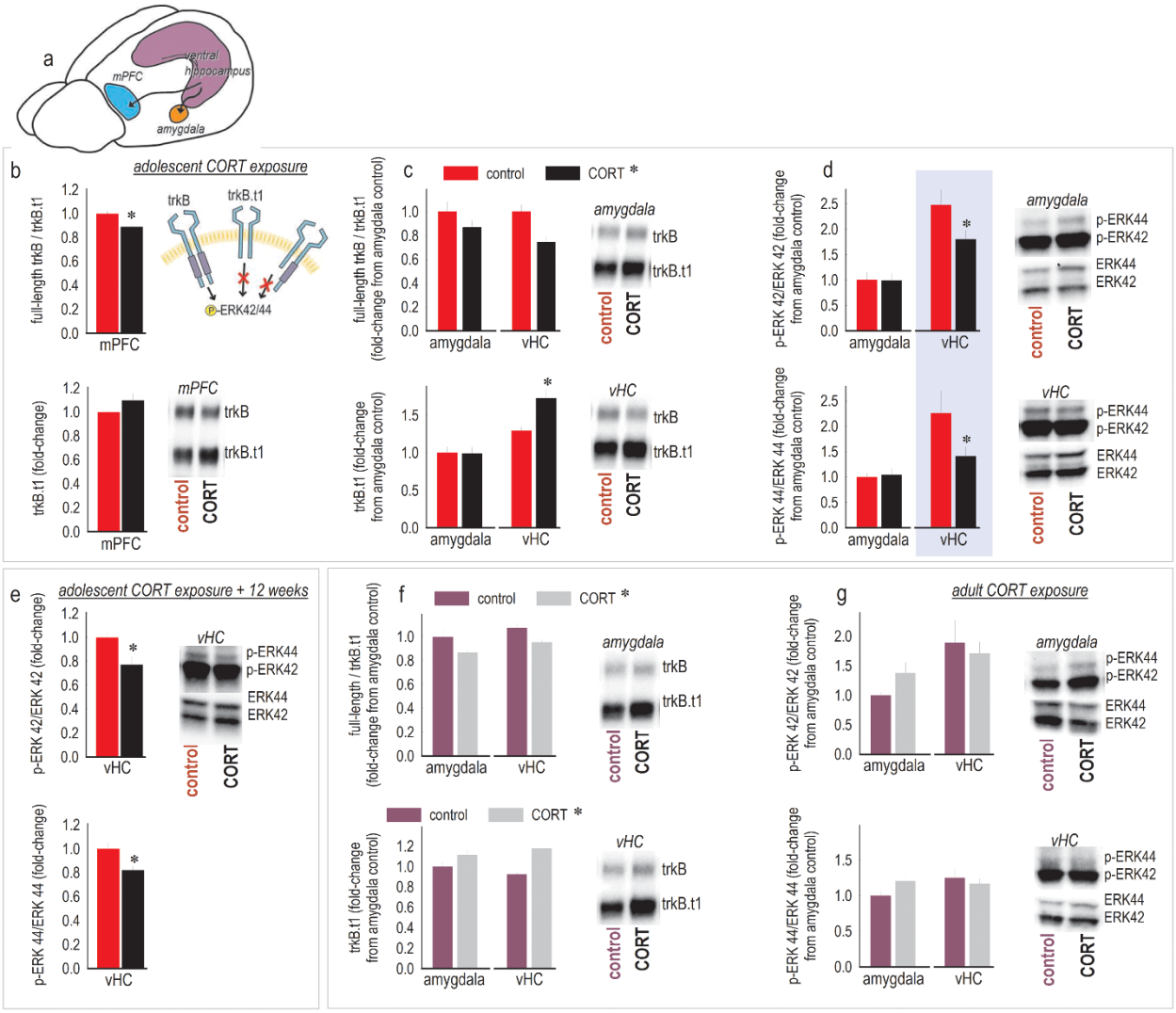

Figure 2-3. Adolescent CORT exposure regulates cortico-limbic trkB and ERK42/44 phosphorylation. (a) Based on our findings, we profiled the neurobiological effects of CORT in a mPFC-ventral hippocampus-amygdala circuit. (b) Brains were first collected at the end of the CORT exposure period. **Top:** Adolescent CORT exposure decreased the ratio of full-length to truncated trkB in the mPFC, which would decrease the ability of full-length trkB to initiate intracellular signaling events, illustrated at right. **Bottom:** trkB.t1 levels alone did not significantly differ. (c) **Top:** Adolescent CORT exposure also decreased the ratio of full-length to truncated trkB in the vHC and

amygdala. **Bottom:** In the vHC, this was accompanied by an increase in overall trkB.t1 levels. (d) **Top:** p-ERK42 levels were higher overall in the vHC than in the amygdala, and CORT reduced vHC p-ERK42 (in planned comparisons, see also Table 2-3). **Bottom:** The same pattern was detected for p-ERK44. (e) To determine whether this effect was long-lasting, we assessed ERK42/44 phosphorylation 12 weeks following adolescent CORT exposure. **Top:** p-ERK42 was reduced in the vHC. **Bottom:** p-ERK44 was also blunted. (f) Next, we tested the effects of subchronic CORT in adult mice. **Top:** CORT decreased trkB:trkB.t1 in the amygdala and vHC, as in adolescent mice. **Bottom:** trkB.t1 was also elevated. (g) Despite these modifications, ERK42/44 phosphorylation was not impacted. Representative blots are adjacent throughout. These and additional analyses are summarized in Table 2-3. n=4-10/group throughout. Bars/symbols=means+SEMs, *p<0.05 *vs.* control within the same brain region. When in the legend, asterisks indicate main effects of CORT. CORT, corticosterone; ERK42/44, extracellular signal-regulated kinase 42/44; mPFC, medial prefrontal cortex; p-ERK, phosphorylated ERK; trkB, tyrosine kinase receptor B; trkB.t1, truncated trkB; vHC, ventral hippocampus.

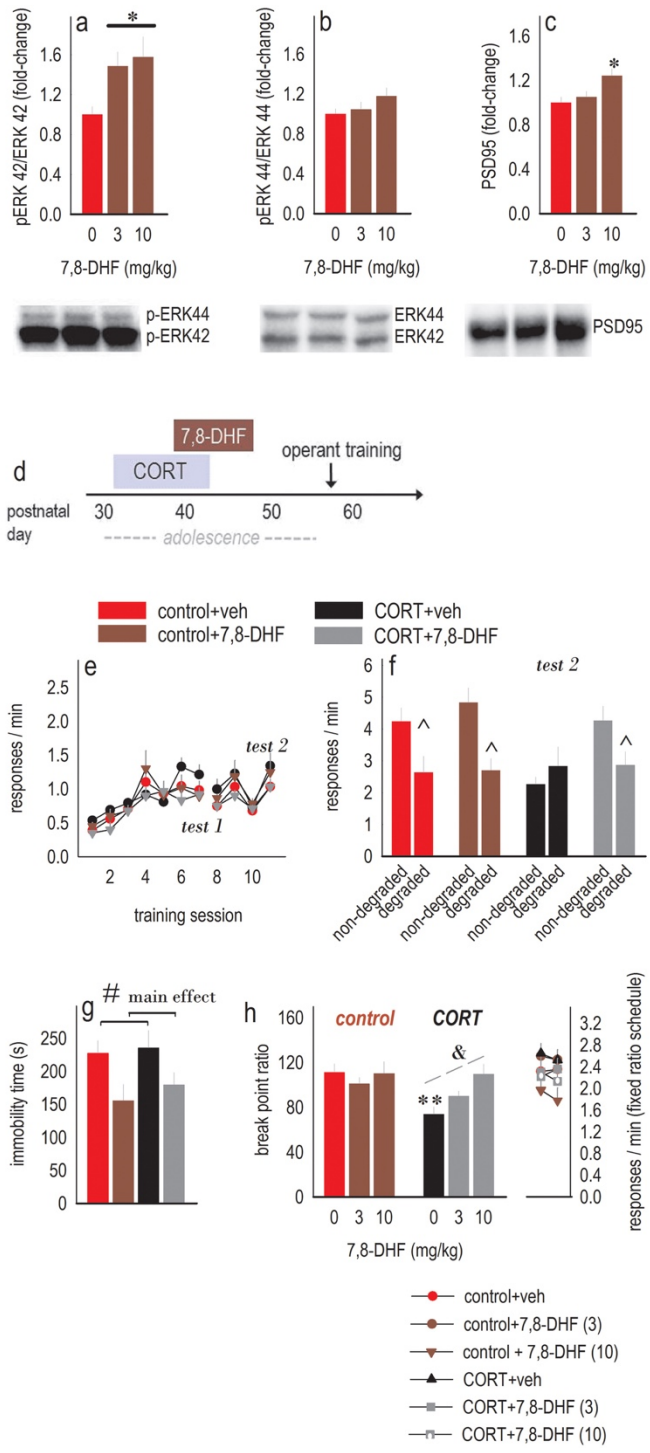

Figure 2-4. Durable blockade of CORT-induced habits and depression-like behavior. (a) Repeated 7,8-DHF treatment increased p-ERK42 in the vHC, (b) but had no effects on p-ERK44. (c) At the highest dose tested, levels of the synaptic marker PSD95 were also increased relative to

the control and 3 mg/kg groups. Representative blots are below. *n*=9-14/group. (d) Experimental timeline. (e) Mice developed food-reinforced instrumental responses without differences between groups. Response acquisition curves represent both responses/min, and breaks in the acquisition curves represent tests for sensitivity to instrumental contingency degradation. (f) As shown in the prior figures, CORT-exposed mice developed habit-based response strategies. 7,8-DHF, 3 mg/kg, blocked these habits, indicated by preferential engagement of the response most likely to be reinforced in the CORT+7,8-DHF group. *n*=5-6/group. (g) 7,8-DHF-treated mice were also less immobile in the forced swim test, a durable antidepressant-like effect. *n*=7/group. (h) Further, 7,8-DHF dose-dependently blocked CORT-induced deficiencies in break point ratios (while having no effects on responding when a fixed ratio 1 schedule was applied; symbols at right). *n*=6-13/group. Bars/symbols=means+SEMs, *$p<0.05$, **$p<0.001$ following CORTx7,8-DHF interaction, ^$p<0.001$ following response choice interactions, &$p=0.002$ *vs.* CORT alone, #$p<0.05$ main effect of 7,8-DHF (no interaction). 7,8-DHF, 7,8-dihydroxyflavone; CORT, corticosterone; ERK42/44, Extracellular signal-Regulated Kinase 42/44; p- ERK, phosphorylated ERK; PSD95, postsynaptic density 95; vHC, ventral hippocampus.

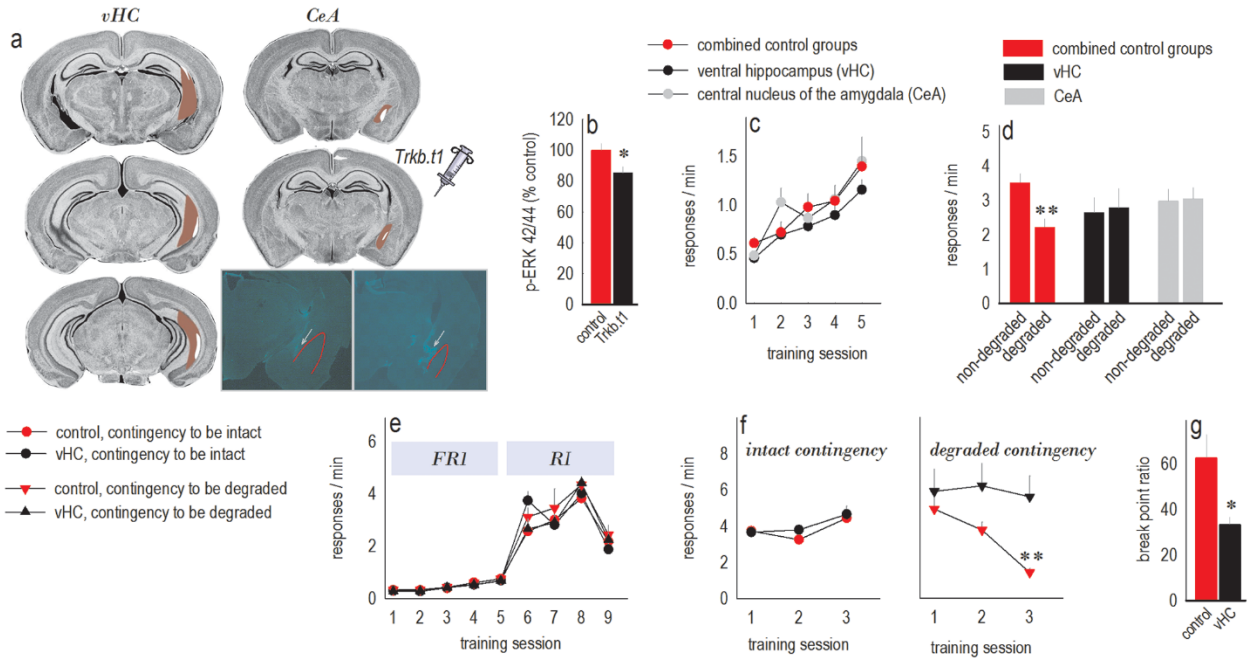

Figure 2-5. Overexpression of truncated trkB induces habits, makes them harder to "break," and causes depression-like behavior. (a) We infused a lentivirus expressing truncated trkB (*Trkb.t1*) or GFP into the vHC and CeA. Large (red) and small (white) infusion sites are represented on images from the Mouse Brain Library (Rosen et al., 2000). All infusion sites are described in Fig S2-3. At bottom right, representative lentiviral GFP expression in the CeA is shown (at gray arrows). The external capsule is highlighted in red, and the image is intentionally over-exposed. (b) In *Trkb.t1*-expressing mice, p-ERK42/44 immunoreactivity was diminished at the infusion site. *n*=7-8/group. (c) All mice acquired the instrumental responses. Response acquisition curves represent both responses/min. (d) *Trkb.t1* in the vHC and CeA blocked sensitivity to instrumental contingency degradation. Thus, selective *Trkb.t1* over-expression recapitulated the long-term effects of adolescent CORT exposure. *n*=6-8/*Trkb.t1* group; total control=23. (e) Another group of mice was first trained using a fixed ratio schedule of reinforcement. Then, to build on our findings reported in (d), an RI schedule of reinforcement was applied, with no interruption in training and no

differences in responding between groups. (f) In reaction to repeated instrumental contingency degradation training, control mice inhibited a response that was unlikely to be reinforced, their habits "breaking" ("degraded contingency," right). By contrast, mice with vHC *Trkb.t1* failed to inhibit responding. Response rates associated with an intact contingency were unaffected (left). Response rates are represented on 2 plots in the interest of clarity, but were compared together by ANOVA. n=9-10/group. (g) Finally, vHC *Trkb.t1* overexpression also decreased responding on a progressive ratio schedule of reinforcement in adulthood, again recapitulating the long-term effects of adolescent CORT exposure. n=9/group. Bars/symbols=means+SEMs, *p<0.05, **$p\leq$0.004. CeA, central nucleus of the amygdala; ERK42/44, Extracellular signal-Regulated Kinase 42/44; p-ERK, phosphorylated ERK; trkB, tyrosine kinase receptor B; trkB.t1, truncated trkB; vHC, ventral hippocampus.

Group	Figure	Age of CORT/stressor exposure	Additional manipulation, if any	Age of testing	End points
1	2-1a,b	CORT: P31-42	n/a	P42 vs. P125	Blood serum CORT and glands
2	2-1c	CORT: P31-42	n/a	adult	Progressive ratio test
3	2-1d	swim stress: P31-P42	n/a	P31 vs. P42	Blood serum CORT
4	2-1e	swim stress: P31-42	n/a	adult	Progressive ratio test
5	2-1f-h, Fig S2-1	CORT: P31-42	n/a	P42 vs. P125	Dendritic spine analysis
6	2-1i	CORT: P31-42	Acute stressor	adult	Forced swim test
7	2-2a,b,f, Table S2-3	CORT: P31-42	n/a	P56	Instrumental contingency degradation, reinforcer devaluation
8	2-2c	CORT: P31-42	n/a	P56	Instrumental contingency degradation with context shift
9	2-2d-f, Table S2-3	CORT: P56-67/68	n/a	P82	Instrumental contingency degradation, reinforcer devaluation
10 F	Fig S2-2	CORT: P31-42	n/a	P56	Instrumental contingency degradation
11	2-2g,h	CORT: P31-42	n/a	P86	Instrumental contingency degradation
12	2-3a-d, Table 2-3	CORT: P31-42	n/a	P42	Western blotting
13	2-3e	CORT: P31-42	n/a	P125	Western blotting
14	2-3f,g	CORT: P56-67	n/a	P67	Western blotting
15	2-4a-c	n/a	7,8-DHF	n/a	Western blotting
16	2-4d-g, Table S2-2	CORT: P31-42	7,8-DHF	adult	Instrumental contingency degradation, followed by forced swim test, followed by locomotor monitoring
17	2-4h	CORT: P31-42	7,8-DHF	adult	Progressive ratio test
18 F	Fig S2-2	CORT: P31-42	7,8-DHF	adult	Instrumental contingency degradation
19	Table 2-4, Table S2-1	CORT: P31-42	7,8-DHF	P125	Western blotting, glands
20	2-5a-d, Fig S2-3	n/a	Trkb.t1 virus	adult	Instrumental contingency degradation, immunostaining
21	2-5e-g, Fig S2-3	n/a	Trkb.t1 virus	adult	Repeated instrumental contingency degradation, followed by progressive ratio

Table 2-1. List of experiments. A summary of experiments reported in this manuscript is provided, with corresponding figure numbers. "Adult" refers to testing completed between 8-12 wk of age. Abbreviations: 7,8-DHF, 7,8-dihydroxyflavone; CORT, corticosterone; F, female mice; P, postnatal day; Trkb.t1, truncated trkB.

			Day 1	Day 3	Day 5	Day 7	Day 9	Day 11	Day 13	Day 15	Day 17
			Mean±SEM	Mean±SEM	Mean±SEM	Mean±SEM	Mean±SEM	Mean±SEM	Mean±SEM	Mean±SEM	Mean±SEM
A. *Adolescence*		control	12.6±0.96	14.0±0.99	14.8±0.93	15.8±0.81	16.9±0.63	17.7±0.44	n/a		
		CORT**	13.6±0.33	15.4±0.33	15.0±0.27	16.1±0.27	16.6±0.20	16.9±0.18	n/a		
B. *Adulthood*		control	22.8±1.01	23.3±1.10	23.6±1.10	23.9±1.07	24.1±1.07	24.1±1.13	n/a		
		CORT **	21.2±0.95	21.3±0.92	21.3±0.98	21.3±0.92	21.3±0.94	21.2±0.93	n/a		
C. *Adolescence*		control	15.1±0.59	15.7±0.49	16.6±0.24	16.8±0.34	17.1±0.39	16.6±0.35	n/a		
		stress	15.8±0.60	16.1±0.47	16.6±0.53	16.6±0.43	16.8±0.45	16.8±0.38	n/a		
D. Adolescence (7,8-DHF) - M	control	veh	16.5±0.48	17.5±0.29	17.8±0.29	18.3±0.34	18.6±0.31	18.9±0.32	18.8±0.41	18.9±0.37	19.6±0.30
		DHF(3)	16.5±1.08	16.7±0.83	17.2±0.56	17.8±0.52	18.3±0.43	18.7±0.39	18.6±0.46	19.2±0.40	19.4±0.42
		DHF(10)	16.5±0.90	17.5±0.76	17.8±0.62	18.1±0.54	18.4±0.55	18.7±0.56	18.8±0.60	19.4±0.59	19.6±0.52
	CORT **	veh	15.9±0.47	16.5±0.35	*16.1±0.34	*16.1±0.37	*16.1±0.36	*16.3±0.34	*16.1±0.32	*16.9±0.33	*17.7±0.34
		DHF(3)	16.6±0.72	16.7±0.69	*16.8±0.62	*16.7±0.49	*16.5±0.52	*16.8±0.50	*16.8±0.51	*17.4±0.59	*18.4±0.55
		DHF(10)	16.9±0.79	17.3±0.38	*17.2±0.37	*17.3±0.39	*17.3±0.48	*17.4±0.47	*17.3±0.49	*18.0±0.51	*19.0±0.53
E. *Adol. (DHF) - F*	control	veh	13.5±0.31	13.4±0.26	13.6±0.17	14.4±0.15	14.2±0.14	14.4±0.16	14.6±0.18	14.6±0.18	14.8±0.15
	CORT	veh	13.5±0.36	13.4±0.28	13.5±0.22	14.1±0.23	14.3±0.26	14.6±0.24	14.4±0.38	14.9±0.40	15.0±0.37
		DHF(3)	13.2±0.42	13.5±0.32	13.8±0.24	14.3±0.27	14.4±0.31	14.7±0.26	14.8±0.31	15.4±0.32	15.4±0.33

Table 2-2. Body weights following CORT, stress, and 7,8-DHF. (Row A) The change in body weight across days of mice exposed to CORT or water during early adolescence depended on CORT status [day x CORT interaction $F_{(5,75)}$=4.1, p=0.003], but post-hoc tests were nonsignificant. **(Row B)** In adult mice, body weight change across days also depended on CORT status [day x CORT interaction $F_{(5,60)}$=12.1, p<0.001], but post-hoc tests were nonsignificant. **(Row C)** Body weight of mice exposed to repeated forced swimming or control manipulation during early adolescence increased across days [main effect of day $F_{(5,65)}$=22.7,p<0.001], with no group differences [main effect and interaction p's>0.05]. **(Row D)** For mice exposed to CORT or water during adolescence and treated with 7,8-DHF (0.0, 3.0, or 10.0 mg/kg), the change in body weight across days depended on CORT status [day x CORT interaction $F_{(8,240)}$=18.1,p<0.001], with CORT impairing normal weight gain (days on which control and CORT groups significantly differed are indicated by asterisks, as determined by post-hoc tests). There were no effects of 7,8-DHF treatment alone, nor any interactions [p's>0.05]. **(Row E)** The average body weight of female mice exposed to

CORT or water during early adolescence and treated with 0.0 or 3.0 mg/kg of 7,8-DHF increased across days [main effect $F_{(8,168)}$=42.1,p<0.001], with no group differences [p's>0.05]. Shaded cells indicate the period of 7,8-DHF or vehicle administration. "M" refers to males, and "F" refers to females. Unit of measure is grams. **refers to interaction effects; *refers to post-hoc comparisons (both p<0.05). 7,8-DHF dosing (in mg/kg) is indicated in parentheses. Abbreviations: Adol., adolescence; 7,8-DHF and DHF, 7,8-dihydroxyflavone; CORT, corticosterone; F, females; M, males; veh, vehicle.

		vHC	Amygdala	mPFC	DMS	VS
CORT as % of control	trkB/trkB.t1	74.4*	86.9*	88.8*	99.3	84.7*
	trkB.t1	133.9*	99.1	109.7	101.5	119.4*
	trkB	92	90.4	97	102	100.9
	p-ERK42	72.8*	99.6	113.6	99.6	136.9
	p-ERK44	62.5*	104.7	104.6	99.3	115.5

Table 2-3. Protein levels following subchronic (11 days) CORT exposure during early adolescence. Levels of full-length and truncated trkB receptor isoforms and phosphorylated ERK42/44 were quantified in various brain regions at the end of the CORT exposure period in adolescents. Values represent the % change relative to control animals. *$p<0.05$ in two-tailed t-tests. Abbreviations: vHC, ventral hippocampus; mPFC, medial prefrontal cortex; DMS, dorsomedial striatum; VS, ventral striatum.

			p-ERK42/ ERK42	p-ERK44/ ERK44
			Mean ± SEM	Mean ± SEM
Adolescence (7,8-DHF)	control	veh	*1.00 ± 0.05	1.00 ± 0.05
		DHF(10)	0.90 ± 0.12	1.05 ± 0.07
	CORT	veh	*0.77 ± 0.06	0.82 ± 0.03
		DHF(10)	1.11 ± 0.21	0.93 ± 0.04

Table 2-4. Protein levels in the vHC of adult mice exposed to CORT during adolescence ± 7,8-DHF (10 mg/kg). 7,8-DHF influenced p-ERK42/ERK42 in CORT-exposed mice [CORT x 7,8-DHF interaction $F_{(1,28)}$=4.2,p=0.05]. Post-hoc tests indicated that control+veh group significantly differed from CORT+veh group as reported in independent experiments (see Table 2-3 and Fig 2-3) (indicated by asterisks, *p<0.05), but 7,8-DHF eliminated this difference. CORT decreased p-ERK44/ERK44, but there was no effect of 7,8-DHF [main effect CORT $F_{(1,27)}$=8.1,p=0.008, no interaction]. Values indicate fold-change from control+veh group. Abbreviations: 7,8-DHF and DHF, 7,8-dihydroxyflavone; CORT, corticosterone; ERK42/44, Extracellular signal-Regulated Kinase 42/44; p-ERK, phosphorylated ERK.

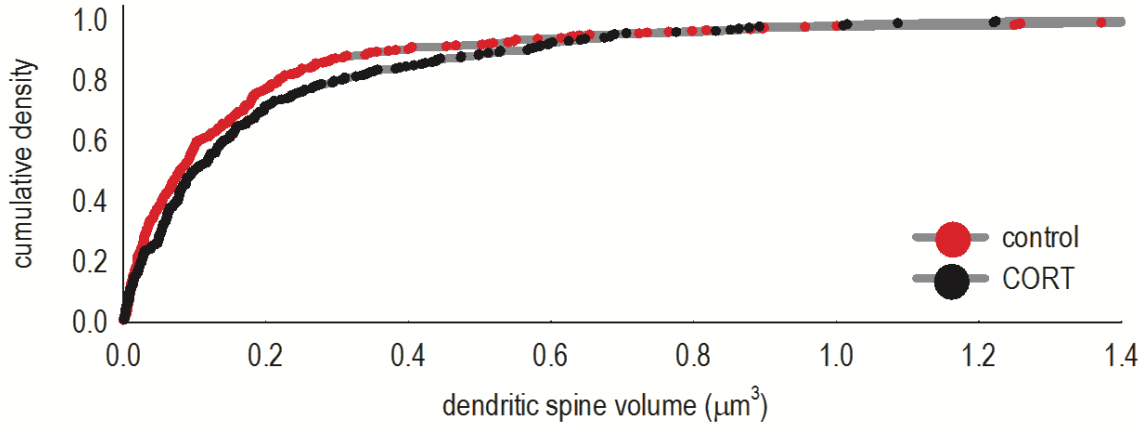

Figure S2-1. Long-term consequences of adolescent CORT exposure on dendritic spine morphologies in the mature mPFC. Dendritic spines on excitatory neurons within the anterior mPFC of adult mice exposed to CORT during adolescence were imaged. While we detected no differences in dendritic spine densities, lengths, or head diameter, dendritic spines from CORT-exposed mice were larger in overall volume. These findings provide evidence of long-term structural effects of adolescent CORT exposure, and notably, glucocorticoid receptor blockade has differential effects, *reducing* dendritic spine head diameters (Swanson et al., 2013). Each symbol represents an individual dendritic spine, and groups were compared by Kolmogorov-Smirnov comparisons, *p=0.03. CORT, corticosterone; mPFC, medial prefrontal cortex.

Figure S2-2. CORT causes habit behavior in female mice, which can be blocked by 7,8-DHF. (a) Experimental timeline. (b) Female mice exposed to CORT during adolescence acquired the instrumental responses in adulthood [*F*s<1]. Response acquisition curves represent both responses/min. (c) As in males, a history of CORT exposure biased responding towards inflexible habit-like behavior [interaction $F_{(1,7)}$=5.6,p=0.05]. n=4-5/group. Notably, habit behavior was detectable at an earlier time point relative to studies using males (Fig 2-2). This is consistent with

habit biases in female mice (Quinn et al., 2007). (d) Experimental timeline. (e) A separate cohort of mice acquired the nose poke responses [Fs$\leq$1]. (f) Control mice preferentially generated the response most likely to be reinforced following instrumental contingency degradation [response t_{14}=3.0,p=0.009], while CORT-exposed mice failed to differentiate between the responses, responding habitually [response t_{14}=0.2,p=0.8]. 7,8-DHF blocked these habits [response t_{12}=2.7,p=0.02]. n=7-8/group. Bars/symbols=means+SEMs, *p<0.05. 7,8-DHF, 7,8-dihydroxyflavone; CORT, corticosterone.

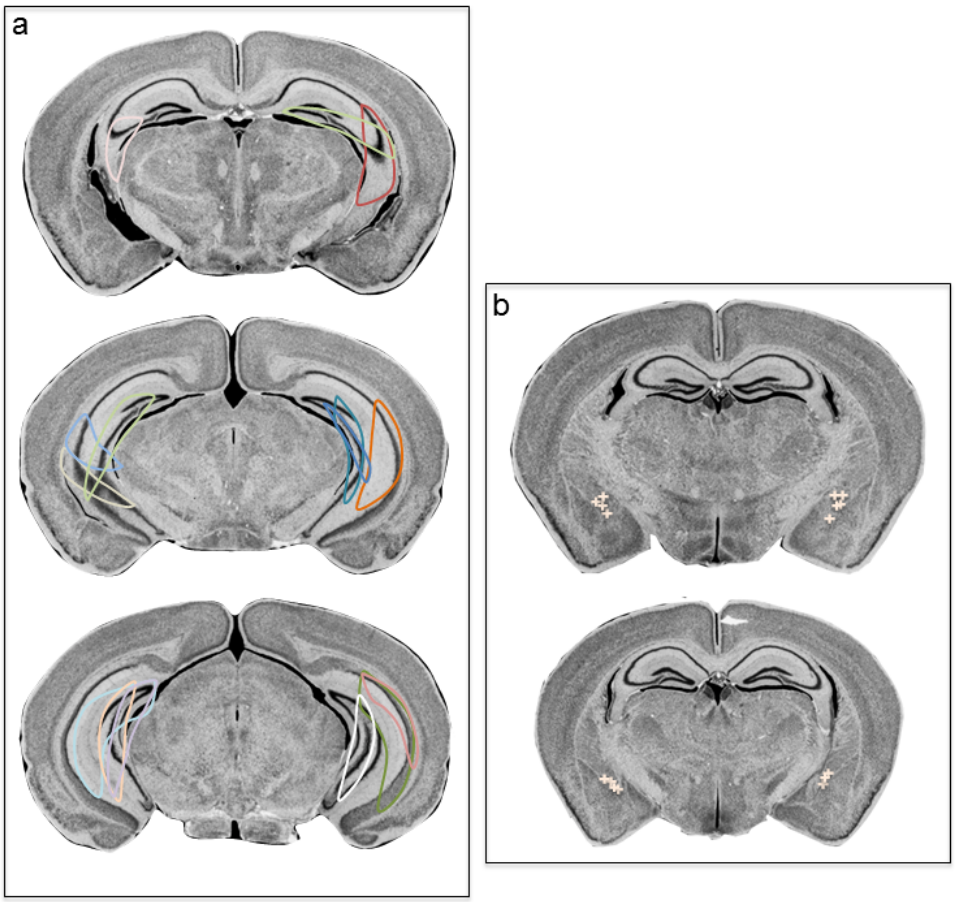

Figure S2-3. Additional histological documentation associated with Fig 2-5. (a) Coronal brain sections from the Mouse Brain Library (Rosen et al., 2000) are shown. Each trace represents the largest detected hippocampal viral vector spread in a given mouse. (b) Separate mice received CeA infusions. The center of each *Trkb.t1*-expressing viral vector spread is indicated. The largest and smallest infusion sites are documented in Fig 2-5. CeA, central nucleus of the amygdala; trkB, tyrosine kinase receptor B; trkB.t1, truncated trkB.

			adrenal	thymus
			Mean ± SEM	Mean ± SEM
Adolescence (7,8-DHF)	control	veh	0.020 ± 0.0011	0.23 ± 0.020
		DHF(3)	0.017 ± 0.0014	0.23 ± 0.014
		DHF(10)	0.016 ± 0.0025	0.22 ± 0.011
	CORT	veh	0.020 ± 0.0017	0.23 ± 0.019
		DHF(3)	0.016 ± 0.0012	0.24 ± 0.012
		DHF(10)	0.019 ± 0.0013	0.22 ± 0.028

Table S2-1. Gland weights in mice with a history of CORT ± 7,8-DHF. When mice were euthanized in adulthood following a history of CORT ± 7,8-DHF treatment (Fig 2-4), adrenal and thymus gland weights did not differ [all *p*'s>0.05]. Values indicate gland weights as a percentage of total body weight. 7,8-DHF dosing (in mg/kg) is indicated in parentheses. 7,8-DHF, 7,8-dihydroxyflavone; CORT, corticosterone.

		hours	1-6	7-12	13-18	19-24
			Mean ± SEM	Mean ± SEM	Mean ± SEM	Mean ± SEM
Adolescence (7,8-DHF)	control	veh	55275 ± 3441	46079 ± 7863	29327 ± 10458	27549 ± 4993
		DHF	37538 ± 2804	32389 ± 3672	26233 ± 11994	32700 ± 10015
	CORT	veh	43568 ± 5628	42631 ± 7505	18455 ± 4943	19427 ± 3534
		DHF	49734 ± 6421	34546 ± 4323	29135 ± 8478	39562 ± 14695

Table S2-2. Locomotor monitoring of mice exposed to CORT during adolescence ± 7,8-DHF. The locomotor activity of adult mice exposed to CORT ± 7,8-DHF (3 mg/kg) as adolescents was monitored over 24 hr following the forced swim test (Fig 2-4). There were no effects of CORT, 7,8-DHF, CORT x 7,8-DHF interactions, or any interactions with time [main effect and interaction *p*'s>0.05]. Units of measure are photobeam breaks, and these findings are consistent with no locomotor effects of repeated 7,8-DHF treatment in rats (Chang et al., 2016). 7,8-DHF, 7,8-dihydroxyflavone; CORT, corticosterone.

		chow	pellet	matched pellet
		Mean ± SEM	Mean ± SEM	Mean ± SEM
Adolescence	control	0.38 ± 0.032	0.74 ± 0.09	0.40 ± 0.00
	CORT	0.41 ± 0.048	0.69 ± 0.12	0.40 ± 0.00
Adulthood	control	0.53 ± 0.052	1.01 ± 0.13	0.50 ± 0.00
	CORT	0.50 ± 0.044	0.97 ± 0.15	0.50 ± 0.00

Table S2-3. Food intake associated with reinforcer devaluation experiments in Fig 2-2. Male mice with a history of CORT exposure during adolescence or adulthood consumed more of the reinforcer pellets ("devalued") than regular chow ("non-devalued") when given free access to each before a probe test conducted in extinction. Thus, in a third condition, the amount of pellets available during the prefeeding period was matched to the amount of chow that the group consumed previously. Intake values are reported in grams. CORT, corticosterone.

CHAPTER 3: ADOLESCENT CORTICOSTERONE AND TRKB PHARMACO-MANIPULATIONS SEX-DEPENDENTLY IMPACT INSTRUMENTAL REVERSAL LEARNING LATER IN LIFE

3.1 CONTEXT, AUTHOR'S CONTRIBUTION, AND ACKNOWLEDGEMENT OF REPRODUCTION

The following chapter presents evidence that corticosteroid exposure and tyrosine receptor kinase B (trkB) manipulations during adolescence have long-term, sex-dependent effects on behavioral flexibility in an instrumental reversal task dependent on the orbital prefrontal cortex (oPFC). The dissertation author contributed to the paper by designing and conducting the experiments, analyzing the data, and writing the manuscript under the guidance of Dr. Shannon Gourley. This chapter is reproduced from Barfield ET, Gourley SL (2017b) Adolescent corticosterone and trkB pharmaco-manipulations sex-dependently impact instrumental reversal learning later in life. *Frontiers in Behavioral Neuroscience* 11:237.

3.2 ABSTRACT

Early-life trauma can increase the risk for, and severity of, several psychiatric illnesses. These include drug use disorders, and some correlations appear to be stronger in women. Understanding the long-term consequences of developmental stressor or stress hormone exposure and possible sex differences is critically important. So-called "reversal learning" tasks are commonly used in rodents to model cognitive deficits in stress- and addiction-related illnesses in humans. Here, we exposed mice to the primary stress hormone corticosterone (CORT) during early adolescence (postnatal days 31-42), then tested behavioral flexibility in adulthood using an instrumental reversal learning task. CORT-exposed female, but not male, mice developed perseverative errors. Despite resilience to subchronic CORT exposure, males developed reversal performance impairments following exposure to physical stressors. Administration of a putative tyrosine kinase receptor B (trkB) agonist, 7,8-dihydroxyflavone (7,8-DHF), during adolescence blocked CORT-induced errors in females and improved performance in males. Conversely, blockade of trkB by ANA-12 impaired performance.

These data suggest that trkB-based interventions could have certain protective benefits in the context of early-life stressor exposure. We consider the implications of our findings in an extended "Discussion" section.

3.3 INTRODUCTION

Reversal tasks assess the ability of mice or rats to flexibly modify behaviors when reinforcement contingencies change. These tasks are commonly used to model behavioral inflexibility associated with addiction and other disorders in humans (for recent review Izquierdo et al., 2017). Typically, animals are trained to associate specific actions or stimuli with reward (e.g., food), then, the association is modified such that a previously non-predictive contingency could be used to obtain reinforcement, while the original contingency is no longer predictive. Thus, these tasks require animals to inhibit a familiar response strategy and deploy a new strategy to obtain reinforcement.

Behavioral inflexibility in these tasks may result from (Ersche et al., 2008; Jentsch et al., 2002; Schoenbaum et al., 2004) and also *predispose* organisms to (Dalley et al., 2007; Belin et al., 2008; Groman et al., 2009) drug-seeking behaviors. For example, in humans, deficits in reversal learning correlate with the severity of cocaine use (Moreno-López et al., 2015), and a "behavioral disinhibition" trait is associated with later substance use disorders (Tarter et al., 2004; Nigg et al., 2006). Further, rats or mice exhibiting low inhibitory control over impulsive responding more rapidly escalate rates of cocaine self-administration and are more prone to developing drug taking characterized as compulsive (Dalley et al., 2007; Belin et al., 2008; Cervantes et al., 2013). Thus, individual differences in behavioral flexibility and inhibitory control may influence the progression from initial drug use to habitual or compulsive drug-seeking (Cervantes et al., 2013).

Adverse experiences early in life are linked with negative psychiatric outcomes in adulthood,

including increased risk for drug use disorders (Fergusson et al., 1996; Kessler et al., 1997; Dube et al., 2003; Green et al., 2010; Afifi et al., 2012) and greater lifetime severity of substance abuse (Hyman et al., 2006; Sacks et al., 2008; Enoch, 2011). Furthermore, several studies report that this association is stronger in women (Widom & White, 1997; MacMillan et al., 2001; Simpson & Miller, 2002; Hyman et al., 2008). The neurobehavioral processes that translate developmental stressor exposure into psychiatric vulnerabilities later in life are incompletely understood, but may involve disruption of prefrontal cortex (PFC)-dependent executive functions, such as behavioral flexibility and inhibitory control (Elton et al., 2014). Indeed, early-life trauma is associated with deficits in inhibitory control (Lewis et al., 2007; Mueller et al., 2010; Skowron et al., 2014; Marshall et al., 2016), and in at least one study, trait impulsivity mediated the relationship between childhood trauma and substance dependence severity in adulthood (Schwandt et al., 2013). Comparing susceptibility to early-life stress-induced inhibitory control deficits between males and females may provide insight into mechanisms of sex differences in risk for stress-related psychopathology.

In the present study, we manipulated levels of the primary glucocorticoid, corticosterone (CORT), and determined long-term consequences in an instrumental reversal task. We exposed mice to exogenous CORT from postnatal day (P) 31-42, corresponding to early adolescence in rodents (Spear, 2000), and a period of considerable structural maturation in the PFC (Shapiro et al., 2017b). Mice were then trained as adults to perform food-reinforced nose poke responses. We found that subchronic CORT exposure induced habit-biased behavior, despite the cessation of CORT exposure, and these findings are reported in Barfield et al. (2017a). We then tested mice in an instrumental reversal task, and the results of those tests are reported here.

In some groups, we stimulated tyrosine kinase receptor B (trkB), the high-affinity receptor for brain-derived neurotrophic factor (BDNF), which regulates dendritic spine structure and function (Yoshii & Constantine-Paton, 2010). BDNF-trkB systems are impacted by stress (Gray et

al., 2013; Numakawa et al., 2013), implicated in addiction-related behaviors (Li & Wolf, 2015), and significantly modify reward-related decision making (Pitts et al., 2016). Loss of neurotrophic support following prolonged exposure to elevated glucocorticoids is thought to contribute to structural and functional alterations in the PFC that are associated with stress-related psychopathology (Duman et al., 1997). Moreover, recent findings implicate down-regulation of BDNF-trkB signaling in the long-term behavioral consequences of adolescent stress exposure (Xu et al., 2016b; Zhang et al., 2017; Barfield et al., 2017a). Thus, we hypothesized that stimulating trkB may have protective benefits in animals exposed to CORT during adolescence, blocking enduring behavioral deficits.

We report sex-dependent long-term consequences of both CORT and trkB manipulations in an instrumental reversal task. Namely, a history of early-adolescent CORT exposure in females, but not males, induced perseverative responding. Despite apparent resilience to early-adolescent CORT, males developed behavioral inflexibilities following repeated stressor exposure or trkB blockade during early adolescence. Further, a putative trkB agonist improved performance in both sexes. We consider the implications of our findings in an extended "Discussion" section.

3.4 MATERIALS AND METHODS

3.4.1 Subjects

Subjects were male and female wild-type C57BL/6 mice (Jackson Labs, Bar Harbor, ME, USA) or mice expressing *thy1*-derived yellow fluorescent protein (YFP; Feng et al., 2000) that were fully back-crossed onto a C57BL/6 background. Mice were not handled, other than for routine veterinary care, until P31. Mice were group-housed, maintained on a 12-h light cycle (0700 on) and provided food and water *ad libitum* except during instrumental conditioning when body weights were maintained at 90%–93% of baseline to motivate responding. Animal numbers for each experiment are indicated in the respective figure captions. This study was carried out in accordance with the

recommendations of the Guide for the Care and Use of Laboratory Animals. The protocol was approved by the Emory University IACUC.

3.4.2 CORT exposure

CORT hemisuccinate (4-pregnen-11β 21-DIOL-3 20-DIONE 21-hemisuccinate; Steraloids) was dissolved in tap water (25 μg/mL free base; Gourley et al., 2008a,b, 2012b). CORT-exposed mice were given CORT in place of normal drinking water, while control mice consumed tap water. CORT solutions were changed every 3 days. Water bottles were weighed daily, and mice weighed every other day to calculate average doses (~5-9 mg/kg/day) of CORT. Mice were exposed to CORT or water from P31 to 42, corresponding to early adolescence in rodents (Spear, 2000). After a 2-week washout period, when mice reached young adulthood (P56), instrumental conditioning began. Timelines are in the figures.

3.4.3 Forced swim stress

Mice were exposed to forced swim stress daily from P31 to 42. Mice were placed in a glass cylinder (24 cm × 15.5 cm diameter) filled with 25°C water in a dimly lit room. After 6 min, mice were dried in a warm cage lined with paper towels, then returned to the home cage. Water was changed between mice. Control mice were handled but not exposed to swim stress.

3.4.4 Instrumental conditioning

Mice were trained to nose poke for food reinforcement (20 mg grain-based pellets; Bio-Serv, Flemington, NJ, USA) using Med Associates conditioning chambers equipped with two nose-poke recesses, a retractable lever, and a food magazine. Responding on each of two nose-poke recesses was reinforced using a fixed ratio 1 (FR1) schedule of reinforcement; 30 reinforcers were available

for responding on each aperture, resulting in 60 reinforcers/session. Sessions ended when mice acquired all 60 reinforcers or at 70 min. Five to seven daily training sessions were conducted. Response acquisition curves represent both nose poke responses/min. Mice were then tested in an instrumental contingency degradation task, followed by a reversal task (see timeline in Figure 3-1A). Given our focus on reversal conditioning here, only reversal performance is shown.

3.4.4.1 Instrumental reversal test

In this task, the reinforced response was "reversed" to a lever press; there were no consequences for generating the previously reinforced nose poke response (Figure 3-1B). Lever pressing was reinforced using an FR1 schedule, with one 25-min session/day for four consecutive days. Lever press rates reflect acquisition of a new response, while nose poke rates reflect inhibition of the previously reinforced response. The percent of correct responses made during session 1 was calculated by dividing total lever presses by total responses (lever presses + nose pokes).

3.4.5 Drugs (dosing and timing)

Male and female mice were administered (*i.p.*) the putative trkB agonist, 7,8-dihydroxyflavone (7,8-DHF; Sigma; 3 mg/kg; dissolved in 17% DMSO and saline; Zhang et al., 2014), or vehicle daily from P39 to 47, overlapping with the end of the adolescent CORT exposure period. This period is marked by significant pruning of dendritic spines on excitatory pyramidal neurons in the mouse orbital PFC (oPFC; Shapiro et al., 2017b). Additionally, expression levels of trkB in the oPFC increase during this time (Shapiro et al., 2017b), potentially facilitating activity-dependent refinement of synaptic connections and stabilizing synapses that are not pruned. This period was also determined based on prior work (Barfield et al., 2017a).

The trkB antagonist, ANA-12 (Sigma; 0.5 mg/kg, 1% DMSO), or vehicle was administered

(*i.p.*) daily from P31 to 42, to match the period of adolescent CORT exposure.

3.4.6 Statistical analyses

Two-tailed statistical analyses with $\alpha \leq 0.05$ were performed using SPSS. Response rates were compared by two-factor (group × session) or three-factor (CORT × 7,8-DHF × session) mixed analysis of variance (ANOVA) with session as a within-subjects (repeated measure) factor. Tukey's *post hoc* tests were used following interactions or main effects between greater than two groups, and results are indicated graphically. The percent of responses that were reinforced ("correct") were compared by one-factor or two-factor (CORT × 7,8-DHF) ANOVA or Student's *t*-tests. In additional comparisons, CORT-exposed mice were split into "vulnerable" and "non-vulnerable" groups based on a median split of percent correct values from session 1 of the instrumental reversal task. Throughout, values >2 SDs from the mean were excluded.

3.5 RESULTS

3.5.1 Early-adolescent corticosteroid exposure in females impairs response inhibition in adulthood

We exposed female and male mice to CORT in the drinking water from P31 to 42, equivalent to early adolescence in humans (Spear, 2000). Some mice also received daily *i.p.* injections of vehicle (17% DMSO and saline) or 7,8-DHF (3 mg/kg) from P39 to P47. In the interest of clarity, only vehicle-treated and non-injected mice are shown in Figure 3-2, and then all injected groups are represented in Figures 3-3, 3-4, respectively.

As adults, female mice acquired the nose poke responses without group differences (main effect and interaction *F*s < 1; Figure 3-2A). When the response requirement was "reversed" to a lever press, females with a history of adolescent CORT exposure made more perseverative errors

than control mice – responding on the previously reinforced nose poke apertures despite non-reinforcement – during session 1 (interaction $F_{(3,69)} = 4.0$, $p = 0.01$; Figure 3-2B). Meanwhile, acquisition of the newly reinforced lever response was unaffected (main effect and interaction Fs < 1; Figure 3-2C).

We also calculated the percentage of total responses that were reinforced during session 1. While this measure did not significantly differ between groups ($t_{(23)} = 1.3$, $p = 0.2$; Figure 3-2D), mice with a history of CORT exposure qualitatively appeared to segregate into two groups. As an exploratory analysis, we applied a median split to CORT mice. CORT-exposed "vulnerable" mice made significantly fewer correct responses relative to both control and CORT-exposed "non-vulnerable" mice (main effect group $F_{(2,22)} = 11.1$, $p < 0.001$; Figure 3-2E). Thus, in females, a history of CORT exposure during early adolescence increases perseverative errors, and considerable individual differences are noted.

We repeated this experiment in male mice. Mice acquired the food-reinforced nose poke responses in adulthood, without group differences (main effect and interaction Fs < 1; Figure 3-2F). In contrast to females, early-adolescent CORT exposure in males did not impact response reversal (errors, main effect and interaction Fs < 1; Figure 3-2G; lever response acquisition, main effect and interaction Fs < 1; Figure 3-2H). There were no differences in the percentage of responses that were reinforced ($t_{(12)} = 0.8$, $p = 0.5$; Figure 3-2I), and a median split of values revealed no differences between control mice and either of the two CORT-exposed subgroups (main effect group $F_{(2,11)} = 2.0$, $p = 0.2$; Figure 3-2J). Thus, females may be especially vulnerable to the long-term effects of CORT exposure during early adolescence on response inhibition.

3.5.2 TrkB stimulation blocks CORT-induced errors

We attempted to block the long-term behavioral consequences of adolescent CORT

exposure in females by administering 7,8-DHF (3 mg/kg) during mid-adolescence (P39–47). A 7,8-DHF-only group was omitted to conserve animal usage. We found no group differences in response acquisition (main effect and interaction Fs < 1; Figure 3-3A). As before, mice with a history of adolescent CORT exposure generated more non-reinforced responses in reversal (main effect group $F_{(2,21)} = 3.6$, $p = 0.046$; session × group interaction $F_{(6,63)} = 2.1$, $p = 0.07$), and treatment with 7,8-DHF mitigated this impairment in response inhibition (Figure 3-3B). Acquisition of the newly reinforced lever press did not differ (main effect and interaction Fs ≤ 1; Figure 3-3C). Groups did not differ in the percentage of responses that were reinforced during session 1 (main effect group $F_{(2,21)} = 1.5$, $p = 0.3$; Figure 3-3D). Further, treatment with 7,8-DHF eliminated the existence of "vulnerable" and "non-vulnerable" populations within the CORT-exposed group (ANOVA with CORT+vehicle and CORT+7,8-DHF mice separated into two subgroups based on a median split (five groups total): main effect group $F_{(4,19)} = 7.9$, $p = 0.001$; Figure 3-3E). Thus, a trkB agonist occludes long-term deficits in response inhibition following early-life CORT exposure.

3.5.3 TrkB stimulation in adolescence improves behavioral flexibility in adulthood

Male mice exposed to water or CORT during early adolescence were also administered either vehicle or 7,8-DHF. As adults, all mice acquired the nose poke responses for food reinforcement, without group differences (main effect and interaction Fs ≤ 1.2; Figure 3-4A). In instrumental reversal, a history of CORT exposure did not impact response inhibition (Fs < 1) or acquisition of the newly reinforced response (Fs < 1), as described above. Nevertheless, 7,8-DHF reduced perseverative responding during session 1 in both control and CORT-exposed animals (session × 7,8-DHF interaction $F_{(3,72)} = 7.1$, $p < 0.001$; Figure 3-4B) and increased responding on the newly reinforced lever in session 1 (session × 7,8-DHF interaction $F_{(3,72)} = 6.6$, $p = 0.001$; Figure 3-4C). 7,8-DHF also increased the percentage of responses that were correct during session 1 (main effect

$F_{(1,24)}$ = 7.7, p = 0.01; CORT × 7,8-DHF interaction $F_{(1,24)}$ = 1.8, p = 0.2; Figure 3-4D), further indicating that stimulation of trkB during adolescence enhances behavioral flexibility in adulthood, well beyond the period of drug treatment.

3.5.4 Adolescent stressor exposure and trkB inhibition impair reversal performance

The apparent resilience of male mice to early-adolescent CORT exposure was somewhat surprising, so we exposed another group of males to swim stress during early adolescence (daily from P31 to 42). In adulthood, we detected no group differences in the acquisition of the nose poke responses (main effect F < 1; interaction $F_{(4,68)}$ = 1.8, p = 0.1; Figure 3-5A). When mice were required to shift responding to a previously non-reinforced lever, control and stressor-exposed mice did not differ in responding on the previously reinforced nose poke apertures (main effect $F_{(1,16)}$ = 1.3, p = 0.3; interaction $F_{(3,48)}$ = 1.3, p = 0.3; Figure 3-5B). However, mice with a history of adolescent stressor exposure responded less on the newly reinforced lever (main effect $F_{(1,16)}$ = 8.1, p = 0.01; session × group interaction $F_{(3,48)}$ = 1.2, p = 0.3; Figure 3-5C), making fewer reinforced responses during session 1 ($t_{(17)}$ = 2.3, p = 0.04; Figure 3-5D). Thus, stressor exposure in early adolescence impairs the ability of male mice to develop novel response strategies in adulthood. This deficiency is similar to those following lateral oPFC (loPFC) ablation (Gourley et al., 2010; summarized in Table 3-1).

Prolonged stressor or glucocorticoid exposure can down-regulate *Bdnf* or BDNF-trkB signaling in the PFC, and these alterations are associated with impairments in PFC-dependent behavioral tasks (Gourley et al., 2009a, 2012b; Xu et al., 2016b; Zhang et al., 2017). Thus, we also tested whether inhibiting trkB during early adolescence would induce reversal deficits in male mice.

Male mice received the trkB antagonist, ANA-12 (0.5 mg/kg), daily from P31 to 42, matching the period of early-adolescent CORT exposure. As adults, mice acquired the nose poke

responses for food reinforcement, without group differences (main effect $F_{(1,12)} = 3.6$, $p = 0.08$; interaction $F_{(4,48)} = 2.2$, $p = 0.09$; Figure 3-5E). ANA-12 impaired response inhibition (interaction $F_{(3,33)} = 10.4$, $p < 0.001$; Figure 3-5F) and also acquisition (interaction $F_{(3,30)} = 4.1$, $p = 0.01$; Figure 3-5G) during session 1. ANA-12-treated mice made fewer correct responses overall during session 1 ($t_{(12)} = 3.1$, $p = 0.01$; Figure 3-5H). Thus, trkB inhibition during adolescence recapitulated the effects of CORT exposure in females (perseverative responding) and also stressor exposure in males (response acquisition impairments). Our findings are summarized in Table 3-1.

3.6 DISCUSSION

Adolescents who experience chronic stress have higher incidences of stress-related psychiatric illnesses and behaviors associated with addiction as adults. These behaviors may result in part from disruption of the PFC-dependent ability to flexibly modify behavior when environmental contingencies change (Elton et al., 2014; Zhang et al., 2016a; see for review Watt et al., 2017). BDNF signaling through trkB regulates cellular maturational processes occurring in the PFC during adolescence (Xu et al., 2000; Shapiro et al., 2017b), and is disrupted by exposure to stress or elevated glucocorticoids (Bath et al., 2013; Numakawa et al., 2013; Suri & Vaidya, 2013). Using an oPFC-dependent instrumental reversal task in mice (see Gourley et al., 2010), we evaluated the long-term consequences of adolescent exposure to CORT, physical stress, trkB stimulation, or trkB inhibition, on behavioral flexibility. In females, adolescent CORT exposure increased perseverative errors in adulthood, and this was blocked by a trkB agonist. By contrast, behavioral flexibility was not impacted by CORT exposure in males, but was enhanced by trkB stimulation, as in females. Males did, however, develop reversal deficits in adulthood following repeated stressor exposure or trkB antagonism during adolescence. These findings add to evidence that adverse events or elevated glucocorticoid levels during adolescence can impair behavioral flexibility later in life. Moreover, our

data suggest that trkB-based interventions may prevent adolescent stress-related behavioral impairments later in life.

3.6.1 Long-term, sex-dependent behavioral consequences of adolescent CORT exposure

The majority of rodent studies examining the long-term neurobehavioral effects of early-life adversity have utilized male subjects, despite evidence for sex differences in the prevalence and severity of stress-related psychiatric illnesses (Kessler et al., 1993; MacMillan et al., 2001; Holbrook et al., 2002; Kuehner, 2003; Becker et al., 2012; Fattore et al., 2014). We report that CORT dissolved in the drinking water of mice during a period equivalent to early adolescence in humans (P31-42; Spear, 2000) impaired performance in an instrumental reversal task in adulthood in female mice, but not males. Specifically, adolescent CORT impaired the inhibition of a previously reinforced response, increasing perseverative errors. Similarly, exposure to stress-level cortisol in young-adult or older-adult female squirrel monkeys increases response inhibition errors in a detour-reaching task that assesses the ability to inhibit prepotent responses when reinforcement contingencies change (Lyons et al., 2000).

We also calculated the percent of responses that were reinforced ("correct") during the first reversal session, revealing a "CORT vulnerable" subgroup (approximately half of the group) with significantly impaired behavioral flexibility relative to a CORT-exposed "non-vulnerable" group and control mice. What mediates this vulnerability is unclear, but one factor may be dopamine D2-family receptors (D2, D3, D4). D2/3 antagonists disrupt reversal performance in monkeys and rodents (Ridley et al., 1981; Kruzich & Grandy, 2004; Floresco et al., 2006; Lee et al., 2007; De Steno & Schmauss, 2009), and variation in D2 receptor density in the ventral PFC (including oPFC; Gourley et al., 2009b) and midbrain (Laughlin et al., 2011) correlates with behavioral flexibility. Furthermore, adolescent stress exposure decreases PFC D2 expression in adulthood (Wright et al., 2008). Whether

vulnerabilities to corticosteroid-related modifications in dopamine D2 levels influence the behavioral patterns reported here could be tested in future experiments.

In contrast to females, males exposed to early-adolescent CORT failed to develop instrumental reversal impairments in adulthood. However, some studies report long-term deficits in reversal learning and extradimensional set-shifting (EDS; a medial PFC (mPFC)-dependent function) in male rodents following social stress during early adolescence (Snyder et al., 2015; Zhang et al., 2016). Accordingly, daily forced swim stress during early adolescence (P31-42) impaired the acquisition of the "reversed" response contingency here, resembling the effects of loPFC lesions (Gourley et al., 2010). These findings suggest that in males exposed to repeated stress during adolescence, components of the stress response besides elevations in CORT (*e.g.*, increased noradrenergic tone) may be responsible for response acquisition deficiencies. Indeed, under conditions of chronic stress in adult rats, stress-induced release of norepinephrine in the mPFC impairs certain forms of behavioral flexibility, while noradrenergic receptor blockade in the mPFC prevents stress-induced deficits (Jett & Morilak, 2013). On the other hand, 3 weeks of CORT exposure in adolescent male mice causes errors in a similar instrumental reversal task (Shapiro et al., 2017a), indicating that males are not entirely resilient to CORT.

One possibility is that higher concentrations of CORT would have triggered reversal deficiencies in males. Females have higher basal CORT levels than males, an effect that develops during puberty (Netherton et al., 2004; McCormick & Mathews, 2007; Stroud et al., 2011). Because puberty in rodents typically occurs around P35 in females and P40 in males (Korenbrot et al., 1977; Evans, 1986), administering exogenous CORT during a time when endogenous levels in females are increasing may result in higher total CORT levels in females. While we did not measure blood serum CORT in these experiments, it is possible that recapitulating "female-like" CORT levels in males may have induced comparable behavioral inflexibility.

Because the PFC undergoes significant structural remodeling during adolescence, and PFC-dependent cognitive functions continue to develop throughout this period, prolonged stressor or CORT exposure during adolescence may be more impactful than exposure in adulthood. Consistent with this possibility, male rats exposed to 5 days of social defeat stress during early adolescence (P28-32), then housed in isolation, exhibit deficits in EDS and reversal learning in an attentional set-shifting task 6 weeks later. However, rats exposed to the same stress in mid-adolescence (P38-47) or adulthood (P70-79) do not show these cognitive impairments (Zhang et al., 2016a). Furthermore, adolescent stress- or CORT-induced alterations in PFC neuronal morphology, like behavioral deficits, can persist into adulthood. For example, social isolation stress during early adolescence (P30-35) in male rats reduces synaptic density in the mPFC in adulthood (Leussis et al., 2008b), suggestive of a long-term loss of dendritic spines, which house the majority of excitatory synapses in the brain. Gourley et al. (2013b) also reported dendritic spine loss on pyramidal neurons in the oPFC immediately following CORT exposure during adolescence (P35-56) that is still evident 1 week after the cessation of CORT. Meanwhile, CORT-induced spine changes in the infralimbic mPFC, hippocampus, and amygdala recover. Consistent with these findings in rodents, early-life adversity in humans is associated with reduced oPFC volume and morphological alterations in adulthood (Holz et al., 2015; Teicher & Samson, 2016a). Our findings add to these and other studies reporting long-term consequences of stressor or CORT exposure during early adolescence.

3.6.2 trkB manipulations in adolescence influence behavioral flexibility in adulthood

One mechanism by which CORT or stressor exposure during adolescence may impact PFC-dependent behaviors later in life is by reducing BDNF-dependent stimulation of trkB (Duman et al., 1997; Blugeot et al., 2011). Consistent with this possibility, the trkB agonist 7,8-DHF blocked perseverative responding in females with a history of adolescent CORT exposure. Additionally,

while a subgroup of vehicle-treated CORT-exposed females was especially vulnerable to impairments in behavioral flexibility, 7,8-DHF eliminated these individual differences, and in males, 7,8-DHF improved performance above control levels.

How might 7,8-DHF have long-term behavioral consequences? One possibility is that it prevents CORT-induced disruptions in PFC synaptic maturation by augmenting or restoring trkB activity. BDNF and trkB increase in adolescence/young adulthood (Webster et al., 2002; Shapiro et al., 2017b), and BDNF-trkB signaling is critical for the maintenance of stabile dendritic spines in the postnatal brain (Vigers et al., 2012). Chronic stress or CORT exposure can reduce PFC BDNF and trkB (*e.g.*, see Gourley et al., 2009a, 2012b), potentially altering the trajectory of dendritic spine maturation (Leussis & Anderson, 2008a; Eiland et al., 2012) and behavioral outcomes. Consistent with this possibility, we found that administration of the trkB antagonist, ANA-12, during early adolescence impaired behavioral flexibility in adulthood.

Our trkB antagonism experiments were performed in males, but it is worth noting that prior reports indicate that knockdown of *Bdnf* beginning in the preadolescent period or in adulthood in female mice produces behavioral consequences that are comparable to, or more severe than, those in male mice. For example, Vigers et al. (2012) report that forebrain-specific knockdown of *Bdnf* (progressive knockdown begins at ~P21) induces depressive-like immobility in the forced swim test and contextual fear generalization in both male and female adult mice. However, Monteggia et al. (2007) report that this knockdown strategy results in depression-like behavior in female, but not male, mice. Similarly, inducible knockdown of *Bdnf* in adulthood increases susceptibility to stress-induced depression-like behavior in female mice only (Autry et al., 2009).

Recent findings in addition to ours interestingly implicate BDNF-trkB in the long-term behavioral consequences of early-adolescent stress. Male rats exposed to chronic mild stress (CMS) during early adolescence (P28-37) exhibit impairments in EDS and decreased levels of BDNF

protein, the ratio of p-ERK42/44 / ERK42/44, and p-CREB in the mPFC 6 weeks after stressor exposure (Zhang et al., 2017). Additionally, EDS is correlated with p-ERK/ERK ratios in mPFC, and the antidepressant duloxetine ameliorates stress-induced alterations in EDS and mPFC BDNF levels in tandem. *Impairing* BDNF-trkB signaling can also alter PFC-dependent functions and drug-taking behaviors. For example, mutant mice with disruption of promoter IV-driven (activity-dependent) BDNF expression are impaired in a spatial reversal task (Sakata et al., 2013). Knock-in mice expressing a homolog of the human *BDNF* gene polymorphism *Val66Met*, which decreases the activity-dependent release of BDNF (Chen et al., 2004), develop excessive and compulsive alcohol drinking despite adverse consequences, and this phenotype is reversible by a trkB agonist (Warnault et al., 2016). Furthermore, early-life stress decreases *Bdnf* exon IV mRNA in the PFC and increases cocaine-induced conditioned place preference (CPP) in periadolescent (P45) mice (Viola et al., 2016). Interestingly, higher CPP scores correlate with *Bdnf* expression, with higher place preference associated with lower *Bdnf*. Thus, by disrupting trkB signaling, early-life stress may increase vulnerability to compulsive drug taking.

3.6.3 Broader considerations: sex differences in reversal performance

Here, we found that adolescent CORT-exposed females and adolescent stress-exposed males exhibited impairments in reversal performance in adulthood; however, the specific behavioral impairments were sexually dimorphic – females failed to inhibit the previously reinforced response, while males were unable to acquire a newly reinforced response (summarized in Table 3-1). In the context of addiction, poor control over perseverative behavior could modulate the progression from recreational drug use to abuse and dependence, increase relapse rates, and impair treatment response (Carroll et al., 2011). Meanwhile, deficits in the acquisition of a "reversed" response contingency

could be comparable to impairments in the ability to adjust behavior according to positive feedback and potentially, to substitute new healthy behaviors for maladaptive ones.

The association between early-life trauma and the risk for, and severity of, drug dependence is stronger in women (Widom & White, 1997; MacMillan et al., 2001; Simpson & Miller, 2002; Hyman et al., 2008), and at least one study reports that a greater proportion of stimulant-dependent women, compared to men, have clinically significant "disinhibition" scores prior to stimulant abuse (Winhusen & Lewis, 2013). In that same study, physical abuse was associated with greater disinhibition in women, but not men. Thus, females may be more vulnerable to chronic stress-induced inhibitory control deficits, in line with our findings. Further, Elton et al. (2014) report that childhood maltreatment results in enduring, sex-dependent changes in the functional connectivity of a network mediating inhibitory behavioral control, also in line with sex-dependent effects of adolescent CORT exposure on perseverative responding here. Future work should examine whether adolescent CORT-induced deficits in response inhibition increase vulnerability to compulsive drug taking later in life. CORT exposure during adolescence increases cue-induced reinstatement of ethanol seeking in adult female, but not male, rats (Bertholomey et al., 2016), suggesting that females are especially vulnerable to adolescent CORT-induced neurobehavioral changes that increase susceptibility to drug relapse later in life.

Our findings may also point to sexually dimorphic stress-related vulnerabilities in specific brain regions, given that lesions of the medial oPFC (moPFC) impair response inhibition in this task (like CORT-exposed females), while loPFC lesions impair response acquisition (like stressor-exposed males; Gourley et al., 2010). These patterns are interesting when considered alongside studies reporting structural changes in the oPFC of substance-dependent males and females. For example, cocaine-dependent females and males exhibit decreased cerebral blood flow in the moPFC and loPFC, respectively (Adinoff et al., 2006). Adolescent females with substance dependence

exhibit decreased cortical thickness and grey matter volume in regions involved in inhibitory control, decision-making, reward, and risk-taking, including the moPFC and dorsolateral PFC (DLPFC; Dalwani et al., 2015; Boulos et al., 2016), while substance-dependent adolescent males show decreased grey matter volume in DLPFC (Dalwani et al., 2011). Regner et al. (2015) report decreased oPFC gray matter volume in abstinent stimulant-dependent women, but not men. However, in a population of predominantly male treatment-seeking individuals with alcohol dependence, loPFC surface area and volume were smaller in individuals who subsequently relapsed in a 1-year period compared to individuals who remained abstinent (Cardenas et al., 2011; Durazzo et al., 2011). Together, these findings suggest that PFC subregions disrupted by both adolescent stress or glucocorticoid exposure and drugs of abuse may be sex-dependent (potentially: loPFC in males and both moPFC and loPFC in females; see Table 3-1).

3.6.4 Conclusion

PFC dysfunction and associated deficits in inhibitory control and behavioral flexibility are hallmarks of stress-related illnesses, including addiction. Substance-dependent individuals report loss of control over drug use and have difficulty modifying behaviors when reward contingencies change, potentially contributing to persistent drug seeking despite adverse consequences (Garavan & Hester, 2007; Everitt & Robbins, 2016). Being able to implement new strategies to promote behavioral change and control impulsive responses may be critical to treatment response – among cocaine-dependent individuals undergoing cognitive-behavioral therapy, higher impulsivity/risk-taking before treatment is associated with poorer treatment retention and higher relapse rates (Carroll et al., 2011).

Substance-dependent individuals exhibit structural and functional alterations in the oPFC, including blood flow abnormalities (London et al., 2000; Adinoff et al., 2006), decreased grey matter

volume (Matochik et al., 2003; Tanabe et al., 2009; Dalwani et al., 2015), and decreased cortical thickness (Boulos et al., 2016). In animal models, adolescent CORT and cocaine exposure result in long-term changes in oPFC neuron structure (Gourley et al., 2012a, 2013b; DePoy et al., 2014, 2016, 2017) that are associated with maladaptive behaviors symptomatic of addiction (Lucantonio et al., 2012). Further, poor inhibitory control may be a *predisposing factor* for addiction (Jentsch & Taylor, 1999; Tarter et al., 2004; Groman et al, 2009; Moffitt et al., 2011), as is a history of early-life adversity (Fergusson et al., 1996; Kessler et al., 1997; Dube et al., 2003; Green et al., 2010; Afifi et al., 2012). These and other findings have led to the hypothesis that early-life adversity may affect addiction liability by impairing oPFC-mediated inhibitory control. That is, in individuals with a history of stressor exposure during sensitive developmental periods like adolescence, repeated drug use may further impair inhibitory control, exacerbating the development, persistence, and severity of addiction. Indeed, social stress during adolescence in rats causes binge-like cocaine self-administration in adulthood (Burke & Miczek, 2015).

Using an oPFC-dependent instrumental reversal task, we find that exposure to CORT during early adolescence induces perseverative errors in female, but not male, mice in adulthood. Further, intervention with a trkB agonist in mid-adolescence, when dendritic spines in the PFC undergo activity-dependent pruning or stabilization (Petanjek et al., 2011; Gourley et al., 2012a; Selemon, 2013; Chung et al., 2017) corrected decision-making strategies in CORT-exposed mice. With the caveat that the sexes were tested in independent cohorts here – precluding direct comparisons between them – we nevertheless argue that further related research may advance our understanding of how sex differences in the neurobehavioral response to adolescent adversities contribute to sex differences in vulnerability to, and clinical course of, stress-related illnesses. Our findings additionally suggest that pharmacotherapies augmenting trkB may ameliorate the enduring consequences of stressful life events occurring in adolescence.

3.7 FUNDING

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3.8 ACKNOWLEDGMENTS

We thank A. Allen for assisting with injections.

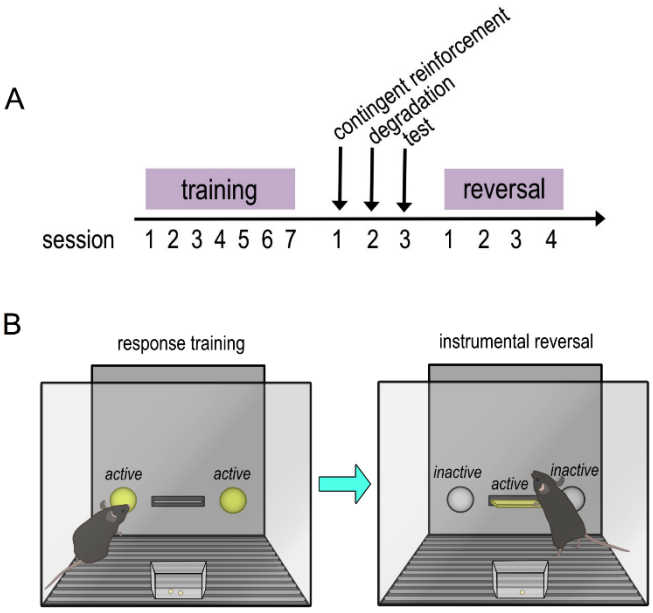

Figure 3-1. Timeline and schematic of instrumental training and reversal task. (A) Timeline of training and tasks. Mice were trained to nose poke in operant conditioning chambers for food reinforcers. Next, an action-outcome contingency degradation task was performed, in which the contingency between an action and outcome are "degraded" by providing food pellets independently of the mouse's actions. The data from these tests are reported in Barfield et al. (2017a). An instrumental reversal task followed, and the data from these tests are reported in the present manuscript. **(B)** Schematic of response training and instrumental reversal. In the instrumental reversal task, the reinforced response was "reversed" from a nose poke on the sides of the chamber to a lever press located in the center of the chamber that had not previously been available. Acquisition of the newly reinforced response (lever press) and inhibition of the previously reinforced response (nose poke) were measured over multiple sessions.

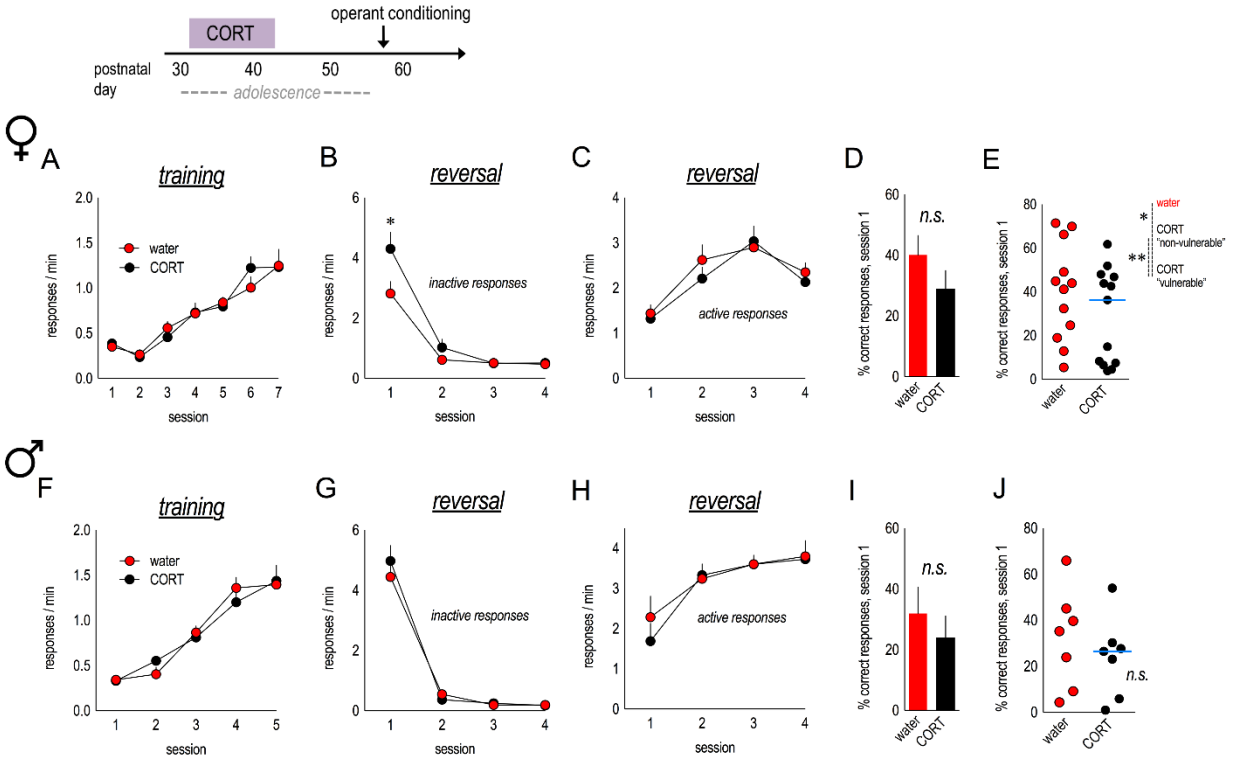

Figure 3-2. CORT exposure in adolescent females induces reversal errors in adulthood. An experimental timeline is at top. **(A)** Female mice with a history of CORT exposure were trained to nose poke for food reinforcers, with no group differences. **(B)** In an instrumental reversal task, CORT-exposed mice made more perseverative errors, generating the previously-reinforced response. **(C)** CORT did not impact the acquisition of the new lever press response. **(D)** The percent of correct responses made during session 1 did not differ between groups. **(E)** However, a median split revealed that CORT-exposed females separated into two distinct subgroups; the "vulnerable" group made significantly fewer correct responses. *n*=12-13/group. **(F)** Male mice exposed to CORT during adolescence acquired the nose poke responses without group differences. **(G)** A history of early-adolescent CORT exposure did not impact inhibition of the previously reinforced, now-inactive, response. **(H)** Acquisition of the newly reinforced response was also not impacted. **(I-J)** The percent of correct responses made during session 1 also did not differ between groups. *n*=7/group. Symbols and bars represent means + SEMs, except for

scatter plots, where symbols represent individual mice, and blue lines indicate the median. *$p \leq 0.05$.

**$p \leq 0.001$.

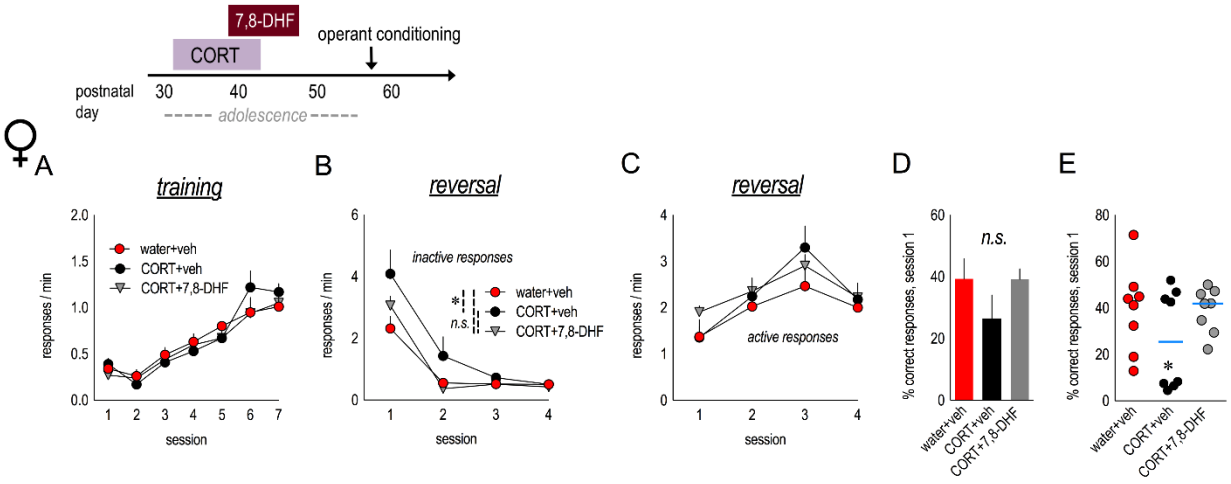

Figure 3-3. TrkB stimulation blocks perseverative errors in CORT-exposed female mice.

Experimental timeline is at top. Female mice were exposed to CORT from P31-42 and administered the trkB agonist, 7,8-DHF, from P39-47. **(A)** As adults, mice were trained to nose poke for food reinforcers, with no differences between groups. **(B)** CORT-exposed mice made more perseverative errors, and 7,8-DHF mitigated this effect. **(C)** Groups generated the newly reinforced response at similar rates. **(D)** There were no gross effects on the percent of correct responses made during session 1. **(E)** CORT-exposed mice were divided into two subgroups on the basis of a median split of percent values, revealing two populations, including a subgroup that made significantly fewer correct responses. 7,8-DHF eliminated these differences. n=8/group. Symbols and bars represent means + SEMs. *$p \leq 0.05$.

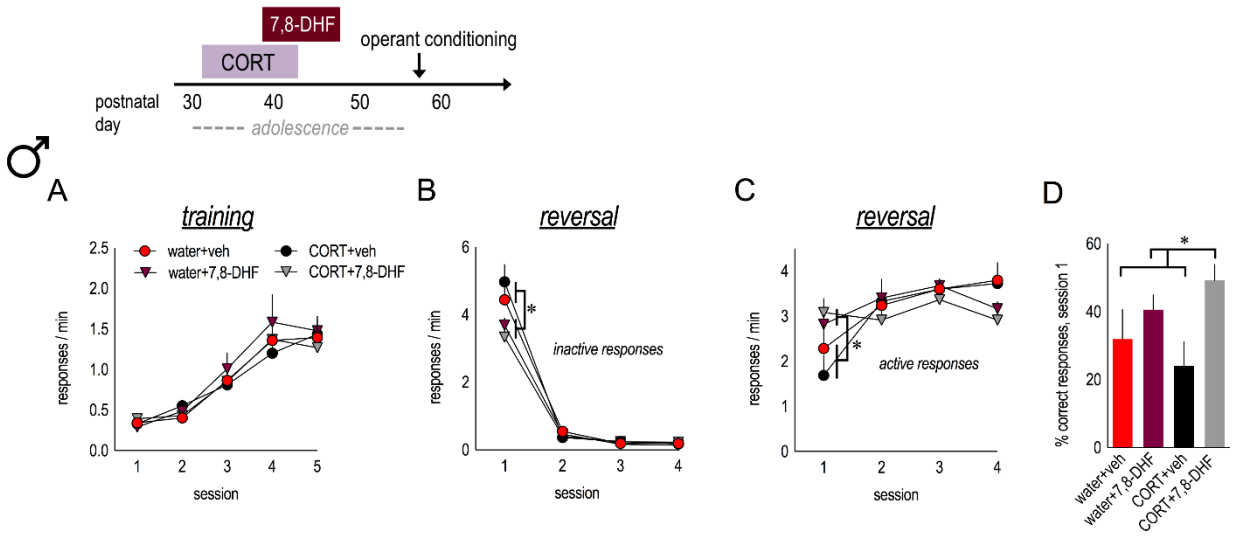

Figure 3-4. TrkB stimulation during adolescence enhances behavioral flexibility in adulthood. Experimental timeline is at top. Male mice were exposed to CORT from P31-42 and administered the trkB agonist, 7,8-DHF, from P39-47. **(A)** Mice acquired the nose poke responses in adulthood, with no group differences. **(B)** In both water and CORT-exposed mice, 7,8-DHF reduced perseverative errors in the first reversal session. **(C)** Further, 7,8-DHF increased active response rates on the first day of reversal. **(D)** 7,8-DHF also increased the percent of correct responses made during session 1 (asterisk signifies a main effect of 7,8-DHF). n=7/group. Symbols and bars represent means + SEMs. *$p \leq 0.05$.

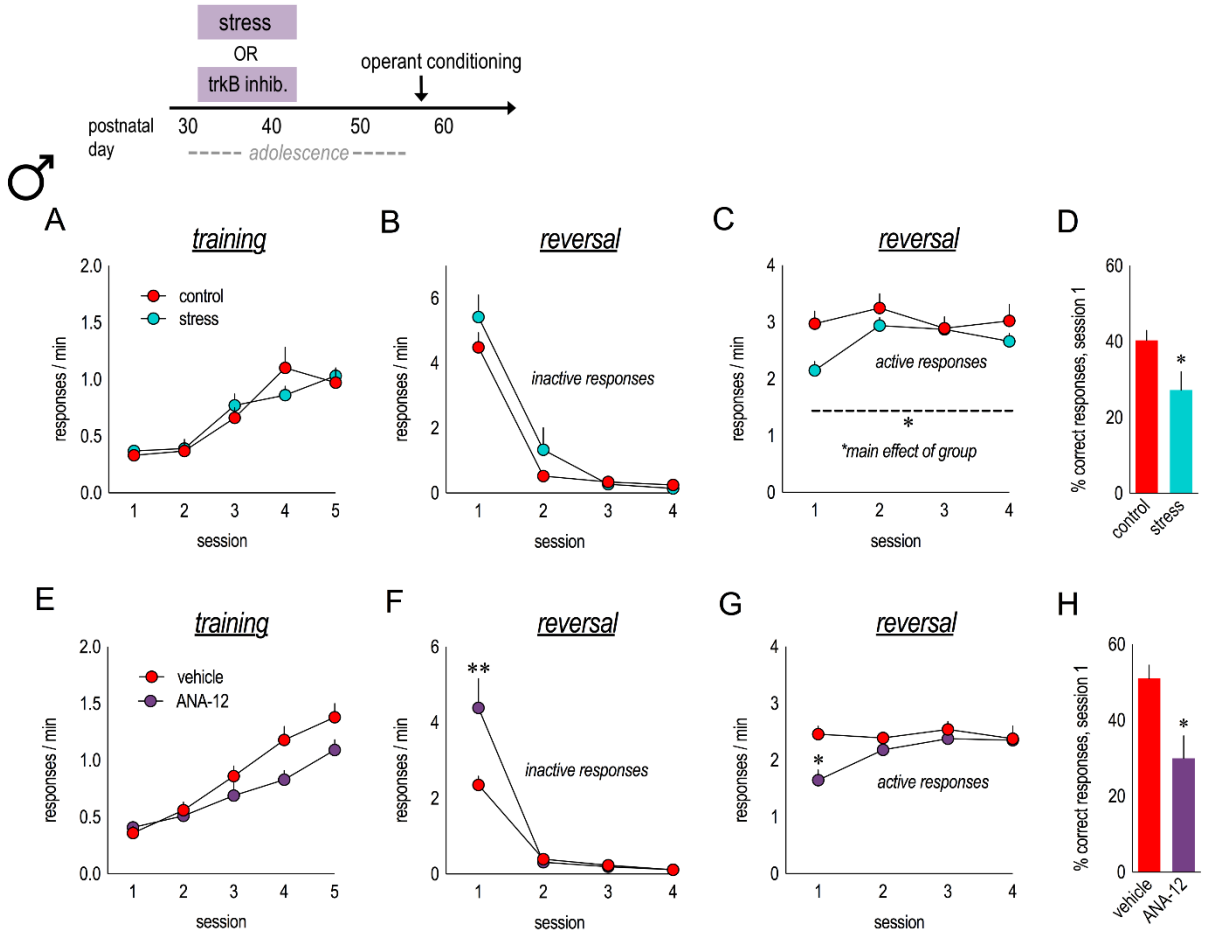

Figure 3-5. Physical stressors and pharmacological inhibition of trkB in adolescence impair instrumental reversal in adulthood. Experimental timeline is at top. **(A)** A history of stressor exposure did not impact responding for food reinforcement during training in adulthood. **(B)** In an instrumental reversal task, both groups performed the now-inactive nose poke response at similar rates. **(C)** By contrast, stressor-exposed mice performed the now-active lever press response at lower rates. **(D)** Adolescent stressor exposure also reduced the percent of correct responses made during session 1. *n*=9-10/group. **(E)** In parallel experiments, mice received daily injections of the trkB antagonist, ANA-12, from P31-42. Mice learned to nose poke for food reinforcement in adulthood, with no group differences. **(F)** TrkB blockade during adolescence increased perseverative errors during the first reversal session. **(G)** ANA-12 also reduced responding on the now-active lever during session 1. **(H)** ANA-12-

treated mice made fewer correct responses during session 1. n=7/group. Symbols and bars represent means + SEMs. *$p \leq 0.05$. **$p \leq 0.001$.

Manipulation	Region	Sex	Reference
Impairments of response acquisition			
Early-adolescent forced swim stress	n/a	M	Figure 3-5C
Early-adolescent trkB antagonism	n/a	M	Figure 3-5G
Lesion	loPFC	M	Gourley et al., 2010
Inhibition of Abl-family kinases	loPFC	M	Gourley et al., 2012a
Bdnf knockdown	loPFC	M	Gourley et al., 2013a
Disconnection of the loPFC and striatum	loPFC	M	Gourley et al., 2013a
Impairments of response inhibition			
Early-adolescent CORT exposure	n/a	F	Figure 3-2B
Early-adolescent trkB antagonism	n/a	M	Figure 3-5F
Lesion	moPFC+loPFC	M	Boulougouris et al., 2007
Lesion	moPFC	M	Gourley et al., 2010

Table 3-1. Failures in response acquisition and errors of perseveration in an instrumental reversal task can be dissociated. Errors of perseveration (repeatedly performing an "old" response, despite reversal) or response acquisition (failing to develop a new strategy) can be dissociated in reversal tasks that rely on spatial or instrumental learning and memory. Here we detected failures in response acquisition and errors of perseveration (highlighted in blue). These deficits are associated with manipulations of the lateral oPFC (loPFC) and medial oPFC (moPFC), respectively (highlighted in gray).

CHAPTER 4: ADOLESCENT CORTICOSTEROID EXPOSURE DETERIORATES VENTRAL HIPPOCAMPAL-ORBITOFRONTAL CORTICAL INPUTS: FUNCTIONAL CONSEQUENCES

4.1 CONTEXT AND AUTHOR'S CONTRIBUTION

The following chapter presents evidence that exposure to corticosterone (CORT) during adolescence impairs the ability to use outcome-predictive associations to guide behavior in appetitive and aversive contexts in adulthood. We also show that adolescent CORT exposure degrades anatomical connectivity between the ventral hippocampus (vHC) and orbital prefrontal cortex (oPFC) in adulthood, necessary for sustaining goal-directed response selection. The dissertation author contributed to the paper by designing and conducting the experiments, analyzing the data, and writing the manuscript under the guidance of Dr. Shannon Gourley.

4.2 ABSTRACT

In an ever-changing and often ambiguous environment, organisms must use previously learned associations between antecedents and outcomes to predict future outcomes and make optimal behavioral choices. Chronic stress can impair one's ability to flexibly adjust behavior when environmental contingencies change, particularly in cases of early-life stressor exposure. Nevertheless, neurodevelopmental mechanisms remain unclear. Previously, we reported that elevated levels of the primary stress hormone, corticosterone (CORT), during early adolescence in mice impair response-outcome-based decision making in a food-reinforced task, biasing response strategies instead towards inflexible, context-elicited habits. Here, we extend these findings to show that adolescent CORT exposure also impedes the appropriate use of contextual cues in aversive circumstances, resulting in fearful behavior (freezing) in neutral contexts. The orbital prefrontal cortex (oPFC) is involved in prospectively calculating likely outcomes, even when they are not observable, and it receives input from the hippocampus. Adolescent CORT caused a loss of dendritic spines on pyramidal neurons in the lateral, but not medial, oPFC (loPFC), and reduced the presence of vHC→loPFC axonal terminals in adulthood. To identify functional consequences, we

selectively and inducibly inactivated vHC→loPFC projections, impairing the ability of otherwise healthy mice to sustainably select actions based on anticipated outcomes. Our findings reveal that vHC→loPFC projections are necessary for action-consequence decision making and suggest that their degradation is involved in the long-term neurobehavioral consequences of adolescent CORT exposure.

4.3 INTRODUCTION

An organism's survival in an ever-changing world requires the ability to predict rewards or threats in the environment. Learned associations between antecedents and outcomes must be used and flexibly updated to orchestrate appropriate behavioral responses. An inability to update these associations when environmental contingencies change can produce maladaptive behaviors resembling core symptoms of a number of psychiatric disorders, including depression, post-traumatic stress disorder (PTSD) and substance use disorder (SUD). For example, in PTSD, fear responses to trauma-relevant contextual cues generalize to other harmless situations or stimuli that partially resemble the initial trauma (Morey et al., 2015). In SUD, continued drug abuse may become increasingly negative, yet individuals struggle to modify behaviors and instead, exhibit persistent drug seeking (Everitt et al., 2001; Garavan & Hester, 2007; Everitt & Robbins, 2016). Stressor exposure, which promotes rigid, outcome-insensitive habits (Kim et al., 2001; Schwabe et al., 2007, 2012; Dias-Ferreira et al., 2009; Schwabe, 2013; Patterson et al., 2013), can trigger or exacerbate disease symptomatology (Admon et al., 2013). Dysfunction in the stress-sensitive circuitry that guides flexible outcome-based behavior may be a common feature of seemingly disparate disorders (Griffiths et al., 2014).

The ventral hippocampus (vHC) and orbital prefrontal cortex (oPFC) are involved in guiding behaviors based on expected outcomes (rewarding or aversive) (Yu & Frank, 2015;

Wikenheiser & Schoenbaum, 2016). The oPFC is thought to generate internal representations of 'task spaces' (Wilson et al., 2014), integrating abstract information about response-outcome and stimulus-outcome relationships. vHC projections are critical for the generation of task spaces in the oPFC. The vHC combines internal state and external environment information, outcome features, context features, rules for obtaining outcomes, and predictive associations into unified representations (Markus et al., 1995; Kennedy & Shapiro, 2009; Vanni-Mercier et al., 2009; Mack et al., 2016) that support integrative coding in the oPFC (Wikenheiser et al., 2017). Goal-relevant contextual information provided by vHC inputs (Komorowski et al., 2013) can also be used by the oPFC to help select appropriate behavioral responses for a given context (Young & Shapiro, 2011; Farovik et al., 2015; Mizumori & Tryon, 2015). Thus, the oPFC and vHC support outcome-based learning and memory necessary for flexible adaptation of behavior.

In humans, early-life adversity promotes the use of inflexible, habit-based learning and memory strategies at the expense of response-outcome strategies in decision making (Patterson et al., 2013; Pechtel & Pizzagalli, 2013) and is associated with volume reductions and neuronal morphological alterations in the oPFC and hippocampus in adulthood (Teicher & Samson, 2016a). Developmental stress or stress hormone exposure in rodents confers similar long-term behavioral deficits (Barfield et al., 2017a; Zhang et al., 2017) and cortico-limbic structural changes (Eiland & Romeo, 2013). Stress hormones could conceivably modify cortico-limbic development, causing decision-making biases that are long-lasting.

Here, we examined the long-term neurobehavioral consequences of prolonged exposure to elevated levels of the primary stress hormone, corticosterone (CORT), during early adolescence in mice. We report that mice with a history of adolescent CORT exposure fail to adjust behavior when outcome-predictive associations change. Deficiencies are long-lasting, and they coincide with the loss of dendritic spines in the lateral oPFC (loPFC), as well as inputs from the vHC. Finally, we

demonstrate that vHC→loPFC inputs are indispensable for sustainably selecting actions based on anticipated outcomes, implicating this projection in chronic behavioral consequences of adolescent CORT exposure.

4.4 MATERIALS AND METHODS

4.4.1 Subjects

Group-housed male wild-type C57BL/6 mice (Jackson Labs) were used, except for dendritic spine imaging experiments, which used mice expressing *thy1*-derived yellow fluorescent protein (YFP; Feng et al., 2000) that were fully back-crossed onto a C57BL/6 background. Mice were maintained on a 12-h light cycle (0800 on) and provided food and water *ad libitum* except during instrumental conditioning when body weights were maintained at ~90% of baseline to motivate responding. Animal numbers for each experiment are indicated in the respective figure captions. Procedures were approved by the Emory University IACUC and carried out in accordance with the recommendations of the *Guide for the Care and Use of Laboratory Animals*.

4.4.2 CORT exposure

4-pregnen-11β 21-DIOL-3 20-DIONE 21-hemisuccinate (Steraloids) was dissolved in tap water (25 μg/mL free base) (Gourley et al., 2008a,b, 2012b; Barfield et al., 2017a). CORT-exposed mice were given CORT in place of normal drinking water, while control mice consumed tap water. Water bottles were weighed daily, and mice weighed every other day to calculate average doses (~5-9 mg/kg/day) of CORT. Every 3 days, water bottles were refilled with fresh tap water or newly prepared CORT solution. Mice were exposed to CORT or water from postnatal day (P) 31-42 or P70-81, corresponding to early adolescence and adulthood in rodents (Spear, 2000). After a washout

period of at least 2 weeks, instrumental conditioning or fear conditioning began. Timelines are provided in the respective figures.

4.4.3 Surgery

Experiments requiring intracranial surgery utilized: a fluorescent anterograde tracer, Fluoro-Ruby (Millipore); a retrograde adeno-associated virus (AAV) expressing Cre (AAVrg-pmSyn1-EBFP-Cre, Addgene); a recombinant AAV encoding a Cre-driven, inhibitory Gi-coupled Designer Receptor Exclusively Activated by Designer Drugs (DREADD) (rAAV5-hSyn-DIO-hM$_4$D(Gi)-mCherry; UNC Viral Vector Core); or a control vector (rAAV5-hSyn-EYFP; UNC Viral Vector Core).

Fluoro-ruby infusions occurred at P49; surgeries for chemogenetic experiments occurred at P49±1 day. Mice were anaesthetized with Ketamine/Dexdomitor (80 mg/kg/0.5 mg/kg, *i.p.*) and placed in a digitized stereotaxic frame (Stoelting). The head was shaved, scalp retracted, and skull leveled.

For fluoro-ruby infusions, 2 burr holes per hemisphere were drilled, and fluoro-ruby (10% solution in distilled water) was infused bilaterally into the vHC (ML±3.6, AP-2.4, DV-4.5 and ML±3.3, AP-2.8, DV-4.8) over 3 min (0.04 µl/site).

For viral vector infusions, 3 burr holes per hemisphere were drilled. A retrograde Cre-expressing virus was first infused bilaterally into the loPFC (ML±1.5, AP+3.0, DV-3.1) over 5 min (0.5 µl/site). A virus expressing a Cre-driven Gi-coupled DREADD or a control vector was then infused bilaterally into the vHC (ML±3.6, AP-2.5, DV-4.5 and ML±3.2, AP-2.7, DV-5.0) over 5 min (0.25 µl/site). This strategy results in the Cre-driven expression of Gi-DREADDs only in vHC neurons that project to the loPFC in experimental mice, while control mice also express Cre, but not DREADDs.

Throughout, needles were left in place for 5 additional min before withdrawal and suturing of the scalp. Mice were revived with Antisedan (1 mg/kg, *i.p.*). Mice infused with fluoro-ruby were euthanized one week after surgery. Mice infused with viral vectors were allowed to recover for at least 2 weeks before behavioral testing.

4.4.4 Drugs

Mice were administered (*i.p.*) the synthetic DREADDs ligand, clozapine-n-oxide (CNO; Sigma-Aldrich; 1 mg/kg, in 2% dimethylsulfoxide (DMSO) and saline). All mice were administered CNO, in order to equally expose all animals to any unintended consequences of CNO, *e.g.*, back-metabolism to clozapine (Gomez et al., 2017).

4.4.5 Instrumental conditioning

Mice were food-restricted and trained to nose poke for 20 mg grain-based food reinforcers (Bio-Serv) using Med Associates conditioning chambers equipped with 2 nose poke recesses and a food magazine. Training was initiated with a fixed ratio 1 (FR1) schedule of reinforcement; mice could earn up to 30 reinforcers for responding on each of 2 apertures (60 reinforcers/session). Sessions ended when mice acquired all 60 reinforcers or at 70 min. Following 6 sessions of FR1 training (1/day), mice were shifted to a random interval (RI) 30-sec schedule of reinforcement for 4 days to bias towards habit-based responding (Dickinson et al., 1983). 30 reinforcers were again available (60 reinforcers/session, 1 session/day), and sessions ended when all 60 pellets had been delivered, or at 70 min. Response acquisition curves represent both responses/min.

Mice were next tested in a response-outcome contingency degradation task, as in our prior reports (*e.g.*, Gourley et al., 2012b; Barfield et al., 2017a). In a 25-min "non-degraded" session, one nose poke recess was occluded, and responding on the other was reinforced using a variable ratio 2

schedule of reinforcement. During a 25-min "degraded" session the next day, the opposite aperture was occluded, and responding on the available aperture produced no programmed consequences. Reinforcers were instead delivered into the magazine at a rate matched to each animal's reinforcement rate on the previous day. Thus, the reinforcement schedule associated with one nose poke was enriched, while the causal relationship between the other response and the outcome was degraded. In effect, responding on one aperture was significantly less likely to be reinforced than responding on the other.

The order of these sessions and the location of the nose poke aperture associated with an enriched or degraded response-outcome contingency were counter-balanced. Response strategies were then assessed in a 5-min choice test conducted in extinction on the following day. Both apertures were available. Preferential engagement of the response that was most likely to be reinforced is indicative of a goal-directed response strategy; by contrast, engagement of both responses equally, or habitually, can indicate a failure in response-outcome learning (Balleine & O'Doherty, 2010). Response preference scores, calculated as the ratio of response rates on the aperture associated with a "non-degraded" contingency / the aperture associated with a "degraded" contingency, are also shown, adjacent to raw response rates.

For experiments utilizing DREADDs, the "non-degraded" session preceded the "degraded" session, and all mice were administered CNO immediately following the "degraded" session, inactivating vHC→oPFC projections during a period of oPFC-dependent response-outcome memory encoding (Zimmermann et al., 2018) in DREADD-expressing mice. On each of the next 2 days, mice underwent 3 successive 5-min choice tests to track response strategies over time. To minimize potential confounding effects of injection stress, all mice were given saline injections (*i.p.*) for several days prior to the contingency degradation procedure.

4.4.6 Fear conditioning

Mice were habituated to the conditioning chambers (context A) (Coulbourn) for 2 days (15 min/day) prior to conditioning. Auditory fear conditioning was conducted in an 8-min session, consisting of a 3-min habituation period, followed by 5 presentations of a 30-s 6kHz tone conditioned stimulus (CS) co-terminating with a 1-s, 0.6 mA footshock (the unconditioned stimulus; US). Twenty-four h after conditioning, mice were placed in a novel context (context B) to assess animals' ability to distinguish between footshock-associated and neutral contexts, and for the purposes of testing animals' memory of the CS-US association. The lighting, scent, and floor texture differed from the initial context. The test session consisted of a 3-min baseline period, followed by 15 CS presentations without the US, with a 30-s inter-tone interval. Tests were conducted 1 day and then 17 days later, also in context B. The percentage of time mice spent freezing prior to tone onset (*i.e.*, context-elicited freezing) and during tone presentations (*i.e.*, CS-elicited freezing) was determined using FreezeFrame software (Coulbourn).

For experiments utilizing DREADDs, CNO was administered to all mice 30 min prior to context B test day 1, following the investigations of Zimmermann et al. (2018) into oPFC contributions to fear-related learning. We measured context- and CS-elicited freezing with CNO on board, and then 1 and 17 days later, to determine if vHC→oPFC inactivation affected subsequent behavior.

4.4.7 Dendritic spine imaging and reconstruction

thy1-YFP-expressing mice exposed to water or CORT-infused water from P31-42 were euthanized following behavioral testing (instrumental conditioning experiments), at P100. Mice were rapidly decapitated, brains were extracted and submerged in chilled 4% paraformaldehyde for 48 h, then transferred to 30% w/v sucrose, and sectioned at 40 μm using a freezing microtome. Dendritic

segments on deep-layer pyramidal neurons in the lateral and medial oPFC (loPFC, moPFC), located between Bregma +2.8-+2.22, were imaged with a spinning disk confocal (VisiTech International) on a Leica microscope.

Between 1-5 independent segments from secondary and tertiary basilar dendritic branches, 15-20 μm in length, within 50-100 μm of the soma, were collected from each animal. Images were processed and dendritic spines were enumerated and reconstructed in 3D by a single blinded rater using Imaris software (described Gourley et al., 2013b). Group sizes were 6-8 mice for the loPFC and 4-5 mice for the moPFC (less than loPFC due to sparseness of YFP signal in this region). Each mouse contributed a single density value (its average) to statistical comparison by *t*-test. Spine densities on neurons located in the rostral loPFC (Bregma +2.8-+2.46) were used for correlation analyses.

4.4.8 Histology and axon terminal quantification

Mice were deeply anaesthetized and transcardially perfused with 4% paraformaldehyde one week following fluoro-ruby infusions, or after behavioral tests. Brains were extracted, stored in chilled 4% paraformaldehyde for 48 h, then stored in 30% w/v sucrose, and sectioned at 40 μm. Sections were mounted and coverslipped with Vectashield Mounting Medium; Vectashield with DAPI was used for sections containing fluoro-ruby. Infusion sites were verified by imaging blue fluorescent protein (BFP, retro-Cre), mCherry (Gi-DREADDs), YFP (control viral vector), or fluoro-ruby. Mice with mis-targeted infusions in at least 1 hemisphere were excluded from all analyses (n=2 from fluoro-ruby experiment, n=2 from DREADDs experiment).

Fluoro-ruby-positive axon terminal punctae in the loPFC and moPFC were imaged using a spinning disk confocal microscope. Z-stacks were collected with a 100x 1.4-NA objective using a 0.2 μm step size, then collapsed into a maximum intensity projection (MIP) using ImageJ. All MIPs

contained 45 serial sections. MIPs were converted to binary images using the threshold tool, then punctae were separated using the Watershed segmentation plugin and quantified using the Analyze Particles command. Eight images were collected per animal for each brain region, and each animal contributed a single density value (its average) to statistical analyses (9-10 mice/group). A single blinded experimenter collected and processed all images.

4.4.9 Statistical analyses

Two-tailed statistical analyses with $\alpha \leq 0.05$ were performed using SPSS. Response rates, response preference ratios, and percentage of time spent freezing were compared by 1- 2- or 3-factor mixed analysis of variance (ANOVA), with session, time, or aperture as a within-subjects (repeated measure) factor, as appropriate. Following interactions, Tukey's *post hoc* tests were applied, and results are indicated graphically. When 2 groups were compared (as for dendritic spine density and punctae density analyses), 2-tailed unpaired *t*-tests were used. Response preference ratios in Fig. 1D were compared using a 1-sample *t*-test against 1 (no preference). Correlations were analyzed by linear regression. Throughout, values +/- 2 standard deviations from the mean were considered outliers and excluded.

4.5 RESULTS

4.5.1 Adolescent CORT exposure has long-term behavioral consequences

In both humans and rodents, glucocorticoid exposure can induce biases towards habit-based behavior, at the expense of goal-directed action (Gourley et al., 2012b; Guenzel et al., 2014). Further, stressor exposure during early developmental periods appears to confer *long-term* habit biases across rodent-primate species (Patterson et al., 2013; Schwabe et al., 2012; Grissom et al., 2012). Despite these homologies, neurodevelopmental mechanisms remain unclear. We recently reported that

elevating levels of the primary stress hormone CORT during adolescence is sufficient to induce habit biases later in life and modify neurotrophin receptor expression throughout hippocampal-prefrontal regions involved in goal-directed action (Barfield et al., 2017a). Here, we attempt to clarify the long-term effects of adolescent CORT on key neurocircuits associated with behavioral deficits.

First, we reanalyzed a behavioral dataset generated for our prior report, in which we exposed mice to CORT in the drinking water from P31-42, equivalent to early adolescence in humans, then trained them to nose poke 2 recesses for food reinforcers as adults (Fig. 1A). We used FR1, then RI, schedules of reinforcement for training and found no side biases, nor group differences, in the acquisition of the food-reinforced nose poke responses ($F_{(1,15)}$=2.7, p=0.12; interaction $F_{(9,135)}$=1.6, p=0.12) (Fig. 1B, reprinted from Barfield et al., 2017a).

Next, we assessed whether mice behaved according to goal-directed "response-outcome" strategies using an instrumental contingency degradation procedure, in which the likelihood of reinforcement associated with one behavior is greatly decreased (Fig. 1A). A "goal-directed" response strategy is to then preferentially engage the other behavior, which remains likely to be reinforced, while habit-based responding is insensitive to response-outcome contingency (Balleine & O'Doherty, 2010). Mice with a history of CORT exposure assumed habit-based strategies, failing to differentiate between the behaviors that were more, or less, likely to be reinforced. Meanwhile, control mice differentiated between the responses, demonstrating goal-oriented response strategies (interaction $F_{(1,13)}$=6.0, p=0.03) (Fig. 1C, reprinted from Barfield et al., 2017a). These same response rates can be converted to preference ratios, which we present for the first time here. Again, mice with a history of CORT exposure failed to differentiate between responses that were more (or less) likely to be reinforced ($t_{(6)}$=0.16, p=0.88 *vs.* 1, no preference), while control mice did differentiate between the two responses ($t_{(7)}$=2.9, p=0.025 *vs.* 1) (Fig. 1D). Thus, mice exposed to CORT in

adolescence failed to differentiate between reinforced and nonreinforced behaviors later in life, instead deferring to familiar response strategies.

We interpret our findings as evidence that adolescent CORT exposure impedes the ability of mice to behave according to outcome expectancies. In this case, behavioral abnormalities should be detectable regardless of task valence (appetitive or aversive). To test this perspective, we next assessed whether adolescent CORT exposure impacted discrimating between threat-predictive (unsafe) and neutral (safe) contexts. As a comparison, we also included mice exposed to elevated CORT during adulthood (from P70-81) (Fig. 1E), and we used a fear conditioning procedure that also assessed the acquisition and memory for auditory CS-US associations. First, five auditory CSs (tone) co-terminated with the US (mild footshock). Freezing increased over time, with no effect of CORT ($F_{(1,22)}$=2.3, p=0.15; CORT*time and CORT*time*age interaction Fs<1) (Fig. 1F). An interaction between age and time ($F_{(4,88)}$=3.2, p=0.017), however, revealed that older mice froze less than younger mice during the last CS presentation (post-hoc p=0.05), irrespective of CORT exposure. Higher CS-elicited freezing in young adult mice relative to more mature mice is consistent with prior reports of age effects on fear conditioning (Morrissey et al., 2011; Pattwell et al., 2012).

One and 18 days later, mice were placed in a novel "safe" context with different lighting, scent, and floor texture. The same CS was then played. We compared freezing prior to the CS to assess context-elicited freezing, and also freezing during CS presentations. Mice exposed to CORT during adolescence generated more context-induced freezing, apparently generalizing the "safe" and "unsafe" contexts (interaction $F_{(1,13)}$=4.9, p=0.046) (Fig. 1G). Interestingly, freezing was elevated only on day 18, well after the initial conditioning (post-hoc for day 18, p=0.044) (Fig. 1G). We found no effect of CORT, test day, or interactions on CS-elicited freezing (Fs<1) (Fig. 1H).

In contrast to mice exposed to CORT in adolescence, mice exposed to subchronic CORT in adulthood did not differ from control mice in context- or CS-elicited freezing (main effect and

interaction *F*s<1) (Fig. 1I-J). Notably, all mice froze more in response to the conditioning chamber at the remote, relative to recent, time point (main effect day $F_{(1,8)}$=30.7, p=0.001) (Fig. 1I) and moderately less to the CS (main effect day $F_{(1,8)}$=7.4, p=0.026) (Fig. 1J).

Collectively, these findings indicate that adolescent CORT exposure triggers habit-based behaviors and fearful reactions to contexts that should otherwise be considered non-threatening, behaviors that are detectable in adulthood, well after CORT exposure.

4.5.2 Long-term CORT-induced dendritic spine loss in the oPFC

The oPFC (particularly the lateral subregion) is essential for outcome-based learning and memory in both reward- and fear-related contexts (see for discussion, Zimmermann et al., 2018), and is susceptible to chronic CORT-induced dendritic spine loss (Gourley et al., 2013b). To determine whether subchronic CORT exposure during adolescence has long-term structural effects in the oPFC, which may contribute to long-lasting behavioral changes, we euthanized the *thy1*-YFP expressing mice from our appetitive conditioning experiments (Fig. 1) and enumerated dendritic spine densities on excitatory deep-layer pyramidal neurons in the loPFC (Figs. 2A,E). As a comparison, we also enumerated spine densities in the moPFC. Dendritic spines in the loPFC were lost in CORT-exposed mice ($t_{(12)}$=2.8, p=0.016) (Figs. 2B,E), while densities in the adjacent moPFC were unaffected ($t_{(7)}$=-0.6, p=0.6) (Fig. 2C). Notably, mice were euthanized over 8 weeks following the cessation of adolescent CORT exposure, indicating that dendritic spine loss in the loPFC is detectable *well* into adulthood. Also notable, 3D reconstruction of dendritic spines revealed that response preference ratios from the choice test (which serve as an index of "goal-directedness") were positively correlated with the density of mature, mushroom-shaped spines in the rostral loPFC (r=0.56, p=0.048) (Fig. 2D). We did not identify correlations with immature spine types or with dendritic spines in the moPFC.

4.5.3 Adolescent CORT exposure diminishes vHC projections to the oPFC

The vHC projects to the PFC (Verwer et al., 1997), including the loPFC (Cenquizca & Swanson, 2007), providing goal-relevant information necessary for guiding outcome-based learning and memory (McKenzie et al., 2016; Wikenheiser et al., 2017) (Fig. 3A). These projections mature during adolescence (Benes et al., 1989; Caballero et al., 2016), making them a potential target of glucocorticoid excess during this period. To assess the long-term effects of elevated CORT during adolescence on these projections, we exposed mice to CORT during early adolescence (from P31-42 as above), then infused the fluorescent tracer, fluoro-ruby, into the vHC at P49 (Fig. 3B). We then collected brains 1 week later, in early adulthood, corresponding with the onset of behavioral studies above.

Fluoro-ruby has both anterograde and retrograde properties, but connectivity between the vHC and loPFC is predominantly unidirectional, with minimal (if any) projections from the loPFC to the vHC (Cenquizca & Swanson, 2007). Accordingly, retrogradely labeled cell bodies in the loPFC were largely absent in our mice, while axonal punctae were abundant. Mice exposed to CORT during adolescence had fewer fluoro-ruby-positive axonal punctae per cubic micron in the loPFC ($t_{(17)}$=3.3, p=0.004) (Figs. 3C,D), consistent with fewer dendritic spines in this region (Fig. 2). Meanwhile, in the moPFC, the density of fluoro-ruby-positive punctae did not differ between groups ($t_{(17)}$=0.4, p=0.70) (Fig. 3E), consistent with no CORT-induced changes in moPFC dendritic spines (Fig. 2). These findings indicate that adolescent CORT exposure alters the anatomical connectivity between the vHC and loPFC in adulthood.

4.5.4 vHC→oPFC projections are involved in outcome-based decision making

Exposure to CORT during adolescence had long-term maladaptive effects on the ability of mice to associate responses with their outcomes (Fig. 1). Adolescent CORT also eliminated dendritic spines in the loPFC (Fig. 2) and projections from the vHC to the loPFC (Fig. 3). To test whether vHC→loPFC connectivity is necessary for outcome-sensitive behaviors, we infused naïve mice with Cre-expressing retrograde viral vectors into the loPFC and Cre-driven inhibitory Gi-coupled DREADDs or a control fluorophore in ventral hippocampal CA1 (Fig. 4A,C). Activation of the DREADD specifically silences vHC projections to the loPFC. Infusion sites are shown in Fig. 4B.

All mice learned to nose poke for food reinforcement, but mice expressing Gi-DREADDs generated lower response rates (main effect $F_{(1,7)}$=7.9, p=0.026) (Fig 4D). This pattern was unexpected, given that the DREADDs ligand, CNO, was not delivered until later in the experiment. Specifically, immediately following response-outcome contingency degradation, during a period of presumed memory formation, all mice were injected with CNO to inactivate vHC→loPFC projections in DREADDs-expressing mice (timeline in Fig. 4A). We performed 5-min choice tests over the next two days (3/day), to track response strategies over time. Groups did differ on day 1 (main effect and interaction Fs<1) (Fig. 4E). On day 2, we discovered a trending main effect of group ($F_{(1,7)}$=4.56, p=0.07; no interaction $F_{(2,14)}$=1.8, p=0.20), with Gi-DREADDs mice showing lower ratios overall (Fig. 4F). Inactivating the oPFC following instrumental contingency degradation has the same consequences (Zimmermann et al., 2017, 2018), strongly suggesting that vHC→oPFC projections are necessary for the stable retention of response-outcome memories. We anticipate that replicating this experiment will greatly strengthen our findings.

Mice were next tested in a fear conditioning procedure (Fig. 4G). Groups did not differ during tone-shock Pavlovian fear conditioning in context A (main effect and interaction Fs<1) (Fig. 4H). The DREADD ligand CNO was delivered the following day, when mice had the opportunity to learn about a new ("safe") context, with the timing of CNO administration based on prior loPFC

inactivation studies (Zimmermann et al., 2018). Unlike in prior investigations, however, and in our appetitive conditioning experiments above, inactivating vHC→loPFC projections did not affect context- or CS-elicited freezing (context freezing: main effect and interaction Fs<1; CS freezing: main effect $F_{(1,7)}$=1.9, p=0.21, no interaction F<1) (Fig. 4I,J, respectively). Thus, inactivation of vHC→loPFC activity, at least during the time point tested here, did not impair the discrimination of threat- or safety-predictive contexts.

4.6 DISCUSSION

We find that excess glucocorticoid exposure during adolescence impairs the ability of adult mice to modify behaviors based on response-outcome relationships, and on cues that should signal threat *vs.* safety. While chronic CORT induces the same behavioral deficiencies in adults (Gourley et al., 2012b; Besnard et al., 2018), we find that *subchronic* exposure triggers behavioral abnormalities in adolescents and yet, is without consequence in adults (Barfield et al., 2017a; Fig. 1). This pattern suggests that adolescents are particularly vulnerable to the long-term behavioral consequences of elevated CORT, causing a deferral to familiar, generalized behavioral patterns, even when they are inefficient or inappropriate.

To understand, neurobiologically, long-term CORT-induced behavioral abnormalities, we focused on the oPFC. The oPFC is conceptualized as building "task spaces," allowing organisms to link behaviors and stimuli with anticipated outcomes, even when these associations are not readily observable (see Introduction), and it is necessary for response-outcome updating in the reward-related task used here (Zimmermann et al., 2017, 2018). As in many brain regions, oPFC neurons are subject to CORT-induced dendritic spine loss, but unlike in other regions, spine loss is long-lasting, detectable well after CORT exposure (Gourley et al., 2013b). Aspects of oPFC development are particularly prolonged relative to more medial structures (van Eden & Uylings, 1985; Shapiro et al.,

2017b), potentially opening a window of vulnerability to disruption by glucocorticoid excess. We find lower dendritic spine densities on layer V neurons in the loPFC of adult mice exposed to CORT as adolescents. The oPFC is innervated by the vHC, itself subject to long-term modifications in neurotrophin receptor systems following adolescent CORT exposure (Barfield et al., 2017a). We examined vHC→oPFC projections, revealing fewer axon terminals in the loPFC. Inducibly inactivating vHFC→oPFC inputs caused response-outcome updating errors, partially recapitulating decision-making abnormalities in CORT-exposed mice. Our findings reveal a CORT-sensitive mechanism (vHFC-oPFC connections) by which the brain updates and sustains response-outcome associations necessary for efficient reward-related decision making.

4.6.1 Adolescent CORT exposure impairs the ability of mice to select actions according to anticipated outcomes

Across rodent-primate species, early-life stress impedes goal-directed behavior, resulting in inflexible habits that are insensitive to goals (Patterson et al., 2013; Schwabe et al., 2012; Pechtel & Pizzagalli, 2013). Furthermore, glucocorticoid exposure is sufficient to induce habit biases in both humans and rodents (Gourley et al., 2012b; Guenzel et al., 2014). We report that subchronic CORT exposure in mice during a period equivalent to early adolescence in humans (P31-42; Spear, 2000), but not adulthood, trigger habit biases in adulthood (Barfield et al., 2017a; Fig. 1). Notably, adolescent CORT-induced habits are context-elicited (Barfield et al., 2017a). Optimal context-based learning and memory notoriously requires the hippocampus, and we hypothesize that habit biases are associated with the vHC in particular. Over the course of instrumental conditioning, organisms can form associations between contexts and response-outcome contingencies (Colwill & Rescorla, 1990; Trask & Bouton, 2014). When familiar contingencies are violated and require updating, the hippocampus detects a mismatch between the expected features of the context (*e.g.*, its association

with known response-outcome contingencies) and actual features, and alerts other brain regions to update response selection strategies (Mizumori, 2013; Mizumori & Tryon, 2015). The vHC is likely responsible for the generation and transmission of these signals, since the ventral, and not dorsal, hippocampus encodes abstract, goal-relevant features of contexts (Komorowski et al., 2013).

If adolescent CORT-induced behavioral abnormalities involve the (mis-)processing of contextual information that allows one to predict likely outcomes, they should be detectable in other tasks requiring context discrimination, and regardless of task valence (appetitive or aversive). Accordingly, we next exposed mice to subchronic CORT during adolescence or adulthood, then fear conditioning in one context and exposure to a second, "safe" context. Mice with a history of adolescent CORT exposure froze twice as much as control mice in a "safe" context. Thus, adolescent CORT exposure impairs context discrimination. Notably, the acquisition, consolidation, and retention of discrete tone-shock pairings were intact. Nevertheless, stressor exposure during adolescence interferes with several types of aversion-based learning and memory later in life, including shock avoidance (Tsoory & Richter-Levin, 2005), the retention of contextual and auditory fear conditioning (Morrissey et al., 2011), and the extinction of tone-shock (Toledo-Rodriguez & Sandi, 2007) and context-shock (Koseki et al., 2009) associations. Despite variabilities in tests, ages, and stressors used across investigations, it seems clear that stressors during adolescence can have lasting effects on fear-related behaviors, and some of these effects could be attributable to CORT. Further experiments could reveal that adolescent CORT exposure impedes the extinction of tone-shock associations in particular, given that prolonged CORT and oPFC inactivation in adults delay fear extinction (Gourley et al., 2009a; Zimmermann et al., 2018).

Interestingly, we found age differences in the acquisition of conditioned fear responses, and the degree to which context- and tone-elicited freezing in the "safe" context changed over time. Mice exposed to CORT during adolescence were tested starting at P61 (early adulthood), while

those exposed as adults were tested at P100 (mid-adulthood). Younger adult mice froze more than older adult mice during auditory fear conditioning, consistent with prior reports (Morrissey et al., 2011; Pattwell et al., 2012). Also, older mice froze more in the "safe" context, regardless of CORT exposure. In contrast, Houston et al. (1999) found that the incubation of generalized freezing *declines* with age. In their study, the "safe" context was fairly distinct from the conditioning context. Meanwhile, our experiments utilized the same conditioning chambers throughout, but the floors, scent, and lighting differed. It may be that disambiguating contextual cues was more difficult in our experiment, taxing aging memory systems.

4.6.2 Enduring loss of dendritic spines and vHC terminals in the loPFC following adolescent CORT exposure

The oPFC can be divided according to anatomical and developmental distinctions. The lateral regions are part of an 'orbital network' that receives inputs from all sensory modalities (Ongur & Price, 2000). Meanwhile, the 'medial network' functions as a visceromotor system because it projects to visceromotor structures in the hypothalamus and brainstem (like the periaqueductal gray). The loPFC and moPFC also differ in maturational trajectories. In humans, white matter volume increases by 23% in the loPFC but only 11% in the moPFC from age 8 to 30. Cortical thickness reaches adult-like levels at age ~23 in the loPFC but much later, at ~40 years, in the moPFC (Tamnes et al., 2010). We found that dendritic spines on deep-layer loPFC pyramidal neurons were lost several weeks after early-adolescent CORT exposure. Further, the density of mature, mushroom-shaped spines (*i.e.*, synapse-containing spines) positively correlated with response preferences, consistent with evidence that response-outcome conditioning is associated with dendritic spine head enlargement in the loPFC (Sharp et al., 2017; DePoy et al., 2016). In contrast, we detected no CORT-induced modifications in the moPFC, despite its involvement in

certain forms of response-outcome decision making (Bradfield et al., 2015; Gourley et al., 2016). CORT exposure during early adolescence may have coincided with a period of significant synaptic remodeling in the loPFC, but not the moPFC. Also, our findings do not preclude the possibility that other moPFC neuron populations, such as cortico-cortical layer II/III neurons or layer V neurons not expressing *thy1*-driven-YFP (see Baker et al., 2018) were affected by adolescent CORT exposure.

The PFC (including the oPFC), vHC, and vHC→oPFC projections mature during adolescence (Andersen, 2003; Goodman et al., 2014; Caballero et al., 2016). PFC dendritic spines and synapses proliferate during childhood and early adolescence, then are pruned throughout adolescence into early adulthood (Bourgeois et al., 1994; Petanjek et al., 2011; Gourley et al., 2012a). The volume of the anterior hippocampus (equivalent to vHC in rodents) decreases during human postnatal development (Gogtay et al., 2006), while dendritic spine density increases in late adolescence (Chen et al., 2018). Moreover, the *uncinate fasciculus*, the white matter tract that connects the anterior hippocampus to the lateral and orbital PFC (Petrides & Pandya, 1988; Kier et al., 2004), continues to develop into adulthood (Lebel & Beaulieu, 2011). Despite considerable evidence that stressors during adolescence impact hippocampal and PFC development (Eiland & Romeo, 2013), we are the first, to our knowledge, to report that stress-comparable levels of CORT (see Barfield et al., 2017a) weaken anatomical connectivity between the vHC and loPFC. Input loss may contribute to adolescent stress-induced disruption of synaptic transmission in a hippocampal-PFC pathway (Koseki et al., 2009).

In addition to their prolonged maturational trajectories, the vHC and PFC (including the oPFC) are enriched in glucocorticoid receptors (GR), which regulate dendritic spine structure and function, in part via interactions with neurotrophin systems (reviewed Barfield & Gourley, in revision). Our finding that adolescent CORT eliminated dendritic spines and vHC-originating terminals in the lateral, but not medial, oPFC may relate to evidence that tyrosine receptor kinase B

(trkB) protein levels are higher in the oPFC relative to medial PFC (which includes the moPFC) during mid-adolescence, potentially serving to stabilize dendritic spines that escape pruning (Shapiro et al., 2017a). CORT-induced loss of trkB or its ligand BDNF in the oPFC (see Gourley et al., 2009a) would be expected to destabilize spines not otherwise destined for pruning.

An interesting area for future studies will be to determine the time course of adolescent CORT-induced spine and vHC→loPFC input loss. One possibility is that CORT triggers the atrophy of long-range vHC projections to the loPFC. With loss of presynaptic input, postsynaptic spines in the loPFC may then be eliminated. In support of this model, vHC lesions in adult rats degenerate terminals and fibers in the loPFC (Halim et al., 2000). Further, neonatal vHC lesions decrease dendritic lengths and dendritic spine densities on layer V pyramidal neurons in the PFC of adult rats (Lipska et al., 2001). Another possibility is that adolescent CORT exposure first induces dendritic spine loss in the loPFC, and apposing presynaptic terminals of vHC neurons subsequently atrophy. Examining these questions may yield critical insight into circuit-level and ontogenetic mechanisms of stressor vulnerability.

4.6.3 vHC→loPFC inactivation weakens response-outcome memory

Humans and animals can learn to associate actions with their outcomes. When contingencies change, and expected outcomes do not match actual outcomes, internal schemas must be updated so that behaviors can be modified. Flexible, goal-directed behavior therefore necessitates learning, updating, and retaining internal representations of outcome-predictive associations. In previous experiments, we trained mice to perform 2 operant responses for food reward, then the contingency between 1 response and its outcome was violated, requiring mice to update response-outcome associations. The loPFC was temporarily inactivated immediately following this session. In a probe test the next day, mice initially preferred the response most likely to be reinforced, evidence that

they learned new response-outcome associations, but this preference decayed, evidence that they could not retain the new memory (Zimmermann et al., 2017, 2018). Here we used Cre-dependent Gi-coupled DREADDs to selectively and inducibly inactivate vHC→loPFC projections, causing an almost indistinguishable decay in response preference. Our findings suggest that a vHC→loPFC pathway is necessary for sustained response-outcome memory.

vHC→loPFC inactivation did not fully recapitulate the behavioral effects of adolescent CORT exposure, given that CORT-exposed mice failed to generate response preferences at *any* point in testing. Other phases of memory formation or retrieval may thus be affected by CORT. Also, CORT may strengthen competing habit-based systems. We nevertheless believe that memory retention is one of several processes affected by CORT because we previously tested CORT-exposed mice following progressively protracted washout periods, and memory retention deficiencies – nearly identical to those induced by vHC→loPFC inactivation – persevered even despite a considerable recovery period (Barfield et al., 2017a).

Importantly, we only inactivated vHC→loPFC projections during a brief window of time immediately following a contingency degradation procedure. Inactivating these projections during the subsequent choice test may too disrupt goal-directed response selection, given that representations of task structure in the loPFC that guide decision-making (including outcome-predictive associations) require real-time interactions between the vHC and loPFC (Wikenheiser et al., 2017). Another possibility is that immediate failures in response-outcome decision making induced by adolescent CORT exposure are due to dysfunction in additional regions, like the prelimbic (PL) cortex (Hart et al., 2014). Hence, concurrent PL and vHC→loPFC damage may be sufficient to induce both immediate and sustained decision-making impairments.

4.6.4 No effects on fear conditioning

Inactivating vHC→loPFC projections prior to placing mice in a novel, "safe" context did not impact the ability of mice to discriminate between threat-predictive or neutral contexts at the time of testing, the following day, or at a remote time point. CS-elicited freezing was also not impacted. Thus, vHC→loPFC projections appear to be uninvolved in these processes. A recent lesion study in rats suggests that the vHC *is* important, however, for disambiguating outcome-predictive contextual information when contexts are very similar (McDonald et al., 2018). Therefore, inactivating this pathway at a remote time point, when detailed memory of the threat-predictive context has faded, may impair the ability of mice to discriminate between threatening and neutral contexts. We plan to test the effects of vHC→loPFC inactivation at different time points in future experiments.

4.6.5 Summary and broader implications

The loPFC is involved in a variety of functions necessary for goal-directed behavior, including encoding outcome expectancies (Schoenbaum et al., 2011), attributing discrepancies between expected and actual outcomes to specific causes (*i.e.*, credit assignment) (Noonan et al., 2017), and forming a cognitive map of task space (Wilson et al., 2014). The vHC, which provides a direct monosynaptic projection to the oPFC (Jay & Witter, 1991; Verwer et al., 1997), transmits mismatch signals to the PFC, triggering learning-related plasticity that updates internal representations of outcome-predictive associations (Penner & Mizumori, 2012; Numan, 2015). Further, recent evidence indicates that inactivation of the ventral subiculum – the primary output of the hippocampus – impaired rats' ability to adjust behavior in response to changes in reward contingency, and concomitantly disrupted the ability of the loPFC to encode integrated representations of task state (Wikenheiser et al., 2017). Considered together with the observation that adolescent CORT exposure here caused loPFC dendritic spine and vHC→loPFC projection

loss, and with the behavioral consequences of chemogenetic vHC→loPFC inactivation, we argue that the vHC→loPFC pathway is: 1) essential for retaining outcome-based memory necessary for goal-directed behavior and 2) exceptionally vulnerable to excess glucocorticoids during adolescence, rendering CORT-exposed organisms biased towards inflexible, habit-based behaviors.

Disruption of maturational trajectories can produce persistent changes in brain structure (Andersen, 2016), setting the stage for psychiatric disease onset (Goodman et al., 2014; Whittle et al., 2014; Teicher et al., 2016b). Psychiatric disorders characterized by maladaptive, outcome-insensitive behaviors (such as inflexible habits) are associated with structural and functional abnormalities in the oPFC and vHC, and in cortico-limbic connectivity (Jackowski et al., 2012; Godsil et al., 2013). In addition to shared dysfunction of vHC→PFC connections, these disorders are precipitated by stress and share the risk factor of early-life adversity. Indeed, there is considerable evidence that chronic stress or adverse experiences during childhood or adolescence increase the risk for, and severity of, depression, PTSD, and SUD throughout life (Cabrera et al., 2007; Norman et al., 2012; Fryers & Brugha, 2013; Copeland et al., 2013). The long-term effects of adolescent glucocorticoid exposure on vHC→oPFC projections may therefore increase risk for psychopathology by disrupting executive functions and behaviors mediated by this connection.

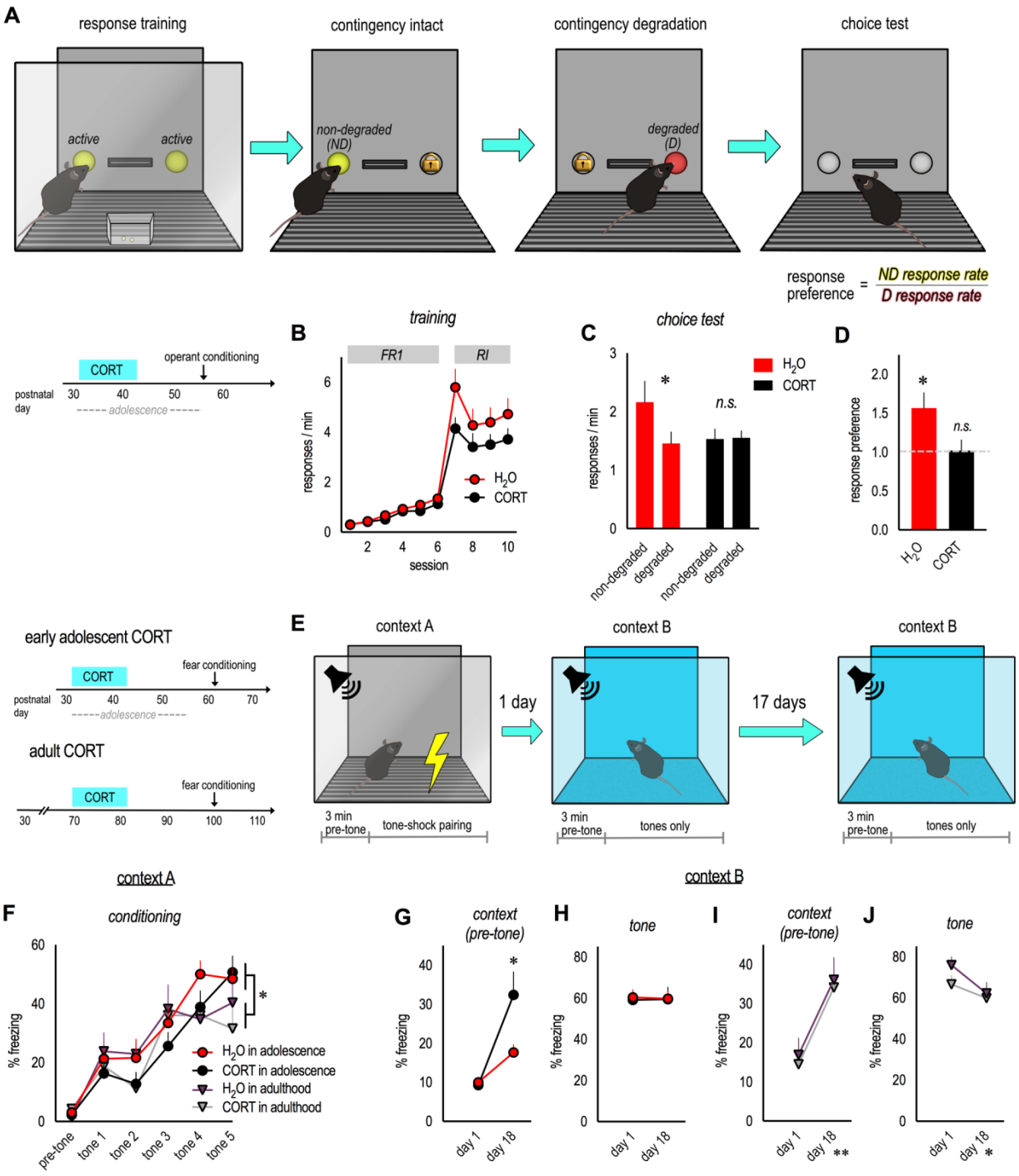

Figure 4-1. Adolescent CORT exposure impairs the ability of mice to update outcome expectancies, regardless of valence, in adulthood. (A) Mice were trained to nose poke for food reinforcers in operant conditioning chambers. Next, the reinforcement schedule associated with one

nose poke response was enriched, while the contingency between the other response and its outcome was 'degraded' by providing food pellets independently of the mouse's responses. The ability to update expectancies and select responses based on anticipated outcomes was then assessed in a brief choice test conducted in extinction. **(B)** Experimental timelines are provided at the left of each row. First, mice exposed to CORT during adolescence acquired the nose poke responses in adulthood without differing from control mice. 'FR1' and 'RI' denotes the schedules of reinforcement used throughout. **(C)** A probe test following instrumental contingency degradation revealed that CORT-exposed mice generated habit-based response strategies, failing to differentiate between behaviors that were ("non-degraded") or were not ("degraded") likely to be reinforced. Meanwhile, control mice preferentially engaged the response most likely to be reinforced. **(D)** Response strategies in the choice test were also characterized by calculating the ratio of 'non-degraded' / 'degraded' response rates, highlighting habit-like responding (preference ratios ~1, no preference) in CORT-exposed mice. n=8-9/group. **(E)** In a separate experiment, mice were fear conditioned. One and then 17 days later, freezing in a neutral "safe" context and in response to the tone CS's were measured. **(F)** Prior CORT exposure did not impact freezing during fear conditioning, but younger mice froze more than older mice during the final CS presentation. **(G)** Adolescent CORT-exposed mice froze more in a neutral "safe" context than age-matched control mice, generalizing a contextually-based fear response. **(H)** Adolescent CORT exposure did not affect CS-elicited freezing. n=7-8/group. **(I)** Adult CORT exposure did not affect context-elicited freezing, and these older mice froze more at the remote time point after conditioning. **(J)** CS-elicited freezing modestly decreased between recent and remote tests, again without group differences. n=5/group. Symbols and bars represent means + SEMs. *$p \leq 0.05$. **$p \leq 0.001$. **B** and **C** are reprinted from Barfield et al. (2017a).

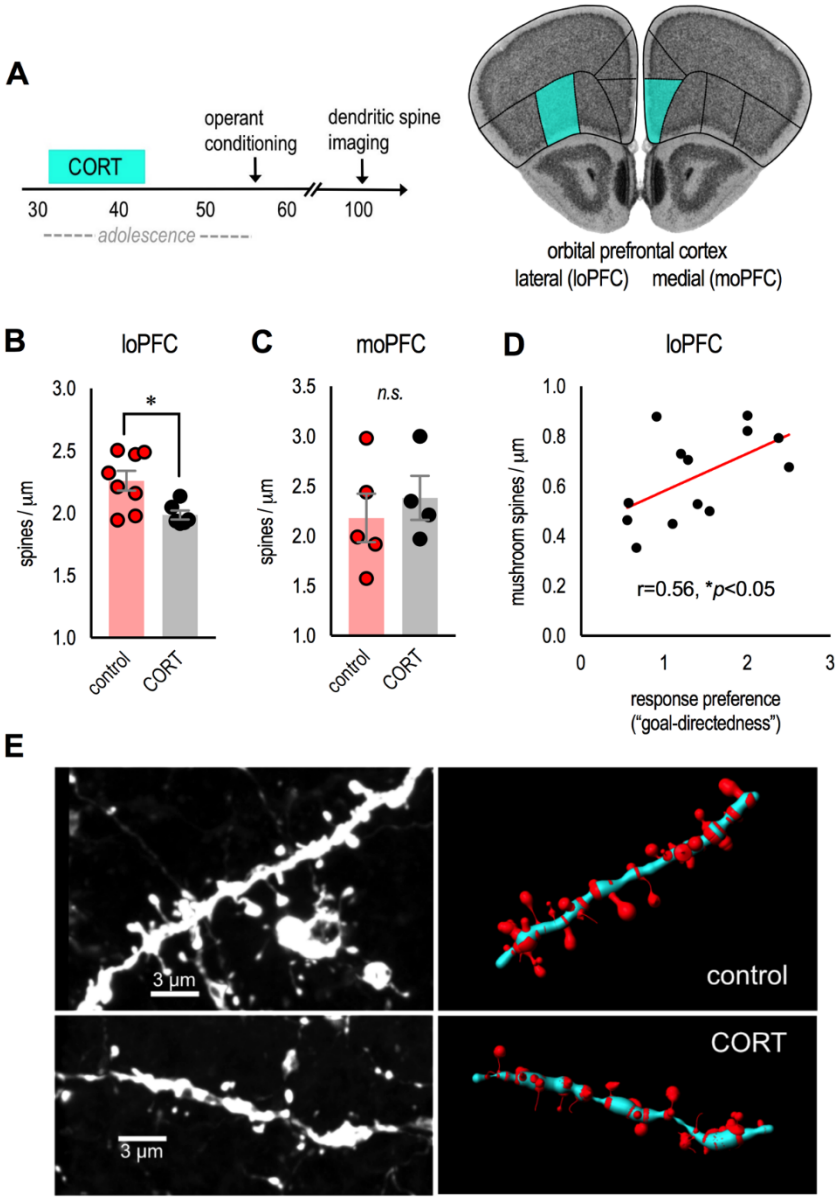

Figure 4-2. CORT induces dendritic spine loss in the loPFC: Correlations with decision-making abnormalities. (A) Experimental time (left). *thy1*-YFP-expressing mice from instrumental conditioning experiments in Fig. 1 were euthanized in adulthood after behavioral testing, and basilar dendrites on excitatory neurons in the loPFC (and, for comparison, moPFC) were imaged. These regions are highlighted on images from the Mouse Brain Library (Rosen et al., 2000). **(B)** Adolescent CORT exposure eliminated dendritic spines in the loPFC (n=6-8/group), **(C)** but not moPFC (n=4-

5/group). **(D)** In the rostral loPFC, the density of mature, mushroom-shaped spines correlated with response preference ratios from Fig. 1D. *n*=6-7/group. **(E)** Representative dendrites from the loPFC (unprocessed image at left, reconstruction at right). Bars represent means + SEMs, symbols represent individual mice. $*p \leq 0.05$.

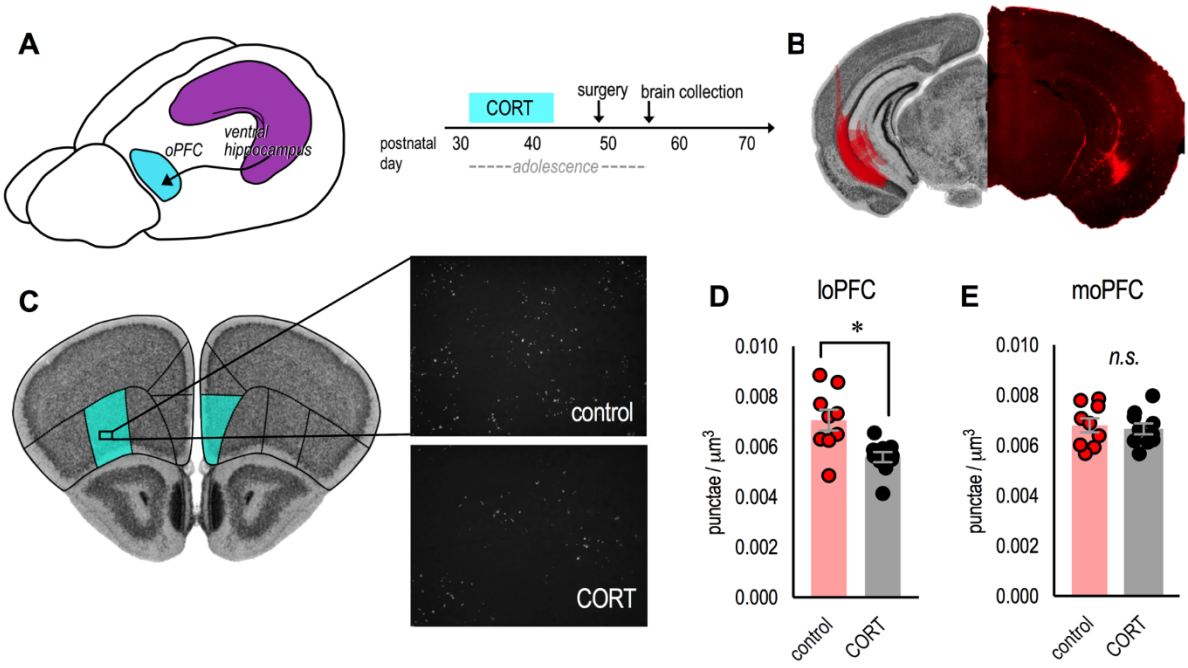

Figure 4-3. Adolescent CORT exposure degrades ventral hippocampal→loPFC input. (A) Mice exposed to CORT from P31-42 received infusions of the anterograde tracer fluoro-ruby into the vHC to characterize vHC projections to the oPFC. **(B) At left:** The spread of fluoro-ruby in the vHC is transposed onto an image from the Mouse Brain Library (Rosen et al., 2000). **At right:** Representative image of fluoro-ruby in the vHC. **(C)** Unprocessed images of fluoro-ruby-positive axon terminal punctae in the loPFC. **(D)** Adolescent CORT exposure reduced the density of vHC terminals in the loPFC, **(E)** but not the moPFC. n=9-10/group. Bars represent means + SEMs, symbols represent individual mice. *$p \leq 0.05$.

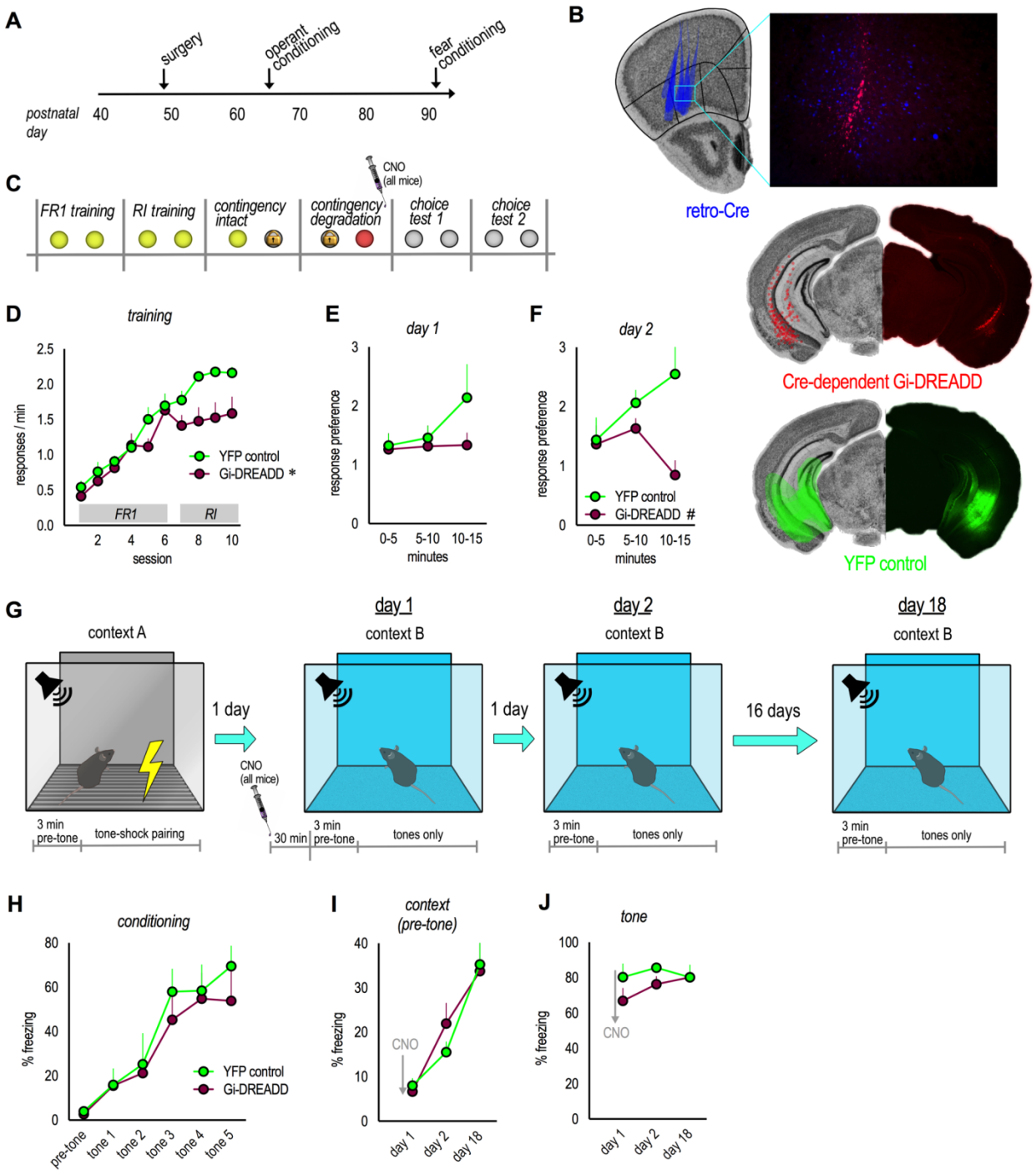

Figure 4-4. Inactivating vHC→loPFC projections weakens goal-directed response selection. (A) Experimental timeline. **(B)** Retro-Cre in the loPFC (blue), combined with a Cre-dependent Gi-DREADD in the vHC (red), was used to inactivate vHC→oPFC projections. Spread

of viral vectors in the loPFC and vHC is drawn in the left hemispheres on images from the Mouse Brain Library (Rosen et al., 2000), and representative infusions are shown at right. **(C)** Schematic of operant conditioning procedures. Mice were trained to nose poke for food reinforcers, then the likelihood that one response would be reinforced was decreased by providing food pellets independently of the mouse's responses ('contingency degradation'). The DREADD ligand CNO was administered immediately following this session. Preferential engagement of the response most likely to be reinforced ('non-degraded') in subsequent choice tests reflects a goal-directed response strategy and requires sustained memory of the updated response-outcome relationships. **(D)** 'FR1' and 'RI' denotes the schedules of reinforcement. All mice acquired the responses, but response rates lagged in DREADDs-expressing mice during the last sessions of RI training. **(E)** Following instrumental contingency degradation, initially, all mice preferentially engaged the response most likely to be reinforced (preference ratios >1). **(F)** However, the following day, this preference decayed in mice in which vHC→loPFC projections were previously inactivated, suggesting weakened retention of response-outcome memory. **(G)** Schematic of fear conditioning procedures. CNO was given 30 min prior to testing in a neutral context. **(H)** Mice acquired conditioned freezing, without group differences. **(I)** Inactivation of vHC→loPFC projections did not affect context-elicited freezing (prior to tone onset) at any of the time points tested. **(J)** CS-elicited freezing was also not affected by vHC→loPFC inactivation. n=4-5/group. Symbols represent means + SEMs. #p=0.07.

CHAPTER 5: CONCLUSIONS AND FUTURE DIRECTIONS

5.1 SUMMARY OF RESULTS

Broadly, the findings described in the previous chapters provide significant insight into the mechanisms through which insults during adolescence impact executive functions later in life. In Chapter 2, we provide novel evidence that subchronic exposure to corticosterone (CORT) in mice during adolescence, but not adulthood, induces persistent impairments in goal-directed decision making, and reduces the expression of signaling proteins downstream of the neurotrophin tyrosine receptor kinase B (trkB) selectively in the ventral hippocampus (vHC), well into adulthood. Importantly, we also show that trkB signaling in the vHC bidirectionally regulates inflexible habits. In Chapter 3, we demonstrate that CORT exposure and trkB manipulations during adolescence have long-term, sex-dependent effects on behavioral flexibility in a task dependent on the orbital prefrontal cortex (oPFC) – which receives inputs from the vHC. We show in Chapter 4 that vHC projections to the oPFC are necessary for sustaining goal-directed response selection, and that adolescent CORT exposure degrades vHC-oPFC anatomical connectivity in adulthood.

In Chapter 2, we additionally validated our exogenous CORT exposure model for use in adolescent mice, showing that it recapitulates many of the effects of chronic stress. For example, adolescent CORT exposure induced depression-like amotivation, altered stressor reactivity, eliminated dendritic spines on pyramidal neurons in the medial PFC (mPFC), and altered neurotrophin receptor expression throughout several cortico-limbic regions. Importantly, it also elevated blood serum CORT to stress-like levels, while leaving diurnal CORT cycling intact.

Prior studies utilizing an identical exogenous CORT exposure technique exposed rodents chronically (duration of exposure ≥ 20 days) (Gourley et al., 2008a,b). We used a *subchronic* duration of CORT (11 days) to test whether exposure during adolescence is more impactful than exposure during adulthood. In support of this perspective, we found that CORT exposure during adolescence, but not adulthood, biased mice towards context-elicited habits later in life. Specifically,

in a food-reinforced operant conditioning task in which the contingency between one response and its outcome was degraded, adolescent CORT-exposed mice failed to differentiate between two responses based on the likelihood of reinforcement in a choice test. Furthermore, these habits were context-elicited. Mice were able to modify responses when the *value* of outcomes changed, though. This pattern of behavioral deficits is consistent with hippocampal damage (see Discussion section of Chapter 4). Accordingly, we also found that CORT exposure during either adolescence or adulthood disrupted the balance of full-length (active) and truncated (inactive) isoforms of the neurotrophin receptor, tyrosine receptor kinase B (trkB), throughout multiple cortico-limbic brain regions. However, the vHC of adolescent CORT-exposed mice showed the most pronounced shift in trkB balance, which favored the inactive isoform. Phosphorylation of the trkB signaling protein, extracellular signal-regulated kinase 1/2 (ERK1/2) was also decreased selectively in the vHC following adolescent CORT exposure, and this reduction persisted well into adulthood. Stimulating trkB-ERK1/2 during adolescence with the trkB agonist, 7,8-dihydroxyflavone (7,8-DHF), blocked long-term habit biases and corrected vHC phospho-ERK1/2 levels in mice exposed to CORT during adolescence. Further, over-expressing inactive trkB in the vHC of naïve mice reduced phosopho-ERK1/2 and induced habit-based behavior. These findings implicate decreased trkB-ERK1/2 in the vHC in enduring habit biases following adolescent CORT exposure.

The vHC projects to the oPFC, and both regions are important for the ability to flexibly modify behaviors when outcome-predictive associations change. In Chapter 3, we explored the long-term effects of adolescent exposure to CORT, physical stress, trkB stimulation, or trkB inhibition, on behavioral flexibility in an instrumental reversal learning task that is sensitive to oPFC and vHC lesions. In this task, the response-outcome requirement to obtain food reinforcement is "reversed," such that animals must inhibit performing a previously reinforced response and adopt a novel response strategy to obtain reinforcement. This task dissociates two types of response errors

that are associated with damage to the lateral or medial subregions of the oPFC, allowing for the identification of distinct neurobehavioral deficits. We found that female mice exposed to CORT during adolescence developed perseverative errors in adulthood (mimicking the effects of medial oPFC lesions), failing to inhibit a previously reinforced response, and this was blocked by a trkB agonist. Behavioral flexibility was not impacted by adolescent CORT exposure in males, but was enhanced by trkB stimulation. Males were, however, vulnerable to reversal deficits following repeated stressor exposure or trkB antagonism during adolescence. These findings indicate that the effects of adolescent CORT on some measures of behavioral flexibility are sexually dimorphic, with potential implications for understanding how sex differences in the neurobehavioral response to adolescent adversities can contribute to sex differences in vulnerability to stress-related illnesses characterized by behavioral inflexibility and inhibitory control deficits. These data also add to our findings from Chapter 3, showing that augmenting trkB ameliorates long-term deficits in the ability to modify behavioral responses when response-outcome contingencies are degraded or reversed.

In Chapter 4, we expand on our findings from the previous two chapters. We further characterize the long-term effects of adolescent CORT on flexible, outcome-based behaviors requiring intact hippocampal-prefrontal function, and we identify enduring changes in the lateral oPFC (loPFC) and its vHC afferents following adolescent CORT. As a follow-up to our findings in Chapter 2 that adolescent CORT exposure biased behavior towards context-elicited habits, we found that adolescent CORT also impeded the appropriate use of contextual cues and previously learned associations in aversive circumstances, resulting in maladaptive fear responses in a neutral context. Further, adolescent CORT-exposed mice had fewer dendritic spines on pyramidal neurons in the lateral, but not medial, oPFC, and fewer vHC→loPFC axon terminals in adulthood. In line with the role of the loPFC (Wilson et al., 2014) and vHC inputs into the loPFC (Wikenheiser et al., 2017) in guiding behaviors based on internal representations of outcome-predictive associations and

goal-relevant contextual features, inducibly inactivating vHC→loPFC projections impaired the ability of mice to sustainably select responses based on expected outcomes, resulting in a deferral to familiar, habit-based responding. These patterns implicate a vHC→loPFC pathway in outcome-based associative learning and memory processes that guide goal-directed behavior. Further, they suggest that degradation of vHC projections to the loPFC may contribute to the long-lasting behavioral consequences of adolescent CORT exposure.

5.2 INTEGRATION OF FINDINGS WITH THE CURRENT LITERATURE

As reviewed in Chapter 1, stress-related disorders, such as depression, are commonly characterized by hormonal stress response abnormalities, deficits in neurotrophin signaling, and synaptic loss in cortico-limbic brain regions. Mounting evidence indicates that these changes are not mutually exclusive. Indeed, signaling through trkB and GR regulates dendritic spine structure and function and the hormonal stress response. Disruption of trkB and GR function (for example, by chronic stress) can negatively impact neuronal architecture in cortico-limbic brain regions and impair regulation of glucocorticoid secretion.

The neurobehavioral consequences of chronic stress vary depending on the developmental timing of stressor exposure. This may be due to temporal and regional differences in the trajectory of synaptic maturation and the expression of trkB and GR across development. Increased levels of trkB and BDNF in the PFC during adolescence or young adulthood, as reported by some studies, may indicate that trkB signaling is especially important for the synaptic changes occurring during this developmental period. Moreover, GR levels in the PFC are markedly increased in late adolescence/young adulthood. Thus, trkB expression in the adolescent PFC may be more susceptible to down-regulation by excessive GR activation with chronic stress.

In Chapter 2, we introduce the idea that exposure of mice to the GR ligand, CORT, for 11 days during early adolescence, but not adulthood, produces long-term behavioral, molecular, and neuroanatomical consequences. In Chapters 2, 3, and 4, we report that adolescent CORT exposure impairs the ability to adjust behavior when environmental contingencies change, in both appetitive and aversive circumstances, in adulthood. Specific behavioral deficits include: impaired goal-directed decision making (*i.e.*, habit biases), perseverative errors in instrumental reversal (in CORT-exposed females but not males), and impaired discrimination of cues signaling threat *vs.* safety. Our findings add to a growing body of work indicating that adverse experiences or elevated glucocorticoids during development can impair flexible, outcome-based behaviors later in life (Koseki et al., 2009; Patterson et al., 2013; Wright et al., 2015; Shapiro et al., 2017a).

The enduring behavioral consequences of stressors or stress hormone exposure during adolescence may be due to long-term changes in neuronal morphology within hippocampal-prefrontal regions that support behavioral flexibility and goal-directed decision making. The oPFC, vHC, and vHC→oPFC projections support outcome-based learning and memory necessary for flexible adaptation of behavior (Yu & Frank, 2015; Wikenheiser et al., 2017), and they continue to mature during adolescence (Lebel & Beaulieu, 2011), which may open a window of vulnerability to insults like excess glucocorticoid exposure. In addition to their prolonged maturational trajectories, the vHC and PFC (including the oPFC) are enriched in glucocorticoid receptors (GR), which regulate dendritic spine structure and function, in part via interactions with neurotrophin systems (reviewed in Chapter 1). For example, excessive GR binding can suppress the expression of trkB, which is essential for normative dendritic spine maturation during adolescence and the maintenance of stable spines (Xu et al., 2000; Gorski et al., 2003). We found that the adolescent vHC is especially vulnerable to enduring CORT-induced disruption of trkB-ERK1/2 signaling. CORT exposure during adolescence also eliminates dendritic spines on pyramidal neurons in the loPFC and

vHC→loPFC projections in adulthood. These findings are in agreement with considerable evidence that stressor or CORT exposure during adolescence in rodents can alter neuronal morphology in the PFC and hippocampus in adulthood (Eiland & Romeo, 2013; Romeo, 2017). Initial reports in humans indicate that early-life stress is associated with reduced integrity of the *uncinate fasciculus*, the white matter tract that connects the anterior hippocampus to the lateral and orbital PFC, later in life (Hanson et al., 2015; Ho et al., 2017). But we are the first, to our knowledge, to report that stress-comparable levels of CORT during adolescence weaken anatomical connectivity between the vHC and loPFC in adulthood.

Lesions or inactivation of the oPFC and vHC disrupt behaviors and executive functions that rely on outcome-based learning and memory, such as goal-directed decision-making (Izquierdo et al., 2004; Lex et al., 2010; Gremel & Costa, 2013; Zimmermann et al., 2017,2018), conditioned fear extinction (Zelinski et al., 2010; Sotres-Bayon et al., 2012; Zimmermann et al., 2018), and threat-safety discrimination (Zelinski et al., 2010; Cullen et al., 2015; Trow et al., 2017; Sarlitto et al., 2018). As described in Chapter 3, we found a positive correlation between goal-directed response selection and the density of mushroom-shaped ("memory supporting") spines in the loPFC, suggesting that persistent habit biases in adolescent CORT-exposed mice are related to enduring loss of dendritic spines. Further, our findings that selective inactivation of vHC→loPFC projections impairs sustained goal-directed response selection, and overexpression of an inactive trkB isoform in the vHC (which decreased p-ERK1/2) induces inflexible habits, additionally implicate disrupted trkB-ERK1/2 signaling and weakened vHC→loPFC connections in the persistent behavioral consequences of adolescent CORT exposure. These findings are consistent with other reports evincing a role for drug-induced remodeling of oPFC neurons and BDNF signaling in the oPFC in habit-like behaviors (reviewed Pitts et al., 2016 and DePoy & Gourley, 2015). Future work focusing on the intersection of BDNF-trkB and glucocorticoid signaling within cortico-limbic regions during

adolescent development may critically inform our understanding of the neurodevelopmental mechanisms that shape neuroplasticity to stress.

5.3 IMPLICATIONS AND FUTURE DIRECTIONS

Organisms must be able to identify causal relationships between actions and their outcomes in order to guide behavior to achieve goals. Impairments in causal awareness or in flexibly updating outcome-predictive associations when contingencies change, can contribute to maladaptive patterns of thought or behavior that are risk factors for, or core symptoms of, various stress-related psychiatric disorders (Negron-Oyarzo et al., 2016). For example, individuals suffering from depression have difficulties planning and working for distant goals. A sense of helplessness in depression has been characterized as an impairment in causal awareness (Griffiths et al., 2014). Depressed individuals may have aberrant beliefs about the causality of their actions in achieving a goal, and so, they may not an initiate an action. They may also attribute outcomes to their current situation or state rather than to their chosen action. Depression is also characterized by cyclical, non-productive thought patterns or actions; *e.g*, depressive rumination, like habits, can be stimulus-elicited, precipitated by stress, and resistant to change (Watkins & Nolen-Hoeksema, 2014). Further, individuals with substance use disorder (SUD) struggle to modify behaviors when reward contingencies change, exhibiting persistent drug seeking despite increasingly negative consequences (Everitt & Robbins, 2016). And in post-traumatic stress disorder (PTSD), fear responses to threat-related stimuli can be persistent and inflexible, and generalize to stimuli signaling safety (Morey et al., 2015).

The psychiatric disorders characterized by shared impairments in behavioral flexibility also share the risk factor of early-life adversity. Moreover, these disorders are characterized by structural and functional alterations in hippocampal-prefrontal regions that are susceptible to chronic stress

(Godsil et al., 2013), and continue to mature during adolescence (Andersen & Teicher, 2008). Adverse experiences during early adolescence increase the risk for chronic, remitting depression, substance abuse, anxiety disorders, and a range of adverse mental health outcomes throughout the lifespan (Martins et al., 2011; Carr et al., 2013; Casement et al., 2015). These and other findings have led to the hypothesis that adverse experiences during adolescence may increase vulnerability to diverse psychiatric diseases by disrupting the maturation of cortico-limbic circuits that guide flexible, outcome-based behaviors, resulting in enduring structural changes that underlie persistent behavioral consequences (Sheth et al., 2017).

Our findings implicate long-term dendritic spine loss in the oPFC and weakened vHC→oPFC connections in inflexible, habit-like behaviors following CORT exposure during adolescence in mice. Similar structural alterations have been reported in humans. For example, teens who experienced childhood maltreatment exhibit reduced cortical thickness of the loPFC (Lim et al., 2017). And early-life adversity is associated with decreased gray matter volume in the oPFC and hippocampus in teenagers (Edmiston et al., 2011) and adults (Dannlowski et al., 2012). Enduring reductions in the structural integrity of the *uncinate fasciculus* (UF), which connects the anterior hippocampus to the lateral and orbital PFC, has also been reported in individuals exposed to early adversity (Hanson et al., 2015; Ho et al., 2017). Interestingly, in these studies, reduced UF integrity mediated the relationship between sensitivity to early-life stress and internalizing symptomatology in adolescents (Ho et al., 2017), and was associated with higher psychological vulnerability to subsequent stressors in young adults (Hanson et al,. 2015). These findings suggest that degraded vHC→oPFC projections may be associated with persistent behavioral consequences. Future work should utilize the DREADDs techniques described in Chapter 4, but in adolescent CORT-exposed mice, to determine whether stimulation of vHC→oPFC connections can restore goal-directed

responding, providing additional support for the contention that these projections regulate behavioral vulnerability to CORT.

We report in Chapters 2 and 3 that the trkB agonist, 7,8-dihydroxyflavone (7,8-DHF), blocks adolescent CORT-induced habits, depressive-like amotivation, perseverative-like responding, and reductions in vHC trkB-ERK1/2 in adult mice. We hypothesize that adolescent CORT impacts long-term behavioral outcomes by reducing trkB signaling in the vHC and oPFC, which may impair the stabilization of dendritic spines and the strengthening of synaptic connections between vHC terminals and loPFC spines. Thus, it is possible that trkB mediates the effects of adolescent CORT exposure on structural outcomes. A key experiment testing this hypothesis will be to examine whether 7,8-DHF blocks the long-term reduction of loPFC dendritic spines and vHC→loPFC projections following adolescent CORT exposure. Positive findings would strengthen the contention that pharmacological interventions that augment trkB activity in adolescents exposed to chronic stress may be particularly efficacious in treating enduring neurobehavioral deficits that increase the risk for psychiatric illnesses throughout life.

Lastly, we report in Chapter 3 that exposure to CORT during adolescence induces perseverative-like responding in an oPFC-dependent instrumental reversal task in female, but not male, mice in adulthood. Specifically, females failed to inhibit a previously reinforced response, even though it was disadvantageous. Individual differences in inhibitory control may predispose organisms to drug-seeking behaviors (Nigg et al., 2006; Cervantes et al., 2013), and may mediate the association between early-life trauma and risk for, and severity of, substance dependence later in life (Schwandt et al., 2013). Moreover, this association is stronger in women (Hyman et al., 2008). Our findings suggest that greater vulnerability to adolescent CORT-induced inhibitory control deficits in females may increase vulnerability to compulsive drug taking in adulthood, and future experiments could directly test this hypothesis by correlating CORT-induced perseverative errors during

instrumental reversal with subsequent patterns of cocaine self-administration. Our findings may also suggest that vulnerability of specific oPFC sub-regions to stress- or CORT-induced dysfunction may be sexually dimorphic, since lesions of the moPFC impair response inhibition and lesions of the loPFC impair response acquisition in this task. Future investigations might enumerate dendritic spines on pyramidal neurons in the loPFC and moPFC of adult female mice with a history of adolescent CORT exposure (we focused on males in Chapter 4 here). While males appear to be relatively resilient to CORT-induced spine loss in the moPFC (but not loPFC), we hypothesize that females may be vulnerable. Such investigations will be an important step in determining how sex differences in the neurobehavioral response to adolescent adversities impact sex differences in vulnerability to stress-related illnesses like SUD later in life.

APPENDIX A: PUBLICATIONS TO WHICH THE AUTHOR HAS CONTRIBUTED

Barfield ET, Gourley SL (in preparation) Adolescent corticosteroid exposure deteriorates ventral hippocampal-orbitofrontal cortical inputs: Functional consequences.

Barfield ET, Gourley SL (in revision) Prefrontal trkb and glucocorticoid systems in stress and developmental contexts. Neurosci Biobehav Rev.

Barfield ET, Gerber KJ, Zimmermann KS, Ressler KJ, Parsons RG, Gourley SL (2017a) Regulation of actions and habits by ventral hippocampal trkB and adolescent corticosteroid exposure. PLoS Biol 15:1-27.

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