The role of the prefrontal cortex in stress and motivation

By

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Abstract

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Stress and motivation are inextricably intertwined. Importantly, stress, especially early in life, is associated with increased substance use and risk of addiction. Furthermore, there is high co-morbidity between stress-induced disorders such as PTSD and motivation disorders such as addiction and depression. This is suggestive of an overlapping neurobiological mechanism of vulnerability. The prefrontal cortex (PFC) is implicated in both stress and motivation circuitry and thus, may be a critical link between the two. It is highly connected to limbic regions, including the amygdala and hippocampus, as well as to regions crucial for motivation and reward such as the striatum and ventral tegmental area (VTA). The PFC is also highly sensitive to stress and has an extended period of experience-dependent plasticity as it continues to develop through adolescence. Thus, it is important to understand PFC circuitry, how it changes over development in response to stressors and how factors such as stressor type and sex might contribute to individual variability following stress exposure.

In this thesis, I explore the role of prefrontal cortex circuitry in both stress and motivated behaviors using rodent models. In chapter one, I introduce the stress response, the relationship between stress and motivated behaviors, and describe the prefrontal cortex systems that connect them. In chapter two, I elaborate on part of this circuitry, describing the anatomy of VTA dopaminergic and GABAergic projections to PFC subregions, as well as to other limbic brain areas. In chapter three, I investigate how responding to the distress of another (vicarious stress) leads to biased motivated helping behavior depending on group membership and I describe the neural circuits underlying this behavior. Further, I address how helping behavior and the neural circuits involved change across development. I present evidence that in-group biases arise over development and that pro-social intent toward ingroup members recruits distinct neural circuits. In chapter four, I explore the effects of an acute traumatic stressor on PFC oligodendrocytes and myelination. I show there are sex-specific differences; females are preferentially affected, displaying stress induced increases in PFC myelin in the short term, yet decreased myelination in the long term relative to controls. Finally, I provide closing thoughts, offer future directions, and elaborate on the relevance of the PFC in human disorders. Overall, this body of work provides insight into stress and motivation circuitry, with a focus on the prefrontal cortex

Dedication

To everyone who has shaped me along this journey, especially my family and friends who were there when I needed them most.

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Chapter 1: Introduction

Part 1: The Stress Response

Defining 'Stressors' – events that induce stress

Stress is a pervasive part of our lives, and indeed, in the lives of all living things. Stress can come from physical activities, like running a marathon, or it can arise from psychological worries, perhaps about performance on an exam, or whether you said the wrong thing during an interview. Stressors can be acute and minor, like the temporary pain from a bee sting, or they can be chronic and life threatening, such as a battle with cancer. In our society, more and more stressors come from social tensions and social interactions. For example, the thought of giving a public speech induces fear and anxiety in many of us. However, not all stress is bad, and indeed, we often seek out stressful situations (watching scary movies, riding roller coasters, skydiving, and rock-climbing, to name a few). Thus, some amount of stress is exciting and beneficial. Yet all of these emotionally charged situations lead to stress.

Definition of 'Stress' aka 'the stress response'

In the 1930s, an endocrinologist named Hans Selye defined stress as the 'nonspecific bodily response to any demands placed on it [the body]' (Selye, 1976). Following Selye's definition, 'stress' and the 'stress response' can be thought of as two interchangeable terms. Further, this definition enforces the idea that stress comes from anything that pushes us out of balance or disrupts homeostasis. While the stressors themselves may be varied, there is a common physiological stress response. Stress allows us to adapt and cope with the stressor: it increases arousal and taps into energy stores, helping us meet the challenge at hand. An optimal amount of stress (which Selye called 'eustress') leads to focus, motivation and enhanced performance (Selye, 1976). On the other hand, distress, or negative stress, can lead to exhaustion and 'wear and tear' on the body (Bruce S McEwen & Seeman, 1999). The effects of stress are diverse and span physiological, behavioral and cognitive realms. Stress can influence everything from cellular structures and patterns of neural activity, to our motivations, desires and decisions.

Detection and appraisal of stressors

Stress starts with detection of a threat, whether real or imagined. For example, we might first hear the rattle of a rattlesnake, a crucial sensory input that gets transmitted to the brain. Alternatively, threats may be more abstract in nature, like a looming thesis deadline. Regardless of the source, once detected, this threat is then subject to appraisal: the brain assesses whether the stressor represents a problem and determines if and how strong a response is warranted. The limbic system, particularly the amygdala, hippocampus, bed nucleus of the stria terminalis (BNST), and prefrontal cortex (PFC), play key roles in evaluating incoming stimuli, assessing the resources available and determining whether a stress response will be initiated (Calhoon & Tye, 2015). This

important assessment step is known to be influenced by many factors, including previous experience, the availability of resources to meet the demand (whether that be physical, psychological or social resources), and the degree of control and predictability over the stressor (Godoy, Rossignoli, Delfino-Pereira, Garcia-Cairasco, & Umeoka, 2018).

The physiological stress response

If a stress response is triggered, there is a coordinated cascade of hormonal and neural activity throughout the central and peripheral nervous systems. Specifically, activation of brainstem and hypothalamic brain regions are involved in initiating this physiological stress response (for a review, see Ulrich-Lai & Herman, 2009).

Autonomic nervous system

First, there is a rapid response driven by the autonomic nervous system, specifically the sympathetic nervous system (SNS). The SNS, named because it was thought to be 'in sympathy' with our emotions, increases arousal levels and rapidly prepares us for action in response to a stressor. This sympathetic response can be triggered by neurons from two hypothalamic nuclei, the paraventricular nucleus (PVN) or the lateral hypothalamus (LH), which project directly to preganglionic neurons in the brainstem and in the spinal column (Ulrich-Lai & Herman, 2009). Additionally, threats can directly or indirectly activate neurons in brainstem nuclei, including in the locus coeruleus (LC) and the rostral ventrolateral medulla oblongata (RVLM), which synthesize the catecholamine norepinephrine (NE) (Guyenet, 2006; Hökfelt, Fuxe, Goldstein, & Johansson, 1974; Marina et al., 2011; Ross et al., 1984; Sawchenko, Li, & Ericsson, 2000). As with the hypothalamus, catecholinergic LC and RVLM neurons project to the spinal cord to act on preganglionic fibers. Once at the level of the spinal cord, the SNS diverges into two major pathways: a sympatho-neural and a sympatho-adrenal medullary (SAM) pathway. In the sympatho-neural system, nerves directly innervate organs and tissue to induce changes, signaling through acetylcholine (ACh) and norepinephrine (NE) through the spinal cord and associated ganglions outside of the CNS (Ulrich-Lai & Herman, 2009). For example, nerves from the superior cervical ganglion innervate the eyes, causing them to dilate and giving us more light to visualize the oncoming threat (Lichtman, Purves, & Yip, 1979). In the SAM, the splanchnic nerve activates the adrenal medulla, triggering the release of epinephrine and norepinephrine into the bloodstream. These hormones act on adrenergic receptors found on organs all across the body. For example, adrenergic receptors on the heart control heart rate and blood pressure and are responsible for that pounding heartbeat you may feel when suddenly startled (Perez, 2006). Together this immediate response to a stressor is commonly referred to as the 'fight or flight' response.

HPA axis

At the same time, there is a slower, longer-lasting response to stress driven by the endocrine system, specifically via the hypothalamic-pituitary-adrenal (HPA) axis (S. M. Smith & Vale, 2006). The detection of a threat triggers a cascade that once again starts in the hypothalamus, yet ultimately ends with the release of glucocorticoid stress hormones from the adrenal gland. As with the autonomic system, information about the stressor gets transmitted to parvocellular neurons in the PVN of the hypothalamus, which synthesize and secrete corticotropin releasing hormone

(CRH). Axons from the PVN release CRH into the portal blood system which transports the hormone to the anterior pituitary. Here, CRH binds to receptors to stimulate the release of adrenocorticotropic hormone (ACTH) into the bloodstream. ACTH receptors are primarily in the adrenal glands, where binding stimulates the release of glucocorticoids (GCs) from the adrenal cortex into the blood. In humans, the GC synthesized at the adrenal cortex is cortisol, while in rodents, it is corticosterone (Dallman & Jones, 1973; Taves, Gomez-Sanchez, & Soma, 2011; see Timmermans, Souffriau, & Libert, 2019 for a review). Almost every cell type in our body contains one or both of the receptors sensitive to GCs: mineralcorticoid receptors (MRs) and glucocorticoid receptors (GRs). The binding of cortisol to these receptors has effects all over the body, primarily driving the mobilization of energy substrates, breaking down proteins and fats to provide energy for our organs (Brillon, Zheng, Campbell, & Matthews, 1995). In addition, cortisol is known to suppress inflammation (a common reason why steroids are prescribed in inflammatory disorders) (Cline & Melmon, 1966). Ultimately, GC activity feeds back to regulate activity of the HPA axis. GCs act on receptors at multiple levels of the axis, including in the hippocampus, hypothalamus and pituitary, to decrease the production of both CRH and ACTH, which ultimately leads to a termination of the HPA response (Herman, McKlveen, Solomon, Carvalho-Netto, & Myers, 2012). Altogether, autonomic and neuroendocrine systems, acting on different time scales, coordinate the primary physiological response to stress.

Additional stress responses in the brain

The effects of stress are widespread throughout the brain. Release of stress signaling molecules, including CRH, GCs and catecholamines induce a coordinated 'symphony' of downstream effects that extends beyond the two systems described above (for a review see Joëls & Baram, 2009).

Corticotropin Releasing Hormone (CRH)

In addition to its role in the HPA axis, CRH has extensive effects throughout extra-hypothalamic brain regions, including in cortico-striatal circuits (Bale & Vale, 2004; Koob, 1999). It is now known that CRH is not only produced in the PVN; it is also synthesized by neurons in the central amygdala (CeA), hippocampus and BNST, among other regions (Yuncai Chen, Bender, Frotscher, & Baram, 2001; Sawchenko et al., 2007; Swanson & Sawchenko, 1983). Thus, levels of CRH in the brain can increase in response to stress independent of CRH release in the PVN and HPA axis responses (Funk, O'Dell, Crawford, & Koob, 2006; Shepard, Barron, & Myers, 2000; Wang et al., 2005). CRH receptors (CRH-1 and CRH-2) are highly enriched in midbrain regions such as the ventral tegmental area (VTA), as well as in limbic regions like the nucleus accumbens (Nac), hippocampus, amygdala and PFC (Chalmers, Lovenberg, & De Souza, 1995; Potter et al., 1994; Van Pett et al., 2000). Activation of CRH receptors can influence everything from neuronal firing patterns and gene expression to stress-related behaviors (Baram & Hatalski, 1998; Koob, 1999, 2008; Regev & Baram, 2014). For example, release of CRH from the CeA enhances emotional memory consolidation (Roozendaal, Brunson, Holloway, McGaugh, & Baram, 2002) while in the BNST, infusions of CRH increase anxiety-like behaviors, an effect blocked by CRH-1 antagonists (Jasnow, Davis, & Huhman, 2004; Sahuque et al., 2006; Tran, Schulkin, & Greenwood-Van Meerveld, 2014). Overall, activation of CRH receptors has been broadly linked

with stress-related learning and memory as well with stress-related anxiety (Merali, Khan, Michaud, Shippy, & Anisman, 2004; Müller et al., 2003).

Glucocorticoids (GCs)

A key part of the stress response is the release of glucocorticoids. As previously mentioned, GC receptors are found all over the body, and indeed, the brain is no exception. Further, the two GC receptors, MR and GR, are located in distinct patterns in the brain, allowing GCs to have region-specific effects (Morimoto, Morita, Ozawa, Yokoyama, & Kawata, 1996). Of importance, MR and GRs have different binding affinities; MR has a high affinity for corticosteroids, meaning they are highly occupied even at low levels of circulating GCs. In contrast, GRs have a lower binding affinity, and become more occupied when GC levels increase, as is observed following a stressor (De Kloet & Reul, 1987; Reul & De Kloet, 1985). Therefore, regions of the brain with high densities of MRs, such as the hippocampus, are thought to be crucial for regulation of baseline HPA tone (Reul & De Kloet, 1985). In contrast, regions with high densities of GRs only begin to respond as GC levels rise (as is observed in the stress response). Many extra-hypothalamic regions have strong responses to GC release and GR binding, including in the hippocampus, amygdala and PFC. Activation of GRs in these brain regions leads to different effects. Importantly, as stress levels rise, many of these regions come online to help regulate the response to stress (B S McEwen, De Kloet, & Rostene, 1986). In particular, activation of GRs in the hippocampus and PFC have an overall suppressive effect, helping regulate and control both the autonomic and neuroendocrine response to stress (Herman et al., 2012). The hippocampus has an especially dense number of GRs and is a crucial extra-hypothalamic site for regulation of the HPA axis (R M Sapolsky, Krey, & McEwen, 1984). In contrast, activation of GRs in the BLA is excitatory, and is thought to enhance the stress response (Duvarci & Paré, 2007). Overall, with higher circulating glucocorticoids, as observed during a stress response, GR receptors are activated across the brain leading to additional downstream responses in the brain.

Catecholamines

Broadly, stress increases the release of catecholamines (norepinephrine and dopamine) in the brain and these neurotransmitters play a crucial role in responding and adapting to stressors (Arnsten, 2009, 2015; Goto, Otani, & Grace, 2007). In particular, it is well known that stress stimulates norepinephrine release from the LC. The LC sends projections to nearly all areas of the brain, leading to release of NE and binding of adrenergic receptors across the brain (Aston-Jones & Cohen, 2005; C. W. Berridge & Waterhouse, 2003). The binding of adrenergic receptors produces diverse effects depending on the particular brain region and receptor (Arnsten, 2015). Less often discussed is a stress-induced release of dopamine (Cuadra, Zurita, Lacerra, & Molina, 1999; Imperato, Angelucci, Casolini, Zocchi, & Puglisi-Allegra, 1992; Puglisi-Allegra, Imperato, Angelucci, & Cabib, 1991). Binding of dopamine receptors across the brain is thought to contribute towards stress-effects on risk-assessment and decision making (as will be discussed more in-depth in a later section).

Interactions between these systems

Interestingly, these signaling systems can interact with one another. For example, activity in the LC is modulated by interactions with CRH (Aston-Jones & Cohen, 2005; Valentino, Foote, & Aston-Jones, 1983; Valentino, Foote, & Page, 1993). Additionally, hippocampal excitability is enhanced by NE, and in a synergistic manner, it is further augmented when GCs are also present (Pu, Krugers, & Joëls, 2007; Roozendaal, McEwen, & Chattarji, 2009; Roozendaal, Okuda, Van der Zee, & McGaugh, 2006). Thus, these systems should be thought of as working in concert in response to stress.

Limbic integration/control of the stress response

Together, through these diverse signaling systems, stress activates key limbic regions which control the physiological stress response. Specifically, the amygdala, hippocampus and mPFC all provide input to the PVN indirectly via activation of GABAergic cells in the BNST (Herman et al., 2012; Ulrich-Lai & Herman, 2009). While the hippocampus and mPFC inhibit the PVN and work to shut off the stress response, the amygdala stimulates PVN neurons involved in stress. There is a balance of excitation and inhibition that works to differentially manage the stress response. These brain areas also act in conjunction; they are part of a complex circuit, functioning to integrate sensory, arousal and attentional information with emotions and memory. Ultimately, the brain works to respond appropriately to the stressor and manage the stress response.

Factors that affect the severity of the stress response

There are a number of factors that affect the severity of the stress response, including one's age and sex.

Age

As limbic brain regions develop with age, our stress response system, including the HPA axis, is dynamic and displays heightened responsiveness to stress at certain timepoints (Kudielka, Buske-Kirschbaum, Hellhammer, & Kirschbaum, 2004). Stress can occur in the early postnatal period, during developmental transitions like adolescence, in adulthood, or at a later age. In the early postnatal time period, stress can alter neural architecture to increase adverse reactions to stressors, leading to toxic stress (Bruce S. McEwen et al., 2015; Shonkoff, Boyce, & McEwen, 2009; Shonkoff et al., 2012). In humans and in rodents, early stressors include changes in maternal care. In humans, children with depressed mothers have heightened HPA axis activity (Lupien, King, Meaney, & McEwen, 2000). Similarly, in rodents, maternal separation leads to reduced GR levels in the hippocampus and changes in the density of CRH binding sites; these alterations are associated with increased anxiety and HPA axis activity (D. Liu et al., 1997; Schulkin, W. Gold, & S. McEwen, 1998). In adolescence as well, there are heightened HPA axis responses (Dahl & Gunnar, 2009; Gunnar, Wewerka, Frenn, Long, & Griggs, 2009) and prolonged release of ACTH and glucocorticoids following stress (Romeo, Karatsoreos, & McEwen, 2006; Romeo & McEwen, 2006). Furthermore, areas of the brain like the amygdala, PFC, and ventral striatum are significantly altered by stressors during the peri-adolescent period (see Eiland & Romeo, 2013 & Tottenham & Galván, 2016 for reviews). Most research on stress focuses on adulthood. There is a

vast body of work demonstrating how stress effects on limbic regions fall along an inverted-U curve, with detrimental changes in structure and function following severe or chronic stress (Robert M. Sapolsky, 2015). Generally, stress responses in the PFC, amygdala, hippocampus and hypothalamus differ in adulthood compared to earlier in life (see Lupien, McEwen, Gunnar, & Heim, 2009 for a review). Lastly, in aging, as our brains become less plastic, we also become less resilient to stressors. High levels of glucocorticoids are associated with hippocampal atrophy and may even contribute to disorders such as Alzheimer's disease (Gilbertson et al., 2002; Kulstad et al., 2005; Lupien et al., 1998). Overall, the severity of the stress response and its effects in the brain differ depending on life stage.

Sex

Sex is also an important biological factor that contributes to individual variation in response to stress (Bangasser & Valentino, 2014). For example, women have a higher risk of developing trauma-related mental health disorders than do men (Bekhbat & Neigh, 2018; Dell'Osso et al., 2013; Ronald C. Kessler et al., 2005; Ronald C Kessler, Sonnega, Bromet, Hughes, & Nelson, 1995; Kline et al., 2013; Steven Betts, Williams, Najman, & Alati, 2013). In addition, women generally have stronger activation of the HPA axis following stressors; for example, there is greater release of ACTH following injections of CRH (J. H. Liu et al., 1983). In animals, sex differences in stress are also observed, although there are conflicting findings depending on the stressor and circumstance. In acutely stressful situations, female rodents tend to fare better than males. They are less sensitive to stress-associated novel or aversive environments; for example, females explore more than males and defecate and freeze less (Handa & Chung, 2019). However, with chronic stress, female animals appear to be more sensitive to stress and over longer periods of time, males adapt more readily, while females fail to adapt over the same time period (Goodwill et al., 2019; Kennett, Chaouloff, Marcou, & Curzon, 1986).

Explanations for these sex differences are only beginning to be uncovered. In particular, sex hormones may contribute towards these effects. For example, women have greater amygdala reactivity to negative emotional stimuli than men (Jennifer S Stevens & Hamann, 2012), a difference that only arises after puberty (Guyer et al., 2008; Pagliaccio et al., 2013). More recently, greater fear processing was observed in females specifically during high estrogen phases (Hwang et al., 2015). Similarly, in animals, treatment with estrogen augments ACTH and GC responses (Beatty, 1979), and estrogen promotes stress sensitivity and structural remodeling in regions of the brain (Shansky et al., 2010). However, low estrogen may also alter stress responses. Low estrogen impairs fear inhibition and extinction (Glover et al., 2012, 2013), and increases amygdala and anterior cingulate cortex (ACC) connectivity (Engman, Linnman, Van Dijk, & Milad, 2016). Thus, there is an emerging idea that, similar to glucocorticoids, the effects of estrogen fall along an inverted U curve; there is an optimal amount of estrogen for appropriately responding to stress.

There are also genetic risk factors that may contribute towards observed sex differences. Specifically, genetic polymorphisms in genes that regulate the cellular stress response, including pituitary adenylate cyclase-activating polypeptide (PACAP) and its receptor, are associated with sex-specific differences in stress. High levels of PACAP are associated with post-traumatic stress disorder (PTSD) in women but not men, and receptor polymorphisms found in women are associated with anxiety and impaired safety signal discrimination (Ressler et al., 2011). This same polymorphism is also associated with greater amygdala reactivity and less coupling of the amygdala with regulatory regions like the hippocampus and the ACC (Pohlack et al., 2015;

Stevens et al., 2014). Further, this specific PACAP receptor polymorphism is located within an estrogen response element and is regulated by estrogen (Ressler et al., 2011). Interestingly, sex-differences in PACAP receptor associated fear responses primarily emerge after puberty, again suggesting that sex hormones play a regulatory role (T Jovanovic et al., 2013). With all of these sex differences, it is important to keep in mind that the types of stressors that men and women are exposed to also differ, and further, there are environmental, psychological and cultural factors that could greatly influence the brain and behavior. Future research will need to carefully consider sex differences and their potential underlying mechanisms.

Interactions of age and sex

These two factors (age and sex) also interact within the context of stress. In humans, experiencing early life trauma (in particular psychological trauma) prior to puberty greatly increases the susceptibility to PTSD in adulthood in females more so than in males (Heim & Nemeroff, 2001). The impact of adolescent stress may also be particularly robust, long-lasting and sex-specific, as stress hormones and sex hormones act in concert during this time to shape future endocrine responses and to shape the development of the brain (Rowson et al., 2019). Thus, both age and sex are important to consider when examining the effects of stress.

Stress and Pathologies - maladaptive stress

Generally, the stress response is an adaptive, coordinated response to stressors. However, severe and chronic stress can lead to maladaptive responses and contribute to a number of disorders. As stress is repeated and persistent, our bodies continue to try to adapt through change, a process known as allostasis. Over a longer period, there is a physiological cost of these adaptive shifts, or a high allostatic load (Bruce S. McEwen, 1998a, 1998b). A high allostatic load occurs when one chronically tries to correct for something in a way that may be maladaptive. Too high of an allostatic load leads to wear and tear on the body, predisposing one to disorders. For example, increased cortisol and inflammatory markers from the stress response may lead to elevated blood pressure or perhaps even hypertension in the long term (Kulkarni, O'Farrell, Erasi, & Kochar, 1998). Overall, prolonged activation of stress response systems is damaging, in both the body and in the brain (Danese & McEwen, 2012).

Many mental illnesses are either directly or indirectly linked to stress, including PTSD, anxiety disorders, major depression disorder (MDD) and substance use disorders (Bruce S McEwen, 2003; Sinha, 2008). PTSD is perhaps the most directly connected to stress, as it is defined by exposure to a traumatic event (American Psychiatric Association, 2013). Severe stress triggers maladaptive plasticity, leading to long-lasting changes in fear and anxiety behavior (Shalev, Liberzon, & Marmar, 2017). Stress, and especially stress early in life, can also indirectly produce vulnerabilities to later developing psychiatric disorders (C. P. Carr, Martins, Stingel, Lemgruber, & Juruena, 2013; Heim & Binder, 2012; Ronald C. Kessler, Davis, & Kendler, 1997; Ronald C Kessler, 1997; E. McCrory, De Brito, & Viding, 2012). In particular, early life trauma increases the risk for major depression and anxiety disorders (Fernandes & Osório, 2015; Heim & Nemeroff, 2001; Spatz Widom, DuMont, & Czaja, 2007). In addition, there is strong evidence linking early-childhood adversity with a risk of addiction (Sinha, 2008). Stressors experienced in critical developmental periods may alter maturation of the brain and stress responses systems,

leading to long-term consequences and this observed vulnerability. Together, these findings point towards overlapping circuitry and mechanisms between stress and psychiatric disorders.

Part 2: Stress and Motivation

As described above, stress is a well-known risk factor in the development of both substance use (Sinha, 2008) and depressive disorders (Spatz Widom et al., 2007). Stress, especially early in life, may have long-lasting impacts on reward and motivation circuitry, leading to predispositions to disorders like addiction. For example, both children and adolescents who have experienced negative life events, including sexual and physical abuse, show increased drug use and abuse (Breslau, Davis, & Schultz, 2003; Dembo, Dertke, Borders, Washburn, & Schmeidler, 1988; M D Newcomb, Maddahian, & Bentler, 1986; Michael D Newcomb & Bentler, 1988; Widom, Weiler, & Cottler, 1999).

More broadly, stress interacts strongly with motivation at a neural and behavioral level (for a review, see Hollon, Burgeno, & Phillips, 2015). Stress can affect everything from our ability to pay attention, to our motivation to get out of bed in the morning. A moderate amount of stress (eustress) can result in enhanced motivation and performance, for example providing motivation to accomplish a task by a deadline (like writing your thesis). In contrast, larger stressors such as unexpected hurdles while trying to complete a project can cause motivation to plummet. Thus, intensity, predictability and length of a stressor are all relevant in understanding the role of stress in motivated behaviors. In addition, there is a reciprocal relationship between stress and reward; exposure to natural rewards can buffer the stress response (Dutcher & Creswell, 2018; Yuan et al., 2019), while a history of drug use can also increase susceptibility to stressors (Covington et al., 2011). Overall, stress, motivation and reward are all deeply intertwined.

In animal models as well, there is evidence to suggest that stress increases drug seeking, drug administration and drug relapse (Mantsch, Baker, Funk, Lê, & Shaham, 2016; Sinha, 2001, 2008). In addition, chronic stress can be used to induce depressive-like symptoms such as reduced pleasure (anhedonia), social withdrawal, behavioral despair and learned helplessness (Russo & Nestler, 2013). Thus, animal models allow us to explore the neural mechanisms connecting stress with motivated behavior.

In the brain, hypothalamic and extrahypothalamic CRH targets motivational pathways, especially limbic and midbrain circuits. Importantly, injections of CRH into the amygdala, BNST, VTA and Nac have all been shown to increase self-administration or reinstatement of drug seeking behaviors (Shaham et al., 1997; Wang et al., 2005; Wang, You, Rice, & Wise, 2007). The VTA in particular is a crucial brain region involved in reward; it is primarily known for its dopaminergic projections, although it also contains glutamatergic and GABAergic neurons (Howard L. Fields, Hjelmstad, Margolis, & Nicola, 2007; Yamaguchi, Qi, Wang, Zhang, & Morales, 2015). Dopamine (DA), a central catecholamine, is both sensitive to stress and plays a role in regulating stress and exerting cognitive and behavioral control in emotional situations (Belujon & Grace, 2015; Cabib & Puglisi-Allegra, 2012). Importantly, firing patterns of VTA neurons, including VTA dopaminergic neurons, are sensitive to both stress and reward. For example, stress induced CRH release modulates firing of VTA neurons (Holly et al., 2016; Holly, Debold, & Miczek, 2015; Holly & Miczek, 2016; Rodaros, Caruana, Amir, & Stewart, 2007; Wang et al., 2005). CRH release into the VTA, especially originating from the BNST (Rodaros et al., 2007), also has been implicated in both reward and anxiety-like behaviors (Jennings et al., 2013).

The VTA has two major pathways that are highly affected by stress: the mesolimbic and mesocortical pathways (for a review, see Holly & Miczek, 2016). In the mesolimbic pathway, VTA DA neurons project to limbic areas like the Nac, amygdala and hippocampus, while in the mesocortical pathway, VTA DA projects to cortical regions such as the mPFC and cingulate cortex (Howard L. Fields et al., 2007). Activation of these pathways are thought to modulate motivational behaviors associated with approach, avoidance and reinforcement of these behaviors (B. T. Chen & Bonci, 2018; Ikemoto & Bonci, 2014; J. D. Salamone & Correa, 2012a). Critically, stress also affects both of these pathways, leading to changes in DA release in the Nac and mPFC (Cuadra et al., 1999; Imperato et al., 1992; Puglisi-Allegra et al., 1991).

Mesolimbic pathway

In the mesolimbic pathway, there is a strong dopaminergic projection from the VTA to the nucleus accumbens. This pathway in particular has been highly implicated in motivated behaviors and reward; dopamine release in the Nac is thought to contribute to both reward seeking and the 'pleasure' one feels upon receiving a reward (K. C. Berridge & Robinson, 1998). Mesolimbic dopamine is also implicated in social aspects of motivated behavior (Gunaydin et al., 2014a; Robinson, Heien, & Wightman, 2002). Due to its overall role in reward, stress activation of this pathway is perhaps not intuitive, as we typically think of stress and aversion as counter to reward. However, different stressors produce opposing outcomes. For example, while acute social defeat increased firing of VTA DA neurons projecting to the Nac, resulting in more DA release (Anstrom, Miczek, & Budygin, 2009), chronic social defeat stress and other severe stressors *reduce* DA tone in the Nac (Mangiavacchi et al., 2001; Miczek, Nikulina, Shimamoto, & Covington, 2011). Similarly, acute activation of CRH receptors on DA terminals in the Nac increases DA release, leading to reward seeking behavior. However, following chronic CRH exposure, CRH no longer leads to DA release, and in fact induces a conditioned place aversion (Lemos et al., 2012). Consequently, the length of a stressor may differentially modulate motivated behavior in this pathway.

Even for chronic stressors, there are discrepancies in findings. For example, one group found that 8-12 weeks of chronic mild stress inhibited VTA DA neurons projecting to the Nac, which led to depressive-like behaviors. This was rescued with optogenetic stimulation (Tye et al., 2013). In contrast, another group found that chronic social stress *increased* firing in this pathway (Chaudhury et al., 2013a), and phasic stimulation during subthreshold defeat was sufficient to induce depressive-like behaviors like reduced sucrose consumption (Friedman et al., 2014). This indicates that not just stressor length but also the stressor type can have an effect on the mesolimbic pathway. Furthermore, the Nac is a heterogenous brain region, containing both D1 and D2 type medium spiny neurons (MSNs) with different inputs, outputs and circuit functions (Surmeier, Ding, Day, Wang, & Shen, 2007). Stress may affect each of these cell types and pathways differently and much remains to be explored (Soares-Cunha, Coimbra, David-Pereira, et al., 2016; Soares-Cunha, Coimbra, Sousa, & Rodrigues, 2016).

Lastly, stress alterations in reward-based decision making are proposed to be modulated through the mesolimbic pathway. In both humans and animals, stress biases us away from making high-effort, high reward choices when a low-reward option is available for less effort (Shafiei, Gray, Viau, & Floresco, 2012; Valentin, Dickinson, & O'Doherty, 2007; for a review, see Hollon et al., 2015). This effect is proposed to be mediated by stress-induced increases in VTA CRH levels, which reduces firing of VTA-Nac dopaminergic neurons, thereby reducing the salience or

rewarding aspect of an action (J. Salamone, Correa, Farrar, Nunes, & Pardo, 2009; J. D. Salamone & Correa, 2012a; Treadway et al., 2012; Wanat, Bonci, & Phillips, 2013). Altogether, stress effects within the mesolimbic pathway can lead to diverse behavioral outcomes.

Mesocortical pathway

Stressors also activate the mesocortical pathway of the VTA, with projections to the mPFC and cingulate cortex (Bannon & Roth, 1983; Ariel Y Deutch, Clark, & Roth, 1990; Thierry, Tassin, Blanc, & Glowinski, 1976). In rodents, social defeat stress leads to increased extracellular dopamine (Cuadra et al., 1999; Tidey & Miczek, 1996) well as extracellular glutamate (Moghaddam, 1993) in the PFC. In fact, the majority of projections from the VTA to the PFC are glutamatergic (Natalia Gorelova, Mulholland, Chandler, & Seamans, 2012; Yamaguchi, Wang, Li, Ng, & Morales, 2011b). Increasingly, the mesocortical pathway from the VTA to the mPFC is thought to be involved in the processing of aversive, stressful stimuli. Painful injections of formalin into the hindpaw increased the AMPA/NMDA ratio (a measure of LTP) in mPFC projecting VTA DA neurons (Lammel, Ion, Roeper, & Malenka, 2011). These neurons are primarily located in the medial, posterior VA, where others had similarly found VTA dopamine neurons that were sensitive to stress (Brischoux, Chakraborty, Brierley, & Ungless, 2009). In addition, these medial VTA neurons receive input from the lateral habenula (Lhb); stimulation of Lhb inputs to these VTA-mPFC projecting cells led to a strong conditioned place aversion which was blocked by injecting a dopamine antagonist into the mPFC (Lammel et al., 2012a). In a more recent study, stimulation of mPFC projecting VTA DA neurons was found to be both anxiogenic and aversive (Gunaydin et al., 2014a). Yet, not all studies have produced consistent findings. Using a rodent model of social defeat stress, one group found that stress-susceptible animals had *decreased* firing rates of VTA neurons projecting to the mPFC, and optogenetically inhibiting this pathway led to enhanced susceptibility to social stress (Chaudhury et al., 2013a). Thus, this study implies that a normally functioning connection from the VTA to the mPFC is crucial for controlling stress as well. These contrasting findings may stem from different stressor types and durations. In addition, VTA neurons may project to specific subregions and populations of cells within the PFC. Future research will need to address these questions.

Together, these findings suggest that distinct subpopulations of VTA dopamine neurons differentially respond to rewarding and aversive stimuli, yet overall, there is more heterogeneity to explore (Lammel, Lim, & Malenka, 2014a).

Part 3: The Prefrontal Cortex (PFC)

PFC Functions and Anatomy

The prefrontal cortex, located in the most anterior part of the frontal lobe, is an essential brain region for executive functioning including: attention, planning, working memory, decision-making, emotional control and coordination of goal-directed behaviors (Dixon, Girn, & Christoff, 2017; Goldman-Rakic et al., 1996; S. P. Wise, 2008). Critically, within all of these functions, the PFC is a region of overlap between stress and motivation circuits.

Region Specific Functions in Humans/Primates

In humans, the PFC is made up of several major regions including: the anterior cingulate cortex (ACC), the orbitofrontal cortex (OFC), the ventromedial PFC (vmPFC) and the dorsolateral PFC (dlPFC). Each of these regions have their own unique functions (Carlén, 2017).

The ACC is involved in emotional processing and integration and is especially activated when experiencing pain of oneself and others (P. N. Fuchs, Peng, Boyette-Davis, & Uhelski, 2014; Jackson, Meltzoff, & Decety, 2005; Jeon et al., 2010; Johansen, Fields, & Manning, 2001; Singer et al., 2004). The ACC also has a role in effort-based decision making, especially in challenging situations where cognitive or emotional conflict arises (Croxson, Walton, O'Reilly, Behrens, & Rushworth, 2009; Lapish, Durstewitz, Chandler, & Seamans, 2008), and in cases where one must decide an optimal choice between options (Kennerley, Walton, Behrens, Buckley, & Rushworth, 2006). Altogether the ACC is considered an important link between emotional and cognitive processing (see Stevens et al., 2011 for a review).

The OFC has been widely recognized for its role in sensory integration and value-based decision making (Kringelbach, 2005; Schoenbaum, Takahashi, Liu, & McDannald, 2011; Wallis, 2012). In strong support of this, neuronal firing patterns in the OFC are sensitive to the value of anticipated rewards. In addition, these neurons are sensitive to one's motivational state. For example, firing is decreased as one becomes satiated and the value of food rewards diminish (Tremblay & Schultz, 1999).

The vmPFC has more affective functions than other prefrontal regions and is critically involved in regulating one's emotions. Much of our early knowledge on this region came from lesion studies. One of the most famous cases was that of Phineas Gage, a railroad worker who damaged his vmPFC when a tamping rod accidentally exploded and passed through his brain. Before his accident, Gage was described as "serious, industrious and energetic". Afterward however, he became irritable, quick-tempered, irresponsible, and thoughtless of others. In short, he showed poor emotional control, resulting in risky decision making (O'Driscoll & Leach, 1998). Later work by Antonio Damasio's group found further evidence that vmPFC damage produces impairments in emotional processing and decision making. Patients with vmPFC damage had almost no physiological responses to stressors such as socially disturbing images, and in general, these patients had reduced emotions such as compassion in shame (Damasio, Tranel, & Damasio, 1990). In short, Damasio concluded they had no 'moral compass' without their emotions guiding them (Damasio, 2007).

Lastly, the dlPFC, the most recently evolved region of the PFC, is critical for complex cognitive functions. It guides our thoughts, attentions and actions using working memory and is responsible for planning and guiding sequences of behaviors aimed at a goal (Joaquin M Fuster, 1991; Goldman-Rakic, 1995). To this end, the dlPFC is also involved in rule learning and rule modification (Miller, 2000). Altogether, the dlPFC provides the most powerful cognitive control over motor behavior.

Homology with rats

The prefrontal cortex is the most recently evolved region of the brain; primates and humans have a larger PFC than other mammals (Teffer & Semendeferi, 2012). Despite differences in size and architecture, mice and rats, commonly used as animal models for human disorders, have a

PFC with functionally homologous regions to primates (Wallis 2012), although the degree to which the rodent PFC is homologous to primates remains debated (see Wise 2008).

In rodents, the medial PFC (mPFC) is comprised of the infralimbic (IL) prelimbic (PL) and anterior cingulate cortices, which are homologous to human Brodmann areas 24b, 32 and 25 respectively (Gabbott, Warner, Jays, Salway, & Busby, 2005; Harry B M Uylings, Groenewegen, & Kolb, 2003; Wallis, 2012; S. P. Wise, 2008). Thus, the rodent IL, sitting more ventrally, can be thought of as homologous to the human vmPFC, while the more dorsal PL can be thought of as homologous to the human dlPFC. Functionally, the rodent IL is responsible for emotional regulation, while the PL is responsible for goal-directed behaviors and working memory (Gourley & Taylor, 2016a; Kesner & Churchwell, 2011; Robbins et al., 1996; Harry B M Uylings et al., 2003). In addition, as in primates, the rodent OFC is involved in reward learning and value decision making (Sul, Kim, Huh, Lee, & Jung, 2010; Wallis, 2012). The rodent OFC is a heterogeneous region (Izquierdo, 2017; Joseph L. Price, 2007), whose subregions (the medial orbital (MO), ventral orbital (VO) and lateral orbital (LO) regions) are only beginning to be pieced apart at a functional level (Fuchs, Evans, Parker, & See, 2004; Hervig et al., 2020).

PFC Connectivity

The PFC is highly interconnected with much of the brain, including cortical, subcortical and brainstem regions. Of particular relevance here, the PFC has dense reciprocal connections with the amygdala and hippocampus, forming a connected circuit that is crucial for processing stress and emotions more broadly (Buijs & Van Eden, 2000; Roozendaal et al., 2009). The PFC itself is comprised mostly of glutamatergic pyramidal neurons, with some inhibitory GABAergic interneurons regulating local circuitry (Ferguson & Gao, 2018). Thus, most of the outputs from the PFC are glutamatergic in nature. The cellular makeup of the PFC and the connectivity of each PFC subregion contributes towards the PFC's unique functions.

The ACC for example receives input from the amygdala and hippocampus as well as input from the OFC, together carrying information regarding valence, value and reward (see Rolls, 2019 for a review). The ACC sends outputs to the striatum (Saleem, Kondo, & Price, 2008) and to the hypothalamus and amygdala; projections to the amygdala especially are thought to control innate fear responses (Jhang et al., 2018; Ortiz et al., 2019). The ACC is also part of a broader 'salience network' which is sensitive to emotionally salient stimuli (Menon, 2015; K. S. Taylor, Seminowicz, & Davis, 2009). As part of this salience network, the ACC has strong projections to the anterior insula and claustrum (Qadir et al., 2018; White et al., 2018), brain regions with roles in attention (Goll, Atlan, & Citri, 2015; S. M. Nelson et al., 2010), and empathy (Gu, Hof, Friston, & Fan, 2013; Mutschler, Reinbold, Wankerl, Seifritz, & Ball, 2013). Therefore, specific functions of the ACC should be considered within a circuit context.

The dlPFC and vmPFC have their own connectivity patterns. Broadly speaking, the dorsal PFC is highly connected with regions involved in cognition and action, while ventral PFC regions are more highly connected with emotional and limbic circuits (Dalley, Cardinal, & Robbins, 2004; Etkin, Egner, & Kalisch, 2011a; Vertes, 2006). For example, the dlPFC projects more to the dorsal striatum, while the vmPFC projects more to ventral striatum and limbic regions (Suzanne N. Haber, 2016). This pattern is observed in rodents as well. The PL primarily projects to the dorsal striatum, Nac core, BNST, VTA, and BLA, while the IL primarily projects to the Nac shell, hypothalamus, CeA and brainstem (including to the PAG, NTS and RVLM)(Carr & Sesack, 2000; Gabbott et al., 2005; Myers-Schulz & Koenigs, 2012; Sesack, Deutch, Roth, & Bunney, 1989;

Vertes, 2004). These IL outputs, especially connections to the posterior hypothalamus and brainstem, are crucial for regulating autonomic and neuroendocrine responses to stress (Diorio, Viau, & Meaney, 1993; Thayer & Brosschot, 2005). These structural connectivity patterns are in line with functional networks; the dlPFC is part of a central executive network (CEN), acting together with the posterior parietal cortex to maintain working memory and to work towards goal-directed actions (Menon, 2010), while the vmPFC is part of a 'default mode network' (DMN) (Raichle, 2015), activated at rest and during mind-wandering. Critically, the dlPFC projects to the vmPFC, allowing regulation over both the DMN and emotional control (Barbas & Pandya, 1989). Similarly, in rodents, there are reciprocal connections between the two homologous PFC regions (the PL and IL) (Vertes, 2004).

The OFC is unique compared to the other PFC subregions; it receives strong input from sensory regions, including from the gustatory and olfactory cortices. The OFC also receives input from limbic and other cortical areas and thus integrates sensory stimuli with emotional/affective information (J L Price, 1999; Joseph L. Price, 2006b, 2006a). OFC, originally defined by its reciprocal connections to the mediodorsal thalamus (Wallis, 2012), has strong outputs to the hypothalamus and amygdala, as well as to the ventral striatum (for a review see Heilbronner, Rodriguez-Romaguera, Quirk, Groenewegen, & Haber, 2016). Specifically, in both primates and in rodents, the medial OFC projects to the ventromedial striatum, while lateral OFC regions (like the LO) project more to central/lateral striatum. In addition, the MO and VO project highly to one another, as well as to the lateral hypothalamus, hippocampus, substantia nigra and to the VTA (Hoover & Vertes, 2011). As a result, some have proposed that the MO and VO may be a functional 'unit' in reward learning and decision making (Price, 2007). Yet, the MO also projects more densely to the BLA and Nac, suggesting a stronger role for the MO in motivation circuits (Hoover & Vertes, 2011). The LO, which receives most of the sensory input, is thought to coordinate with the VO and MO, linking sensory events to motivation. Lastly, each of these OFC regions has strong outputs to other parts of the PFC. For example, the OFC projects to the dlPFC; thus, sensory and emotional information processed in the OFC also influence cognitive processes in the dlPFC (Hoover & Vertes, 2011).

Together, via this complicated circuitry, the PFC influences everything from our emotions to our actions. Sensory and affective information gets integrated with cognitive information to inform goal directed behaviors.

Development and Plasticity of the PFC

In the younger years, the PFC is one of the last regions to fully develop, displaying a long period of experience dependent plasticity (Bryan Kolb et al., 2012). This heightened plasticity makes the PFC especially sensitive to the effects of stress, especially early in childhood and during adolescence (Bruce S. McEwen & Morrison, 2013). Indeed, adolescence is also a period when many psychiatric disorders onset (Hoftman & Lewis, 2011). While the PFC is most notable for its protracted development, like many regions of the brain, the PFC remains plastic throughout life. However, during aging**,** neurons in the PFC structurally change, becoming less plastic and less resilient to withstanding stress (Bloss, Janssen, McEwen, & Morrison, 2010). Thus, development of the PFC is important to consider across the lifespan.

Development of the PFC continues into our twenties, at a slower, prolonged pace relative to other brain regions (Fuster, 2002). Throughout childhood and into adolescence there is a loss of grey matter due to synaptic pruning (Gogtay et al., 2004), while at the same time there is a growth

of dendrites and an increase in myelination and white matter volume (Mrzljak, Uylings, Van Eden, & Judáš, 1991; Sowell, Thompson, Holmes, Jernigan, & Toga, 1999; Sowell et al., 2002). Many of the cellular changes that occur in the PFC during adolescence can be explored using rodent models (see Caballero, Granberg, & Tseng, 2016 for a review). For example, pruning of PFC glutamatergic synapses parallels an increase in GABAergic synapses during adolescence (Caballero, Thomases, Flores-Barrera, Cass, & Tseng, 2014; Cressman et al., 2010). In addition to pruning, synaptic stabilization is also observed in pyramidal neurons in the rodent PFC (Boivin, Piekarski, Thomas, & Wilbrecht, 2018; C. M. Johnson et al., 2016). Rodents are also useful for studying developing brain regions and cell-type specific innervation of the PFC. Interestingly, dopamine innervation of the PFC increases through adolescence and into early adulthood (Benes, Vincent, Molloy, & Khan, 1996; Kalsbeek, Voorn, Buijs, Pool, & Uylings, 1988). Many of these cellular changes observed in development are subregion specific. For example, the dlPFC is the last region to show developmental pruning of synapses, and thus, the dlPFC is thought to have the longest period of structural plasticity during development (Bourgeois, Goldman-Rakic, & Rakic, 1994; Huttenlocher & Dabholkar, 1997).

Structural changes in the PFC also parallel changes in functional connectivity, as well as behavioral changes (Casey, Giedd, & Thomas, 2000; E. E. Nelson & Guyer, 2011). For example, PFC to amygdala connectivity, measured via functional MRI of coupling between the regions, develops over childhood to young adulthood and corresponds with increased emotional regulation (Arruda-Carvalho & Clem, 2015; Gee, Gabard-Durnam, et al., 2013; Gee, Humphreys, et al., 2013). In addition, increased vmPFC activation and circuitry develops along with enhanced self-evaluation and self-awareness (Pfeifer et al., 2013). Adolescence also comes with increased risk-taking, a behavior that is related to the development of frontostriatal circuitry (Casey, Jones, & Somerville, 2011; Tottenham, Hare, & Casey, 2011). Lastly, adolescence is a time period of enhanced social development, and many of these developing PFC circuits are thought to contribute to social behaviors (Bicks, Koike, Akbarian, & Morishita, 2015; E. E. Nelson & Guyer, 2011).

In adolescence specifically, there are massive changes in hormones and their receptors, which may contribute towards sex differences in brain development, including in the PFC (for reviews see (Piekarski et al., 2017) & (Shaw, Dupree, & Neigh, 2020). For example, cortical thinning in the OFC and ventral PFC occurs sooner in girls compared to boys (Raznahan et al., 2010). Recent studies have also suggested that the increase in PFC synapses observed in adolescence may peak sooner for females compared to males (Drzewiecki, Willing, & Juraska, 2016). Similar structural shifts are observed for myelination as well; myelin basic protein (MBP) expression in the OFC peaks sooner in females than in males. Further, this effect was specifically dependent on estrogen (Darling & Daniel, 2019). Indeed, many of these sex differences may arise due to the timing of puberty, which occurs sooner in females than in males (Lenroot et al., 2007; M. D. Wheeler, 1991). Altogether, these shifts in timing may parallel sex differences in sensitivity to environmental stimuli (Page & Coutellier, 2018).

Overall, the unique development and plasticity of the PFC is important to keep in mind as we consider how the environment shapes PFC circuits.

The Role the PFC in Stress

The prefrontal cortex both plays a critical role in regulating stress and is affected directly by stress itself. Furthermore, these connections with stress make the PFC a crucial region for stress related disorders.

The PFC in the Control of Stress

The PFC (especially the mPFC) is highly connected to limbic regions and provides critical executive (top-down) control over the stress response (for reviews, see (Gratton & Sullivan, 2005; McKlveen, Myers, & Herman, 2015). The PFC is responsible for control of both autonomic and neuroendocrine stress responses, as well as stress behaviors.

Regulation of the ANS

One of the first findings on the PFC's regulation of the ANS comes from patient studies; specifically, patients with vmPFC damage fail to mount an autonomic stress response in response to emotionally salient stimuli (Bechara, Tranel, Damasio, & Damasio, 1996; Damasio et al., 1990). For example, these patients do not demonstrate the expected changes in heart rate and skin conductance (sweating). In rodents, stimulation of the ventral PFC (the IL) also leads to sympathetic responses (D A Powell, Watson, & Maxwell, 1994; Donald A Powell, Maxwell, & Penney, 1996), via connections to brainstem circuits (McKlveen et al., 2015). Thus, in both humans and rodents, the ventral PFC specifically is responsible for control of the ANS.

Regulation of the HPA axis

The mPFC also plays a role in controlling stress via the HPA axis. As touched on earlier, the PFC has a high density of GRs (Cintra et al., 1994; B S McEwen et al., 1986), with especially high binding in primates during stress (Sánchez, Young, Plotsky, & Insel, 2000). It is thought that activation of these receptors in the PFC during a stress response plays a role in modulating the HPA axis. Activation of the mPFC leads to decreases in ACTH and GC levels (Diorio et al., 1993). In contrast, lesioning the mPFC increases CRH production in the PVN (Herman et al., 1989). mPFC regulation over the PVN comes from indirect projections to intermediate regions like the BNST that in turn regulate the PVN. Generally, dorsal mPFC regions like the PL are thought to be responsible for the bulk of HPA axis regulation. Stimulation of the PL inhibits HPA axis responses to psychogenic stressors and regulates the duration (but not peak) of GC secretion (Diorio et al., 1993). This inhibition is mediated by glutamatergic projections from the PL to the anterior BNST, which sends GABAergic projections to inhibit the PVN (Radley, Gosselink, & Sawchenko, 2009). In contrast, IL stimulation increases the HPA axis response and is more responsive to physical (compared to psychogenic) stressors (Radley et al., 2006; Ron M Sullivan & Gratton, 1999). Yet, there is some debate as to whether the IL stimulates or suppresses stress-induced increases in GCs. A more recent study found that impaired GRs in IL lead to HPA hyper-reactivity under acute stress situations (McKlveen et al., 2013). Together, these findings provide evidence that the mPFC exerts top-down control of the HPA axis.

Regulation of Stress Behaviors

In addition to regulating the physiological stress response, the PFC also regulates stress behaviors such as fear and anxiety, where there are either concrete or indefinite threats respectively (for a review, see (Calhoon & Tye, 2015; Tovote, Fadok, & Lüthi, 2015). Different PFC subregions uniquely contribute towards these fear and anxiety behaviors. Specifically, the PL and IL have opposing roles in conditioned fear; the PL is activated during fear conditioning, while the IL is activated during fear extinction (Do-Monte, Manzano-Nieves, Quiñones-Laracuente, Ramos-Medina, & Quirk, 2015; Milad & Quirk, 2002; Sierra-Mercado, Padilla-Coreano, & Quirk, 2011; Vidal-Gonzalez, Vidal-Gonzalez, Rauch, & Quirk, 2006b). The PL and IL project to different parts of the amygdala to regulate fear behavior. The PL sends excitatory projections to the BLA, which in turn stimulates the CeA and provokes a fear response. In contrast, the IL sends projections to inhibitory intercalated (ITC) cells, which inhibit the CeA and suppress fear behavior (Vidal-Gonzalez et al., 2006b). In addition, the vmPFC in humans (homologous to the IL in rodents) responds to and is crucial for the learning of safety signals, contributing to its control over fear behavior (Christianson et al., 2012; Tanja Jovanovic, Kazama, Bachevalier, & Davis, 2012; Sangha, Robinson, Greba, Davies, & Howland, 2014).

In contrast to fear, anxiety represents a state of hyperarousal in the absence of a direct threat (Calhoon & Tye, 2015). Here, the IL cortex has a larger role than other PFC subregions; lesions of the IL have anxiolytic effects (Gonzalez et al., 2000; Lacroix, Broersen, Weiner, & Feldon, 1998; Ron M. Sullivan & Gratton, 2002), while lesions of the PL produce no change in anxiety-like behavior (Lacroix et al., 1998). In a more recent study, increased excitability in the IL (using a GABA-A antagonist) also led to anxiogenic behaviors such as decreased time exploring the open arm of an elevated plus maze. Finally, depletion of dopamine (DA) in the IL is anxiogenic (Espejo & Miñano, 1999), indicating these effects may be modulated through midbrain and dopamine circuits. Overall, more IL activity is thought to correspond with increased anxiety. This is perhaps linked with the role of the IL in the sympathetic nervous system, though more research would need to confirm this connection.

Appraisal and Cognitive Control of Stress

Regulation of stress also occurs via cognitive influences relayed from the PFC. The PFC is involved in both cognitive appraisal as well as in the perception of control over one's environment. With cognitive appraisal, the PFC helps evaluate a stressor as a challenge instead of as a threat. Re-appraisal training takes advantage of this idea; subjects are taught to view the stressor as a challenge and to view the stress responses as helpful in meeting it. The dorsal ACC and vmPFC are activated during stress reappraisal, and greater vmPFC activity correlates with decreased amygdala activity during reappraisal strategies (Kalisch, 2009).

Similar to appraisal, the perception of control over a stressor reduces the stress response. It has long been known that lack of control over a stressor leads to learned helplessness and depressive-like behaviors (Maier & Seligman, 1976; Seligman, 1972; Seligman & Weiss, 1980). Animals that are trained with inescapable stressors such as footshocks will not escape when giving the chance; instead they choose to give up and accept the shock, assuming that a behavioral response will not change the outcome. In contrast, having control over a stressful situation prevents learned helplessness, and even can prevent harmful effects of future stressors, an idea known as 'behavioral immunization' (Maier & Watkins, 2010). These effects of cognitive control are now

known to be driven by the PFC, and in particular via the vmPFC. If the vmPFC is lesioned, this stress resilience no longer occurs. Similarly, stimulation of the vmPFC is sufficient to induce stress resilience (Amat et al., 2005). These effects are mediated by vmPFC inhibitory control over brainstem and limbic areas like the dorsal raphe nucleus. Through this pathway, the vmPFC controls firing in the amygdala (Amat et al., 2005; Maier & Watkins, 2010). More recently, control over a stressor was found to increase plasticity in the mPFC, increasing the excitability of output neurons (Varela, Wang, Christianson, Maier, & Cooper, 2012). Thus, following the experience of behavior control, future stressors are able to activate the PFC more easily, allowing for better top-down regulation of stress. Overall, the protective effects of cognitive appraisal and control are mediated through PFC pathways.

Stimulation of the PFC to enhance control over stress

Knowing that the PFC is involved in controlling stress, we can aim to boost PFC performance in order to enhance resilience and foster better control over stress. For example, stimulation of the mPFC, either with optogenetics or deep brain stimulation (DBS) produces anti-depressant effects (Covington et al., 2010; Hamani, Diwan, Isabella, Lozano, & Nobrega, 2010; Hamani, Diwan, Macedo, et al., 2010; Mayberg et al., 2005). In humans, non-invasive transcranial magnetic stimulation (TMS) of the dlPFC is used as a treatment of major depression (George et al., 2010; Levkovitz et al., 2015; O'Reardon et al., 2007). Stimulation of the PFC through behavioral changes like exercise and meditation can similarly boost stress regulation (Best, 2010; Dickenson, Berkman, Arch, & Lieberman, 2012; Tang, Hölzel, & Posner, 2015). For example, meditation enhances PFC functioning while simultaneously inhibiting the amygdala, leading to better control over stress. (Deepeshwar, Vinchurkar, Visweswaraiah, & Nagendra, 2015; Dickerson, Lally, Gunnel, Birkle, & Salm, 2005; Etkin, Egner, Peraza, Kandel, & Hirsch, 2006; Tang et al., 2009; Tang, Tang, & Posner, 2016).

Altogether, the PFC, through interactions with other brain regions, produces cognitive control over the physiological and behavioral responses to stress.

The PFC is affected by stress

The PFC, while highly evolved and complex, is also extremely vulnerable to the effects of stress (Bruce S McEwen & Gianaros, 2011).

Structural Plasticity

Acute and chronic stress lead to structural plasticity of both neurons and glia in the PFC. While there is evidence in humans that stress and stress-disorders are associated with structural changes such as decreased volume in the PFC (Ansell, Rando, Tuit, Guarnaccia, & Sinha, 2012; Bremner, 2002; Drevets et al., 1997; Moreno, Bruss, & Denburg, 2017), much of what we know about the effects of stress comes from animal models (Hariri & Holmes, 2015; Bruce S. McEwen et al., 2015). Stress, especially chronic stress, leads to large changes in the morphology and functioning of mPFC neurons, including reduced mPFC spine density and dendritic atrophy (Cerqueira, Mailliet, Almeida, Jay, & Sousa, 2007; Cerqueira, Taipa, Uylings, Almeida, & Sousa, 2006; Cook & Wellman, 2004; Liston et al., 2006; Radley et al., 2006, 2004). It also appears that PFC layers that receive limbic input, such as from the hippocampus, are selectively vulnerable to

these effects of stress (Cerqueira et al., 2006). Further, these structural changes are associated with functional consequences; altered morphology such as changes in spines and branching correlate with impaired working memory (Hains et al., 2009) and impairments on other executive functioning tasks (Liston et al., 2006). Interestingly, these effects are specific to the medial region of the PFC and in fact, the opposite effect is observed in the OFC. These same stressors result in increased OFC branching and spine density (Liston et al., 2006).

Stress effects in the PFC are not limited to neurons; glia are also altered. In human patients with depression, there is observed glial cell loss and reduced cell size (Rajkowska, 2000; Rajkowska & Stockmeier, 2013). In rodents, chronic stress leads to atrophy of astrocytes (Tynan et al., 2013) and glial loss in the PFC is also sufficient to induce depressive-like behaviors (Banasr & Duman, 2008). Changes in oligodendrocytes and myelination have also been observed following chronic stressors such as social defeat stress and social isolation (Lehmann, Weigel, Elkahloun, & Herkenham, 2017; J. Liu et al., 2012; J. Liu, Dietz, Hodes, Russo, & Casaccia, 2018; J. Liu et al., 2016). These changes are functionally relevant; rescue of PFC myelination using clemastine was sufficient to rescue social behavior following stress (J. Liu et al., 2016). Similarly, ablation of oligodendrocyte progenitor cells was sufficient to induce depressive-like behaviors (Birey et al., 2015). Together, these studies suggest that glial cells such as oligodendrocytes and the myelin they produce are functionally relevant for stress behaviors.

Lastly, as previously discussed, the PFC is important for control over the stress response. Following chronic stress, GC receptors across the PFC are altered. Specifically, there is reduced GR expression in the mPFC following chronic stress (Chiba et al., 2012; Mizoguchi, Ishige, Aburada, & Tabira, 2003). Experimentally inhibiting GR expression in the mPFC also increases depressive-like behaviors and increases HPA axis responses to stress (McKlveen et al., 2013). Thus, a reduction in mPFC GRs following stress likely contributes to reduced negative feedback and control over the stress response.

Functional plasticity

Changes in PFC structure following stress leads to alterations in functional connectivity across the brain. In humans, chronic stress impairs connectivity in PFC networks, including in frontoparietal networks that mediate attention (Liston, McEwen, & Casey, 2009) and in PFC connections with the amygdala which aid in emotional control (P. Kim et al., 2013). Further, these changes correlate with impairments on attentional and emotional tasks (P. Kim et al., 2013; Liston et al., 2009). In rodents, chronic stress reduces excitatory synaptic transmission and glutamate receptor expression within the mPFC; an effect dependent on GRs (Yuen et al., 2012). Reduced mPFC spines and dendritic complexity are also thought to contribute towards this reduced excitability and PFC functioning (Cerqueira et al., 2007; Radley et al., 2004). Generally speaking, in both humans and rodents, stress weakens prefrontal cortex networks, shifting one from a state of reflective cognitive control into more reflexive, habitual control over behavior (Arnsten, 2009, 2015).

There are several possible mechanisms for reduced functioning of PFC circuits following stress. One proposed mechanism revolves around catecholamine release and receptor binding. Adrenergic receptors (alpha-1, alpha-2 and beta receptors) are found all across the PFC (Aston-Jones & Cohen, 2005; C. W. Berridge & Waterhouse, 2003). With moderate levels of stress, catecholamine release in the PFC remains low, such that catecholamines like NE bind primarily to alpha-2 receptors; these receptors have a higher binding affinity and strengthen PFC functioning

and regulation of emotional and limbic regions. In contrast, high levels of stress increases catecholamine release in the PFC significantly and this begins to engage low-affinity alpha-1 and beta receptors. Binding of these receptors rapidly reduces the firing of PFC neurons, weakens synaptic efficiency, and altogether impairs cognitive abilities (for a review, see Arnsten, 2015). In addition, chronic stress increases the complexity of PFC GABAergic interneurons, which could shift the balance of excitation and inhibition towards a more inhibitory profile (Gilabert-Juan, Castillo-Gomez, Guirado, Moltó, & Nacher, 2013). Regardless of the specific mechanism, high levels of stress impair PFC networks.

Behavioral consequences of stress that are dependent on the PFC

Behaviorally, stress leads to poor executive functioning in both humans and rodents (Liston et al., 2009, 2006). High levels of stress and binding of catecholamines to alpha-1 receptors impairs executive functions like dlPFC driven working memory and vmPFC driven self-control (Arnsten, 2009; Cerqueira et al., 2007; Qin, Hermans, van Marle, Luo, & Fernández, 2009; Ramos & Arnsten, 2007). In addition, stress impairs a crucial function of the PFC: regulation of the HPA axis, and cognitive control over stress (Shansky & Lipps, 2013). Further, functional and structural changes such as those described above correlate with greater symptom severity (Cerqueira et al., 2007; Liston et al., 2009). Overall, more changes contribute towards worse executive functioning and an overall underperformance of the PFC (for a review, see (Roozendaal et al., 2009).

Timing of Stress Exposure Modulates Stress Effects on the PFC

The timing of stress (whether early in life or in old age) differentially affects PFC structure and function. Early maturation of the PFC is highly influenced by the environment; this includes the effects of environmental stressors. Stress early in life has large effects on PFC development (Eiland & Romeo, 2013; Gratton & Sullivan, 2005; Bryan Kolb, Harker, Mychasiuk, de Melo, & Gibb, 2017; Tottenham & Galván, 2016; Zhang, Chrétien, Meaney, & Gratton, 2005). In humans, adverse childhood events (ACEs) such as childhood trauma are associated with smaller PFC volume and greater HPA axis activity (Ansell et al., 2012; Carrion & Wong, 2012; Danese & McEwen, 2012; Edmiston et al., 2011). Chronic stress in childhood (such as poverty) also leads to functional connectivity changes, with less PFC control over the amygdala (P. Kim et al., 2013; Nooner et al., 2013). In rodents, early stress also alters neural activity and morphology within the mPFC (Baudin et al., 2012; Chocyk et al., 2013; Helmeke, Ovtscharoff Jr, Poeggel, & Braun, 2001; A Muhammad, Carroll, & Kolb, 2012; Arif Muhammad & Kolb, 2011; Ovtscharoff & Braun, 2001; Thomas, Delevich, Chang, & Wilbrecht, 2020). Interestingly however, many stress induced changes in mPFC morphology are reversible in younger animals. For example, after a rest period of three weeks, in young animals, dendritic arbors and spines recover (Bloss et al., 2011). However, in middle aged or older rats, spines fail to recover over time (Bloss et al., 2010). Yet, a recovery in young animals is not always observed; for example, social isolation in adolescence reduced synaptic markers in the mPFC; this effect persisted into adulthood (Leussis & Andersen, 2008; Leussis, Lawson, Stone, & Andersen, 2008). Thus, while the young PFC may undergo more changes following stress, in some cases it can also recover more easily, indicating that the young brain is both highly susceptible and highly resilient to stress.

Stress earlier in life can also lead to changes in PFC glia. In one study, adolescence was found to be a sensitive period for stress-induced changes in PFC myelination. Specifically, social

stress during adolescence decreased myelin basic protein levels in the mPFC (Makinodan, Rosen, Ito, & Corfas, 2012). In a more recent study, maternal separation for 3 hours a day during first 3 weeks of life also led to reduced myelination in the mPFC; this effect also lasted into adulthood (Youjun Yang et al., 2017). Overall, stress at different ages can alter PFC neurons and glia, with lasting implications for behavior.

Sex Differences in Stress Effects on the PFC

In addition to timing, sex is another important factor to consider when discussing stress effects on the prefrontal cortex. For example, in humans, mPFC damage affects stress responses differently in men and women (Buchanan et al., 2010). In rodents, stress can shorten PFC dendrites in males but not females (Garrett & Wellman, 2009). However, mPFC projections to the BLA were susceptible to stress-induced changes in branching in only female rodents (Shansky, Hamo, Hof, McEwen, & Morrison, 2009). This susceptibility in females was found to be mediated by estrogen (Shansky et al., 2010), indicating that estrogens may potentiate stress-induced plasticity. This work in rodents mimics findings in humans and primates demonstrating that the dlPFC in particular is highly affected by estrogen levels in women (Shanmugan & Epperson, 2014) and by estrogen depletion (Hao et al., 2007, 2006; Rapp et al., 2003). With early life stress (ELS) (prior to adolescence), female mice displayed earlier changes in PFC parvalbumin (PV) positive interneurons than males; this shift in timing also corresponded with disrupted social behavior (Holland, Ganguly, Potter, Chartoff, & Brenhouse, 2014). Similarly, in a more recent study, ELS altered PV+ neurons in the OFC of females but not males, leading to sex-specific impairments in reversal learning (Goodwill et al., 2018). Social stressors as an adolescent however impacted male animals more than females, with males showing long-lasting changes in myelin proteins in the PFC (Leussis & Andersen, 2008). Altogether, sex interacts with the timing and specific type of stressor to produce a diverse array of changes in PFC structure and function.

Role of the PFC in Stress-related Disorders

Severe and chronic stress clearly alters PFC functioning, and indeed, the PFC is implicated in many stress-related disorders, including anxiety disorders and PTSD (for reviews see (Duval, Javanbakht, & Liberzon, 2015 & Shin & Liberzon, 2010). Structurally, PTSD patients have reduced PFC volume and grey matter density (Bremner, 2002) in both the ACC (Corbo, Clément, Armony, Pruessner, & Brunet, 2005; Kasai et al., 2008; Schuff et al., 2011; Woodward et al., 2006) and in the medial PFC (Yunchun Chen et al., 2012; Weber et al., 2013). Reduced volume in the PFC is also associated with greater PTSD symptom severity (Yunchun Chen et al., 2012; Weber et al., 2013; Woodward et al., 2006). Further, PFC volume is connected to the HPA axis and the stress response; in both youths and adults with post-traumatic stress symptoms, higher cortisol levels are correlated with reduced PFC volume (Carrion & Wong, 2012). Similar to PTSD, there is decreased ACC grey matter in patients with anxiety disorders such as panic disorder and phobia (Linares et al., 2014; Pannekoek, van der Werff, Stein, & van der Wee, 2013); however, changes in volume have not been studied in connection with anxiety symptom severity.

Functionally, both anxiety and stress disorders like PTSD show a common hypoactivation in ventromedial prefrontal regions, suggesting impairments in the top-down control of emotions and stress (Admon et al., 2013; Koenigs & Grafman, 2009). In PTSD especially, reduced mPFC activity is associated with greater symptom severity (Admon et al., 2013; Dickie, Brunet, Akerib,

& Armony, 2008; L. M. Shin et al., 2004). There is also less connectivity between the mPFC and the amygdala (Hayes, Hayes, & Mikedis, 2012; Tanja Jovanovic et al., 2012; Jennifer S. Stevens et al., 2013), supporting the idea that in these disorders, the mPFC struggles to control the arousal and fear generated by the amygdala. Further, there is impaired connectivity in frontoparietal networks like the central executive network (CEN) (Sripada et al., 2012), indicative of impaired executive functioning and cognitive control. Interestingly, in contrast with findings in the mPFC, patients with anxiety and PTSD demonstrate hyperactivation of the dorsal ACC; this activity is also associated with greater autonomic arousal and reported anxiety (Ramage et al., 2013; L. M. Shin & Liberzon, 2010). The ACC is part of the salience network, responding to threats in the environment. These disorders are characterized by hypervigilance and hyper responsiveness to negative stimuli, so it is fitting that hyperactivation of the ACC and the broader salience network is observed (Akiki, Averill, & Abdallah, 2017). Overall, stress disorders like PTSD and anxiety are associated with structural and functional changes in the PFC that ultimately lead a hyperresponsiveness to threats and impaired emotional control.

The Role of the PFC in Motivation

Functions of the PFC in motivated, goal-directed behaviors

In both humans and rodents, the PFC is crucial for goal-directed behaviors and decision making. Goal-directed behavior and motivated behavior can be thought of as simultaneous terms; both humans and animals are motivated to act for a given purpose. Most commonly, motivation is thought of in relation to achieving a reward such as a food reward or perhaps a drug. However, motivation can also apply to social situations; we can be motivated to seek out social contact, or to act to help another individual. In humans, wide-spread activity is observed across the PFC during goal-directed behaviors of all kinds (Raichle & Gusnard, 2005). The PFC helps integrate information about salience, context and value (coming from the VTA, amygdala, hippocampus and OFC, among other regions). It then uses this information to make decisions and guide actions, sending projections to the amygdala and striatum to ultimately influence behavior. In a simplified summary, three major regions (the PFC, amygdala and striatum) work together as part of a "triadic model" to create a functional network for goal-directed behaviors (Ernst & Fudge, 2009).

Functions of the PFC in motivation for food or drug rewards

It is hard to study goal directed behaviors like food and drug seeking in humans; thus, much of what we know comes from animal models. The OFC is responsible for encoding the sensory properties of rewards (Rolls, Critchley, Verhagen, & Kadohisa, 2010) and generally encodes reward value (Padoa-Schioppa & Assad, 2006). The higher value reward, the more OFC activity is observed (for a review, see Wallis, 2012). The OFC then sends that information to other parts of the cortex such as the mPFC. Within the mPFC, there are distinct and often opposing roles for the dorsal and ventral subregions (the PL and IL respectively, in rodents). Generally, the dorsal mPFC is involved in goal directed behaviors, like instrumental responding for food (Corbit & Balleine, 2003), while the ventral PFC is involved in response inhibition and stimulus-response habits (see Gourley & Taylor, 2016 for a review). This dichotomy in reward seeking behavior is similar to that observed in fear behaviors (Peters, Kalivas, & Quirk, 2009). A study in 2008 by Ishikawa and colleagues nicely demonstrates this dichotomy; inhibiting the dorsal mPFC reduced

cue-evoked reward seeking, while inhibiting the ventral mPFC led to inappropriate responding to the unrewarded cue (Ishikawa, Ambroggi, Nicola, & Fields, 2008). Thus, activation of the PL drives goal-directed approach behaviors (acting like a go-signal), while the IL typically inhibits inappropriate responses (acting as a stop-signal).

Similar findings are observed for drug seeking behaviors. Specifically, lesions of the PL inhibit drug-induced conditioned place preferences (CPPs) (Thomas M. Tzschentke & Schmidt, 1999) and reduce drug-reinstatement (Capriles, Rodaros, Sorge, & Stewart, 2003a). Furthermore, following withdrawal, cues previously associated with a drug activate the PL (West, Saddoris, Kerfoot, & Carelli, 2014). These effects are thought to be mediated by dopamine. The PL receives a dense dopaminergic projection, similar to the human dlPFC (Thomas M. Tzschentke, 2001) and blocking DA receptors in the PL is sufficient to prevent CPPs for drugs such as morphine (De Jaeger et al., 2013). In contrast with the PL, activation of the IL inhibits drug seeking behaviors (Peters, LaLumiere, & Kalivas, 2008b). In general, the IL is highly involved in drug extinction (in parallel to its role in fear (Peters et al., 2009). These opposing roles of the PL and IL in drug seeking are thought to be due to their specific projection patterns. The PL sends more outputs to the dorsal striatum and to the Nac core, while the IL sends more outputs to the ventral striatum, specifically to the Nac shell (Öngür & Price, 2000). Projections of the PL are particularly involved in the reinstatement of drug seeking; inactivation of PL terminals in the Nac and in the BLA decreases reinstatement (M. Stefanik & Kalivas, 2013; M. T. Stefanik, Kupchik, & Kalivas, 2016). Likewise, projections from the IL to the Nac shell are thought to be necessary for extinction of drug seeking behavior (Peters et al., 2008b). These projection patterns are important to consider as the field continues to pull apart PFC circuits involved in motivation.

The final PFC subregion, the ACC, plays a less direct role in goal-directed reward seeking behaviors. While there is some evidence that projections from the VTA to the ACC are involved in drug induced place preferences (Narita et al., 2010a), the ACC is more highly involved in responses guided by reward expectancy, especially when there is a forced choice or other conflict between choices (Judith Schweimer & Hauber, 2005). The ACC is especially important when evaluating how much effort to expend for a specific reward (Walton, Bannerman, Alterescu, & Rushworth, 2003). Lesions of the ACC also lead to impaired discriminated approach, with more errors when making choices (Bussey, Everitt, & Robbins, 1997). Altogether, these PFC subregions work in concert to guide appropriate goal-directed behaviors.

Functions of the PFC in socially motivated behaviors

The PFC also has a role in the top-down control of social behaviors, including social motivation (for reviews, see Bicks et al., 2015; Ko, 2017). Social motivation is thought to be just one component of complex social cognition (the mental operations that guide social behavior; (Millan & Bales, 2013). It involves social orienting, motivated social approach, interaction, social reward, and empathic behaviors (Chevallier, Kohls, Troiani, Brodkin, & Schultz, 2012). In both humans and in rodents, the mPFC is highly involved in the moderation of goal-directed social behaviors (Amodio & Frith, 2006; Ko, 2017; Krueger, Barbey, & Grafman, 2009; E. Lee et al., 2016; Lin, Adolphs, & Rangel, 2011; Yaling Yang & Raine, 2009). In humans, the vmPFC plays a critical role in social reward, motivation and social value; patients with lesions in the vmPFC show impairments in each of these categories (Anderson, Bechara, Damasio, Tranel, & Damasio, 1999). Top down projections from the mPFC to the amygdala and hypothalamus are proposed to coordinate these goal-directed social behaviors (Insel & Fernald, 2004). Studies in rodents allow

us to better pull apart the functions of specific PFC regions and circuits (Yizhar, 2012). For example, activity specifically in the mPFC corresponds with social-approach behavior (E. Lee et al., 2016). In contrast social interaction broadly activates all subregions of the PFC (Y. Kim et al., 2015). However, lesioning of the OFC disrupts a particular kind of social interaction: play behavior (Pellis et al., 2006). Lesions of the ACC also disrupts social memory and decreased social interest in animals (Rudebeck et al., 2007). More recent studies in rodents have also begun to dissect the signaling pathways involved in social behavior (Liang et al., 2015), however this is a burgeoning field, and these is much to be done.

Related to social motivation, the study of empathic behaviors has recently increased, especially in animal models (for a review, see Meyza, Bartal, Monfils, Panksepp, & Knapska, 2017). Here too, the PFC is known to play a role. In humans, lesions of the vmPFC produces apathy and decreased prosocial behavior (Barrash, Tranel, & Anderson, 2000; Krajbich, Adolphs, Tranel, Denburg, & Camerer, 2009). The ACC is also involved in emotional contagion in both humans (Singer et al., 2004) and rodents (Jeon et al., 2010; S. Kim, Mátyás, Lee, Acsády, & Shin, 2012). Prosocial behaviors such as helping behavior and harm aversion have more recently been explored in animal models (Bartal, Decety, & Mason, 2011; Bartal, Rodgers, Bernardez Sarria, Decety, & Mason, 2014; Bartal et al., 2016; Hernandez-Lallement et al., 2020). In a study from this past year, the ACC was found to be crucial for harm aversion in rodents; harm aversion was abolished with deactivation of the ACC (Hernandez-Lallement et al., 2020). Part of this thesis will explore the role of the PFC in a socially motivated prosocial task, assessing helping behavior in rodents.

Overlap of Stress and Motivation in the PFC

Generally, stress impairs motivated, goal-directed behavior and biases us towards habitual responding (Schwabe & Wolf, 2009, 2010); for a review see Hollon et al., 2015). There is an overarching idea that this bias towards habitual responding is mediated by stress-induced inhibition of the PFC (C. shan R. Li & Sinha, 2008). For example, a shift toward habitual behaviors following stress corresponds with atrophy in the mPFC (Dias-Ferreira et al., 2009). This shift is also dependent on GC and NE actions within the PFC (Arnsten, 2009; Arnsten, Wang, & Paspalas, 2012), and can be blocked by administration of a beta-adrenergic receptor, propranolol, prior to the stressor (Schwabe, Höffken, Tegenthoff, & Wolf, 2011). Similarly, concurrent activation of GC and NE systems is sufficient to promote habit formation and to disrupt goal-directed decisions driven by the PFC (Schwabe, Tegenthoff, Höffken, & Wolf, 2012). These findings have also been replicated in rodents. Chronic unpredictable stress causes habit formation, as well as alterations in corticostriatal circuits associated with habit vs. goal behavior (Braun & Hauber, 2013; Dias-Ferreira et al., 2009). Interestingly, similar changes in corticostriatal structure and function are observed in humans during stress-associated habitual behavior, suggesting overlap between human and rodent neurobiology (Soares et al., 2012).

Stress can also directly affect drug seeking behaviors which are perhaps goal-directed initially but become habitual over time. Increases in stress produces impaired self-control and increases in impulsivity, both of which are associated with a risk of substance abuse (Hayaki, Stein, Lassor, Herman, & Anderson, 2005; Sinha, 2001; Wills, Walker, Mendoza, & Ainette, 2006). In rodents, it is well known that stress increases drug seeking and relapse (Mantsch et al., 2016), and importantly, inactivation of the dorsal mPFC blocks this stress-induced reinstatement (McFarland, Davidge, Lapish, & Kalivas, 2004). These effects may be mediated by the mesocortical DA

system, which modulates activity in the PFC during stress (Ariel Y Deutch et al., 1990; R M Sullivan & Gratton, 1998; Thierry et al., 1976). For example, injecting a DA antagonist in the PL prevents foot-shock induced cocaine reinstatement, implicating the mesocortical pathway in stress-induced relapse (Capriles et al., 2003a). Furthermore, social defeat stress alters feedback from the PFC to these same aminergic pathways, contributing towards stress-induced self-administration of drugs (Miczek, Covington, Nikulina, & Hammer, 2004). In a more recent study, stress-induced cocaine seeking was found to be dependent on a CRH regulated DA projection from the VTA to the PL. Significantly, inhibiting this pathway prevented stress induced cocaine seeking. Further, injecting a CRH1 receptor antagonist into the VTA recapitulated this effect, preventing shock-induced drug seeking (Vranjkovic et al., 2018). Overall, it's thought that stress activates VTA DA projections to the dorsal mPFC, which, in concert with impaired feedback, induces reinstatement via a glutamatergic projection to the Nac core (McFarland et al., 2004; Miczek et al., 2004).

Role of the PFC in motivation disorders

The PFC is known to be involved in disorders of motivation, including in major depressive disorder (MDD) and addiction (Goldstein & Volkow, 2011; Koenigs & Grafman, 2009; Russo & Nestler, 2013). Indeed, stress also plays a critical role in both of these disorders, further highlighting an overlap between stress and motivation neurobiology.

Major Depressive Disorder (MDD)

In patients with MDD, there is reduced PFC volume, including both grey and white matter, in the OFC and mPFC (Bremner et al., 2002; Drevets, Price, & Furey, 2008; Drevets et al., 1997; Lai, Payne, Byrum, Steffens, & Krishnan, 2000). Patients with depression not only have reduced numbers of neurons (Rajkowska, O'Dwyer, Teleki, Stockmeier, & Miguel-Hidalgo, 2007) and reduced numbers of excitatory synapses (Duric et al., 2013) but they also have glial cell loss and reduced glial cells sizes (Rajkowska, 2000; Rajkowska & Stockmeier, 2013). Both oligodendrocytes and astrocytes are impaired, specifically within the vmPFC (Rajkowska et al., 2018, 2015). These structural changes translate to functional effects. Specifically, MDD patients demonstrate reduced blood oxygenation levels during executive functioning tasks (Taylor Tavares et al., 2008) and altered functional connectivity, especially within the ventral PFC (for review see Mulders, van Eijndhoven, Schene, Beckmann, & Tendolkar, 2015). Interestingly, deep brain stimulation (DBS) of white matter tracts in the ventral PFC, particularly those adjacent to the subgenual cingulate cortex, can induce remission of otherwise treatment-resistant depression (Mayberg et al., 2005). In animal models as well, stimulation of the vmPFC produces anti-depressive effects, such as reductions in anhedonia and increases in social interaction (Covington et al., 2010; Hamani, Diwan, Isabella, et al., 2010; Hamani, Diwan, Macedo, et al., 2010; Hamani et al., 2012). One hypothesis is that treatments such as DBS, as well as TMS, exert antidepressant effects by activating descending PFC pathways (Mayberg, 2009). A recent study tested this hypothesis using a rodent model; indeed, stimulation of descending projections from deep layers of the PL cortex was sufficient to induce anti-depressant effects (Kumar et al., 2013). These findings are promising and help us better understand the complex role of the PFC in depression, which ultimately could lead to improved treatments.

Addiction

The PFC is also highly involved in addiction (for a review, see Goldstein & Volkow, 2011). Structural imaging has identified reduced PFC thickness across addiction populations, especially in the dorsolateral PFC (Chanraud et al., 2007; Chanraud, Pitel, Rohlfing, Pfefferbaum, & Sullivan, 2010; Fein et al., 2002). Greater changes in dlPFC structure are also associated with longer duration and severity of drug use, and correlate with worse executive functioning over time (Chanraud et al., 2007). Functionally, long term drug use is associated with decreased PFC activity, which in turn is associated with higher craving (Kober et al., 2010). When trying to suppress cravings, activity in ventral PFC regions involved in inhibitory control is inversely correlated with inhibition of limbic regions (Volkow et al., 2010). Generally, with less vmPFC activity, cravings cannot be suppressed as well. Further, those experiencing addiction demonstrate greater impulsivity, which is associated with lateral PFC dysfunction (Crews & Boettiger, 2009). Overall, the reduced self-control observed in addicts is associated with reduced PFC functioning, and greater likelihood of relapse (Seo & Sinha, 2014). These impairments in PFC regions involved in executive control are also mirrored by enhancements in areas involved in value and salience. For example, the OFC is highly activated during craving (Kufahl et al., 2008). This fits with prior literature, as the OFC is activated for highly valued and expected rewards (in this case, drugs) (Wallis, 2012). Similarly, the ventral ACC is highly activated when addicts observe drug-related cues (Garavan et al., 2000), suggesting they are hyper attuned to stimuli in the environment associated with drug abuse. Lastly, as with MDD, stimulation studies have provided additional insights into the functional role of the PFC in addiction. In particular, enhancing activity in the dorsolateral PFC either through TMS or tDCS reduces craving and inhibits drug-cued reactivity in addicts (Conti & Nakamura-Palacios, 2014; Shen et al., 2016). As we continue to extend our knowledge of the prefrontal cortex and its role in addiction, we can better treat substance use disorders, helping suppress inappropriate drug seeking and preventing relapse.

Part 4: Introduction Summary

In this introduction, I have reviewed the stress response, explained the relationship between stress and motivated behaviors, and described the prefrontal cortex systems that connect the two. A central theme of this dissertation will be to explore the role of prefrontal cortex circuitry in both stress and motivated behaviors, using rodent models. The PFC is highly connected to regions involved in emotional circuitry (such as the amygdala and hippocampus) as well as regions crucial for motivation and reward (such as the striatum and VTA). In chapter two, I will elaborate on part of this circuitry, describing VTA projections to PFC subregions, as well as to other limbic brain areas. Another critical theme will be to explore the links between stress and motivation across development; the PFC has a prolonged period of plasticity and may be especially sensitive to stress during peri-adolescence. In chapter three, I will describe the neural circuits involved in a prosocial behavior driven by various distress and how they change across development and under different conditions. Finally, in chapter four, I will explore the effects of an acute traumatic stressor on PFC oligodendrocytes and myelination, describing effects both in the short and long term and how they might relate to fear and avoidance behaviors. Overall, this body of work will add to a growing literature relating stress and motivation circuitry.

Chapter 2: Relative contributions and mapping of ventral tegmental dopamine and GABA neurons by projection target in the rat

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Abstract

The ventral tegmental area (VTA) is a heterogeneous structure that contains not only dopamine (DA) but also GABA and glutamate neurons that project to many different target brain regions. Here we combined retrograde tracing with immunocytochemistry against tyrosine hydroxylase (TH) or glutamate decarboxylase (GAD) to systematically compare the proportion of dopaminergic and GABAergic VTA projections to ten target nuclei. We investigated the VTA projections to anterior cingulate, prelimbic, and infralimbic cortex; nucleus accumbens core, medial shell, and lateral shell; anterior and posterior basolateral amygdala; ventral pallidum; and periaqueductal gray. Overall, there was a strong *non*-dopaminergic component to VTA efferents; in projections to all regions except the nucleus accumbens core, non-DA neurons made up more than 50% of the projection. Additionally, GABA neurons contributed no more than 20% to each projection, with the exception of the projection to the ventrolateral periaqueductal gray, where the GABAergic contribution approached 50%. Therefore, there is likely a significant glutamatergic component to many of the VTA's projections. We also found that VTA cell bodies retrogradely labeled from the various target brain regions had distinct distribution patterns within the VTA, including in the locations of DA and GABA neurons. Despite this patterned organization, VTA neurons comprising these different projections were intermingled and never limited to any one sub-region. The anatomical information we provide here will aid in both the interpretation and the guidance of behavioral studies. A better understanding of VTA sub-populations, and especially the contribution of non-DA neurons to projections, will be critical for future work.

Keywords: ventral tegmental area, dopamine, GABA, retrograde, immunocytochemistry

List of Abbreviations

ABLA
: Anterior basolateral amygdala

ACC
: Anterior cingulate cortex

AL
: Anterior-Lateral

AP
: Anterior-Posterior

BLA
: Basolateral amygdala

CIN
: Cholinergic interneuron

Contra
: Contralateral

CPP
: Conditioned place preference

DA
: Dopamine

DV
: Dorsal-Ventral

FITC
: Fluorescein

GABA
: Gamma-aminobutyric acid

GAD
: Glutamic Acid Decarboxylase

IL
: Infralimbic cortex

IPF
: Interpeduncular fossa

Ipsi
: Ipsilateral

ML
: Medial-Lateral

mPFC
: Medial prefrontal cortex

MT
: Medial terminal nucleus of the accessory optic tract

NAc
: Nucleus accumbens

NAcC
: Nucleus accumbens core

NAcLs
: Nucleus accumbens lateral shell

NAcMs
: Nucleus accumbens medial shell

PBLA
: Posterior basolateral amygdala

PBP
: Parabrachial pigmented area

PL
: Posterior Lateral

PN
: Paranigral nucleus

PrL
: Prelimbic cortex

TH
: Tyrosine Hydroxylase

vlPAG
: Ventrolateral periaquaductal gray

VMAT2
: Vesicular monoamine transporter-2

VP
: Ventral pallidum

VTA
: Ventral tegmental area

Introduction

The VTA is critically involved in the neural processes that underlie motivation and reward (H. L. Fields, Hjelmstad, Margolis, & Nicola, 2007; Morales & Margolis, 2017; R. A. Wise & Rompre, 1989). For example, stimulation of VTA dopamine (DA) neurons can produce positive reinforcement and conditioned place preference (Adamantidis et al., 2011; Ilango et al., 2014; Steinberg et al., 2014; Tsai et al., 2009). In addition, blocking glutamatergic drive onto VTA neurons is sufficient to prevent the rewarding effects of systemically administrated drugs of abuse such as morphine and cocaine (G. C. Harris & Aston-Jones, 2003; G. C. Harris, Wimmer, Byrne, & Aston-Jones, 2004). Both these lines of evidence indicate that the firing of VTA neurons produces positive reinforcement.

However, while the VTA is thought of as a primarily dopaminergic region, almost half of its constituent neurons are *not* dopaminergic (Margolis, Lock, Hjelmstad, & Fields, 2006; Root et al., 2016; Swanson, 1982; Yamaguchi, Wang, Li, Ng, & Morales, 2011) and DA neurons are actually in the minority in most VTA projections (Swanson, 1982). While there is evidence for local GABAergic connections (S. W. Johnson & North, 1992; Omelchenko & Sesack, 2009), GABA, as well as glutamate, VTA projection neurons are clearly more common than initially hypothesized, and these neurons are sufficient to drive behavior independently from DA (Carr & Sesack, 2000; Gorelova, Mulholland, Chandler, & Seamans, 2012; Yamaguchi et al., 2011; for a review see Morales & Margolis, 2017). For instance, VTA GABA neurons fire in response to aversive stimuli and cues predicting a subsequent reward (J. Y. Cohen, Haesler, Vong, Lowell, & Uchida, 2012; Tan et al., 2012). The fact that DA neurons fire in response to the same cues is inconsistent with the possibility of the cue responsive GABA neurons being local interneurons and raises the possibility that they are actually projection neurons. Furthermore, stimulating GABA projections from the VTA to the nucleus accumbens (NAc) enhances associative learning (Brown et al., 2012). The sheer number of non-DA VTA projection neurons and the prima facie evidence for their role in behavior, independent of DA neurons, calls for more careful characterization of these subpopulations.

Rather than being a homogeneous population, the VTA contains multiple sub-regions: the parabrachial pigmented area (PBP), paranigral nucleus (PN), and the midline interfascicular nucleus, and rostral and caudal linear nuclei. Distinct subsets of VTA neurons also project to different target structures, with little axon collateralization (Lammel, Lim, & Malenka, 2014; Margolis, Lock, Hjelmstad, et al., 2006; Swanson, 1982). For example, there are diverse VTA projections to the anterior cingulate cortex (ACC), medial prefrontal cortex (mPFC), nucleus accumbens (NAc), ventral pallidum (VP), basolateral amygdala (BLA), and ventrolateral periaqueductal gray (vlPAG) (Carr & Sesack, 2000; Fallon, Schmued, Wang, Miller, & Banales, 1984; Fields et al., 2007; Margolis, Lock, Chefer, et al., 2006; Pierce & Kumaresan, 2006; Swanson, 1982). Although tracing studies have been done for many of these projections individually, here we have examined the projections to each of these nuclei in parallel, and quantified not only DA but also GABA contributions, using the same methodology. A number of prior studies focused on the distribution of axon fiber terminals within target nuclei, which does not provide information about the distribution of cell bodies within the VTA (Beckstead, Domesick, & Nauta, 1979; Kirouac & Pittman, 2000). This information is important because there is evidence that there are differences in behavioral contributions of the VTA along the anterior-posterior (AP) and medial-lateral (ML) axes (Beier et al., 2015; Ikemoto, 2007; Lammel et al., 2012; Taylor et al., 2014; reviewed in Barrot 2014).

Here we systematically investigated two aspects of the topography of VTA projection neurons. First, we quantified the distribution of the cell bodies within the VTA that contribute to each investigated projection. Second, we determined the proportion of those neurons that expressed either tyrosine hydroxylase (TH), the rate-limiting enzyme of DA production, or the 67 kDa isoform of glutamate decarboxylase (GAD), the enzyme that converts glutamate to GABA. We defined the VTA as the entire region overlapping with the A10 group, and to facilitate comparisons to AP and ML behavioral differences, we specifically examined projections in three distinct VTA regions of interest in horizontal brain slices: posterior lateral, anterior lateral and medial VTA, at multiple dorsal ventral (DV) levels. Consistent with prior reports, the neurons comprising different projections are concentrated in particular sub-regions of the VTA, yet even within these sub-regions, the proportions of dopaminergic and GABAergic neurons contributing to each projection varied widely. Interestingly, we also found novel differences in the VTA projections to adjacent target regions that have not been discriminated in prior studies, such as the anterior and posterior BLA, and between sub-regions of the mPFC. Together, these data show distinct patterns of projection neuron distributions and their phenotypes arising from the VTA.

Materials and Methods

All experimental procedures conformed to National Institutes of Health and Ernest Gallo Clinic and Research Center animal care policy standards and were approved in advance by the University of California, San Francisco Institutional Animal Care and Use Committees.

Injection of tracer

Most experiments were completed in male Sprague Dawley rats (n = 80 Harlan), weighing 275-375g. In a small subset of experiments, younger animals weighing 80 - 100g (n = 10 Harlan) were used; we previously demonstrated physiological properties, including those that sort with projection target, of VTA neurons in rats of this age are not different from observations in adult rats. (Margolis, Mitchell, Ishikawa, Hjelmstad, & Fields, 2008). Therefore, all animals were analyzed together. Rats were anesthetized with isoflurane and mounted onto a stereotaxic frame (Model 900 stereotaxic frame, David Kopf Instruments, USA). The skull was exposed and a craniotomy was performed unilaterally above the stereotaxic coordinates of one of the projection nuclei of the VTA (schematic in Figure 1a). No obvious hemispheric differences were detected, so all data for a specific nucleus was pooled. For animals undergoing protocol A (Table 1), a stainless steel 26 gauge guide cannula (C315GS-4/SPC, PlasticsOne Inc.) was then lowered to the chosen stereotaxic coordinates. A stainless steel 33 gauge injection cannula (C315FDS-4/SPC, PlasticsOne Inc.), connected by tubing to a 1 ml Hamilton syringe, was front-filled with retrograde tracer, Neuro DiI (7% in EtOH; Biotium), and then attached to an infusion pump. The injection cannula was inserted into the guide cannula, and 0.3 µl of Neuro DiI was injected at a rate of 0.1 µl/min. For animals undergoing protocol B, a nanoliter injector (Nanoject II, Drummond Scientific Co.) was prepared with a glass capillary injector tip front-filled with Neuro DiI. The glass injector tip was lowered to the chosen stereotaxic coordinates, and then 70-100 nl of Neuro DiI was injected at a rate of 18-20 nl/15 seconds (72-80 nl/min) (Table 1). Surgical coordinates for all tracer injections are reported in Table 2.

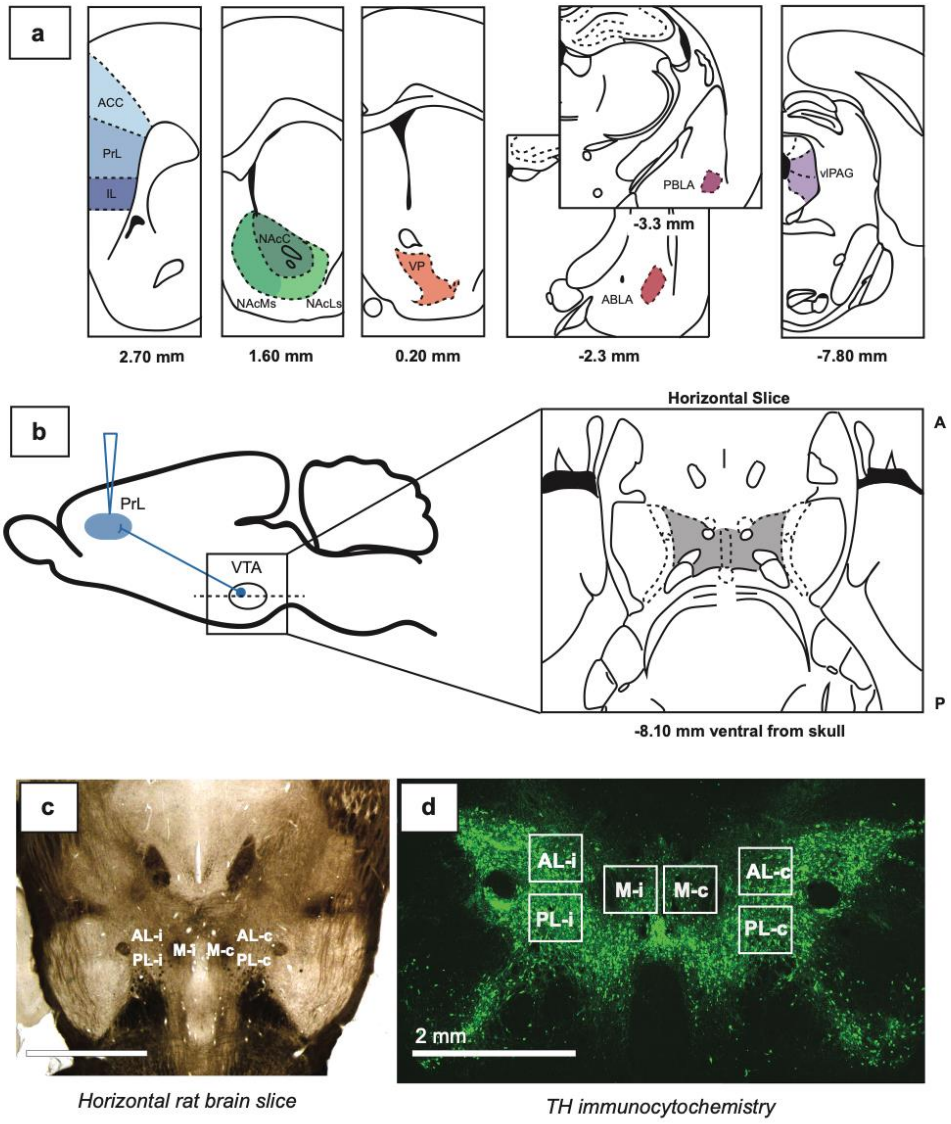

Figure 2-1. Methods Summary.

(a) Representations of coronal sections of the target nuclei receiving retrograde tracer injections. Coordinates are anterior-posterior from bregma (Paxinos & Watson, 1998). For surgical coordinates, see Table 2. (b) Seven days after tracer injection (left), horizontal sections of the VTA were made for analysis of the distributions of retrogradely labeled neurons within the VTA (right) (-8.10 mm ventral from the skull surface) (Paxinos & Watson, 1998). (c) Example brightfield image of a horizontal slice containing the VTA, 50 μm thick. The sampling regions are indicated (-8.10 mm ventral) Scale bar 2 mm. (d) Fluorescent image of a horizontal slice containing the VTA, 50 μm thick, immunocytochemically labeled for TH (green) and depicting the sampling regions (-8.10 mm ventral).

Table 2-1. Protocol details across animals

Protocol Name	Tracer Injection Method	Perfusion Method	Image Collection	Number of Animals by target
A	Injection cannula connected to 1 ml Hamilton syringe	10% formalin	Zeiss LSM510 META	ACC: 4 PrL: 4 NAcC: 1 VP: 2 vlPAG: 3
B	Nanoject injector	4% paraformaldehyde	Zeiss Axioskop 2	ACC: 1 PrL: 5 IL: 7 NAcC: 2 NAcMs: 3 NAcLs: 3 VP: 4 ABLA: 3 PBLA: 5 vlPAG: 3

ACC, anterior cingulate cortex; PrL, prelimbic cortex; IL, infralimbic cortex. For other abbreviations, see list.

Perfusion and histology

At least seven days after the surgery (and no more than 14 days), rats were deeply anesthetized with sodium pentobarbital 200 mg/kg (Euthasol ®, Vibrac AH Inc.), and transcardially perfused with saline solution, followed by either 10% buffered formalin (245-684, Fisher Scientific) or fresh 4% paraformaldehyde. Brains were removed, and post-fixed for a minimum of two and maximum of four hours in either 10% formalin or 4% paraformaldehyde (protocol A or B, respectively).

Coronal brain slices (50 µm) containing the injection region were cut using a Vibratome (Leica VT1000S), with the exception of the vlPAG, where horizontal sections were cut due to proximity to the VTA. Alternating slices were stained with cresyl violet in order to better visualize brain structures, while unstained slices provided a better visualization of DiI spread. All slices containing DiI were then mounted on glass slides, cover-slipped (Vectashield H-1000, Vector Laboratories Inc.), and imaged using a stereomicroscope (Zeiss Stemi 2000-C) at 6.5X magnification. Injections were considered on target if the densest area of dye fell within the target brain region with little to no (less than 5%) spread into neighboring regions (Figure 1a and see panels a and b in Figures 3-12). Animals that were off target were not included in the analysis.

If the injection was on target, horizontal brain slices (50 µm) containing the VTA were cut using a Vibratome (Leica VT1000S). VTA slices spanning the dorsal to ventral extent of the VTA (approximately 7.60 mm to 8.42 mm ventral from skull surface according to Paxinos & Watson, 1998) were immunocytochemically processed for either TH or GAD (Figure 1b-d). Every second slice was processed, and among these, labeling was alternated between TH and GAD. In each

animal, the initial, most ventral VTA slice was chosen by the geometry of the interpeduncular fossa (IPF) (approximately 8.60 mm to 8.42 mm ventral to bregma). Slices were considered dorsal to the VTA when there were no TH(+) cell bodies detected in either of the lateral counting regions. This approach yielded approximately 4 slices per antibody treatment per animal.

Table 2-2. Surgical Coordinates for Unilateral Tracer Injections.

Nucleus Targeted	Coordinates, from bregma (DV from skull surface)
ACC	AP = +1.6mm; DV = -3.5mm; ML = ±0.6mm
PrL	AP = +3.2mm; DV = -4.2mm; ML = ±0.8mm
IL	AP = +2.7mm; DV = -5.8mm; ML = ±0.8mm
NAcC	AP = +1.7mm; DV = -6.6mm; ML = ±1.6mm
NAcMs	AP = +1.7mm; DV = -7.5mm; ML = ±0.7mm
NAcLs	AP = +1.7mm; DV = -8.1mm; ML = ±2.0mm
ABLA	AP = -2.3mm; DV = -8.7mm; ML = ±4.8mm
PBLA	AP = -3.3mm; DV = -8.3mm; ML = ±4.8mm
VP	AP = -0.2mm; DV = -8.5mm; ML = ±2.4mm
vlPAG	AP = -7.8mm; DV = -6.0mm; ML = ±0.6mm

For all abbreviations, see list.

Immunocytochemistry

Immunofluorescence was used to detect TH, the rate-limiting enzyme in the production of DA, or the 67 kDa isoform of GAD, the enzyme that converts glutamate into GABA. All immunocytochemical reactions were done on free-floating sections. Antibodies were diluted in 0.1M PBS (pH 7.4). Sections were incubated for 2 hr in 5% normal goat serum (5% in 0.1 M PBS; S-1000, Vector Laboratories Inc.), then incubated overnight at 4°C in either rabbit anti-TH 1:200 (AB152, EMD Millipore) or mouse anti-GAD67, clone 1G10.2 1:200 (MAB5406, EMD Millipore) (Table 3a). For a subset of sections, tissue was maintained in primary antibody at room temperature for the first two hours of the anti-GAD67 incubation. Following rinses in PBS (6 x 10 min), sections were incubated for two hours in secondary antibody (Table 3b), either Fluorescein (FITC)-conjugated goat anti-rabbit 1:500 (111-095-03, Jackson ImmunoResearch Labs Inc.), or biotinylated goat anti-mouse 1:500 (BA-9200, Vector Laboratories), followed by FITC-conjugated Avidin D (SP-2040, Vector Laboratories). Sections were then rinsed in PBS (6 x 10 min), mounted on glass slides, and cover-slipped (Vectashield H-1000, Vector Laboratories Inc.).

Antibody characterization

The polyclonal rabbit anti-TH antibody used here to immunolabel DA neurons in the VTA has been used previously in our laboratory (Berthet et al., 2014; Hjelmstad, Xia, Margolis, & Fields, 2013; Margolis, Coker, Driscoll, Lemaître, & Fields, 2010; Margolis, Toy, Himmels, Morales, & Fields, 2012). It has also been characterized in the VTA of zebra finches (Gale, Person, & Perkel, 2008). This antibody detects a single band of 62 kDa on Western blots in most species, which corresponds to TH (manufacturer's technical information). The immunolabeling pattern of this antibody within the VTA here (Figure 1d) was very similar to that in another study in which

VTA DA neurons in the Wistar rat were immunolabeled with the same antibody (K. Yang et al., 2009). This antibody appears in the Journal of Comparative Neurology Antibody Database.

The monoclonal mouse anti-GAD67 antibody used here to immunolabel GABA neurons in the VTA has also been used previously in our laboratory (Margolis, Lock, Chefer, et al., 2006; Margolis et al., 2012; Margolis, Lock, Hjelmstad, et al., 2006). This mouse antibody against GAD has been shown to react with the 67 kDa isoform of GAD from rat, mouse, and human (manufacturer's technical information). It displayed no detectable cross reactivity with the 65 kDa isoform of GAD on Western blots of rat brain lysate compared to antibody AB1511 (Chemicon) that reacts with GAD65 and GAD67 (Biancardi, Campos, & Stern, 2010). This antibody appears in the Journal of Comparative Neurology Antibody Database.

Table 2-3. Primary Antibodies Used for Immunocytochemistry

Antibody name	**Immunogen**	**Manufacturer, cat number, reference number, host**	**Dilution**
Anti-tyrosine hydroxylase antibody	Denatured tyrosine hydroxylase from rat pheochromocytoma (62kDa). UniProt Number: P04177	EMD Millipore, Billerica, MA Cat# AB152, RRID:AB_390204 (Rabbit polyclonal IgG)	1:200
Anti-GAD67 antibody, clone 1G10.2	Recombinant GAD67 protein (67kDa). UniProt Number: Q99259	EMD Millipore, Billerica, MA Cat #MAB5406, RRID:AB_2278725 (Mouse monoclonal IgG2A)	1:200

Table 2-4. Secondary Antibodies Used for Immunocytochemistry

Conjugate and host	**Against**	**Dilution**	**Supplier**
Fluorescein (FITC) (Goat)	Anti-rabbit (H + L)	1:500	Jackson ImmunoResearch, West Grove, PA Cat# 111-095-003
Biotin (Goat)	Anti-mouse (H + L)	1:500	Vector Laboratories Cat# BA-9200
Fluorescein (FITC) - Avidin D		1:500	Vector Laboratories Cat# SP-2040

Microscopic imaging and cell counting

Immunostained sections were examined and images for analysis were obtained using a confocal microscope (Zeiss LSM510 META; LSM Image Browser, Zeiss) or a widefield fluorescence microscope (Zeiss Axioskop 2; Neurolucida, MBF Bioscience) (Table 1). Images for figures were obtained on a Nikon confocal microscope (Nikon Ti, Micro-Manager). For cell counting, we placed three sampling windows within the VTA per hemisphere and counted on both ipsilateral and contralateral sides. Specifically, one sampling window was in the anterior lateral VTA (AL), one in the posterior lateral VTA (PL), and one in the medial VTA (M) (Figure 1c and d). Each counting window was 368 x 368 µm. The medial terminal nucleus of the accessory optic

tract (MT) and the IPF were used as landmarks to consistently localize counting regions across slices and animals. The rostral border of the VTA was taken as the level of the caudal tip of the mammillary tract. Counting of DiI(+) cells and quantification of co-localization with TH(+) or GAD(+) was manually completed in ImageJ (NIH). A subset of animals was blind counted by two or more experimenters in order to ensure that cell counting was consistent across observers.

Cells were considered DiI(+) if there was punctate labeling in the red channel distributed throughout the soma and very proximal dendrites that was higher than background fluorescence, sufficient to readily detect neuronal shape, and if this pattern was absent in the FITC fluorescent channel. To estimate the contribution of each counting window to the overall projection, the percentage of DiI was calculated as the number of DiI(+) cells in that window divided by the total number of DiI(+) cells for that animal. To determine the percentage of VTA DA or GABA neurons that project to a specific brain region, we counted the number of DiI(+) neurons within each sampling window, and determined the proportion of those cells that were co-labeled for either TH or GAD. Co-labeling data are presented as overall percent for each target region in Figure 2 (e.g. (Total DiI(+)&TH(+))/(Total DiI(+))) as well as percent co-labeled within a given window (e.g. Figure 3e and Figure 3g). All results are presented as mean ± S.E.M.

Results

We labeled VTA projections with the retrograde transport of the tracer DiI, a lipophilic label that is passively incorporated into the plasma membrane of cells, and then quantified the distribution of labeled neurons within the VTA. Furthermore, we quantified the co-localization of immunocytochemical labeling for TH or GAD in DiI(+) neurons as markers for DA and GABA neurons, respectively. We investigated VTA projections to anterior cingulate cortex (ACC), prelimbic cortex (PrL), infralimbic cortex (IL), nucleus accumbens core (NAcC), nucleus accumbens medial shell (NAcMs), nucleus accumbens lateral shell (NAcLs), ventral pallidum (VP), anterior basolateral amygdala (ABLA), posterior basolateral amygdala (PBLA), and the ventrolateral periaqueductal gray (vlPAG) (Figure 1a, Table 2). We completed analysis of 3-12 animals per projection (average five animals). All injections were unilateral into either the left or right hemisphere; data were grouped together as we observed no hemispheric differences. We counted labeled neurons within six sampling windows (three ipsilateral, three contralateral) in each analyzed horizontal slice through the dorsal-ventral (DV) axis of the VTA (Figure 1d). We made horizontal sections as they facilitate direct comparison of medial, anterior lateral and posterior lateral regions. While raw cell counts are highly dependent upon injection volume, tracer diffusion, and anatomy of injection site, the highest overall cell counts were seen in VTA projections to the VP (110 ± 14 cells ipsi, 20 ± 9 cells contra) and ACC (99 ± 19 ipsi, 50 ± 5 contra, on average). The projections from the VTA to the NAc sub-regions and to the vlPAG also had relatively higher counts, with 60-75 cells in the ipsilateral projection, on average (NAcC: 67 ± 8 cells ipsi, 7 ± 3 cells contra; NAcMs: 75 ± 18 cells ipsi, 16 ± 7 cells contra; NAcLs: 60 ± 16 cells ipsi, 2 ± 1 cells contra; vlPAG: 66 ± 18 cells ipsi, 49 ± 14 cells contra). In contrast, the sparsest overall counts were in VTA projections to the PrL (31 ± 5 ipsi, 6 ± 1 contra) and IL (26 ± 4 ipsi, 3 ± 1 contra).

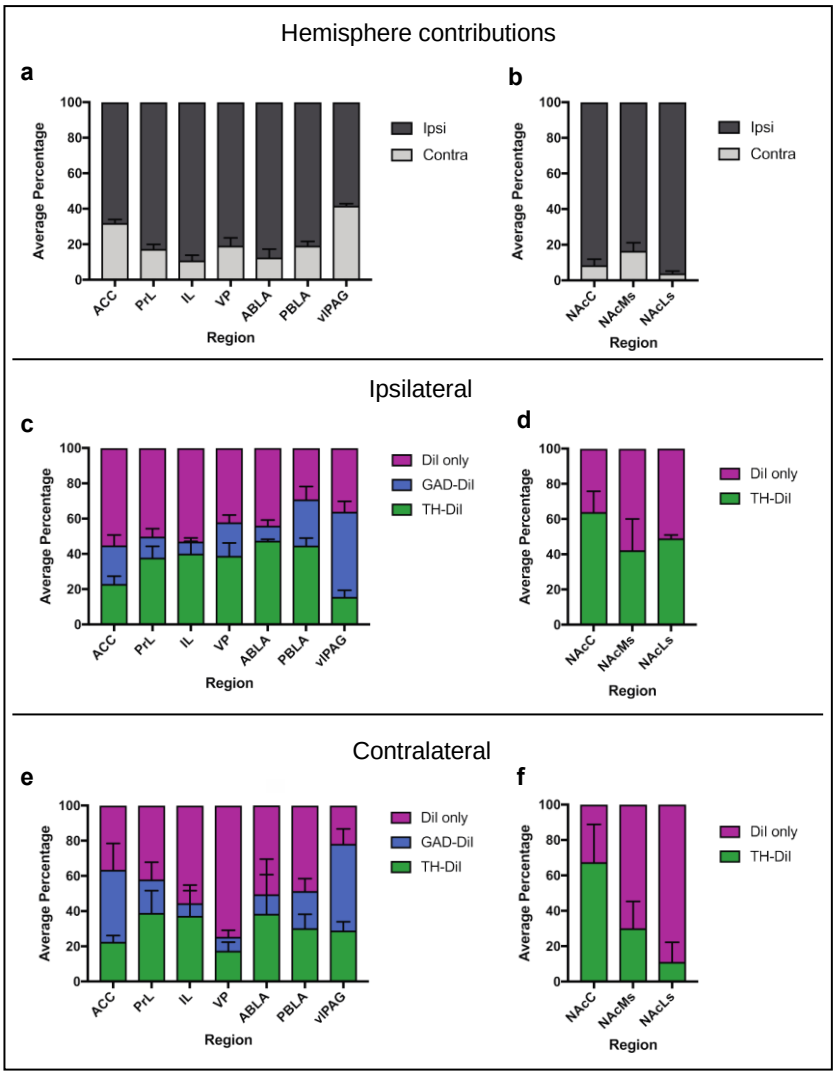

Figure 2-2. Overall quantification of retrogradely labeled VTA neurons for each projection. Laterality, and dopaminergic and GABAergic contributions are shown. (a) Ipsilateral (ipsi) and contralateral (contra) VTA projection densities for ACC, PrL, IL, VP, ABLA, PBLA, and vlPAG. (b) Ipsilateral and contralateral VTA projection densities for NAcC, NAcMs, and NAcLs. (c) The proportions of ipsilateral retrogradely labeled neurons that were co-labeled with either TH (TH-DiI) or GAD (GAD-DiI) for ACC, PrL, IL, VP, ABLA, PBLA, and vlPAG. (d) The proportions of ipsilateral retrogradely labeled VTA neurons that project to the NAc sub-regions and were co-labeled for TH (TH-DiI). (e) The proportions of contralateral retrogradely labeled neurons that were co-labeled with either TH (TH-DiI) or GAD (GAD-DiI) for ACC, PrL, IL, VP, ABLA, PBLA, and vlPAG. (f) The proportions of contralateral retrogradely labeled VTA neurons that project to the NAc sub-regions and were co-labeled for TH (TH-DiI). All results are presented as mean ± S.E.M.

Cell counts to the ABLA and PBLA fell in the middle of the range (ABLA: 46 ± 8 cells ipsi, 7 ± 3 cells contra; PBLA: 55 ± 5 cells ipsi, 14 ± 2 cells contra). Sparser projections, especially contralateral projections, produced some variability in the quantification of TH and GAD co-localization in some cases.

Cortical projections

The medial walls of the rodent frontal cortex are involved in emotional and reward related behaviors (Etkin, Egner, & Kalisch, 2011b; T M Tzschentke, 2000). This region consists of the anterior cingulate cortex (ACC) and the medial prefrontal cortex (mPFC). Previous studies of VTA projections have not differentiated prelimbic (PrL) and infralimbic (IL) cortices within the mPFC (D B Carr & Sesack, 2000; Chandler, Lamperski, & Waterhouse, 2013; A. Y. Deutch et al., 1991; Fallon et al., 1984; Seroogy, Dangaran, Lim, Haycock, & Fallon, 1989), but since the PrL and IL have different projection targets and are implicated in different roles in fear expression and drug seeking behavior (Gourley & Taylor, 2016b; Moorman, James, McGlinchey, & Aston-Jones, 2015; Peters, LaLumiere, & Kalivas, 2008a; Vidal-Gonzalez, Vidal-Gonzalez, Rauch, & Quirk, 2006a), we investigated differences between them here.

Anterior cingulate cortex

Ipsilateral projections from the VTA to the ACC were modestly denser than contralateral projections: 68 ± 2% of DiI labeled VTA cell bodies were ipsilateral to the injection site (Figure 2a, Figure 3c). DiI(+) cells were relatively evenly distributed across counting windows within the ipsilateral or contralateral sides (Figure 3c). For both ipsilateral and contralateral connections, 23 ± 4% of ACC-projecting VTA neurons were co-localized with TH labeling (Figure 2c,e); the highest percentages of DiI(+)-TH(+) neurons were localized in the dorsal lateral regions (Figure 3d,e). Among contralateral projections, ACC-projecting neurons in the posterior-lateral VTA had a greater percentage of TH co-labeling (32 ± 4%) with lower co-labeling rates in the anterior-lateral and medial regions (13 ± 5% and 10 ± 2% respectively) (Figure 3e). Interestingly, many of the contralateral ACC-projecting VTA neurons were co-localized with GAD (41 ± 15%) compared to the ipsilateral projection (22 ± 6%) (Figure 2c,e). For both ipsilateral and contralateral ACC-projecting VTA neurons, the DiI co-localization with GAD was markedly enriched in the more dorsal slices compared to ventral slices, where little co-localization was detected (Figure 3f,g).

Prelimbic cortex

In contrast to ACC-projecting VTA neurons, projections from the VTA to the PrL were largely ipsilateral (82 ± 3%, Figure 2a, Figure 4c). Throughout the DV extent of the VTA, more DiI labeled cell bodies were located in midline regions, both ipsilateral and contralateral to the injection site. Looking at the distribution along the DV axis, a greater number of PrL-projecting neurons were located in the ventral VTA (Figure 4c). A similar percentage of ipsilateral and contralateral PrL-projecting neurons were co-localized with TH (38 ± 6% ipsi and 39 ± 13% contra) (Figure 2c,d,e). Not only were PrL-projecting neurons most numerous in midline structures, co-localization with TH was also highest here, especially at the mid-dorsal level. In fact, on the contralateral side, the only PrL-projecting TH(+) neurons were along the midline (Figure 4e). Overall, a much lower percentage of PrL-projecting cells were co-localized with GAD (12 ± 5% ipsi and 19 ± 10% contra) compared to ACC-projecting VTA neurons (Figure 2c,e,f). The percentage of GAD(+) PrL-projecting neurons was slightly higher in midline VTA regions, especially in the contralateral ventral VTA (Figure 4g).

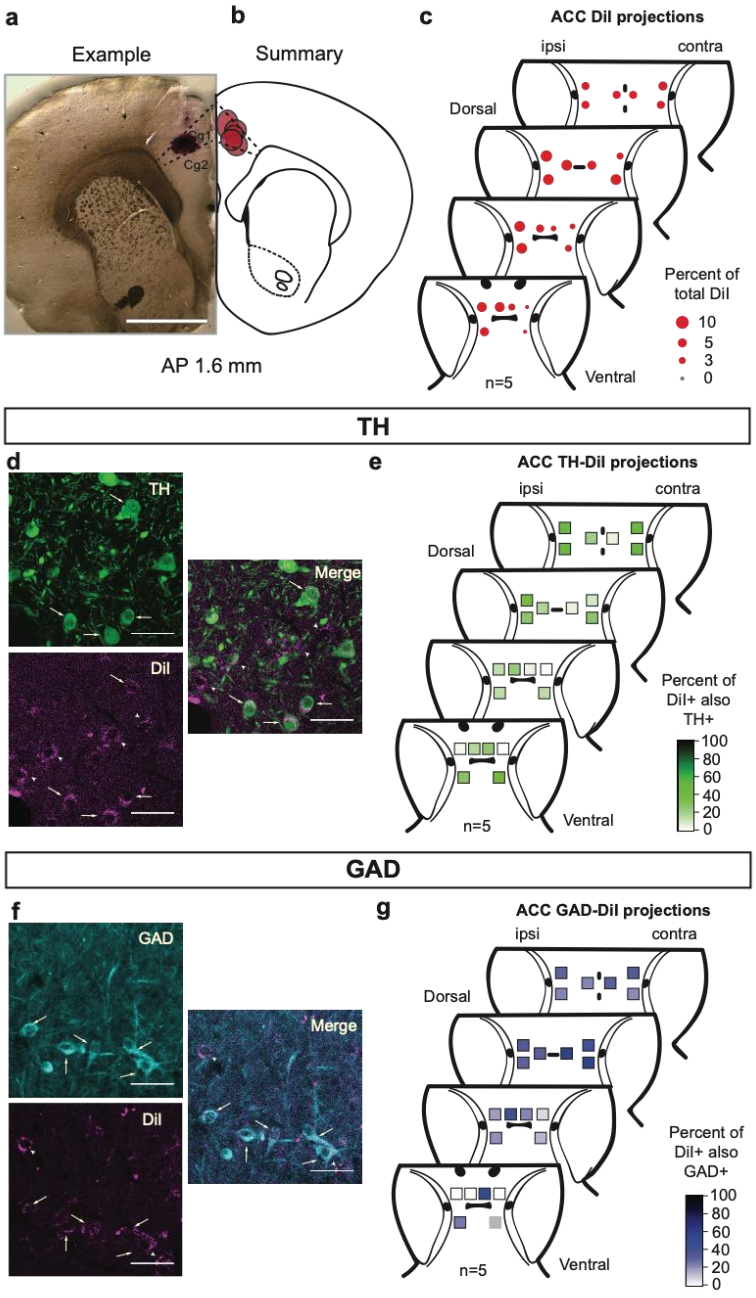

Figure 2-3. Projections from the VTA to the anterior cingulate cortex (ACC). (a) Example DiI injection site in the ACC (AP 1.6 mm). Scale bar 2 mm. (b) Summary of all DiI injection sites (red circles) in the ACC (AP 1.6 mm) (Paxinos & Watson, 1998). n=5 (c) Retrogradely labeled neurons were detected in all sampling regions throughout the DV extent of the VTA. Dot size indicates the mean percentage of retrogradely labeled neurons within that sampling window compared to the total projection. The slice depths from dorsal to ventral are approximately -7.8, -8.0, -8.2 and -8.4 mm. (d) Example images of DiI labeled cells (magenta) and immunofluorescent labeling for TH (green). Scale bar 50 μm. Arrows indicate TH-DiI co-labeled neurons. Arrowheads indicate DiI only neurons. (e) Within each sampling region, the color indicates the percentage of DiI neurons co-labeled with TH. (f) Representative images of DiI filled cells and immunofluorescent labeling for GAD. Scale bar 50 μm. Arrows indicate GAD-DiI co-labeled neurons. Arrowheads represent DiI only neurons. (g) Within each sampling region, the color indicates the percentage of DiI neurons co-localized with GAD.

Infralimbic cortex

Projections from the VTA to the IL were mostly unilateral, with 89 ± 3% of DiI labeled cells located ipsilateral to the injection site (Figure 2a, Figure 5c). The densest location of DiI(+) neurons was the medial VTA. In contrast to the PrL projection, a greater percentage of IL-projecting neurons were located in the more dorsal VTA compared to the ventral VTA (Figure 5c). A similar percentage of ipsilateral and contralateral IL projecting cells were co-localized with TH (40 ± 7% ipsi, 37 ± 17% contra) (Figure 2c,d,e). Interestingly, the percentage of ipsilateral IL-projecting TH(+) neurons was highest in posterior lateral regions (62 ± 6%), distributed fairly

evenly across the DV axis. On the contralateral side, TH(+) IL-projecting cells were located exclusively in the medial VTA, but evenly distributed across the DV slices. (Figure 5e). Only seven percent of all IL-projecting neurons were co-localized with GAD (Figure 2c,e,f). There was a uniquely high proportion of ipsilateral GAD(+) IL-projecting neurons in the posterior lateral VTA, in the mid-ventral slice, where little to no co-localization was detected in any other sampling region (Figure 5g).

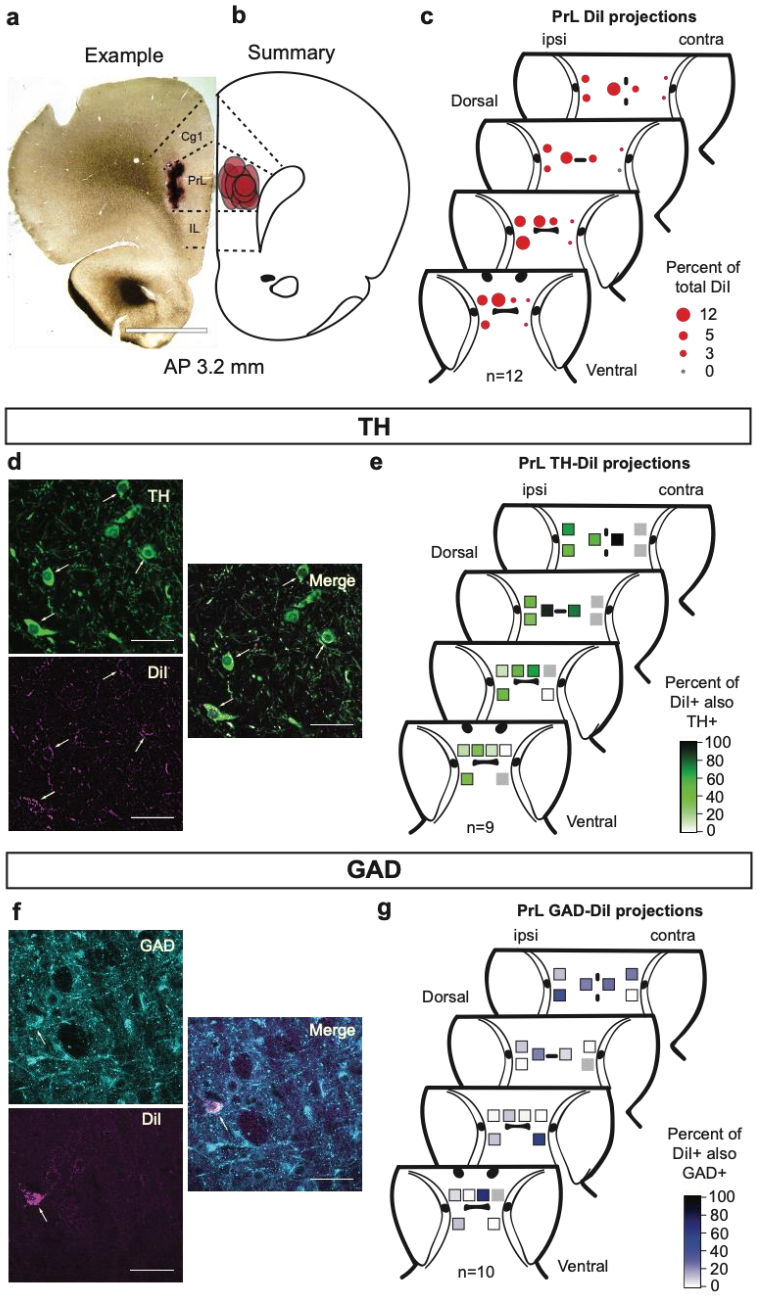

Figure 2-4. Projections from the VTA to the prelimbic cortex (PrL).
(a) Example DiI injection site in the PrL (AP 3.2 mm). Scale bar 2 mm. (b) Summary of all DiI injection sites (red circles) in the PrL (AP 3.2 mm) (Paxinos & Watson, 1998). n=12 (c) Retrogradely labeled neurons were detected in all but one sampling region throughout the DV extent of the VTA. Dot size indicates the mean percentage of retrogradely labeled neurons within that sampling window compared to the total projection. The slice depths from dorsal to ventral are approximately -7.8, -8.0, -8.2 and -8.4 mm. (d) Representative images of DiI labeled cells (magenta) and immunofluorescent labeling for TH (green). Scale bar 50 µm. Arrows indicate TH-DiI co-labeled neurons. (e) Within each sampling region, the color indicates the percentage of DiI neurons co-labeled with TH. (f) Representative images of DiI filled cells and immunofluorescent labeling for GAD. Scale bar 50 µm. Arrow indicates GAD-DiI co-labeled neuron. (g) Within each sampling region, the color indicates the percentage of DiI neurons co-localized with GAD.

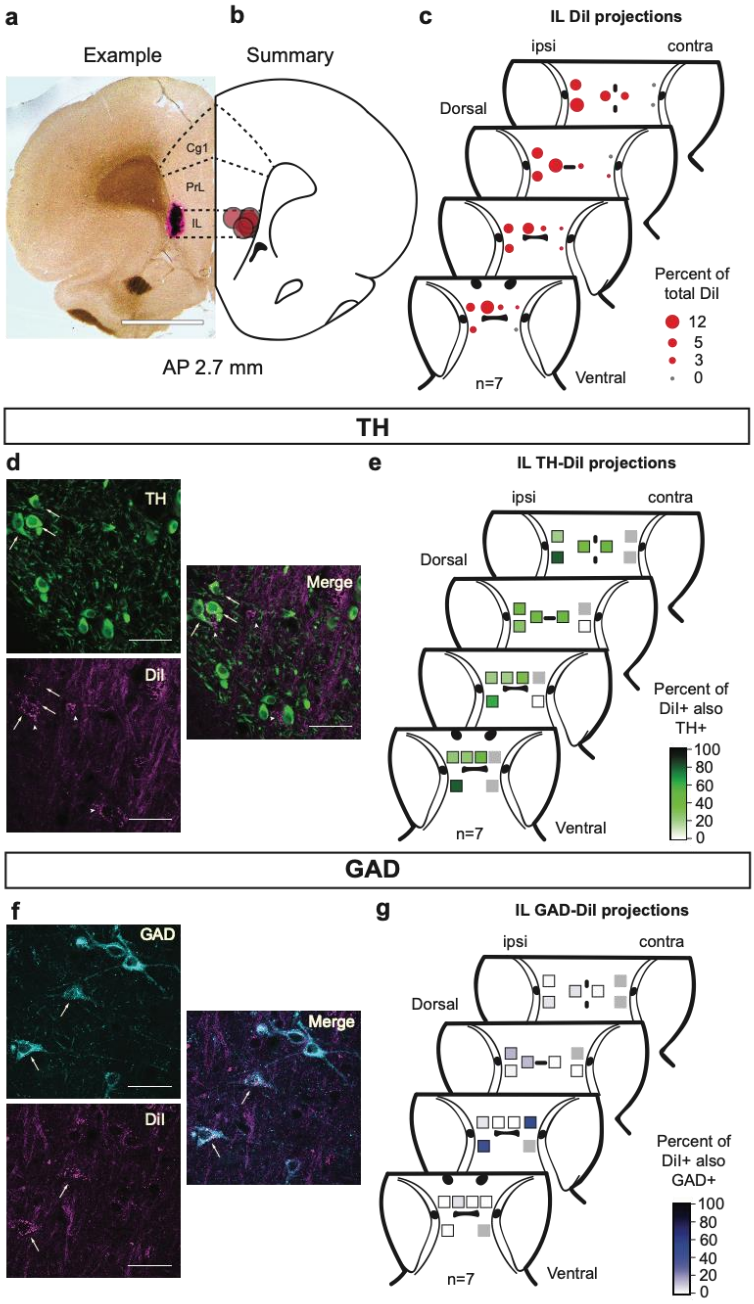

Figure 2-5. Projections from the VTA to the infralimbic cortex (IL). (a) Example DiI injection site in the IL (AP 2.7 mm). Scale bar 2 mm (b) Summary of all DiI injection sites (red circles) in the IL (AP 2.7 mm) (Paxinos & Watson, 1998). n=7 (c) Retrogradely labeled neurons were detected in all sampling regions ipsilateral to the injection site throughout the DV extent of the VTA. Dot size indicates the mean percentage of retrogradely labeled neurons within that sampling window compared to the total projection. The slice depths from dorsal to ventral are approximately -7.8, -8.0, -8.2 and -8.4 mm. (d) Representative images of DiI filled cells (magenta) and immunofluorescent labeling for TH (green). Scale bar 50 μm. Arrows indicate TH-DiI co-labeled neurons. Arrowheads indicate DiI only neurons. (e) Within each sampling region, the color indicates the percentage of DiI neurons co-labeled with TH across animals. (f) Representative images of DiI filled cells and immunofluorescent labeling for GAD. Scale bar 50 μm. Arrows indicate GAD-DiI co-labeled neurons. (g) Within each sampling region, the color indicates the percentage of DiI neurons co-localized with GAD.

Nucleus Accumbens

Within the NAc, the core and shell are neuroanatomically distinct regions (A.Y. Deutch & Cameron, 1992; Zahm, 1998) that play different roles in motivated and reward related behavior (Bassareo, Cucca, Frau, & Di Chiara, 2015; Floresco, Montes, Tse, & van Holstein, 2018; Ito, Robbins, & Everitt, 2004). In addition, differences have previously been found in the mouse with respect to the distribution of VTA projections to the medial and lateral NAc shell, with medial VTA neurons projecting preferentially to the medial shell and lateral VTA neurons projecting to lateral shell (Lammel et al., 2008). These sub-regions of the NAc shell may also play distinct roles

in reward related behavior and hedonic impact (Lammel et al., 2012b; Pecina & Berridge, 2005). Thus, we examined VTA projections to each region separately.

No staining for GAD was done for projections to the NAc sub-regions, as based on prior work in our lab and others, we expect the majority of the non-DA neurons to be GABAergic (Margolis, Lock, Chefer, et al., 2006; Van Bockstaele & Pickel, 1995; Yamaguchi et al., 2011). When we previously quantified TH and GAD contributions to the NAc projection with a similar counting approach for larger injections that included the NAc medial shell and core, the sum of TH(+) (66 ± 10%) and GAD(+) (25 ± 5%) neurons accounted for the vast majority of the total projection (Margolis, Lock, Chefer, et al., 2006). There is a sparse glutamatergic projection that accounts for that remaining <10%, and arises mostly from the medial VTA (Yamaguchi et al., 2011a). Thus, in the current study, we infer that most non-DA neurons are GABAergic in the lateral VTA, where non-DA neurons in the medial VTA may be either GABAergic or glutamatergic.

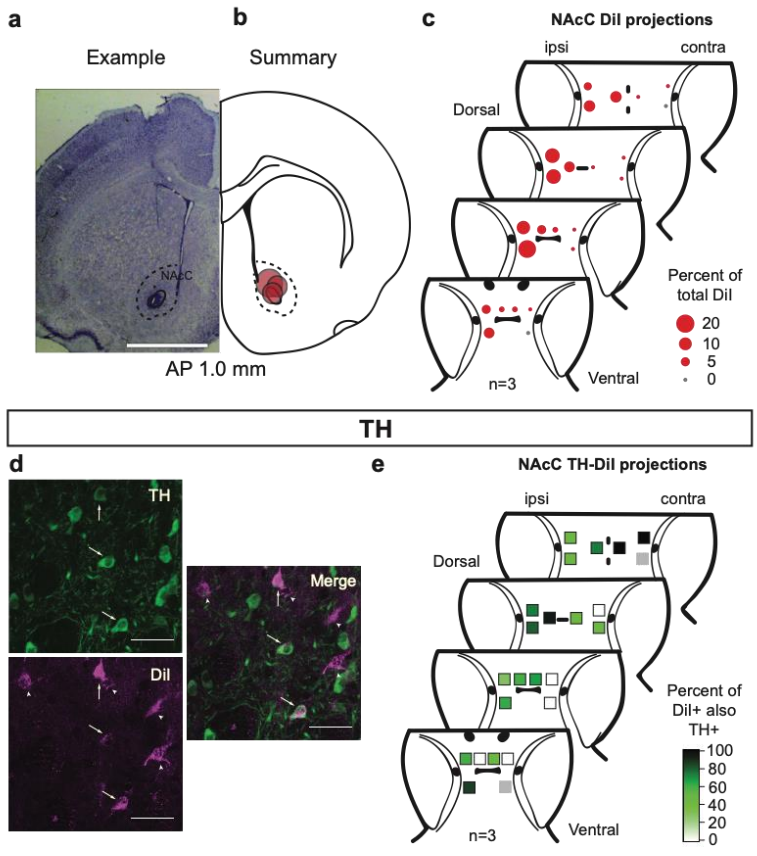

Figure 2-6. Projections from the VTA to the nucleus accumbens core (NAcC).
(a) Example DiI injection site in the NAcC (AP 1.0 mm). Scale bar 2 mm. (b) Summary of all DiI injection sites (red circles) in the NAcC (AP 1.0 mm) (Paxinos & Watson, 1998). n=3 (c) Retrogradely labeled neurons were detected in all but two sampling regions throughout the DV extent of the VTA. Dot size indicates the mean percentage of retrogradely labeled neurons within that sampling window compared to the total projection. The slice depths from dorsal to ventral are approximately -7.8, -8.0, -8.2 and -8.4 mm. (d) Representative images of DiI filled cells (magenta) and immunofluorescent labeling for TH (green). Scale bar 50 µm. Arrows indicate TH-DiI co-labeled neurons. Arrowheads indicate DiI only neurons. (e) Within each sampling region, the color indicates the percentage of DiI neurons co-labeled with TH.

Nucleus Accumbens Core

Projections from the VTA to the NAcC were highly unilateral, with 92 ± 3% of DiI labeled cells located ipsilateral to the injection site (Figure 2b, Figure 6c). The highest density of this projection was located in the lateral regions of the middle slices along the DV axis (Figure 6c). NAcC-projecting cells showed the highest percentage of co-localization with TH of any brain region in this study (64 ± 12% ipsi, 67 ± 21% contra) (Figure 2d,f). In the ipsilateral VTA, high

percentages of TH(+) projection neurons were observed across many of the sampling windows and DV slices, except in the ventral medial VTA. On the contralateral side, TH(+) NAcC-projecting cells appear slightly enriched in medial regions (66 ± 6%) across the DV axis, while among lateral sampling regions a TH(+) contribution was only observed in the dorsal half of the VTA (Figure 6d,e).

Nucleus Accumbens Medial Shell

Most retrogradely labeled NAcMs-projecting VTA neurons were located ipsilateral to the injection site (83 ± 5%) (Figure 2b, Figure 7c). The majority of this projection was located in the mid to ventral VTA, but retrogradely labeled neurons were relatively evenly distributed within each DV plane examined; this projection was markedly sparse in the most dorsal slice of the VTA (Figure 7c). Forty-two ± 18% of all ipsilateral and 30 ± 15% of all contralateral NAcMs-projecting neurons were co-localized with TH (Figure 2d,f). The percentage of ipsilateral NAcMs-projecting TH(+) cells was distributed fairly evenly across the sampling windows throughout the VTA. On the contralateral side, TH(+) NAcMs-projecting neurons were enriched in the medial VTA (28 ± 3%), similar to NAcC projections (Figure 7d,e).

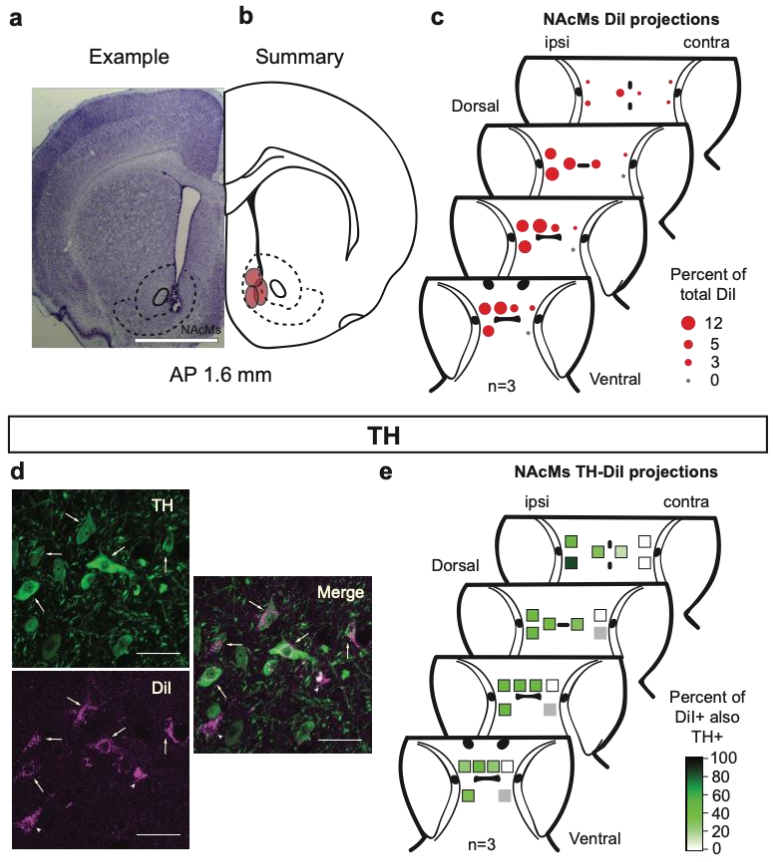

Figure 2-7. Projections from the VTA to the nucleus accumbens medial shell (NAcMs). (a) Example DiI injection site in the NacMs (AP 1.6 mm). Scale bar 2 mm. (b) Summary of all DiI injection sites (red circles) in the NAcMs (AP 1.6 mm) (Paxinos & Watson, 1998). n=3 (c) Retrogradely labeled neurons were detected in all ipsilateral sampling regions throughout the DV extent of the VTA. Dot size indicates the mean percentage of retrogradely labeled neurons within that sampling window compared to the total projection. The slice depths from dorsal to ventral are approximately -7.8, -8.0, -8.2 and -8.4 mm. (d) Representative images of DiI filled cells (magenta) and immunofluorescent labeling for TH (green). Scale bar 50 µm. Arrows indicate TH-DiI co-labeled neurons. Arrowheads indicate DiI only neurons. (e) Within each sampling region, the color indicates the percentage of DiI neurons co-localized with TH.

Nucleus Accumbens Lateral Shell

Projections from the VTA to the NAcLs were almost entirely unilateral, with 96 ± 1% of DiI labeled cells located ipsilateral to the injection site (Figure 2b, Figure 8c). Projection neurons were most dense in the lateral VTA regions, especially in the middle slices of the DV axis (Figure 8c). Similar to findings in the NAcMs projection, approximately 49 ± 2% of all ipsilateral NAcLs-projecting VTA neurons were TH(+). In contrast, only 11 ± 11% of the small number of contralateral projections were TH(+) (Figure 2d,f). On the ipsilateral side, the highest percentage of TH(+) neurons was localized in the lateral regions in the mid-ventral VTA (Figure 8d,e).

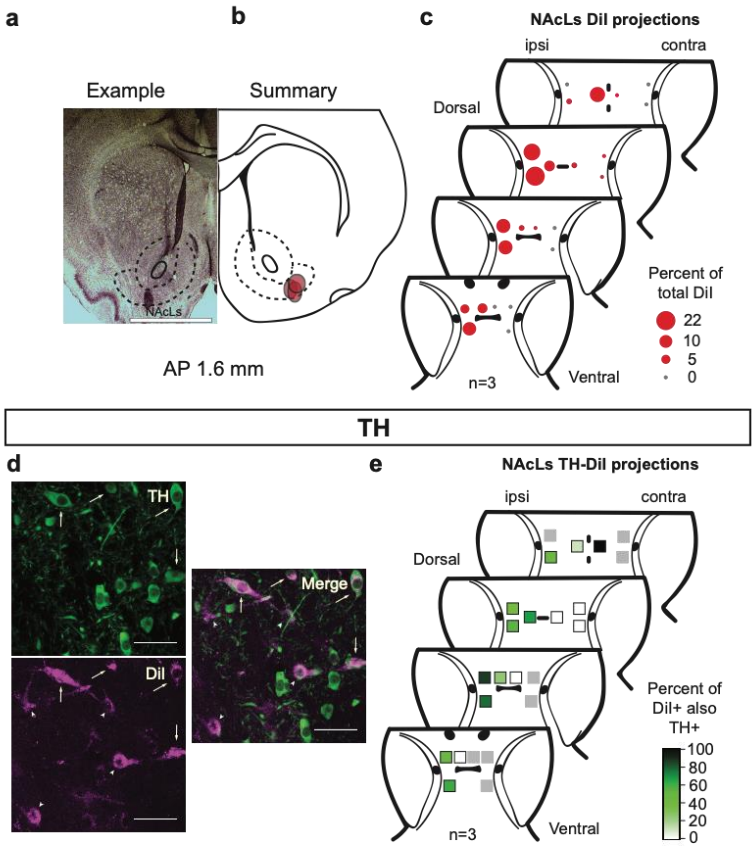

Figure 2-8. Projections from the VTA to the nucleus accumbens lateral shell (NAcLs). (a) Example DiI injection site in the NAcLs (AP 1.6 mm). Scale bar 2mm. (b) Summary of all DiI injection sites (red circles) in the NAcLs (AP 1.6 mm) (Paxinos & Watson, 1998). n=3. (c) Retrogradely labeled neurons were detected in all sampling regions throughout the DV extent of the VTA. Dot size indicates the mean percentage of retrogradely labeled neurons within that sampling window compared to the total projection. The slice depth from dorsal to ventral is approximately -7.8, -8.0, -8.2 and -8.4 mm. (d) Representative images of DiI filled cells (magenta) and immunofluorescent labeling for TH (green). Scale bar 50 µm. Arrows indicate DiI, TH co-labeled neurons. Arrowheads indicate DiI(+), TH(-) neurons. (e) Within each sampling region, the color indicates the percentage of DiI neurons co-localized with TH.

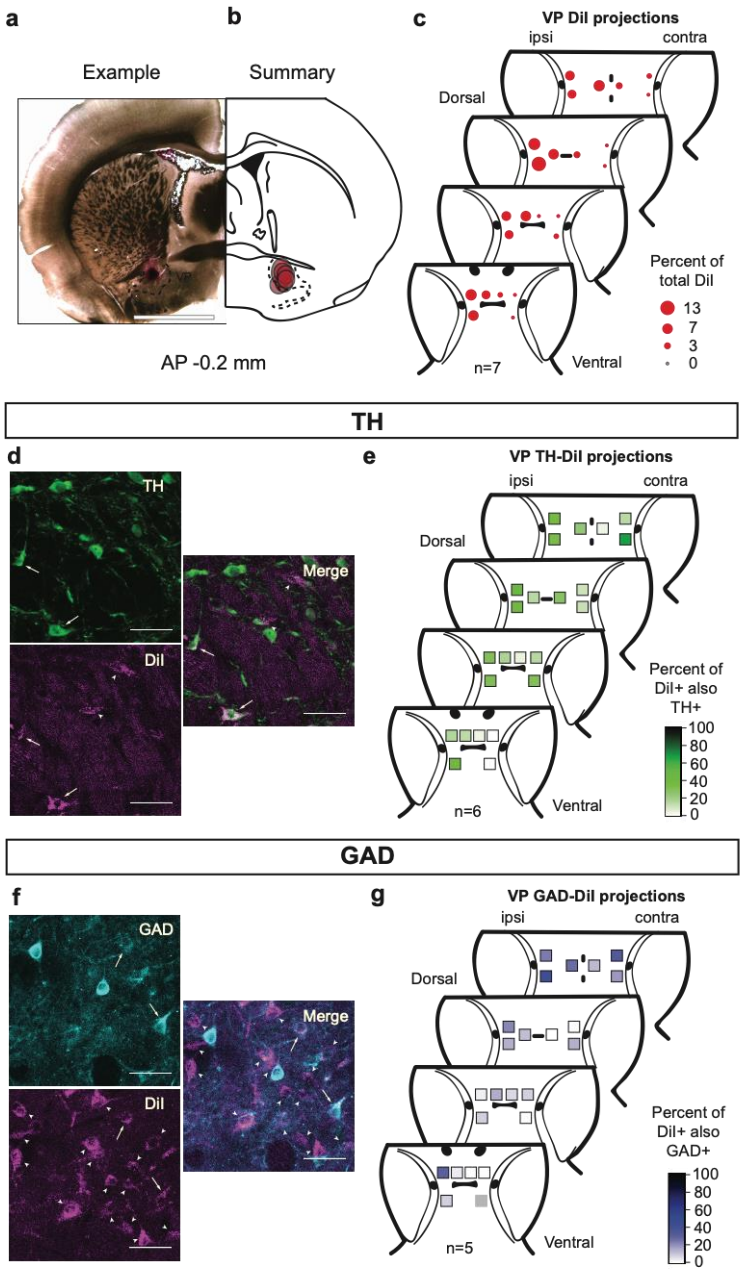

Figure 2-9. Projections from the VTA to the ventral pallidum (VP) (a) Example DiI injection site in the VP (AP -0.2 mm). Scale bar 2 mm. (b) Summary of all DiI injection sites (red circles) in the VP (AP -0.2 mm) (Paxinos & Watson, 1998). n=7 (c) Retrogradely labeled neurons were detected in all sampling regions throughout the DV extent of the VTA. Dot size indicates the mean percentage of retrogradely labeled neurons within that sampling window compared to the total projection. The slice depths from dorsal to ventral are approximately -7.8, -8.0, -8.2 and -8.4 mm. (d) Representative images of DiI filled cells (magenta) and immunofluorescent labeling for TH (green). Scale bar 50 µm. Arrows indicate TH-DiI co-labeled neurons. Arrowheads indicate DiI only neurons. (e) Within each sampling region, the color indicates the percentage of DiI neurons co-labeled with TH. (f) Representative images of DiI filled cells and immunofluorescent labeling for GAD. Scale bar 50 µm. Arrows indicate GAD-DiI co-labeled neurons. Arrowheads represent DiI only neurons. (g) Within each sampling region, the color indicates the percentage of DiI neurons co-localized with GAD.

Ventral pallidum

The VP is an important output of the VTA and plays a significant role in driving motivated behaviors (Hubner & Koob, 1990; Richard, Ambroggi, Janak, & Fields, 2016; K. S. Smith, Tindell, Aldridge, & Berridge, 2009). The VP is made up of unique sub-regions such as the ventromedial and dorsolateral VP; these regions were too small to differentiate via retrograde tracing approaches (Root, Melendez, Zaborszky, & Napier, 2015).

Ipsilateral projections from the VTA to the VP were much denser than contralateral projections (81 ± 4% ipsi) (Figure 2a, Figure 9c). The density of DiI filled cells was not notably different between the three sampling windows or across the DV axis (Figure 9c). A higher proportion of ipsilateral VP-projecting VTA neurons were co-labeled with TH (39 ± 7%) compared to the contralateral projection (17 ± 5%) (Figure 2c,d,e). On the ipsilateral side, the percentage of TH(+) was slightly higher in the lateral VTA compared to medial regions throughout the DV extent. On the contralateral side, VP-projecting TH(+) neurons were also located mostly in the lateral regions. These contralateral projections arose mostly from the dorsal VTA (Figure 9e). The percentage of VP-projecting VTA neurons co-localized with GAD was twice as high on the ipsilateral compared to the contralateral side (19 ± 4% ipsi, 8 ± 4% contra) (Figure 2c,e,f). The average proportion of ipsilateral GAD(+) projection cells was similar across the sample regions, though interestingly, higher percentages of GAD(+) cells were found in dorsal compared to ventral VTA slices. On the contralateral side, higher percentages of GAD(+) cells were also found in the dorsal VTA (Figure 9g).

Basolateral Amygdala

We examined projections of the VTA to different sub-regions of the BLA, the anterior and posterior BLA (ABLA and PBLA, respectively), as these two regions may play different roles in reward related behavior and send distinct outputs to sub-regions of the mPFC and NAc (Kantak, Black, Valencia, Green-Jordan, & Eichenbaum, 2002; J. Kim, Pignatelli, Xu, Itohara, & Tonegawa, 2016; McLaughlin & Floresco, 2007; Wright, Beijer, & Groenewegen, 1996).

Anterior Basolateral Amygdala

Projections from the VTA to the ABLA were mostly unilateral, with 87 ± 5% of DiI labeled cells located ipsilateral to the injection site (Figure 2a, Figure 10c). The density of ipsilateral DiI projections was greatest in the anterior-lateral VTA, especially in the middle regions of the DV axis. The small contralateral projection from the VTA to the ABLA was greatest in the medial VTA (Figure 10c). Forty-seven ± 1% of all ipsilateral and 38 ± 31% of all contralateral ABLA projecting neurons were co-localized with TH, the variability on the contralateral side being driven by the small number of neurons contributing to that projection (Figure 2c,d,e). On the ipsilateral side, the highest percentages of TH(+) neurons were observed in the lateral regions, whilst on the contralateral side, TH(+) retrogradely labeled neurons were limited to medial ventral VTA (Figure 10e). Overall, a small percentage of ABLA-projecting neurons were co-localized with GAD (9 ± 3% ipsi, 11 ± 11% contra) (Figure 2c,e,f). On the ipsilateral side, GAD(+) cells were found almost exclusively in the posterior lateral regions in ventral VTA slices. On the contralateral side, GAD(+) projection neurons were also only located in lateral regions of the VTA (Figure 10g).

Posterior Basolateral Amygdala

Projections from the VTA to the PBLA were mostly ipsilateral (81 ± 2%) (Figure 2a, Figure 11c). The highest densities of projection neurons were located in the anterior lateral regions of the VTA, while the small contralateral projection was slightly greater in the medial VTA (Figure 11c). Similar to the ABLA projections, 45 ± 4% of all ipsilateral and 30 ± 8% of all contralateral PBLA-projecting VTA neurons were co-localized with TH (Figure 2c,d,e). The percentage of ipsilateral PBLA-projecting TH(+) cells was distributed fairly evenly across the sampling windows and across the DV axis. In the contralateral VTA, the highest percentage of TH(+) neurons was located in anterior-lateral regions (Figure 11e). Interestingly, VTA neurons projecting to the PBLA had a higher percentage of GAD(+) neurons (26 ± 7% ipsi, 21 ± 7% contra) compared to projections to the ABLA (9 ± 4% ipsi, 11 ± 11% contra) (Figure 2c,e,f). These GAD(+) neurons were similarly distributed across all sample windows and across the DV axis (Figure 11g).

Ventrolateral Periaqueductal Gray

Although the vlPAG is classically thought of a region involved in the control of pain (Millan, 2002), it has reciprocal connections with the VTA (Beitz, 1982; Omelchenko & Sesack, 2010) and may be more generally involved in reward, especially in opioid mediated reward pathways (Flores, Galan-Rodriguez, Ramiro-Fuentes, & Fernandez-Espejo, 2006; Motta, Carobrez, & Canteras, 2017; Olmstead & Franklin, 1997).

Intriguingly, projections from the VTA to the vlPAG were only slightly denser on the ipsilateral than on the contralateral side (58 ± 1% ipsilateral) (Figure 2a, Figure 12c). The vlPAG had the strongest contralateral projection of all the brain regions in this study. Ipsilateral and contralateral vlPAG-projecting neurons were distributed fairly evenly across the sampling windows, and across the DV axis (Figure 12c). Interestingly, slightly more contralateral vlPAG-projecting VTA neurons were co-localized with TH (29 ± 5%) compared to the ipsilateral projection (16 ± 4%) (Figure 2c,d,e). Just as the numbers of DiI(+) cells were similar across all regions, the proportion of those projections that were co-localized with TH was also relatively evenly distributed across the VTA regions and depths, on both the ipsilateral and contralateral sides. (Figure 12e). Of all the brain regions we analyzed, VTA projections to the vlPAG had the highest percentage of co-labeling with GAD, approaching 50% (48 ± 6% ipsi, 49 ± 9% contra) (Figure 2c,e,f). A similar pattern was seen in both the ipsilateral and contralateral VTA: GAD(+) projection cells were enriched in lateral and absent in medial VTA in the most ventral slices, but more evenly distributed in the rest of the DV extent of the VTA (Figure 12g).

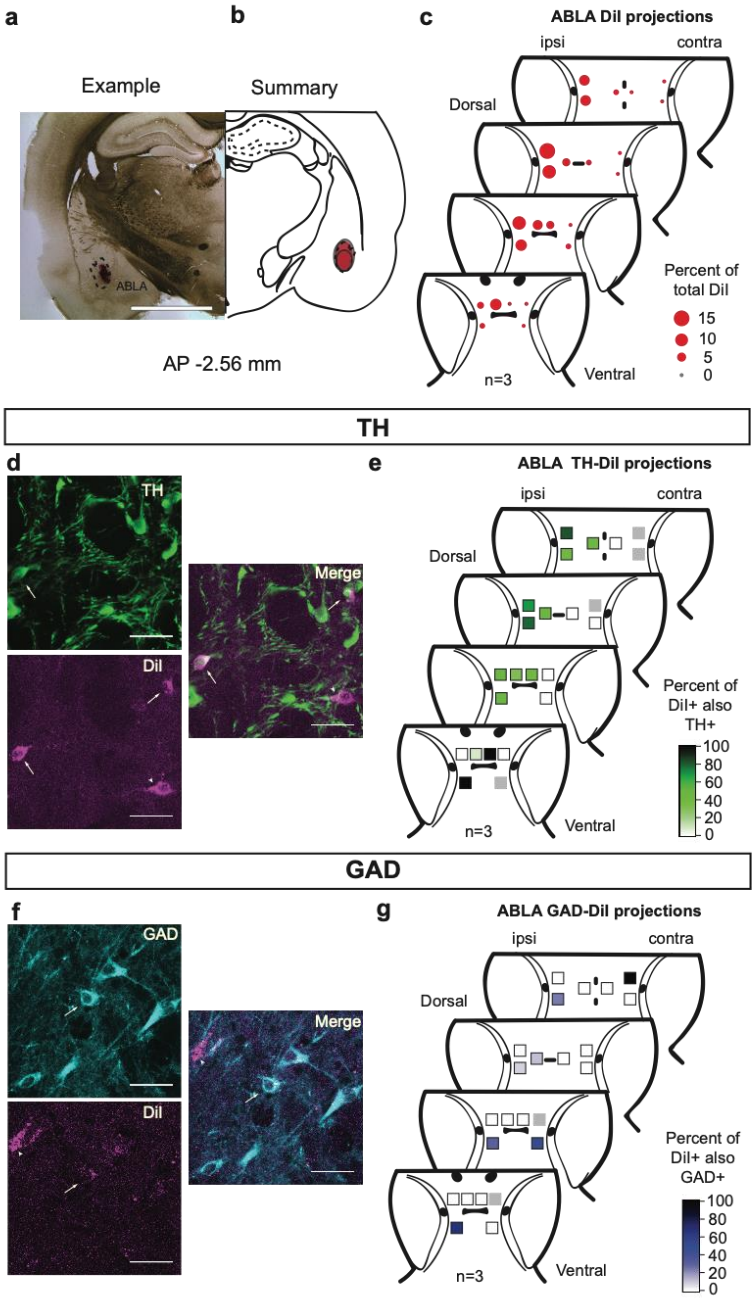

Figure 2-10. Projections from the VTA to the anterior basolateral amygdala (ABLA).
(a) Example DiI injection site in the ABLA (AP -2.56 mm). Scale bar 2 mm. (b) Summary of all DiI injection sites (red circles) in the ABLA (AP -2.56 mm) (Paxinos & Watson, 1998). n=3. (c) Retrogradely labeled neurons were detected in all sampling regions throughout the DV extent of the VTA. Dot size indicates the mean percentage of retrogradely labeled neurons within that sampling window compared to the total projection. The slice depths from dorsal to ventral are approximately -7.8, -8.0, -8.2 and -8.4 mm. (d) Representative images of DiI filled cells (magenta) and immunofluorescent labeling for TH (green). Scale bar 50 μm. Arrow indicates TH-DiI co-labeled neuron. Arrowheads indicate DiI only neurons. (e) Within each sampling region, the color indicates the percentage of DiI neurons co-labeled with TH. (f) Representative images of DiI filled cells and immunofluorescent labeling for GAD. Scale bar 50 μm. Arrows indicate GAD-DiI co-labeled neurons. Arrowhead represents DiI only neuron. (g) Within each sampling region, the color indicates the percentage of DiI neurons co-localized with GAD.

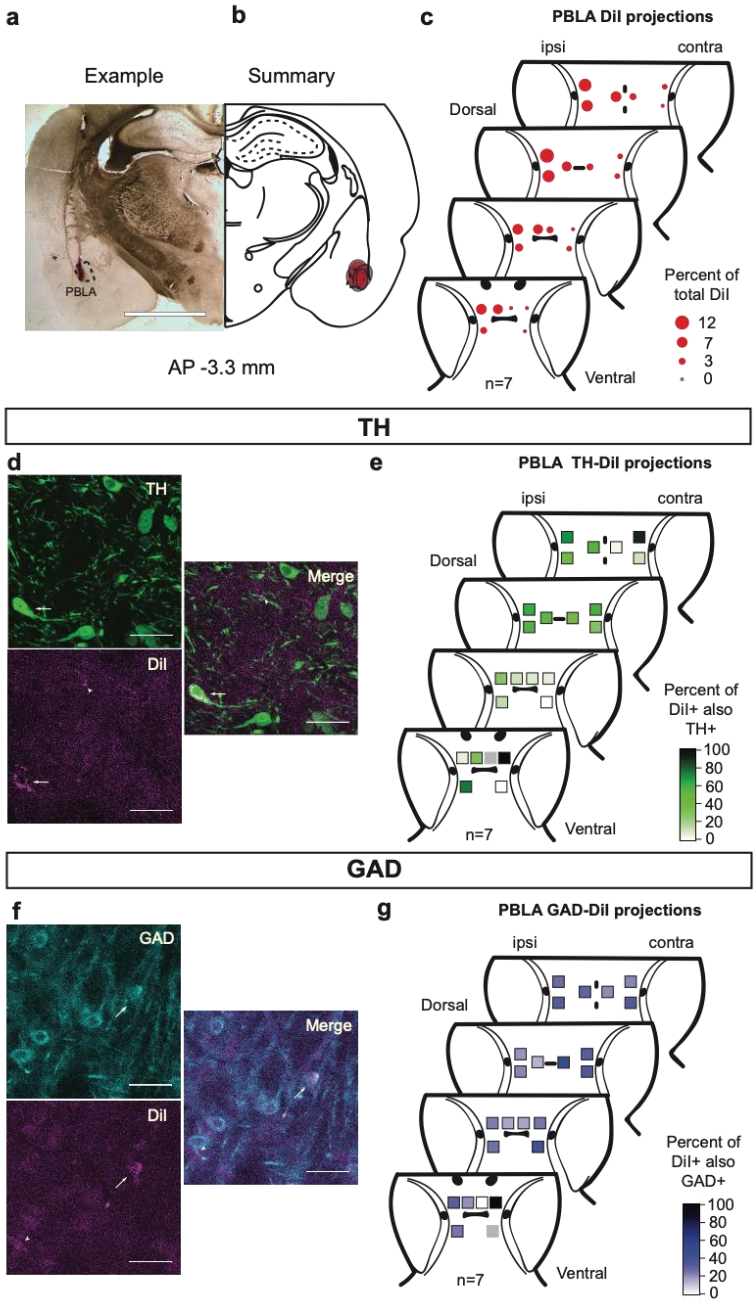

Figure 2-11. Projections from the VTA to the posterior basolateral amygdala (PBLA). (a) Example DiI injection site in the PBLA (AP -3.3 mm) Scale bar 2 mm. (b) Summary of all DiI injection sites (red circles) in the PBLA (AP -3.3 mm) (Paxinos & Watson, 1998). n=7 (c) Retrogradely labeled neurons were detected in all sampling regions throughout the DV extent of the VTA. Dot size indicates the mean percentage of retrogradely labeled neurons within that sampling window compared to the total projection. The slice depths from dorsal to ventral are approximately -7.8, -8.0, -8.2 and -8.4 mm. (d) Representative images of DiI filled cells (magenta) and immunofluorescent labeling for TH (green). Scale bar 50 μm. Arrow indicates TH-DiI co-labeled neuron. Arrowhead indicates DiI only neuron. (e) Within each sampling region, the color indicates the percentage of DiI neurons co-labeled with TH. (f) Representative images of DiI filled cells and immunofluorescent labeling for GAD. Scale bar 50 μm. Arrow indicates GAD-DiI co-labeled neuron. Arrowhead represents DiI only neuron. (g) Within each sampling region, the color indicates the percentage of DiI neurons co-labeled with GAD.

Discussion

Here we systematically investigated the anatomical contributions and distributions of DA and GABA neurons within the VTA that project to ten different target nuclei. We described the location of these projection neurons within the different sub-regions of the VTA, adding more resolution than previously provided. Additionally, we differentiated VTA projections to regions that have not been investigated separately in prior studies, such as to the anterior and posterior BLA. We found that these efferent projections of the VTA varied not only in their somatic locations within the VTA but also in their dopaminergic and GABAergic content.

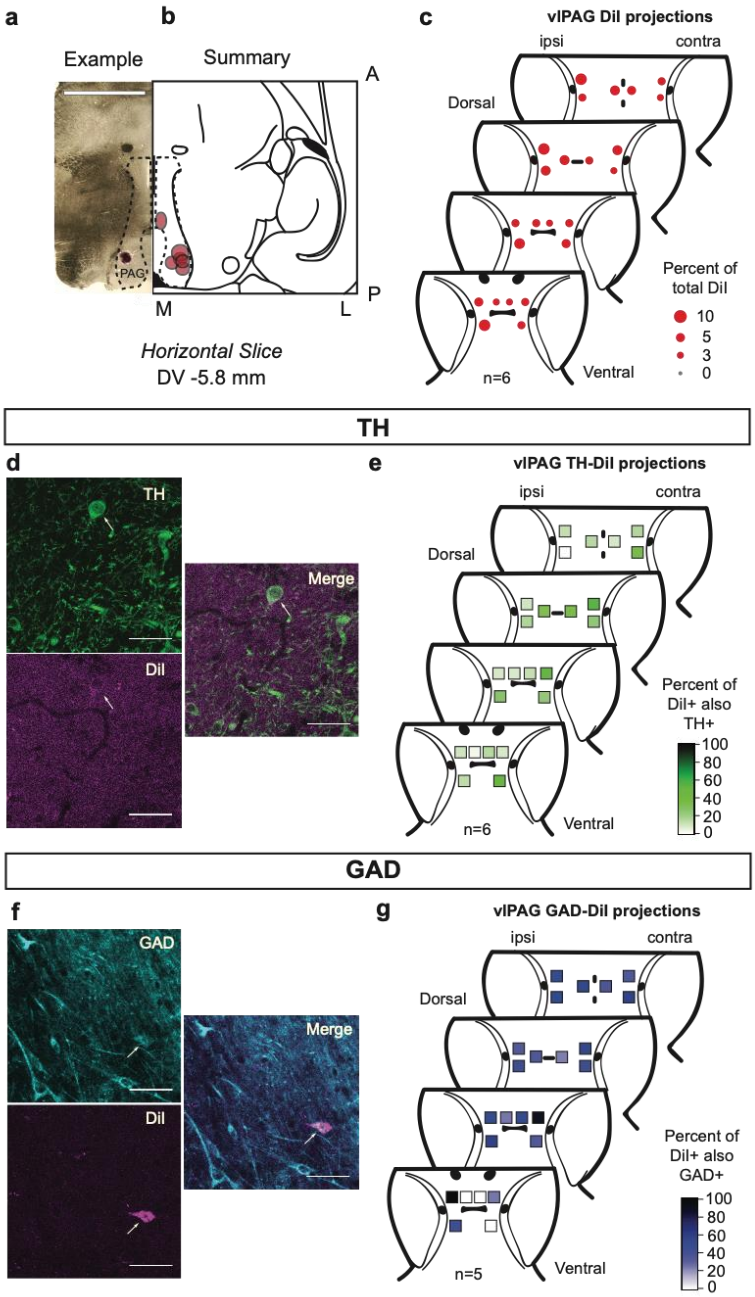

Figure 2-12. Projections from the VTA to the ventrolateral periaqueductal gray (vlPAG). (a) Example DiI injection site in the vlPAG (DV -5.8 mm). Scale bar 2 mm. (b) Summary of all DiI injection sites (red circles) in the vlPAG (DV -5.8 mm) (Paxinos & Watson, 1998). n=6 (c) Retrogradely labeled neurons were distributed relatively evenly across both ipsilateral and contralateral sampling regions throughout the DV extent of the VTA. Dot size indicates the mean percentage of retrogradely labeled neurons within that sampling window compared to the total projection. The slice depths from dorsal to ventral are approximately -7.8, -8.0, -8.2 and -8.4 mm. (d) Representative images of DiI filled cells (magenta) and immunofluorescent labeling for TH (green). Scale bar 50 μm. Arrow indicates TH-DiI co-labeled neuron. (e) Within each sampling region, the color indicates the percentage of DiI neurons co-labeled with TH. (f) Representative images of DiI filled cells and immunofluorescent labeling for GAD. Scale bar 50 μm. Arrow indicates GAD-DiI co-labeled neuron. (g) Within each sampling region, the color indicates the percentage of DiI neurons co-localized with GAD.

Looking broadly at the differences between projections to all of the target regions, we observed an unusually high rate of contralateral VTA projections to the vlPAG and ACC, while the the NAcC and NAcLs had almost exclusively ipsilateral contributions (Figure 2a,b). We observed the greatest proportion of TH(+) neurons within the projection to the NAcC (Figure 2d,f) and the greatest proportion of GAD(+) neurons within the projections to the vlPAG and to the contralateral ACC (Figure 2c,e). The projection containing the smallest percentage of TH(+) somata was to the vlPAG while the smallest percentages of GAD(+) somata were observed in the ABLA and IL projections (Figure 2c,e). While distribution patterns were distinct between projections, each target

region received inputs from neurons within every ipsilateral sampling region, indicating that VTA neurons in near proximity to one another can project to different targets. Also, sub-regions of the same target structure (i.e. ABLA vs. PBLA) and adjacent structures (i.e. PFC areas) receive inputs from different parts of the VTA with different neurotransmitter content. Together, our findings reveal a patterned but intermixed organization of the VTA.

Technical considerations

Several methodological points should be considered in the interpretation of our results. First, the very dorsal portions of the linear nuclei were not represented in our data due to the geometry of the VTA following horizontal sectioning. Overall however, this represents a minor proportion of all VTA/A10 neurons. Horizontal sections provided a better layout of the VTA for comparing projections across the ML and AP axes, which have been compared in a variety of behavioral studies (reviewed in Sanchez-Catalan, Kaufling, Georges, Veinante, & Barrot, 2014 and Lammel et al., 2014, and see below). Horizontal slices of the VTA are also commonly used for *ex vivo* electrophysiology experiments, as many efferent and afferent projections of the VTA remain more intact when sectioned along the horizontal plane (Calabresi, Lacey, & North, 1989; Williams, North, Shefner, Nishi, & Egan, 1984). In the current study, a horizontal layout is not only informative for our anatomical analysis, but also allows us to more directly compare our results with VTA electrophysiology work.

Another technical issue inherent in any experiment that relies on microinjection of a reagent is incomplete coverage of the target nucleus. In the present study, DiI injections were always smaller than the entire brain region of interest in order to prevent spread to neighboring regions. Additionally, shapes of brain regions are often irregular (such as the VP). For both of these reasons, some portions of the projections may not be represented in our counts. That said, smaller injections targeting different parts of certain brain regions enabled us to detect differences between VTA projections to the ABLA and PBLA, and among the projections to the NAcC, NAcMs, and NAcLs Furthermore, we adjusted the volumes of DiI injected in order to maximize coverage of the target nucleus and minimize spread outside that target. However, since injections and tracer uptake never capture an entire projection, we chose to normalize our data and do not draw strong conclusions from differences in absolute counts between projection target groups.

Lastly, there are inherent limitations of immunocytochemistry techniques that may have impacted our results. For example, there is always the possibility that protein levels were too low to be immunocytochemically detected in a subset of neurons, and therefore our percentages may be derived from underestimates of the true population. This is an unlikely issue for TH detection, given reports of virtually complete overlap of TH immunocytochemistry with genetic reporters in the VTA (Margolis et al., 2010; Witten et al., 2011). On the other hand, there is a small subset of VTA neurons that express TH but not the vesicular monoamine transporter (VMAT2), making these neurons capable of synthesizing DA, but not packaging it into vesicles by the expected mechanism (X. Li, Qi, Yamaguchi, Wang, & Morales, 2013). Therefore, it is possible that a small portion of TH(+) neurons do not synaptically release DA, as has been observed in the VTA projection to the lateral habenula (Root et al., 2014). Somatic GAD labeling is less reliable, and may contribute to an underestimate of the GABAergic component of the projections studied here. Identifying GAD colocalization is also confounded by high signal in terminals, which hinders accurate counting by obscuring the difference between signal inside and outside the retrogradely labeled neurons. Therefore, undercounting GABA neurons may account for some of the "unidentified" DiI(+) neurons reported here. On the other hand, there are also many glutamate

neurons in the VTA that likely contribute to these VTA projections (N. Gorelova et al., 2012; Hnasko, Hjelmstad, Fields, & Edwards, 2012; Morales & Root, 2014; Yamaguchi et al., 2011a). There is no existing immunocytochemical target for labeling VTA glutamatergic somata, as protein markers such as vesicular glutamate transporters are located in terminals. Additionally, as of yet, there is no transgenic rat model to enable reporter expression selectively in glutamate neurons in the VTA. We hypothesize that the non-TH and non-GAD DiI filled cells in our study are primarily glutamatergic. Moreover, the percentages of DiI(+) neurons that were non-DA and non-GABA is consistent with prior glutamate studies in projections to the NAc and PFC (N. Gorelova et al., 2012; Yamaguchi et al., 2011a). As tools become available, future work should involve a similar characterization and quantification of glutamatergic projection neurons in the VTA.

Comparison with the literature

Comparison of methodologies

A variety of approaches have been used to map projections from the VTA to its target nuclei. In addition, rat and mouse anatomy may differ. For example, Taylor et al. (2014) analyzed VTA anatomy in the mouse and used a Cre-dependent mouse line to identify GABAergic neurons rather than using immunocytochemistry. Here, we implemented a retrograde tracing methodology and primarily compare our results to other rat studies that used similar methods (Kirouac, Li, & Mabrouk, 2004; Klitenick, Eutch, Churchill, & Kalivas, 1992; Margolis, Lock, Chefer, et al., 2006; Swanson, 1982; Yamaguchi et al., 2011). Studies that use anterograde-tracing methods eliminate the possibility of cell counting, but do map the relative density of terminal fibers in VTA target regions (Beckstead et al., 1979). While we did not systematically compare the overall numbers of VTA cells projecting to each region, we do see similar density patterns as prior reports. For example, using anterograde tracing, a dense projection to the VP and a sparse projection to the mPFC have previously been reported (Beckstead et al., 1979; S. R. Taylor et al., 2014). Additionally, a dense bilateral projection to the vlPAG has been reported with anterograde methods (Beitz, 1982; Kirouac & Pittman, 2000). However, Aransay and colleagues (2015), using single axon anterograde tracing, did report higher rates of sparse collateralization of some VTA neurons. These neurons innervate not only the cerebral cortex but also basal forebrain regions such as the VP and amygdala. Additionally, several VTA neurons were found to innervate forebrain structures and collateralize to brainstem structures and the hypothalamus (Aransay, Rodríguez-López, García-Amado, Clascá, & Prensa, 2015). This group of neurons may coincide with the small population of collateralizing VTA neurons reported in dual retrograde tracing studies (Loughlin & Fallon, 1984; Margolis, Lock, Chefer, et al., 2006; Swanson, 1982). Further, rabies mediated trans-synaptic tracing, and the combination of anterograde and retrograde tracers, have been used towards the goal of mapping input-output relationships of VTA circuits (Beier et al., 2015; Lammel et al., 2012b). For example, Beier et al (2015) recently found that VTA DA neurons that project to the medial or lateral NAc receive different inputs from various brain regions. Overall, where there is data to compare, the patterns observed with these alternate techniques are consistent with our findings, indicating that in the VTA, cell body counts using retrograde tracing corresponds well with the existing anterograde literature.

Comparison of dopaminergic projections

Where previous data exists, the proportion of DA neurons in each projection observed here is largely consistent with the existing literature in the rat (Kirouac et al., 2004; Klitenick et al., 1992; Margolis, Lock, Chefer, et al., 2006; Swanson, 1982; Yamaguchi et al., 2011) but was overall lower than percentages reported in the mouse (S. R. Taylor et al., 2014). For all of the subsequent comparisons, the reported percentages refer to ipsilateral projections, unless otherwise indicated. Projections to cortical regions were approximately 20-40% dopaminergic. Specifically, the projection to the ACC was around 23% TH(+), consistent with findings from Swanson (1982). A slightly higher percentage of TH(+) neurons (approx. 40%) was found in projections to the PrL and IL. This is consistent with prior reports in the rat (Margolis, Lock, Chefer, et al., 2006; Yamaguchi et al., 2011), but much less than the more than 70% DA reported in the mouse (S. R. Taylor et al., 2014). Some of these differences might be due to the precise injection locations and counting regions used in the current study. VTA projections to the NAc contained a lower percentage of DA compared to several studies in the rat and mouse, which reported near 85-90% DA (Swanson, 1982; S. R. Taylor et al., 2014). However, the close to 65% TH(+) neurons observed in the projection the NAc core is consistent with prior findings in our own laboratory following injections covering the core-medial shell border (Margolis, Lock, Chefer, et al., 2006) and was greater than the reported 50% in a more recent study in the rat (Rodríguez-López, Clascá, & Prensa, 2017). The current study is the first to differentiate the percentage of DA in projections to the medial vs. lateral NAc shell in the rat. We found a similar percentage of between 40-50% TH(+) neurons in both of these projections, much less than what was reported in the mouse (S. R. Taylor et al., 2014), but similar to a study in the rat (Rodríguez-López et al., 2017). Overall, however, projections to the NAc and its sub-regions contained the highest percentages of DA neurons, consistent with overall patterns previously reported for both rats and mice (Swanson, 1982; S. R. Taylor et al., 2014; Yamaguchi et al., 2011a). The percentage of TH(+) neurons in VTA projections to the VP (approx. 40%) and BLA (approx. 50%) fell within the range of prior rat work (Klitenick et al., 1992; Swanson, 1982) but again was less than what has been reported in the mouse (S. R. Taylor et al., 2014). This is the first study to differentiate VTA projections specifically to the anterior and posterior BLA; a similar percentage of TH(+) projections was found in both cases. Lastly, consistent with the literature, we found that VTA the projection to the vlPAG has a significant contralateral contribution (Aransay et al., 2015; Beitz, 1982; Kirouac et al., 2004). While one study reported a lack of DA contribution to this projection (Kirouac et al., 2004), we found that a small percentage of TH(+) neurons (approximately 15% ispi, 30% contra) projects to the vlPAG, consistent with more recent work (Aransay et al., 2015).

Comparison of GABAergic projections

Few prior studies have systematically examined VTA GABA projections, especially in the rat. The relatively small contribution of GABA neurons to each projection we observed is largely consistent with prior reports in both the rat and mouse (S. R. Taylor et al., 2014; Yamaguchi et al., 2011a). Although Yamaguchi and colleagues (2011) did not identify GABA directly, around 10% of projections to the mPFC were non-dopaminergic and non-glutamatergic, therefore inferred to be GABAergic. This percentage is consistent with our findings in projections to both the PrL and IL. However, we detected a higher percentage of GAD(+) neurons in the projection to the ACC, especially from the contralateral VTA (approx. 40% contra, 20% ipsi). Prior work from our lab showed that approximately 25% of projections from the VTA to the NAc are GABAergic

(Margolis, Lock, Chefer, et al., 2006). This is relatively similar to the 36% reported by others, and reports of a minimal glutamate-only projection to the NAc (Van Bockstaele & Pickel, 1995; Yamaguchi et al., 2011a). Thus, we expect the majority of the non-DA neurons (approx. 40%) projecting to the NAc to be GABAergic. To our knowledge, this was the first study to quantify the contribution of GABA neurons to the VTA projections to the VP, ABLA, and PBLA in the rat. Approximately 20% of projections to the VP were GAD(+), a much greater percentage than reported in the mouse (S. R. Taylor et al., 2014). Interestingly, a greater percentage of GAD(+) neurons was found to project to the posterior BLA (~25%) compared to the anterior BLA (~10%), suggesting AP organization of VTA inputs to the amygdala. Lastly, we found a robust GABA projection from the VTA to the vlPAG: close to 50% of the projection is GAD(+), more than the previously reported 32% (Kirouac et al., 2004). Importantly, this GABA projection arose from the VTA itself; we did not include projections from the rostromedial tegmental nucleus in this study, which also provides a dense GABA projection to the lateral PAG (Aransay et al., 2015).

Topographic organization of the VTA

We found here that while topographical distributions comprising each of the investigated projections differ, it was rare for a projection to be localized exclusively to a sub-region of the VTA. Consistent with prior studies, dorsal-ventral differences were observed in the VTA projection to the mPFC (Moore & Bloom, 1978; Swanson, 1982). PrL-projecting and IL-projecting VTA neurons were located in more ventral and more dorsal regions, respectively. This is consistent with reports that the ventral VTA projects to more dorsal target regions, while the dorsal VTA projects more ventrally (Moore & Bloom, 1978). However, we found that ACC projection neurons were distributed relatively evenly throughout the DV axis, in contrast to Swanson's finding that cingulate-projecting neurons were located preferentially in the ventral VTA (1982). This difference may be related to tracer injection location, as our ACC injections were anterior to those analyzed by Swanson (1982). Projections to the mPFC were located more robustly in the medial VTA, consistent with prior reports of a denser projection located in the midline VTA nuclei (Chandler et al., 2013; Yamaguchi et al., 2011a). Interestingly, although a greater percentage of TH(+) PrL-projecting neurons were found in midline areas, consistent with prior findings (Swanson, 1982), TH(+) IL-projecting neurons were enriched in the posterior-lateral VTA. Intriguingly, we found higher percentages of GAD(+) neurons in projections to the ACC compared to the PrL and IL, especially in the dorsal VTA. These observations are also consistent with the possibility that largely non-overlapping populations of VTA neurons project to these different sub-regions of PFC. This has been demonstrated for orbital frontal cortex, mPFC, and ACC by Chandler and colleagues (2013), who used simultaneous labeling by different fluorescent retrograde tracers to analyze collateralization of VTA projections to the PFC, and detected few dual labeled neurons. Our observations suggest this is the case for even finer subdivisions of PFC, e.g. PrL and IL.

In projections to the NAc, we also see a graded topographic organization, similar to what has been previously described, with medial VTA neurons projecting more to the medial NAc shell and lateral VTA neurons projecting more to the lateral NAc shell (Ikemoto, 2007; Rodríguez-López et al., 2017). With single cell axon tracing techniques, dense arborization has been observed in axons targeting the NAc core and shell, with little branching to the adjacent NAc sub-regions, consistent with these projections being largely separate (Rodríguez-López et al., 2017). Although there have been reports of a similar medial-lateral topographical organization for DA neurons (Ikemoto, 2007), we found that TH(+) neurons projecting to the NAc and its sub-regions were

relatively evenly distributed throughout the VTA, consistent with other findings (Swanson, 1982; S. R. Taylor et al., 2014; Yamaguchi et al., 2011a). In projections to the VP, although the overall distribution of neurons was fairly even across the VTA, a higher percentage of TH(+) neurons was located laterally, similar to prior reports (Klitenick et al., 1992; S. R. Taylor et al., 2014). Interestingly, a novel dorsal-ventral difference was observed in GAD(+) projections to the VP, with more VP-projecting GABA neurons located in the dorsal VTA.

Dense projections to both the ABLA and PBLA were found in the lateral VTA, a very consistent finding across the literature in both rats and mice (Aransay et al., 2015; Loughlin & Fallon, 1983; Swanson, 1982; S. R. Taylor et al., 2014). More specifically, we found that ipsilateral projections arise mostly from the anterior lateral VTA, while contralateral projections arise from the medial VTA. Higher percentages of TH(+) neurons projecting to the BLA were also observed in more lateral VTA, consistent with prior findings (Swanson, 1982). Interestingly, not only was there a marked difference in the contribution of GAD(+) neurons to the ABLA and PBLA projections, there was also a difference in their topographical organization. While there was an overall lower percentage of ABLA-projecting VTA neurons that were GAD(+), we found that these comprised more of the projection from the lateral and ventral VTA; GAD(+) projections to the PBLA were more evenly distributed.

Lastly, VTA projections to the vlPAG were fairly evenly distributed throughout the VTA. Dopaminergic lateral PAG-projecting neurons have previously been reported in the PBP, which is partially overlapping with our more dorsal lateral sampling areas (Aransay et al., 2015). In addition to observing TH(+) projections in the lateral VTA, we also observed TH(+) projections to the vlPAG from the medial VTA; these projections were evenly distributed in all sampling windows and across the DV axis. The strong GABAergic projection to the vlPAG has been previously reported (Kirouac et al., 2004); we found that the GAD(+) neurons contributing to this projection were evenly distributed in the dorsal VTA, but enriched in lateral regions in the ventral VTA.

Functional implications

Projections of the VTA to target nuclei play a functional role in motivation and reward (H. L. Fields et al., 2007; Morales & Margolis, 2017). The fact that individual VTA neurons rarely collateralize to multiple projection targets (Margolis, Lock, Chefer, et al., 2006; Swanson, 1982) raises the possibility that these different projections may contribute to different aspects of behavior. A large body of research has been devoted to understanding the contribution of DA neurons to reward related behaviors (Lammel, Lim, et al., 2014b; J. D. Salamone & Correa, 2012b; Volkow, Wise, & Baler, 2017). However, only a slight majority of VTA neurons is actually TH(+) in the rat (Kirouac et al., 2004; Klitenick et al., 1992; Margolis, Lock, Chefer, et al., 2006; Swanson, 1982; Yamaguchi et al., 2011), and as we show here, most VTA projections are composed primarily of *non*-DA neurons. In this context, DA-independent VTA contributions to behavior are not surprising. Importantly, DA-independent VTA reward has been observed; for example, intra-VTA morphine can produce a DA-independent conditioned place preference (CPP) (Hnasko, Sotak, & Palmiter, 2005; Nader & van der Kooy, 1997). Non-DA projections have been implicated in aversion; stimulation of VTA glutamate neurons projecting to the lateral habenula or to the medial shell of the NAc is sufficient to produce aversion (Qi et al., 2016; Root et al., 2014). Clearly, more work needs to be done on the GABAergic and glutamatergic projections of the VTA in order to understand their contributions to behavior.

Functional roles of VTA dopamine projections

The behavioral role of DA in many of the target regions considered here has been intensely studied. In the cortex, D1 receptor activation is implicated in effort-based decision-making (Rushworth, Walton, Kennerley, & Bannerman, 2004; J. Schweimer & Hauber, 2005; Walton et al., 2003). DA levels in the ACC increase with either electrical stimulation of the VTA or microinjection of a mu opioid receptor agonist into the VTA (Narita et al., 2010b). In behavioral studies, PrL and IL are often considered together as the "mPFC", potentially generating inconsistent results. For instance, in one study, optogenetic stimulation of mPFC-projecting DA neurons was aversive and anxiogenic (Gunaydin et al., 2014b) but in another study, *inhibition* of the mPFC-projecting DA neurons promoted susceptibility to a subsequent social defeat stressor (Chaudhury et al., 2013b). Differentiating functional effects of VTA projections to the PrL and IL, rather than the mPFC as a whole, remains an interesting question, and may resolve seemingly paradoxical observations (for a review see Moorman et al., 2015). For example, injection of a DA antagonist into the PrL but not IL attenuates the stress-induced reinstatement of drug seeking behavior (Capriles, Rodaros, Sorge, & Stewart, 2003b).

Considerable evidence suggests that mesolimbic DA projections from the VTA to the NAc play an important role in reinforcement and in the rewarding effects of drugs of abuse (Ikemoto, 2007, 2010; J. D. Salamone, Correa, Mingote, & Weber, 2005). The NAc core and NAc shell may play unique roles in behavior. For instance, animals intra-cranially self-administer DA agonists into the NAc shell, but not the NAc core, indicating that the NAc shell drives reward related behaviors (Ikemoto, 2007, 2010; McBride, Murphy, & Ikemoto, 1999). Specific functional roles of VTA projections to the medial vs. lateral NAc shell are only just beginning to be parsed apart (Ikemoto, Quin, & Liu, 2005; Lammel et al., 2012b; Pecina & Berridge, 2013; R. Shin, Qin, Liu, & Ikemoto, 2008).

The BLA is involved in forming associations between sensory cues and rewarding or aversive stimuli (Davis, 1992; Gallagher, 2000; LeDoux, 1996; McGaugh, 2002; Meil & See, 1997). Here, too, researchers have found a role for DA. Lesions of the VTA decrease DA content of the amygdala (Fallon & Moore, 1978) and DA receptor blockage in the BLA attenuates the conditioned reinstatement of cocaine seeking behavior (Kruzich & See, 2001). More specific roles for VTA dopamine projections to the BLA remain to be explored; in particular, we do not yet know how DA projections to the anterior compared to the posterior BLA contribute to behavior.

Blockade of DA receptors in the VP reduces ethanol self-administration into the VTA (Ding, Ingraham, Rodd, & McBride, 2015). Furthermore, 6-hydroxydopamine lesions of the VP block cocaine-induced CPP (Gong, Neill, & Justice, 1996). The VP is now recognized as a reward region in its own right rather than just as a motor output nucleus (Root et al., 2015) and is thought of as an output for limbic signals, as it is receives convergent inputs from reward related brain areas including the PFC, NAc, and amygdala (Grove, 1988; Klitenick et al., 1992; Maurice, Deniau, Menetrey, Glowinski, & Thierry, 1997; Zaborszky, Gaykema, Swanson, & Cullinan, 1997). Thus, VTA projections to the VP are well situated to influence reward processing downstream of other areas.

Functional roles of VTA GABA projections

The development of genetically tagged mouse lines has relatively recently enabled the selective study of the behavioral contributions of VTA GABA and glutamate neurons that are intermixed with DA neurons. Local stimulation of VTA GABA neurons reduces reward consumption, but little is known about how the projections contribute to this behavior (Van Zessen, Phillips, Budygin, & Stuber, 2012). Brown and colleagues (2012) demonstrated that stimulation of NAc-projecting VTA GABA neurons inhibits cholinergic interneurons (CINs) within the NAc, and promotes stimulus-outcome learning. However, this function may still involve DA, as CINs regulate DA release in the striatum through their action on nicotinic receptors on DA axon terminals (Cachope et al., 2012; Threlfell & Cragg, 2011; Zhou, Liang, & Dani, 2001).

The function of GABA projections to many other VTA targets remains unknown; however, one interesting pattern we detected was a greater percentage of GABA projections to the PBLA compared to the projection to the ABLA. These two regions of the amygdala have recently been shown to contain different populations of neurons that have opposing effects on behavior: neurons in the ABLA respond to negative stimuli and can disrupt reward-seeking behaviors when activated, while neurons in the PBLA respond to positive stimuli and are critical for reward seeking and associative conditioning (J. Kim et al., 2016). Stimulation of these PBLA neurons can also elicit a CPP (J. Kim et al., 2016). Our anatomical detection of these VTA GABA populations, as well as those that project to the ACC and vlPAG, among others, provide especially interesting future directions for investigating how the VTA contributes to motivated behavior.

Functional implications of VTA topography

The regional heterogeneity of the VTA also has important implications for behavior. Differences between the anterior and posterior VTA have been described (for a review see Sanchez-Catalan et al., 2014), and the anatomical information we provide here is consistent with such a pattern within the VTA. For example, rats will self-administer drugs of abuse including opiates, ethanol, and nicotine into the posterior, but not the anterior, VTA (Ding et al., 2015; Ikemoto, Qin, & Liu, 2006; Zangen, Ikemoto, Zadina, & Wise, 2002). Understanding the distribution of projection neurons and their different cell types enables the formulation of predictions regarding the circuitry involved in such behaviors. For instance, we found that projections to the IL and ACC arising from the posterior-lateral VTA contain a greater percentage of TH(+) neurons. We also observed a higher percentage of GAD(+) neurons in projections to the ABLA in the posterior-lateral VTA. Different distributions of VTA cell types (DA, GABA, or glutamate) along the AP axis, as well as differences in the target regions those cells project to, may together be important factors driving the observed behavioral differences between the anterior and posterior VTA.

The medial-lateral and dorsal-ventral organization of VTA projections is also important for behavioral outcomes. For example, our current study and others suggest a topographical organization of projections from the medial-lateral VTA to corresponding medial-lateral NAc regions (Ikemoto, 2007; Lammel et al., 2008; Rodríguez-López et al., 2017; reviewed in Lammel et al., 2014). This medial-lateral distribution is behaviorally relevant, as the medial and lateral NAc shell may have functionally separate roles (Lammel et al., 2012b; Pecina & Berridge, 2005). We also found that projections to the PrL and IL were located more densely in the medial VTA, and therefore may contribute to behaviors attributed to the medial VTA; yet PrL and IL projections are

differentiated by their location in the DV axis. These topographical distributions within the VTA are important not only in regard to where to target microinjections of drugs or viruses for behavioral studies, but also with respect to the sampling of neurons for both *in vivo* and *ex vivo* electrophysiology. Overall, the heterogeneity of the VTA, both in projection target and neurotransmitter content, is an important element to be considered for understanding the functional roles of the VTA.

Conclusions

We demonstrate here that a majority of VTA projections have a significant non-dopaminergic component. We also report the distribution of the somata within the VTA that give rise to these different projections, and the proportion of those projections that are dopaminergic or GABAergic. Building on the existing literature, we found that the cell bodies contributing to these projections show unique patterns of localization within the VTA, and variation in their neurotransmitter content. Importantly, these data indicate that projections to different brain regions arise from intermixed populations of neurons across the VTA, yet adjacent projection targets likely receive inputs from different populations of VTA neurons. Together, these observations indicate an organized but intermixed structure to the VTA, including the many non-dopaminergic projection neurons therein, whose behavioral contribution is only beginning to be explored.

Chapter 3: An ingroup bias for pro-social behavior in adult but not young rats is associated with a distinct neural network

** This chapter is adapted from a recent submission. It has not yet been published.

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Abstract

Pro-social behavior, like helping others, is biased towards members of the same group across species. Here, the neural mechanisms underlying ingroup bias were investigated in rats tested for helping behavior. Adults released ingroup, but not outgroup members trapped inside a restrainer. Juveniles released all rats, regardless of group identity. Brain-wide neural activity, indexed by expression of the early-immediate gene c-Fos, identified a neural pattern associated with group identity, and another pattern associated with age. The nucleus accumbens, part of the reward network, was a central hub for the ingroup, a result validated by *in-vivo* calcium imaging. A projection from the cingulate cortex to the accumbens correlated with helping. Thus, ingroup bias emerges in adulthood, with pro-social intent towards ingroup members recruiting a distinct neural circuit.

One Sentence Summary: Ingroup bias for pro-social behavior emerges in adulthood in rats and recruits distinct neural circuits in response to a distressed ingroup member.

Introduction

Pro-social actions that benefit others are a building block of life in social groups (Wilson, 2012). Multiple species across the phylogenetic spectrum demonstrate pro-social behavior (Dugatkin, 1997) typically towards affiliated others (Hamilton, 1964). In humans, an empathy bias towards ingroup members is thought to underlie reduced pro-social motivation towards members of other groups (Cikara, Botvinick, & Fiske, 2011). Encouragingly, children are malleable in their biases towards outgroup members (Skinner & Meltzoff, 2019), and exposure to a diverse environment during childhood can reduce biases in adulthood (Telzer, Humphreys, Shapiro, & Tottenham, 2013; Zuo & Han, 2013). Understanding the neural development of ingroup bias can thus provide insights into the flexibility of this biological mechanism.

To this end, pro-social behavior was examined in adult and juvenile rats. Rodents, a highly social species, experience stress in response to observing others in distress (Meyza et al., 2017), console distressed mates (Burkett et al., 2016), and act for the benefit of others (Hernandez-Lallement et al., 2020; Marquez, Rennie, Costa, & Moita, 2015; Sato, Tan, Tate, & Okada, 2015; Schaich Borg et al., 2017). In a helping test, rats are typically motivated to release a conspecific trapped inside a restrainer, and learn to help without prior training or reward, and in the absence of social contact after helping (Bartal et al., 2011). Importantly, adult rats will release a trapped conspecific of the same strain ("ingroup" member), but will not help a rat of an unfamiliar strain ("outgroup" member) (Bartal et al., 2014), demonstrating an ingroup bias for pro-social behavior. A shift of ingroup membership is observed in rats fostered at birth with the outgroup strain, suggesting that pro-social motivation is flexible and can be modulated by social experience.

Results

Here, helping behavior was studied in juvenile and adult albino Sprague-Dawley (SD) rats tested with trapped SD cagemates ('adult ingroup', n=8, 'juvenile ingroup', n=13, Fig. 1A), or with rats of the unfamiliar black-caped Long-Evans (LE) strain ('adult outgroup', n=16, 'juvenile outgroup' n=8). During the Helping Behavior Test (HBT), a free rat was placed in an arena containing a conspecific trapped inside a restrainer, for daily 1-hr sessions over a two-week period. The free rat could release the trapped rat by opening the restrainer door with its snout. After 40 minutes, if the rat had not opened the door it was opened half-way by the experimenter. This was typically followed by the trapped rat's exit and aimed to prevent learned helplessness. On the final session, the restrainer was latched shut such that all rats had an objectively similar experience for imaging neural activity. Sessions were video-recorded and processed for movement analysis (Fig. 1B).

Adult and juvenile rats tested with ingroup members demonstrated helping behavior, as expressed by a significant increase in % door-opening (Cochrans' Q, $p<0.01$) and reduced latency to door-opening (Friedman, $p<0.05$) along the days of testing (Fig. 1C-D, Movie S1). In line with past observations of an ingroup bias in rats, door-opening behavior was rarely observed in the adult outgroup condition (Cochrans' Q, Friedman, $p>0.05$, Fig. 1E-F). Strikingly, unlike adults, juvenile rats robustly released trapped outgroup members, as expressed by increased % door-opening (Cochrans' Q, $p<0.001$) and decreased door-opening latency (Friedman, $p<0.01$, Fig. 1E-F, Movie S2). On the final session, juveniles spent more time near the trapped outgroup member than did adults (ANOVA, $p<0.01$, Bonferroni $p<0.01$, Fig. 1G), and juveniles in the outgroup were more

active than other conditions (ANOVA, $p<0.01$, Bonferroni $p<0.01$, Fig 1H, Movie S3). Activity was directed at the trapped rat, as indicated by circling of the restrainer, and a significant correlation between activity and time spent near restrainer (Pearson's, $p<0.01$). Thus, rats of all ages were motivated to help trapped ingroup members, and juveniles helped regardless of group identity.

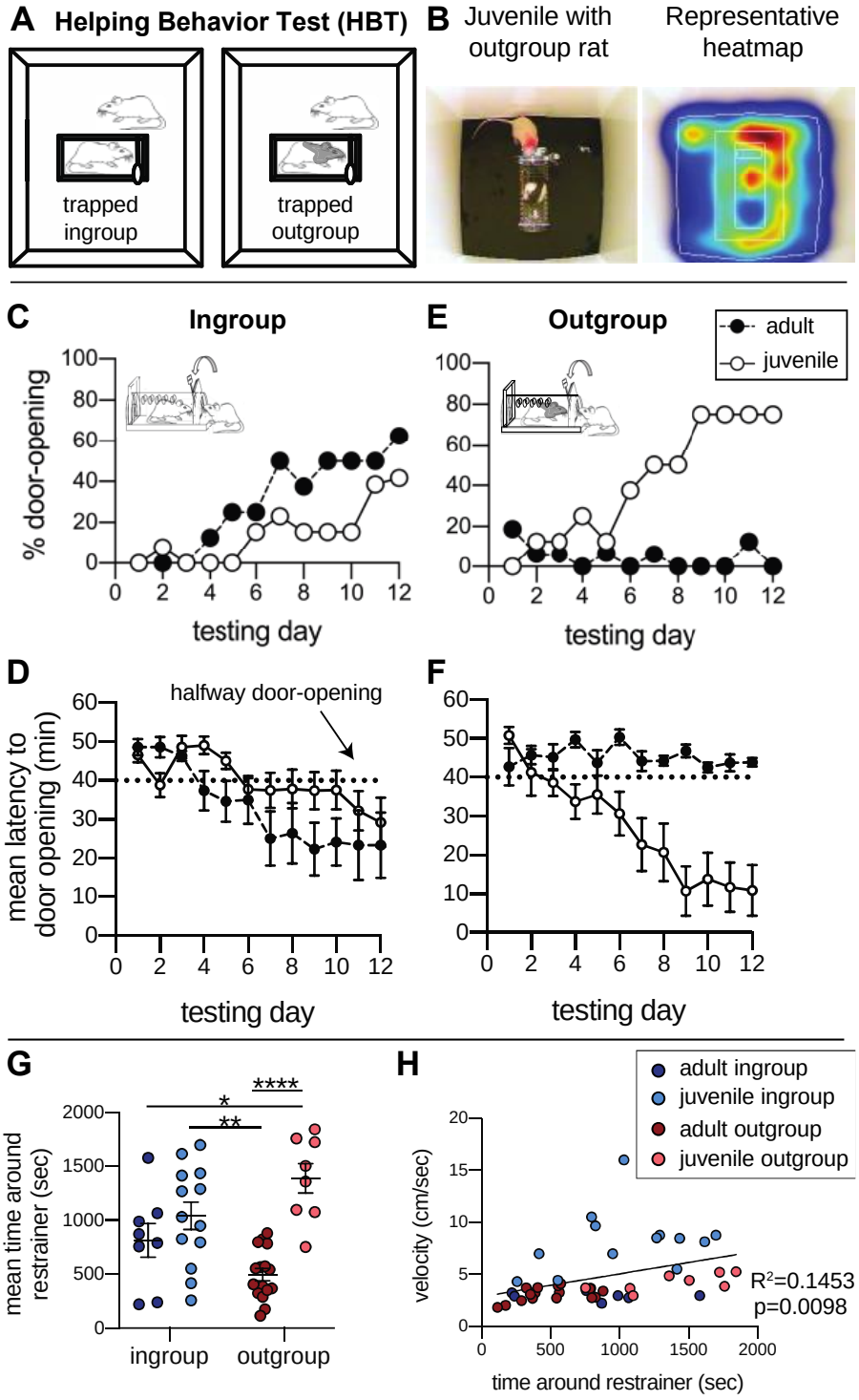

Figure 3-1. Behavior in the helping behavior test (HBT).
(A) Diagram of the HBT (B) Representative movement pattern of a juvenile tested with an outgroup member depicted by a heatmap of the rat's location along the session. Helping behavior is expressed by % of door-openings and latency to open for the ingroup (C-D) and outgroup (E-F) across testing sessions. The dashed line indicates the half-way door-opening by the experimenter. (G) Analysis of movement patterns is presented as the time rats spent near the trapped rat on the final day of testing. This measure was correlated with activity levels (H).

To map brain-wide activation involved in the HBT, the immediate early-gene c-Fos was quantified as an index of neural activity across conditions (n=84 sampled brain regions per rat, Fig. 2A-D, Fig. S1, Table S1; Guzowski et al., 2005). c-Fos expression was measured following the final testing session and reflects neural activity in the presence of a trapped rat. Two overarching patterns of neural activity were identified for the four HBT conditions using multivariate task partial least-square (PLS) analysis. One pattern (latent variable LV1, $p<0.001$) was associated with increased neural activity in the ingroup compared to the outgroup conditions. Permutation bootstrapping tests identified a broadly dispersed network of brain regions that significantly contributed to this contrast (Fig. 1E), including the anterior insula (AI), and anterior cingulate cortex (ACC), regions associated with aversive arousal for others' distress in humans (Uddin, Nomi, Hebert-Seropian, Ghaziri, & Boucher, 2017) and rats (Rogers-Carter & Christianson, 2019). The second pattern (latent variable LV2, $p<0.001$) indicated an effect of age on neural activity patterns (Fig. 1F). Juvenile brains were marked by significantly increased activity in the prefrontal cortex and reduced activity in the hippocampus and hypothalamus compared to adults. Notably, as helping behavior (helpers vs. non-helpers) did not produce a significant latent variable ($p>0.1$), nor reveal differences in c-Fos activity (ANOVA, $p>0.1$, Fig. S2), motivational state was best delineated by the test condition rather than by door-opening. In sum, neural activity patterns were determined primarily according to group-identity and age.

In addition, to identify condition-specific neural activity, c-Fos expression of the four HBT conditions was compared to a baseline of untested adult SD rats, which accounts for non-specific c-Fos expression ("untested", n=10, Fig. 3A-D). A common set of regions was significantly active across all conditions compared to the untested baseline (ANOVA, $p<0.001$, Dunnett's $p<0.05$, Fig S3, Table S2), including the piriform cortex (Pir), prelimbic cortex (PrL), and orbitofrontal cortex (ventral and lateral; VO and LO). These regions thus participate in the response to a trapped conspecific independently of pro-social intent. Conversely, a subset of regions was selectively active in the ingroup conditions compared to baseline (Dunnett's, $p<0.05$), including the medial orbitofrontal cortex (MO), dorsal endopiriform cortex (DEn), basomedial amygdala (BMA), lateral septum (LS), and nucleus accumbens shell and core (NacSh and NacC). The Nac, part of the brain's motivation and reward network (Carelli, 2002), participates in social reward in humans (S N Haber & Knutson, 2010) and rodents (Gunaydin et al., 2014a). To assess whether Nac activity was a general response to the presence of an ingroup member, a control condition was conducted whereby non-trapped ingroup members were divided across a wire mesh, providing a similar level of contact as the HBT ("2 free", n=7). Significantly less activity was observed in the Nac in the "2 free" condition compared to the HBT ingroup (ANOVA, Bonferroni, $p<0.05$, Fig. 3E), indicating that Nac activity is a response to the trapped state of the ingroup member. A significant correlation between Nac activity and door-opening in adults (Fig. 3F-G) supported the idea that the Nac reflects pro-social motivation. Yet, correlation data in and of itself is not sufficient evidence, especially given limited sample size and the possibility of false discoveries. This prompted further analyses. A network analysis based on inter-region correlations of c-Fos expression further identified the Nac as a central hub for the adult ingroup condition; it was highly correlated with numerous brain regions and essential for network connectivity (Fig. S4, Table S3). Together, the c-Fos absolute numbers, as well as the correlation and network analysis all point to the Nac as a central area activated in the ingroup condition.

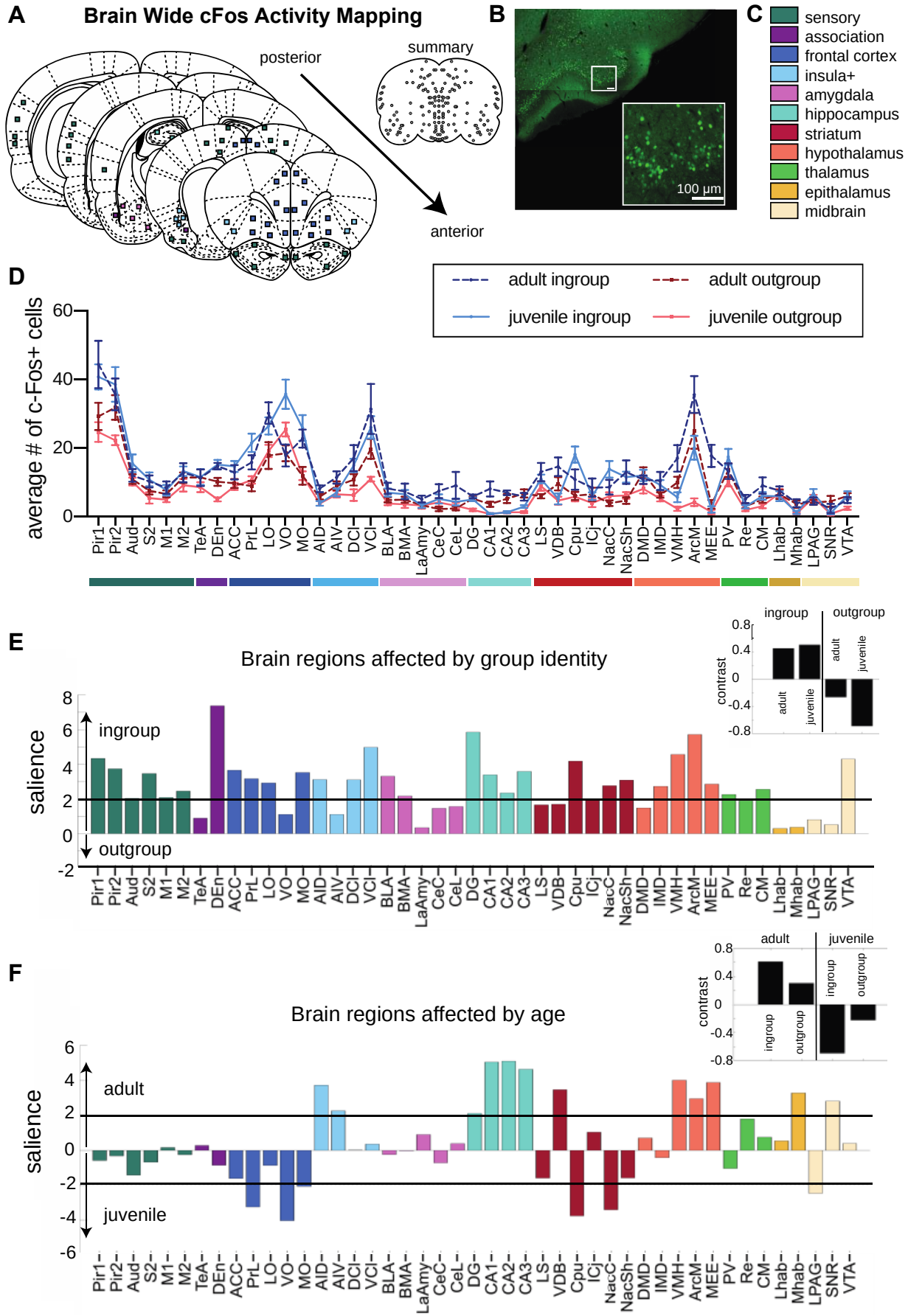
A
Brain Wide cFos Activity Mapping
posterior
summary
anterior
B
100 µm
C
sensory
association
frontal cortex
insula+
amygdala
hippocampus
striatum
hypothalamus
thalamus
epithalamus
midbrain
D
adult ingroup
adult outgroup
juvenile ingroup
juvenile outgroup
average # of c-Fos+ cells
Pir1 Pir2 Aud S2 M1 M2 TeA DEn ACC PrL LO VO MO AID AIV DCI VCI BLA BMA LaAmy CeC CeL DG CA1 CA2 CA3 LS VDB Cpu ICj NacC NacSh DMD IMD VMH ArcM MEE PV Re CM Lhab Mhab LPAG SNR VTA
E
Brain regions affected by group identity
salience
ingroup
outgroup
contrast
F
Brain regions affected by age
adult
juvenile

Figure 3-2. Neural regions associated with the Helping Behavior Test.

(ABOVE) (A) A diagram of brain regions sampled for c-Fos expression. (B) A representative image of c-Fos signal sampled in the piriform cortex. (C) Legend of brain region categories coded by color. (D) Number of c-Fos+ cells per region (mean±SEM). Significant latent variables reveal that group identity (E) and age (F) determine neural activity patterns. The salience represents the z-score of boot-strapping tests, with regions crossing the black threshold lines significantly ($p < 0.05$) contributing to the contrast depicted in the inset (black bars). The directionality of the bars is congruent with the contrast graphs, as demonstrated by the arrows on the axis bar. All regions were more active for ingroup than outgroup members, but several regions (e.g. VO) were more active for juvenile than adult rats.

Next, to explore the Nac's response to a trapped rat *in vivo*, neural activity was recorded during the HBT via fiber photometry for adult SD rats tested with trapped SD strangers (n=8). To tag firing neurons, an AAV virus driving the expression of the genetically-coded calcium indicator GCamp6m under the hSyn promotor was injected into the Nac, and an optic fiber was implanted at the same location (Fig. 4A, Fig. S5, Fig. S6A). Calcium signal was recorded by a photoreceptor, and fluorescence intensity was analyzed as previously described (Lerner et al., 2015) (Fig. S6B). Approach to the trapped rat was measured as the moment of entry into the zone around the restrainer. Thus, this measure captures a similar movement across all rats. When rats approached a trapped ingroup member (Fig. 4B), activity significantly increased (Wilcoxon ranked-sum test, $p<0.05$, Fig. 4C, Movie S4). Activity was not changed when these same rats approached a trapped outgroup member (measured on a single session), or an empty restrainer (Wilcoxon, $p>0.05$, Fig. 4C). Additionally, Nac activity during the entire session was significantly higher when rats tested with ingroup members were in the area around the restrainer rather than outside this zone (repeated measures ANOVA, $p<0.01$, Bonferroni $p<0.01$), an effect not observed for outgroup members (Fig. 4D). A peak in activity observed at the moment of door-opening indicates that door-opening itself was a salient event (Fig. S6C). Cranberries, a non-social reward, were placed in the restrainer on the last session. Activity significantly decreased when rats ate a cranberry, evidence that the Nac was active during seeking, rather than reward acquisition (Wilcoxon, $p<0.05$, Fig. S6D). These data provide further support for selective Nac activation for ingroup members and suggest that pro-social ingroup approach is correlated with increased Nac activity.

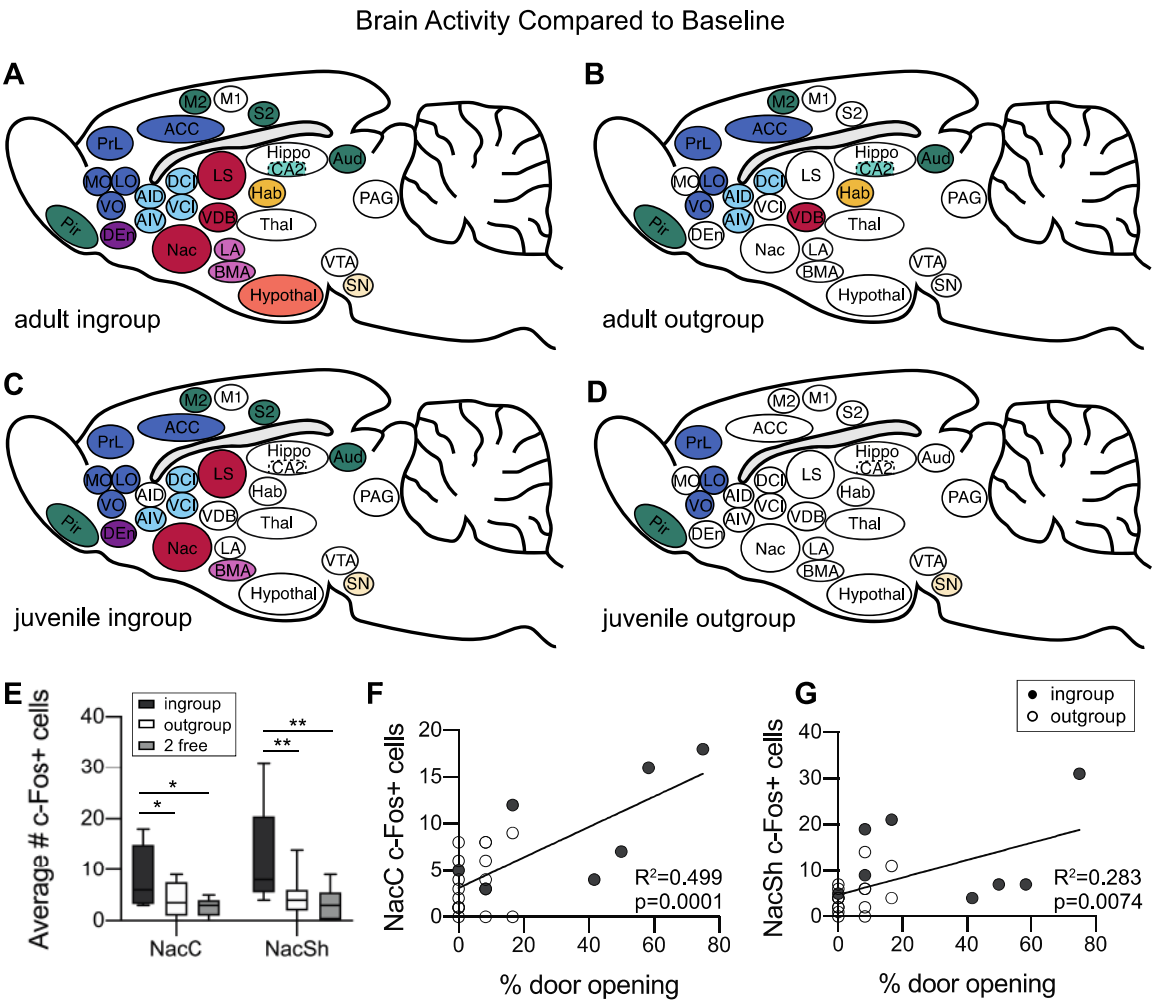

Figure 3-3. Condition-specific neural activity.
(A-D) Colored regions on brain diagrams indicate regions significantly active in each condition compared to an untested baseline. (E) Nac activity was higher in the ingroup condition compared to a control group with two rats freely exploring across a mesh divider ('2 free'). c-Fos in the NacC (F) and NacSh (G) was positively correlated with door opening behavior in adult rats.

To identify Nac inputs that promote pro-social behavior, structural projections were marked by injecting the retrograde tracer Fluoro-Gold (FG) into the Nac prior to the HBT (n=13, Fig. 4E, Fig. S6E-F). Co-labeling of FG^+ and $c\text{-}Fos^+$ cells mark cells that were active during the HBT and structurally projected to the Nac. All sampled regions of the frontal cortex showed substantial structural projections (Fig. S6G) and co-labeling with c-Fos (Fig. 4F-G). A significant positive correlation between the co-labeled cells and door-opening was uniquely observed in the projection from the ACC to the Nac (Fig. 4H), whereas ACC $c\text{-}Fos^+$ cell numbers as a whole did not correlate with behavior (Fig. S6H). This finding suggests that this specific sub-population of cells may participate in pro-social behavior. Yet, this relationship is descriptive only. Overall, the current manuscript aims to give a broad overview of the neural circuitry involved in pro-social intent and provides a base for future work that will aim to dissect these circuits and understand their causal contribution to behavior.

Discussion

This study demonstrates that while adult rats do not show pro-social behavior towards trapped outgroup members, juvenile rats are not biased in this manner, and are motivated to help all rats regardless of group membership, indicating that ingroup bias is acquired along development.

A distinct neural pattern was associated with the response to trapped ingroup members, including regions typically associated with empathy and reward in humans. This pattern may reflect activity associated with movement related to door-opening. Yet this is unlikely, as no helping occurred on the c-Fos day, and the neural activity in the juvenile outgroup rats was different despite previous helping.

Differences in brain activity between adult and juvenile rats are informative for understanding the neural mechanism involved in ingroup bias. Specifically, reduced activity was observed in the hippocampus in young rats compared to adults, including in the CA2, which participates in social recognition (Hitti & Siegelbaum, 2014). It is thus possible that social mapping is less specific in the juvenile brain. This idea is in line with research showing that discrimination based on social identity emerges in the amygdala in adulthood in humans (Telzer et al., 2013) and mice (Bergan, Ben-Shaul, & Dulac, 2014).

An alternative explanation of door-opening behavior is that rats are acting primarily for social contact. While accumulating evidence shows that social contact is not required for helping in rats (Bartal et al., 2011, 2014; Cox & Reichel, 2019), this interpretation cannot be ruled out by the present dataset. However, regardless of motivation, door-opening data clearly indicate a pro-social ingroup bias in adult but not juvenile rats, opening a path for elucidating the neural mechanism and development of this bias.

Methodological limitations should also be considered. c-Fos is an indirect index of neural activity, which does not provide direct access to neural firing, and is suspected to be influenced by other neural events, such as plasticity. Moreover, this index provides high spatial but low temporal resolution, which may be critical in a complex social situation where different events occur over an hour-long period. Finally, although c-Fos was sampled from 84 regions covering most major brain areas, it is still but a fraction of the entire brain. Thus, these data provide a partial picture of the rats' neural response and should be interpreted with this caveat in mind. It is nonetheless encouraging that the Nac emerged as a key region across several measures. The increase in c-Fos+ cells observed in the ingroup condition was mirrored by the in vivo calcium signal, and both measures were associated with behavior (helping and approach respectively).

Understanding the empathy gap for outgroup members is a major goal for society: why do we help some, but remain impervious to the suffering of others? The neural network activated in response to a trapped ingroup member includes regions associated with empathic arousal and pro-social behavior in humans. The similarity of the human literature with the network observed in this study is highly encouraging for a continued investigation into the biological mechanism of pro-social behavior based on rodent models.

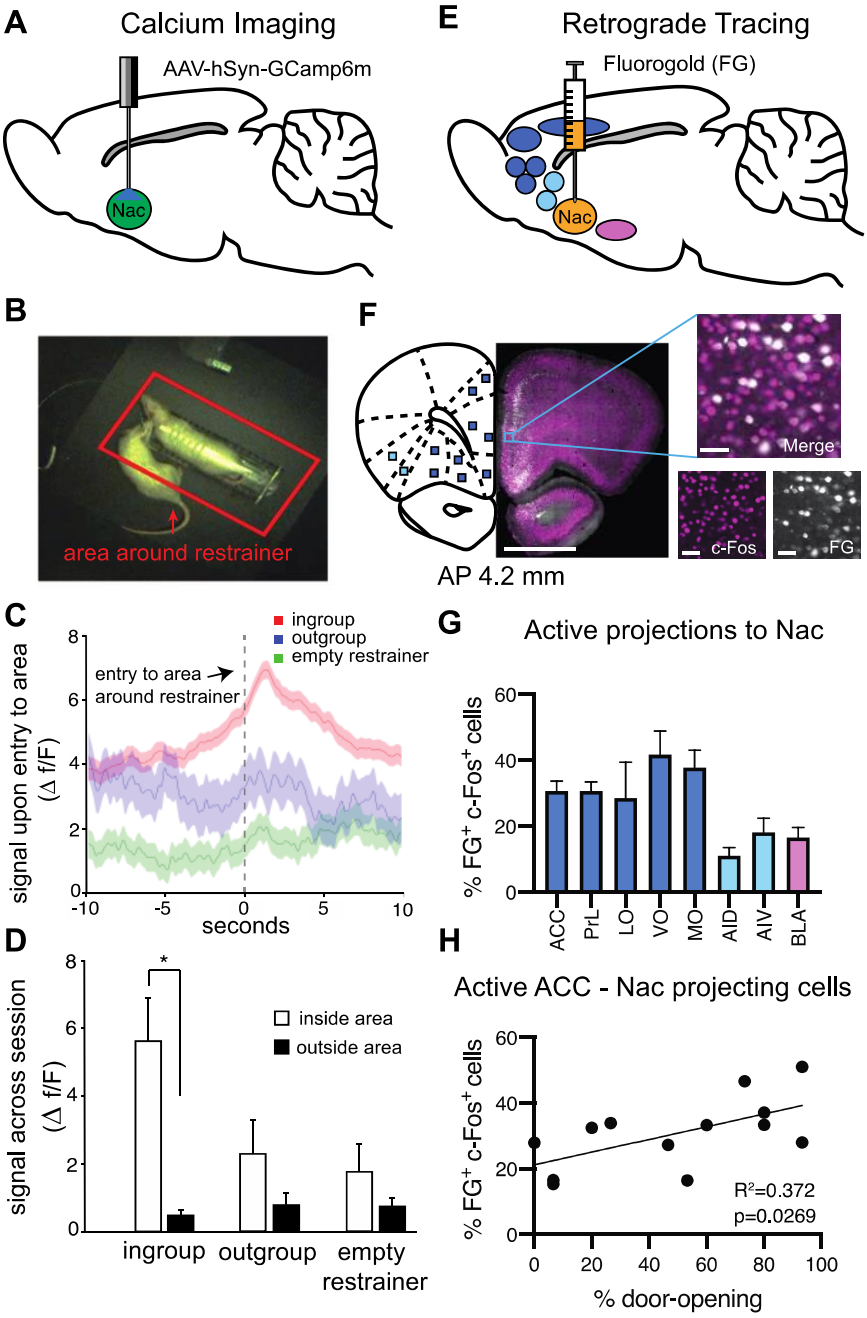

Figure 3-4. Activity in-vivo and in structural projections to the Nac.
(A) Diagram depicting location of virus injection and optic-fiber implant for fiber photometry recordings. (B) Top view of testing arena. The area around the restrainer is depicted by the red rectangle (C) Mean activity (Δ f/F) across rats and testing sessions increased when rats approached a trapped ingroup member (red) but not a trapped outgroup member (blue) or an empty restrainer (green). Point of entry into the area around the restrainer is indicated by the dashed line. (D) Mean activity averaged across the whole session was significantly higher for the ingroup when the rat was in the area around the restrainer. (E) Diagram depicting the retrograde tracer, Fluorogold (FG), injected into the Nac. (F) Representative section depicting tracer and c-Fos labeling: (FG^+, white) ($c\text{-}Fos^+$, magenta). Co-labeled cells (merged) were quantified in regions of interest. (G) % co-localized $FG^+/c\text{-}Fos^+$ for each region. (H) A positive correlation between the number of co-localized cells and helping behavior emerged for the ACC.

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Supplementary Materials:

Materials and Methods

Figures S1-S6

Tables S1-S3

Movies S1-S4

References (*1-14*)

Supplementary Materials

for

An ingroup bias for pro-social behavior in adult but not young rats is associated with a distinct neural network

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This PDF file includes:

Materials and Methods
Figs. S1 to S6
Tables S1 to S3
Captions for Movies S1 to S4

Other Supplementary Materials for this manuscript include the following:

Movies S1 to S4

Materials and Methods

Animals

Rat studies were performed in accordance with protocols approved by the Institutional Animal Care and Use Committee at the University of California, Berkeley. Rats were kept on a 12-hour light-dark cycle and received food and water *ad libitum*. In total, 83 rats were tested across all experiments. For experiments with adults, male Sprague-Dawley rats (age p60-p90 days) were used as the free rats (Charles River, Portage, MI). Adult male Long-Evans rats were used as trapped strangers (Envigo, CA). For experiments with juveniles, male and female Sprague-Dawley rats were born in-house at UC Berkeley. Animals were separated by sex and weaned at p21, then were housed in pairs one week later at p28. Male Long-Evans rats (p28) housed in pairs were purchased from Charles River, as our Long-Evans breeders did not get pregnant as expected. All rats that were ordered were allowed a minimum of 5 days to acclimate to the facility prior to beginning testing.

Helping Behavior test (HBT)

The helping behavior test (HBT) was performed as described previously (Bartal et al., 2011). Animals were handled for 5 days and tested for boldness 4 times. Animals received three daily habituation sessions to the arena, followed by a 15-minute session of open-field testing in the same arena on the following day. For the helping test, rats were placed into arenas with a trapped Sprague-Dawley cagemate ('ingroup') or Long-Evans stranger ('outgroup') inside a restrainer at the center of the arena. If the free rat did not open the restrainer after 40 minutes, the door was opened half-way by the experimenter. Both rats remained in the arena for a total 60 minutes. If the free rat opened the door before the half-way opening it was counted as a door-opening. Rats were tested over 12 days. All juveniles began the first day of restrainer testing at approximately p32. Following a delay of typically one week, rats underwent three more test days. On this last day of testing, the restrainer was latched throughout the 60-minute session, and rats were perfused immediately following behavioral testing. 'Helpers' were defined as rats who opened the restrainer on at least two of the final three sessions. Sessions were video recorded with a CCD color camera (KT&C Co, Seoul, Korea) connected to a video card (Geovision, Irvine, CA) that linked to a PC. Movement data were analyzed using Ethovision video tracking software (Noldus Information Technology, Inc. Leesburg, VA).

Control conditions

In the "2 free" condition a wire mesh was placed in the arena, with the test rat on one side and the partner on the other side. Neither rat was trapped. Rats were either cagemates or strangers. For the "untested" baseline condition, adult rats were used. These rats did not undergo the helping behavior test and c-Fos measurements reflect time in the homecage.

HBT for the fiber photometry experiment

A variation on the HBT was performed to allow a within-subject comparison to the control conditions. To this end, rats were exposed to a Long-Evans stranger for one test session (on day 7), and also had a 10-minute exposure to the closed empty restrainer at the beginning and end of each testing session. Furthermore, on the last session, three dried cranberries were placed inside the restrainer instead of a trapped rat, to record the neural activity involving a non-social reward.

Immunohistochemistry

On the last day of testing, animals were sacrificed within 90 minutes from the beginning of the session, at the peak expression of the early immediate gene product c-Fos. Rats were transcardially perfused with 0.9% saline and freshly made 4% paraformaldehyde in phosphate buffered saline (PBS). Brains were sunk in 30% sucrose as a cryoprotectant and frozen at -80$^{\circ C}$. They were later sliced at 40 μm and stained for c-Fos. Sections were washed with 0.1M tris-buffered saline (TBS), incubated in 3% normal donkey serum (NDS) in 0.3% TritonX-100 in TBS (TxTBS), then transferred to rabbit anti-c-Fos antiserum (ABE457; Millipore, 1:1000; 1% NDS; 0.3% TxTBS) overnight. Sections were then incubated in Alexa Fluor 488-conjugated donkey anti-rabbit antiserum (AF488; Jackson, 1:500; 1% NDS; 0.3% TxTBS). Sections were briefly washed in 0.1M TBS again. Sections were further stained in DAPI (1:40,000) for ten minutes if they did not contain the retrograde tracer FG and were then washed for a further 15 minutes (3x5'). Tissue that contained FG could not be stained for DAPI as both dyes are excited by UV fluorescence and their spectra overlap. Lastly, all slides were coverslipped with DABCO, dried overnight and stored at 4$^{\circ C}$ until imaged.

Immunostained tissue was imaged at 10x using a wide field fluorescence microscope (Zeiss AxioScan) and was processed in Zen software. Regions of interest (250 x 250μm squares) were placed across the whole brain (Fig. S1) and closely followed the methods performed in (Sadananda, Wohr, & Schwarting, 2008; A. L. Wheeler et al., 2013). A custom written script in ImageJ V2.0.0 (National Institute of Health, Bethesda, MD) was used to quantify immunoreactive nuclei (either c-Fos+ and/or FG+ cells), followed by manual checks and counting by multiple individuals who were blind to condition; consistency for counts across individuals was verified by a subset of samples. The threshold for detection of positive nuclei was set at a consistent level for each brain region, and only targets within the size range of 25–125 mm2 in area were counted as cells. Manual verification was targeted at identifying gross errors in the ImageJ scripts. For instance, in some cases the script falsely identified > 100 cells within the counting square, which usually occurred when there was high background staining. This type of error occurred in ~15% of the samples, which were then manually corrected. All means are reported as mean ± SEM. Furthermore, 39 values for cell counts were removed from the dataset as outliers. The outliers were defined as those that were more than two standard deviations higher or lower than the group mean and further fell outside of the observed range for all conditions.

Fiber photometry calcium signal recordings

Rats underwent unilateral injections of 1μL of virus (AAV-hsyn-GCamp6m) into the right hemisphere of the nucleus accumbens (AP: + 2.0, ML: + 1.0, DV: -7.2) and were implanted with an optic fiber patch cord with a numerical aperture of 0.48, and 400μm core (Doric Lenses). Six

weeks were allowed for virus infection, and upon signal detection rats began testing in the HBT while neural activity was recorded. Rats were removed from the experiment in cases where no signal was detected or due to failed implants. An LED emitting 470nm light was used for the Ca^{2+} signal and 405nm light was used as a control signal to remove movement artifacts. GCaMP fluorescence was collected by the same fiber; light passed through a dichroic lens with a GFP emission filter and was registered by a photoreceiver. Synapse software (TDT) was used to demodulate the brightness from the 470nm and 405nm excitation and synchronize it with the video data. For analysis, signal was normalized to the median of the session, and represented as Δf/F, after a least-square linear fit was applied via a custom MATLAB code. After testing was complete, rats were transcardially perfused with 0.9% saline and 4% paraformaldehyde in PBS. Brains were sunk in 30% sucrose as a cryoprotectant, frozen at -80 ^{oC} and sliced at 40μm. Representative sections containing the Nac were mounted on slides and imaged in order to determine location of the implant and virus spread. This protocol followed that previously performed by: (Lerner et al., 2015). In order to line up entry to the point around the restrainer, the synchronized videos were analyzed in Ethovision (Noldus Information Technology, Inc. Leesburg, VA). Using Matlab code, each frame of entry (where "in the zone" changed from 0 to 1) was identified, matched to the neural data via the synchronized time stamps, and used as the 0 point. Time stamp of door-opening events were identified manually and used as input for the Matlab code at a separate analysis.

Retrograde tracing

Rats were anesthetized with 3-5% isoflurane and mounted onto a stereotaxic frame. The skull was exposed and a small hole was made above the determined stereotactic coordinates on the right hemisphere (AP: + 2.0, ML: + 1.0, DV: -7.2; from Bregma (Paxinos and Watson 1998)). A Hamilton Syringe containing the retrograde tracer Fluorogold (FG, Fluorochrome, 4% in saline) was used to administer 200nL of dye into the nucleus accumbens. Rats were allowed 1-2 weeks to recover from surgery prior to starting the behavioral task. Following behavior, histology was performed as described above. The number of immunostained cells co-labeled for FG and c-Fos were manually counted in ImageJ (NIH), as well as the overall number of FG^+ and c-Fos^+ cells. Co-labeled cells represent neurons that were active during the task and that project to the Nac. The number of c-Fos^+ cells was counted using an ImageJ script and then manually checked by hand. A subset of images was counted by two or more experimenters in order to ensure that cell counting was consistent across all observers.

Statistical analysis

Task Partial Least Square (PLS) analysis

Task PLS is a multivariate statistical technique that is used to identify optimal patterns of functional activity that differentiate conditions. Task PLS is used in the analysis of brain region activity to describe the relationship between experimental conditions and functional activity (McIntosh, 1999; McIntosh, Bookstein, Haxby, & Grady, 1996). PLS identifies similarities and differences between groups by locating regions where activation varies with the experimental condition. Through singular value decomposition, PLS produces a set of mutually orthogonal latent variable (LV) pairs. One element of the LV depicts the contrast, which reflects a commonality or difference between conditions. The other element of the LV, the brain region

salience, identifies brain regions that show the activation profile across tasks, indicating which brain areas are maximally expressed in a particular LV.

Statistical assessment of PLS was performed by using permutation testing for LVs and bootstrap estimation of standard error for the brain region saliences. For the LV, significance was assessed by permutation testing; resampling without replacement by shuffling the test condition. Following each resampling, the PLS was recalculated. This was done 500 times in order to determine whether the effects represented in a given LV were significantly different to random noise. For brain region salience, reliability was assessed using bootstrap estimation of standard error. Bootstrap tests were performed by resampling 500 times with replacement, while keeping the subjects assigned to their conditions. This reflects the reliability of the contribution of that brain region to the LV. Brain regions with a bootstrap ratio greater than 2.55 (roughly corresponding to a confidence interval of 99%) were considered as reliably contributing to the pattern. Missing values were interpolated by the average for the test condition. An advantage to using this approach over univariate methods is that no corrections for multiple comparisons are necessary because the brain region saliences are calculated on all of the brain regions in a single mathematical step.

Other statistical tests

In addition to the PLS analysis described above, a one-way ANOVA was conducted on the c-Fos data to compare between the 4 HBT conditions and baseline for each brain region. To correct for multiple comparisons, a Dunnett's post-hoc test was conducted. This correction was specifically designed to compare multiple treatments to a control condition and is thus appropriate for comparing the test conditions to the untested baseline. A one-way ANOVA with a Bonferroni post hoc correction was used to compare NacC and NacSh c-Fos between the adult ingroup, adult outgroup and '2-free' conditions. 2-way ANOVAs were used to compare the pattern of animals' movements during testing. To compare the photometry signal in the time before and after entry to the area around the restrainer, the signal in the second before entry to the zone (the 0 point) was compared to the signal in the second after entry to the zone around the restrainer for the ingroup, outgroup and empty restrainer data. Wilcoxon signed-rank, a non-parametric test, was used in order to avoid assumptions about signal distribution. The comparisons of total session time spent in and outside the zone around the restrainer were conducted with a repeated measures ANOVA for the three conditions together, where the repeated measure was time in the zone and time outside the zone, as these were dependent samples for each animal. Note that the dataset used for the time series analysis and the total time analysis are not the same, and thus the y axis values are different. The time series PSTH graph includes only the 10 seconds before and after each point of entry, whereas the total time bar graphs include the entire dataset for each session, split by location. Changes across days to helping behavior, including % door-opening and latency to door-opening, were examined using Wilcoxon signed rank test and Friedman test respectively. Pearson's correlations were used for all correlation analyses; corrections for multiple comparisons were not run so as to avoid type 2 errors correcting for correlations with 45 brain regions.

Network analysis

Generating network graphs

To obtain network graphs of the c-Fos data, pairwise Pearson correlation coefficients were determined between the number of c-Fos+ cells for all pairs of brain regions (Fig. S4D). The top 10% correlations from these matrices were used to generate network maps (Fig. S4E). The threshold cutoff for the correlation matrices was based on scale-free network characteristics (Fig. S4A-B). To this end, all the possible correlation thresholds were enumerated and applied to the correlation matrices of c-Fos data. Correlation values higher than the cutoff were set to one and the corresponding brain regions were considered connected for that threshold cutoff. This resulted in a brain region network for each correlation threshold cutoff. Scale free topology index and percent connectivity was computed for each network based on the WGCNA tutorial (Langfelder & Horvath, 2008). The results were plotted as functions of correlation threshold cutoffs. A positive value of the scale-free topology index indicates a scale-free network. A correlation threshold cutoff of 10% top correlations was the transition point where the network demonstrates scale-free network characteristics (with a positive scale-free topology index), whereas a higher cutoff would result in an unstable network with few connections. Additionally, the small-worldness of brain networks was examined for the ingroup and outgroup maps (Fig. S4C). For this, the entire range of possible correlation threshold cutoffs was enumerated to compute two metrics: small-worldness and network density. The small-worldness is calculated in the same way as is discussed in (Humphries & Gurney, 2008); the network density is defined in (Wasserman & Faust, 1994).

Identification of central hubs

Central hubs were determined by ranking all brain regions according to two parameters: the betweenness value, representing the number of times all regions must pass through the ROI in order to reach other regions via the shortest path, and the number of connections (degrees) with other regions. The 20% top-ranking regions were then identified; brain areas that were in the top 20% for both categories were considered to be central hubs of the network (Fig. S4F).

Additional strategy for identifying central regions

The networks presented are based on one set of parameters (threshold, clustering algorithm, weights), and as such, has limited validity. In order to increase the robustness of our conclusions, an additional analysis was conducted using an alternative strategy. Identification of primary regions was based here on the prevalence of each region across multiple tests conducted with different configurations. A series of 40 multinomial logistic regression tests compared the HBT ingroup and outgroup conditions to a reference group using different parameters for threshold, clustering and weighting of the network for each test (Table S3), as a customized ensemble clustering algorithm (Vega-Pons & Ruiz-Shulcloper, 2011). In each run of this implementation (Fig. S4G), brain regions were clustered using the selected subset of data (cluster group), and the first principle component (eigenvector) of each cluster was computed. These were considered as the representative “eigen-region” for the clusters they were derived from. Subsequently, the eigen-regions were fed into multinomial logistic regression (Hilbe, 2009; Zalaquett & Thiessen, 1991) to determine whether they are significantly different between the ingroup and the reference group or the outgroup and the reference group. The reference group

itself was varied between using the untested condition and a broader reference group containing the c-Fos data from rats tested for a brief exposure with a trapped rat (3 days), the trapped rats themselves, and the rats in the "2 free" and "untested" conditions (total n=48). The standard p-value cutoff of 0.05 was used after Bonferroni correction. The detail setups of the 40 combinations of algorithms and parameters are listed in Table S3. Louvain clustering (Blondel, Guillaume, Lambiotte, & Lefebvre, 2008) was done with the igraph package in R (Csardi & Nepusz, 2006) and Dynamic tree cut of hierarchical clustering was done with the WGCNA package in R (Langfelder & Horvath, 2008). "Soft power" was defined as described in (Langfelder & Horvath, 2008). Each cluster was classified as significantly different than the reference for the ingroup, outgroup, both or none. The number of occurrences per region in each category was quantified. A high ratio of occurrences in the ingroup only category indicates that this region was uniquely important for the ingroup condition. Specifically, the NacSh occurred in >80% of tests in clusters that were significant for the ingroup condition, but not the outgroup condition (Fig. S4H). Practically no clusters emerged as significant only for the outgroup condition (1/344 clusters, or 0.29%).

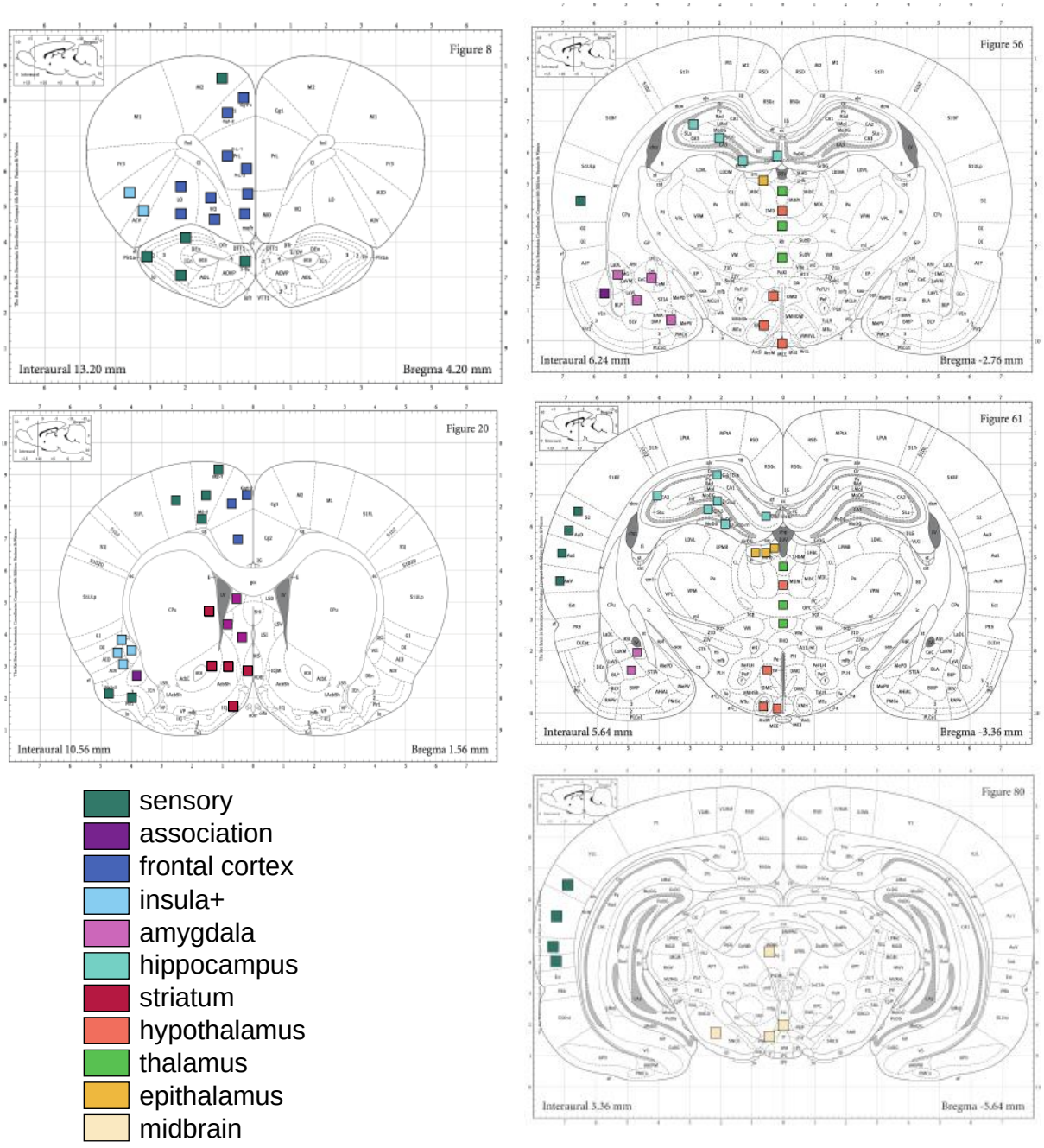


Figure 3-S-1. Regions of interest (ROIs) for c-Fos analysis.
Rat Atlas map with 84 sampled brain regions (According to the Paxinos & Watson Rat Brain Atlas). ROIs are color coded based on the legend in Figure 3-2. Some ROIs (e.g. multiple samples of the same region on different slices) were merged into the final list of 45 areas depicted in Figure 3-2.

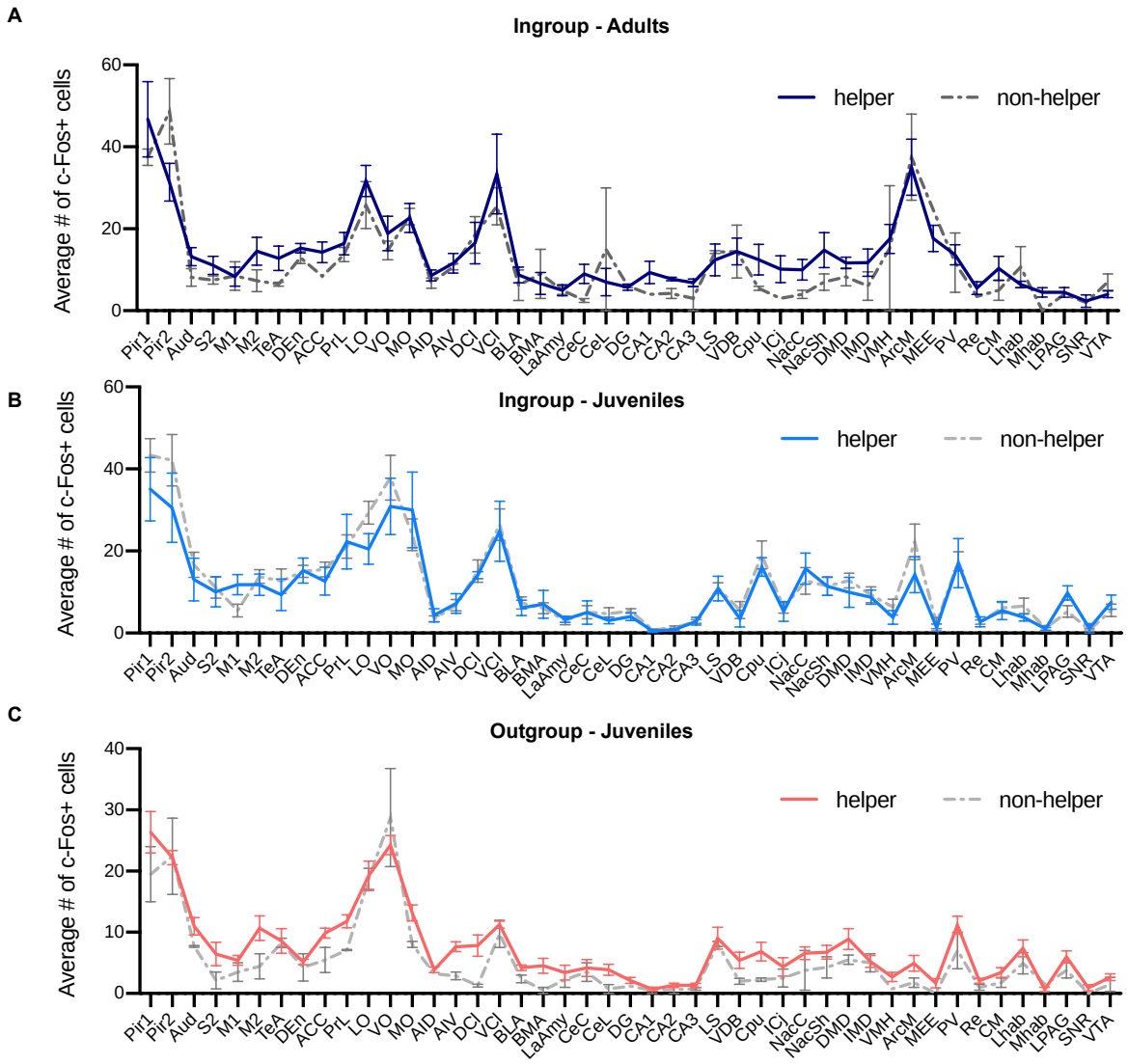
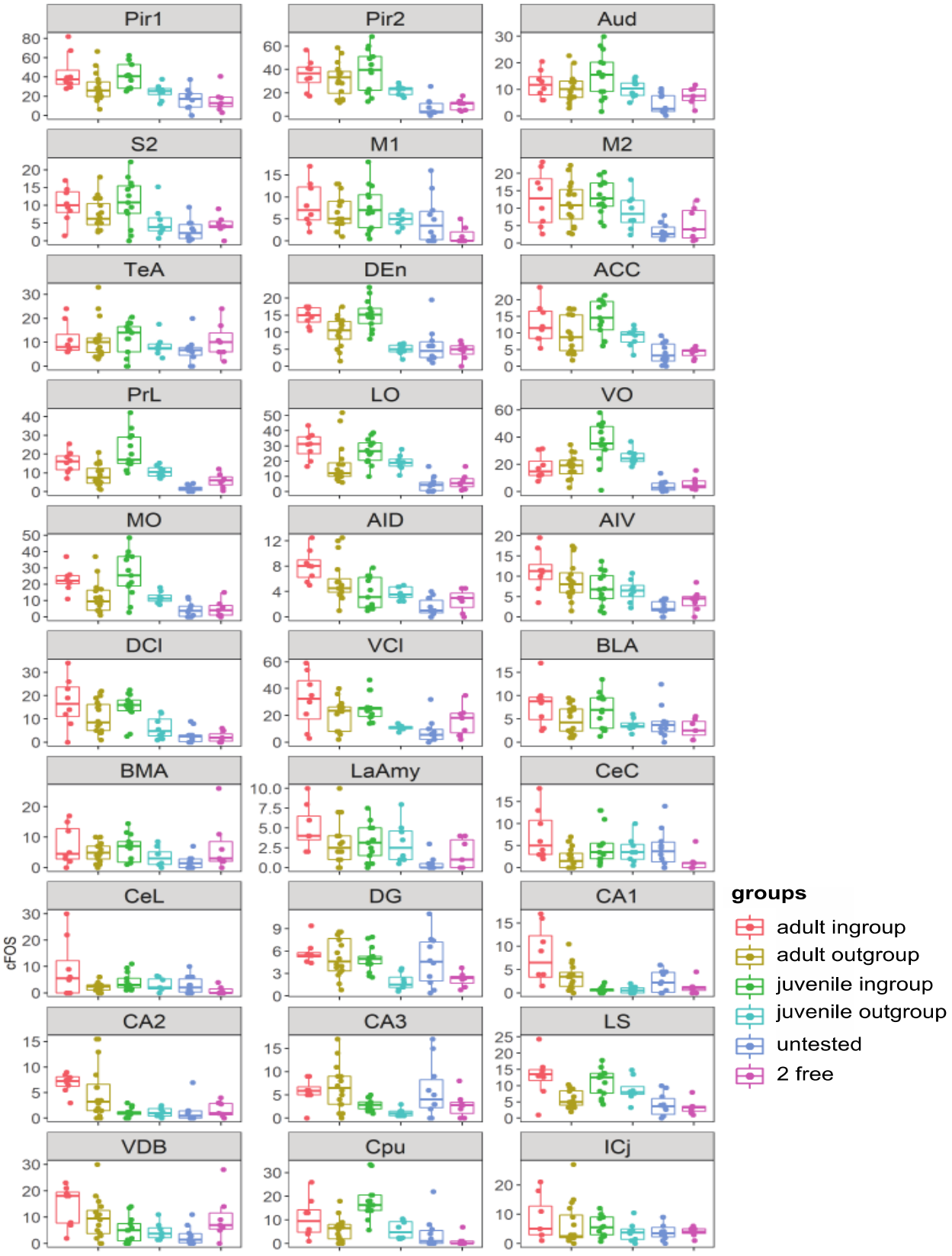


Figure 3-S-2. c-Fos in helpers vs. non-helpers.
Rats defined as consistent helpers were compared against those that did not consistently open by the end of testing. The average number of c-Fos+ cells for each brain region was compared for: (A) adults tested with ingroup members (B) juveniles tested with ingroup members and (C) juveniles tested with outgroup members. Similar patterns of c-Fos were observed for each condition. All adults tested with outgroup members were non-helpers and thus, there was no comparison group and they are not shown.


Pir1
Pir2
Aud
S2
M1
M2
TeA
DEn
ACC
PrL
LO
VO
MO
AID
AIV
DCl
VCl
BLA
BMA
LaAmy
CeC
CeL
DG
CA1
CA2
CA3
LS
VDB
Cpu
ICj
cFOS
groups
adult ingroup
adult outgroup
juvenile ingroup
juvenile outgroup
untested
2 free

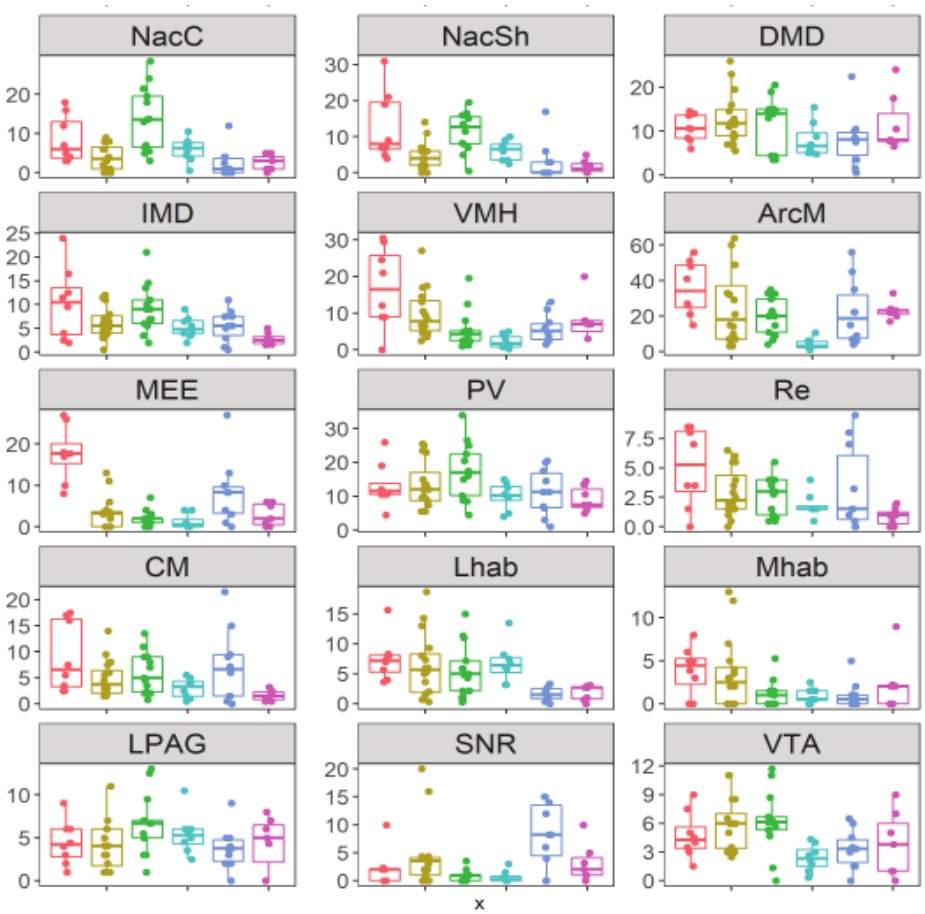


Figure 3-S-3. Box plots of c-Fos data in all brain regions across all test groups.
Center bars mark the median. Lower and upper edges correspond to the 25th and 75th percentiles. Descriptions of the brain region abbreviations can be found in Table S1. Data points are jittered along the x-axis to avoid overlaps. X: experimental groups; Y: c-Fos+ cell numbers.

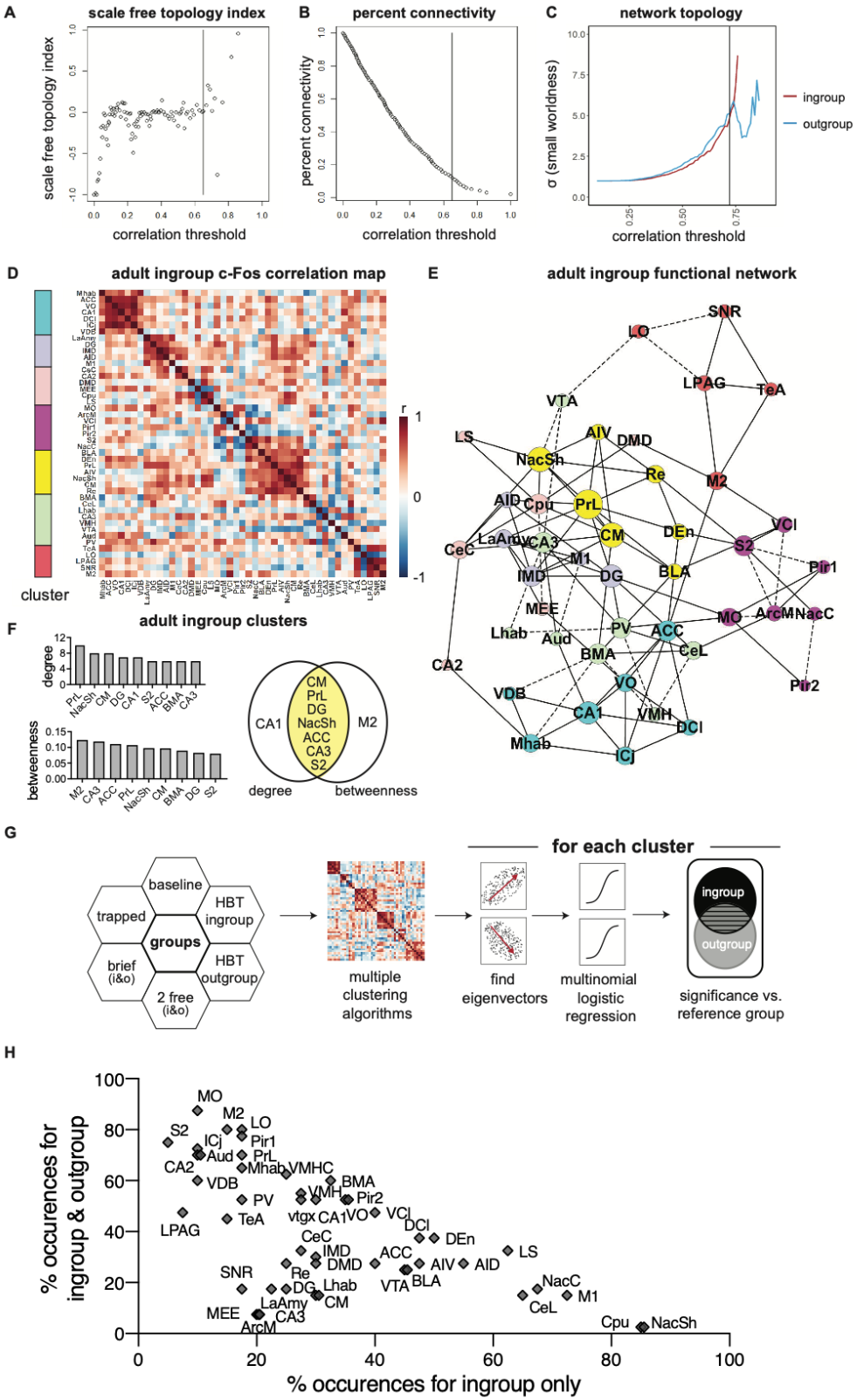

A
scale free topology index
scale free topology index
correlation threshold
B
percent connectivity
percent connectivity
correlation threshold
C
network topology
σ (small worldness)
correlation threshold
ingroup
outgroup
D
adult ingroup c-Fos correlation map
cluster
r
1
0
-1
E
adult ingroup functional network
SNR
LO
LPAG
TeA
VTA
LS
AIV
DMD
NacSh
Re
M2
AID
Cpu
PrL
VCl
CeC
LaAmy
CA3
CM
DEn
S2
M1
Pir1
IMD
DG
BLA
MEE
MO
ArcM
NacC
Lhab
Aud
PV
ACC
CeL
BMA
CA2
VO
Pir2
VDB
CA1
VMH
DCl
Mhab
ICj
F
adult ingroup clusters
degree
betweenness
PrL
NacSh
CM
DG
CA1
S2
ACC
BMA
CA3
M2
CA3
ACC
PrL
NacSh
CM
BMA
DG
S2
CA1
M2
CM
PrL
DG
NacSh
ACC
CA3
S2
degree
betweenness
G
for each cluster
baseline
trapped
HBT ingroup
groups
brief (i&o)
HBT outgroup
2 free (i&o)
multiple clustering algorithms
find eigenvectors
multinomial logistic regression
ingroup
outgroup
significance vs. reference group
H
% occurences for ingroup & outgroup
% occurences for ingroup only
MO
M2
LO
S2
ICj
Pir1
Aud
PrL
CA2
Mhab
VMHC
VDB
BMA
VMH
Pir2
PV
vtgx
CA1
VO
VCl
TeA
DCl
LPAG
CeC
DEn
IMD
ACC
LS
SNR
DMD
AIV
AID
Re
VTA
BLA
NacC
DG
Lhab
M1
LaAmy
CM
CeL
MEE
CA3
Cpu
NacSh
ArcM

Figure 3-S-4. Network analyses.
(A-C) Network parameters used for selecting a threshold. The 10% top-ranking correlation values were used in the network map. This threshold was determined according to the network parameters. Increasing the threshold results in a fragmented network, that is overly scale-free and reduced in connectivity. Decreasing the threshold undermines the small-worldness of the network. (A) Scale free topology index shows the correlation threshold cutoff of the transition to scale free network. (B) The percent connectivity graph represents the correlation threshold for 10% connectivity. (C) The small-worldness of the network for the HBT ingroup and outgroup condition is displayed for each correlation threshold. Solid line is displayed at the top 10% correlation (R2) for all measures. (D) Louvain clustered heat map of pair-wise correlation values for the adult ingroup condition. Bar on left visualizes the identified clusters. (E) Thresholded network map shows the top 10% of positive (solid lines) and negative (dashed lines) correlations. Colors represent clusters identified by the Louvain algorithms on left. (F) Central hubs of the adult ingroup network. To identify central hubs, brain regions were ranked by degree and betweenness. The top 20% of regions are shown for each parameter. Brain areas appearing in the top 20% of both parameters were classified as central hubs. Venn diagrams (below) show central hubs (in yellow center), of top-ranking regions for centrality measures degree and betweenness. (G-H) A series of multiple logistic regression tests compared adult ingroup and outgroup conditions to all other groups. (G) Diagram describing the pipeline of the analysis. (H) Regions uniquely observed for the adult ingroup condition (x axis) are contrasted with regions observed for both adult ingroup and outgroup conditions (y axis). Regions to the right of the dashed line best identify the adult ingroup condition based on brain activity.

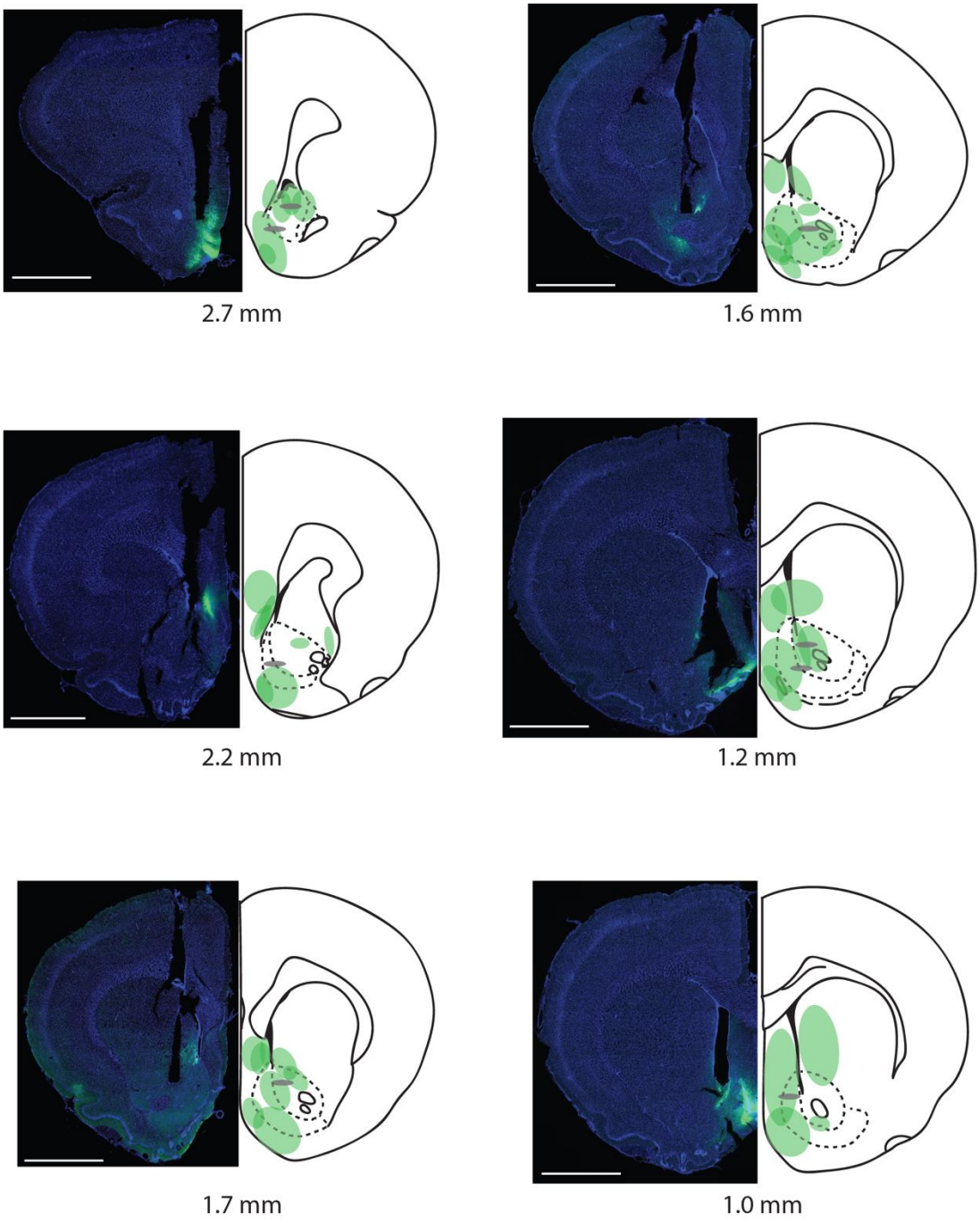


Figure 3-S-5. Summary of fiber photometry injections and implants.
Virus infection, spread and optical fiber placements are shown for all 8 animals, across the anterior-posterior (AP) extent of the nucleus accumbens (AP coordinates are from Paxinos & Watson, 1998). Representative images are shown on the left, while the summaries are shown on the right. DAPI is in blue, GCamp6m virus in green. The ends of the fibers are represented by gray ovals. Scale bar: 2mm.

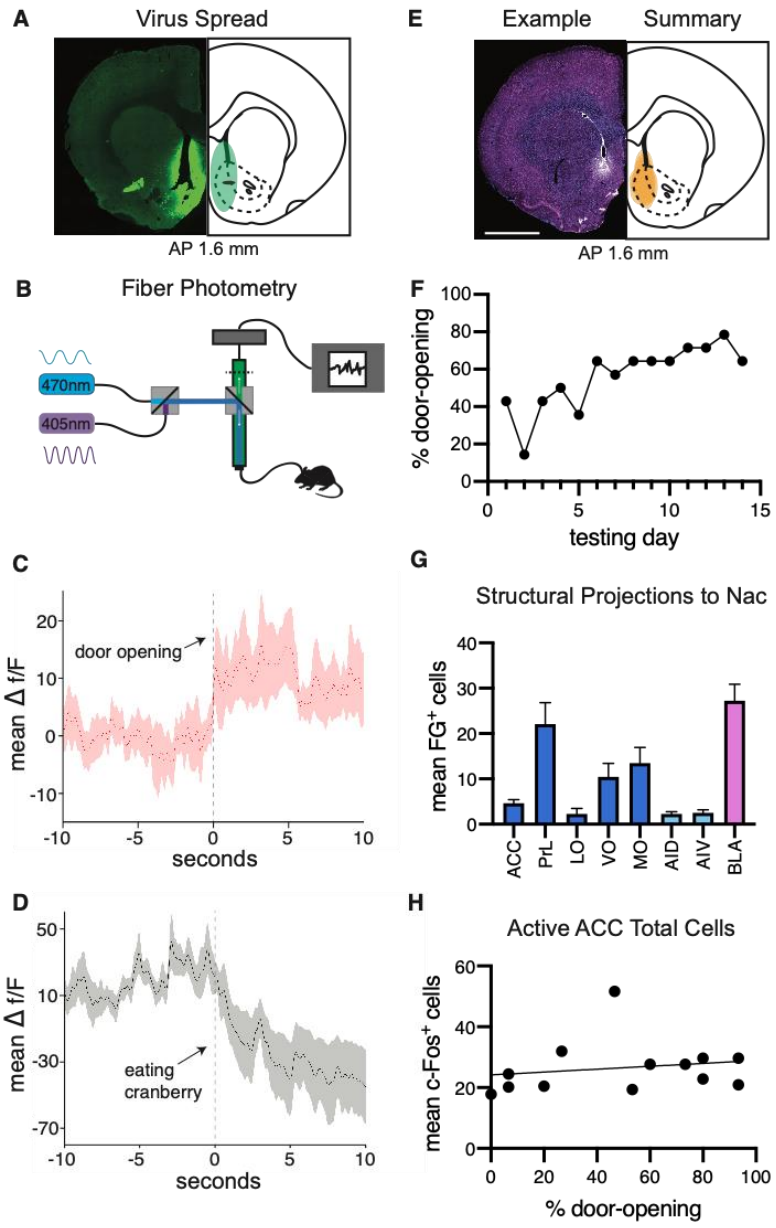


Figure 3-S-6. Additional fiber photometry and retrograde tracing data.
(A) Example of virus spread in one animal. (B) Fiber photometry rig schematic. See methods for a more detailed description. (C) Activity peaked at the moment of door-opening for the ingroup trials. (D) Signal declined when rats were eating a cranberry. (E) A representative Fluorogold (FG) injection and a summary of tracer spread in all animals. c-Fos in magenta and DAPI in blue. Coordinates are anterior-posterior (AP) from bregma (Paxinos & Watson, 1998). (F) Most rats injected with FG learned to open the restrainer and released the trapped rat, as indicated by increased % of door-openings across testing sessions. (G) Number of FG+ cells for each ROI. (H) No correlation was observed between c-Fos+ cells and helping behavior in the anterior cingulate cortex (ACC).

Region	Description
Pir1	Piriform cortex
Pir2	Piriform cortex
Aud	Auditory cortex
S2	Secondary somatosensory cortex
M1	Motor cortex
M2	Motor cortex
TeA	Temporal association cortex
DEn	Dorsal endopiriform nucleus
ACC	Anterior cingulate cortex
PrL	Prelimbic cortex
LO	Lateral orbitofrontal cortex
VO	Ventral orbitofrontal cortex
MO	Medial orbitofrontal cortex
AID	Dorsal agranular insula
AIV	Ventral agranular insula
DCl	Dorsal claustrum
VCl	Ventral claustrum
BLA	Basolateral amygdala
BMA	Basomedial amygdala
LaAmy	Lateral amygdala
CeC	Central amygdaloid nucleus, capsular
CeL	Central amygdaloid nucleus, lateral
DG	Dentate gyrus
CA1	CA1 of hippocampus
CA2	CA2 of hippocampus
CA3	CA3 of hippocampus
LS	Lateral septum
VDB	Nucleus vertical limb diagonal band
Cpu	Caudate putamen
ICj	Islands of Calleja
NacC	Nucleus accumbens core
NacSh	Nucleus accumbens shell
DMD	Dorsomedial hypothalamic nucleus, diffuse
IMD	Intermediodorsal thalamic nucleus
VMH	Ventromedial hypothalamic nucleus
ArcM	Medial arcuate hypothalamus
MEE	Medial eminence
PV	Paraventricular thalamic nucleus
Re	Reuniens thalamic nucleus
CM	Central median thalamic nucleus
Lhab	Lateral habenula
Mhab	Medial habenula
LPAG	Periaqueductal gray
SNR	Substantia nigra, reticular
VTA	Ventral tegmental area

Table 3-S-1. Brain regions analyzed.
Detailed list of brain regions used in the figures.

Brain Region	**Adult Ingroup - Untested**	**Adult Outgroup - Untested**	**Juvenile Ingroup - Untested**	**Juvenile Outgroup - Untested**
Pir1	MD=28.386.96 ******p=0.001***	MD=13.186.04 *p=0.105*	MD=24.726.21 ******p=0.001***	MD=8.576.96 *p=0.539*
Pir2	MD=28.116.59 *******p=0.000***	MD=24.305.65 *******p=0.000***	MD=31.035.88 *******p=0.000***	MD=14.696.59 *p=0.094*
Aud	MD=7.522.83 ****p=0.036***	MD=6.412.44 ****p=0.039***	MD=11.052.56 *******p=0.000***	MD=5.702.83 *p=0.152*
S2	MD=7.242.47 ****p=0.018***	MD=4.562.15 *p=0.120*	MD=7.842.24 *****p=0.004***	MD=2.402.47 *p=0.722*
M1	MD=3.492.28 *p=0.355*	MD=1.641.97 *p=0.814*	MD=2.722.07 *p=0.487*	MD=-0.032.36 *p=1.000*
M2	MD=9.422.73 *****p=0.004***	MD=7.942.34 *****p=0.005***	MD=9.852.44 ******p=0.001***	MD=5.762.73 *p=0.122*
TeA	MD=5.503.64 *p=0.531*	MD=4.613.23 *p=0.407*	MD=4.703.38 *p=0.430*	MD=1.753.64 *p=0.965*
DEn	MD=8.742.08 *******p=0.000***	MD=4.121.80 *p=0.085*	MD=9.181.89 *******p=0.000***	MD=-1.012.08 *p=0.965*
ACC	MD=8.872.42 *****p=0.002***	MD=5.632.08 ****p=0.031***	MD=10.622.20 *******p=0.000***	MD=4.792.42 *p=0.159*
PrL	MD=14.033.12 *******p=0.000***	MD=7.112.71 ****p=0.038***	MD=19.762.78 *******p=0.000***	MD=8.903.12 ****p=0.022***
LO	MD=25.474.85 *******p=0.000***	MD=13.064.27 ****p=0.013***	MD=21.704.40 *******p=0.000***	MD=14.374.85 ****p=0.017***
VO	MD=13.814.92 ****p=0.024***	MD=14.404.27 *****p=0.005***	MD=31.724.39 *******p=0.000***	MD=21.384.92 *******p=0.000***
MO	MD=18.424.59 ******p=0.001***	MD=7.473.99 *p=0.195*	MD=21.514.10 *******p=0.000***	MD=7.544.59 *p=0.293*
AID	MD=6.521.28 *******p=0.000***	MD=4.191.06 *****p=0.001***	MD=2.221.10 *p=0.146*	MD=2.161.23 *p=0.242*
AIV	MD=9.101.99 *******p=0.000***	MD=6.661.73 ******p=0.001***	MD=4.671.78 ****p=0.039***	MD=4.161.99 *p=0.127*
DCl	MD=14.113.31 *******p=0.000***	MD=7.802.84 ****p=0.029***	MD=11.973.06 ******p=0.001***	MD=3.303.31 *p=0.705*
VCl	MD=23.495.92 ******p=0.001***	MD=11.805.08 *p=0.078*	MD=18.115.48 *****p=0.007***	MD=2.995.92 *p=0.960*
BLA	MD=3.811.77	MD=.471.53	MD=2.631.62	MD=-.571.77

	p=0.109	*p=0.993*	*p=0.296*	*p=0.991*
BMA	MD=5.472.00 ***p=0.030***	MD=3.081.76 *p=0.246*	MD=4.621.82 ***p=0.048***	MD=1.722.00 *p=0.796*
LaAmy	MD=4.501.26 ****p=0.003***	MD=2.701.10 *p=0.057*	MD=2.631.15 *p=0.083*	MD=2.631.26 *p=0.126*
CeC	MD=2.931.99 *p=0.387*	MD=-2.311.73 *p=0.470*	MD=0.6181.81 *p=0.990*	MD=-0.4441.99 *p=0.998*
CeL	MD=6.002.49 *p=0.065*	MD=-0.712.19 *p=0.992*	MD=1.192.26 *p=0.955*	MD=0.062.49 *p=1.000*
DG	MD=1.101.15 *p=0.730*	MD=0.440.98 *p=0.973*	MD=0.211.04 *p=0.999*	MD=-2.851.15 *p=0.053*
CAI	MD=5.611.49 ****p=0.002***	MD=1.141.29 *p=0.778*	MD=-1.701.35 *p=0.521*	MD=-1.731.49 *p=0.587*
CA2	MD=5.651.56 ****p=0.003***	MD=3.971.34 ***p=0.017***	MD=-0.031.42 *p=1.000*	MD=-0.071.56 *p=1.000*
CA3	MD=-.292.02 *p=1.000*	MD=.691.67 *p=0.981*	MD=-3.161.80 *p=0.246*	MD=-4.811.95 *p=0.057*
LS	MD=8.802.12 *****p=0.001***	MD=1.711.75 *p=0.720*	MD=6.791.85 ****p=0.002***	MD=4.642.04 *p=0.087*
VDB	MD=11.612.99 *****p=0.001***	MD=6.612.57 ***p=0.044***	MD=2.162.77 *p=0.844*	MD=1.672.99 *p=0.944*
Cpu	MD=6.533.23 *p=0.146*	MD=1.782.77 *p=0.911*	MD=13.862.93 *****p=0.000***	MD=1.473.23 *p=0.972*
ICj	MD=4.262.77 *p=0.347*	MD=2.292.40 *p=0.733*	MD=1.742.51 *p=0.888*	MD=-0.242.77 *p=1.000*
NAcC	MD=6.062.62 *p=0.080*	MD=1.372.25 *p=0.925*	MD=11.312.38 ******p=0.000***	MD=3.432.62 *p=0.487*
NAcSh	MD=9.992.75 ****p=0.002***	MD=1.672.36 *p=0.880*	MD=8.572.49 ****p=0.004***	MD=3.242.75 *p=0.576*
DMD	MD=2.652.90 *p=0.751*	MD=4.742.45 *p=0.172*	MD=3.882.52 *p=0.340*	MD=-0.032.80 *p=1.000*
IMD	MD=4.812.28 *p=0.120*	MD=0.691.98 *p=0.989*	MD=3.652.05 *p=0.225*	MD=-0.3132.28 *p=1.000*
VMH	MD=11.163.08 ****p=0.003***	MD=4.102.64 *p=0.338*	MD=-.212.75 *p=1.000*	MD=-3.563.08 *p=0.589*
ArcM	MD=13.508.23 *p=0.289*	MD=2.887.13 *p=0.981*	MD=-2.087.65 *p=0.996*	MD=-18.008.23 *p=0.103*

MEE	MD=9.383.12 ***p=0.017***	MD=-4.792.67 *p=0.230*	MD=-6.292.91 *p=0.114*	MD=-7.042.91 *p=0.066*
PV	MD=1.943.62 *p=0.948*	MD=2.143.21 *p=0.884*	MD=6.133.25 *p=0.187*	MD=-1.133.62 *p=0.993*
Re	MD=1.841.22 *p=0.363*	MD=-0.291.06 *p=0.996*	MD=-0.491.09 *p=0.973*	MD=-1.411.22 *p=0.590*
CM	MD=1.832.36 *p=0.842*	MD=-2.292.02 *p=0.604*	MD=-1.212.10 *p=0.938*	MD=-4.172.36 *p=0.235*
Lhab	MD=5.802.05 ***p=0.023***	MD=4.731.76 ***p=0.033***	MD=4.171.89 *p=0.101*	MD=5.102.05 *p=0.053*
Mhab	MD=2.861.40 *p=0.143*	MD=2.531.17 *p=0.112*	MD=0.1251.23 *p=1.000*	MD=-0.131.35 *p=1.000*
LPAG	MD=0.681.59 *p=0.977*	MD=0.3211.36 *p=0.998*	MD=2.901.46 *p=0.157*	MD=1.691.53 *p=0.630*
SNR	MD=-5.962.35 ***p=0.049***	MD=-3.892.01 *p=0.175*	MD=-7.352.15 ****p=0.005***	MD=-7.562.27 ****p=0.006***
VTA	MD=1.441.38 *p=0.666*	MD=2.651.22 *p=0.108*	MD=2.821.31 *p=0.111*	MD=-0.941.38 *p=0.892*

Table 3-S-2. One-way ANOVA results, with Dunnett's multiple comparisons tests comparing each group to the untested baseline.
The mean difference (MD) +/- the SEM of the difference are displayed for each comparison, as well as the p-value compared to untested. Statistically significant results are in bold. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.

clustering group	reference group	Weighted	method	parameter
all	rest	No	Louvain clustering	percentile rank<=10%
all	rest	No	Louvain clustering	p<=0.001
all	rest	No	Louvain clustering	|r|>=0.4
all	rest	No	Dynamic tree cut	percentile rank<=10%
all	rest	No	Dynamic tree cut	p<=0.001
all	rest	No	Dynamic tree cut	|r|>=0.4
all	rest	Yes	Louvain clustering	softPower=6
all	rest	Yes	Dynamic tree cut	softPower=6
all	untested	No	Louvain clustering	percentile rank<=10%
all	untested	No	Louvain clustering	p<=0.001
all	untested	No	Louvain clustering	|r|>=0.4
all	untested	No	Dynamic tree cut	percentile rank<=10%
all	untested	No	Dynamic tree cut	p<=0.001
all	untested	No	Dynamic tree cut	|r|>=0.4
all	untested	Yes	Louvain clustering	softPower=6
all	untested	Yes	Dynamic tree cut	softPower=6
rest	rest	No	Louvain clustering	percentile rank<=10%
rest	rest	No	Louvain clustering	p<=0.001
rest	rest	No	Louvain clustering	|r|>=0.4
rest	rest	No	Dynamic tree cut	percentile rank<=10%
rest	rest	No	Dynamic tree cut	p<=0.001
rest	rest	No	Dynamic tree cut	|r|>=0.4
rest	rest	Yes	Louvain clustering	softPower=6
rest	rest	Yes	Dynamic tree cut	softPower=6
rest	untested	No	Louvain clustering	percentile rank<=10%
rest	untested	No	Louvain clustering	p<=0.001
rest	untested	No	Louvain clustering	|r|>=0.4
rest	untested	No	Dynamic tree cut	percentile rank<=10%
rest	untested	No	Dynamic tree cut	p<=0.001
rest	untested	No	Dynamic tree cut	|r|>=0.4
rest	untested	Yes	Louvain clustering	softPower=6
rest	untested	Yes	Dynamic tree cut	softPower=6
untested	untested	No	Louvain clustering	percentile rank<=10%
untested	untested	No	Louvain clustering	p<=0.001
untested	untested	No	Louvain clustering	|r|>=0.4
untested	untested	No	Dynamic tree cut	percentile rank<=10%
untested	untested	No	Dynamic tree cut	p<=0.001
untested	untested	No	Dynamic tree cut	|r|>=0.4
untested	untested	Yes	Louvain clustering	softPower=6
untested	untested	Yes	Dynamic tree cut	softPower=6

Table 3-S-3. Combinations of clustering algorithms, parameters and subsets of data used in Figure 3-S-4. “Cluster group” was used to obtain the cluster assignments for each brain region. “Reference group” was the control group in the multinomial logistic regression. The “Weighted” column indicates whether weights (correlations) between brain regions are taken into account while doing clustering. “All”: all groups; “rest”: all conditions except ingroup and outgroup; “baseline”: untested baseline condition. The thresholds of percentile rank, p value, and absolute r value in the parameter column were applied to the covariance matrix of the c-Fos data in cluster group before feeding it into the selected clustering algorithm.

Movie S1.
Adult and juvenile rats help ingroup members.

Movie S2.
Juveniles but not adults help outgroup members.

Movie S3.
Activity patterns on the final day of testing.

Movie S4.
Nac activity increases when approaching a trapped ingroup member.

Chapter 4: Exposure to acute traumatic stress as a juvenile alters myelination in the rodent prefrontal cortex

Abstract

Stress early in life can have a major impact on brain development, and there is increasing evidence that early stress confers vulnerability for later developing psychiatric disorders. In particular, during peri-adolescence, brain regions crucial for emotional regulation such as the prefrontal cortex (PFC) are still developing and are highly sensitive to stress. Stress effects on PFC myelin and oligodendrocytes (OLs) are beginning to be explored as a novel and underappreciated mechanism underlying psychopathologies, yet there is little research on the effects of acute stress during peri-adolescence. Here, we used a rodent model to test the hypothesis that acute stress as a juvenile would induce changes in PFC OLs and myelin. Male and female juvenile Sprague Dawley rats (p26) were exposed to three hours of severe stress (restraint stress with exposure to a predator odor). Subsequent fear and anxiety-like behavior was assayed either one-week later, or in adulthood (p90), to test for short- or long-term effects respectively, and to assess individual responses to stress. While composite behavioral scores were not altered in males or females in either the short or long term, stress-exposed male animals displayed impaired fear extinction one week after the stressor. Brain sections containing the PFC were then analyzed for OL and myelin markers using immunohistochemistry and fluorescent microscopy. We found that, while there were few changes in animal behavior following stress, there were sex-specific changes in myelination and OLs. A single stressor as a juvenile increased myelination in PFC regions one week later in females, but not males. In the long-term, stress-exposed females showed reduced PFC myelination, while male animals had no change. Acute stress also decreased PFC OLs in the short-term for females, perhaps contributing towards the observed long-term decrease in myelin. Counter to our hypothesis, we did not observe correlations of PFC OLs or myelin with avoidance and fear behaviors following stress, indicating that stress-effects on PFC myelin were not associated with the behavioral measures captured by our study. Overall, our findings suggest that the juvenile PFC is vulnerable to a single traumatic stressor, and that there are both immediate and long-term effects on myelination in a sex-specific manner. However, the functional impact of these biological changes remains to be determined. These findings, as well as future studies in rodents will inform our knowledge of how traumatic stressors impact human prefrontal cortex development and mental health.

Introduction

Stress early in life can have a major impact on brain development and behavior. In particular, stressful experiences from infancy through adolescence are associated with an increased risk of later developing psychiatric disorders (C. P. Carr et al., 2013; Ventriglio, Gentile, Baldessarini, & Bellomo, 2015). For example, it is well known that childhood trauma increases the risk for developing depression and anxiety (Heim & Nemeroff, 2001). Large cohort studies examining Adverse Childhood Experiences (ACEs) confirm that individuals with more ACEs are at a higher risk for mental health disorders (Hughes et al., 2017). Yet, individuals in similar stressful environments can have very different responses to stress; only a subpopulation demonstrates vulnerability, while others demonstrate resilience (Compas & Phares, 1991; Ronald C Kessler et al., 1995; Bruce S McEwen & Stellar, 1993). In part, these individual differences may be explained by biological sensitivities to context and the environment (Ellis & Boyce, 2008). Furthermore, other factors such as sex may play a role. For example, females are more susceptible to developing PTSD and anxiety (Breslau, 2009; Ronald C Kessler et al., 1995; McLean & Anderson, 2009). Thus, a major goal is not only to understand the neurobiological effects of early life stress but also to understand the biological factors that contribute to individual variability.

Early life stress leads to physiological changes in the body, as well as changes in the central nervous system (Bolton, Short, Simeone, Daglian, & Baram, 2019). In particular, stressors experienced during the peri-adolescent time period may have a significant impact on brain maturation and development. Adolescence, defined by the onset of puberty (~age 10 in humans, ~p36 in rodents), is a major period of experience-dependent plasticity and thus, the brain is particularly sensitive to environmental stimuli such as stressors. This in turn could contribute towards the onset of psychiatric disorders such as anxiety and depression, which often appear around this time (Eiland & Romeo, 2013; Gee & Casey, 2015; Ronald C. Kessler et al., 2007, 2005). Importantly, during peri-adolescence, brain regions that play a role in the stress response, such as the amygdala, hippocampus and prefrontal cortex, are still developing and are highly sensitive to stress (Bruce S. McEwen et al., 2015; Popoli, Yan, McEwen, & Sanacora, 2012; Roozendaal et al., 2009; Spear, 2000).

In humans, early life stress leads to reduced hippocampal and amygdala volume as well as alterations in the frontal cortex and anterior cingulate cortex (Andersen et al., 2008; R. A. Cohen et al., 2006; Hanson et al., 2013; Teicher, Samson, Polcari, & McGreenery, 2006). In one specific longitudinal study, cortisol levels and PTSD symptomatology of children who experienced maltreatment predicted subsequent reductions in hippocampal volume (Carrion, Weems, & Reiss, 2007). In addition to structural changes in grey matter, early life stress also produces changes in functional connectivity. In particular, there is elevated amygdala reactivity to emotional stimuli after early stress, and weaker amygdala – PFC connectivity (Gee et al., 2013; McCrory et al., 2013; Nooner et al., 2013; see Tottenham & Galván, 2016 for a review). In rodents, structural changes in the hippocampus, amygdala and prefrontal cortex are also observed (Eiland & Romeo, 2013; Isgor, Kabbaj, Akil, & Watson, 2004; O. Oztan, Aydin, & Isgor, 2011; Ozge Oztan, Aydin, & Isgor, 2011). In one study, chronic variable stress throughout development (p28-56) led to few immediate changes but decreased hippocampal volume three weeks prior to the end of the stressor, suggesting a delayed effect on brain plasticity. These hippocampal changes were associated with impaired memory (Isgor et al., 2004). The majority of studies examining the effects of stress on the prefrontal cortex and amygdala have focused on adult animals. Experiencing stress as an adult leads to atrophy of pyramidal neurons in the PFC (Liston et al., 2006; Radley et al., 2004) and

hypertrophy of neurons in the amygdala (Vyas, Mitra, Shankaranarayana Rao, & Chattarji, 2002). The effect of stress on the PFC and amygdala have only recently been explored during peri-adolescence. In 2012, Eiland and colleagues found that chronic restraint stress (p20-41) in male and female rats reduced pyramidal neuron complexity in the PFC and hippocampus but increased neuronal complexity in amygdala. Furthermore, these changes were associated with elevated depressive-like behaviors (Eiland, Ramroop, Hill, Manley, & McEwen, 2012). Thus, in both humans and rodents, there is a growing body of literature suggesting that early stress leads to changes in developing limbic brain regions, including the hippocampus, amygdala and PFC.

Much of the literature has focused on the effects of peri-adolescent stress on neurons and neuronal plasticity. However, stress effects on glia are beginning to be explored. Specifically, oligodendrocytes (OLs) and the myelin they produce have been shown to be sensitive to stress, not only in white matter, but also in grey matter regions. (Makinodan et al., 2012; Saul, Helmreich, Rehman, & Fudge, 2015); for a review see: (Monje, 2018). In addition, OLs and myelin have been implicated in a number of mental health disorders, including schizophrenia, depression and PTSD, suggesting they play a functional role in mood (Chao, Tosun, Woodward, Kaufer, & Neylan, 2015; Falkai et al., 2016; P. Lee & Fields, 2009; Ma et al., 2007; Nave & Ehrenreich, 2014; Regenold et al., 2007; Sokolov, 2007; Tham, Woon, Sum, Lee, & Sim, 2011); for reviews see: Birey et al., 2017; Fields, 2008). Chronic stress early in life can also alter myelination in both humans and rodents. In humans, both institutionalization and early child abuse alter white matter in the PFC; further, these changes correlate with cognitive deficits (Hanson et al., 2013; Lutz et al., 2017). In mice, chronic social isolation around the peri-adolescent period leads to increased depressive-like behaviors, reduced myelin basic protein (MBP) and hypomyelination of the PFC (Leussis & Andersen, 2008; Makinodan et al., 2012). This effect is only observed when the stressor occurs during the juvenile period, suggesting there may be a critical window for stress effects on PFC myelination (Makinodan et al., 2012). Together, these data indicate that altered myelination may be a novel and underappreciated mechanism by which psychopathologies emerge.

Much of this work has focused on chronic stress. Less is known about the effects of acute, traumatic events. In adults, PTSD patients have increased hippocampal myelin compared to trauma exposed controls (Chao et al., 2015). Interestingly, this increase in hippocampal myelin is positively correlated with PTSD symptom scores, suggesting that vulnerability to stress-induced disorders is related to hippocampal myelin. In a recent study from our lab, similar findings were observed in rodents. Specifically, OLs and myelin in the dentate gyrus of the hippocampus positively correlated with avoidance behaviors following exposure to acute severe stress. In addition, myelin levels in the amygdala correlated with contextual fear learning (Long et al., *in preparation*). Together, these findings suggest that, in adults, OLs and myelin are associated with individual vulnerability following acute stress.

A critical question is whether or not changes in OLs and myelin are observed following acute traumatic stress earlier in life, during the juvenile period. In particular, the PFC is a key brain region of interest, as chronic stress robustly alters PFC myelin, and the PFC is highly plastic during the peri-adolescent phase. Thus, in this study, we sought to explore whether acute stress as a juvenile will induce changes in PFC OLs and myelin. Furthermore, we aimed to assess both short and long-term consequences, behaviorally and anatomically. As the peri-adolescent stage is a period of heightened experience-dependent plasticity, we predicted that acute stress would result in altered myelination in developing limbic brain regions such as the PFC. In particular, in line with the chronic stress literature we hypothesized that there would be decreased PFC myelination following acute stress. Lastly, little is known about how PFC myelin and OLs relate to individual

differences following trauma. Therefore, we sought to address if behavioral differences following traumatic stress were associated with PFC myelin and OLs. In addition, we examined whether there are sex differences following exposure to acute severe stress. Sex is an important biological factor that contributes to individual variation in response to stress. To test these questions, we first exposed male and female juvenile rats to an acute, severe stressor. Animals then underwent a battery of fear and anxiety-like assays either one week later, or as an adult, in order to test for short- or long-term changes respectively, and to assess individual responses to stress. We then analyzed oligodendrocyte and myelin markers in the PFC to examine the effects of stress on glial plasticity. Lastly, we assessed whether myelin and OL markers in the PFC correlated with behavioral measures, with the hypothesis that animals that with the greatest behavioral changes would also display the biggest changes in PFC myelin, relative to controls.

Materials and Methods

Animals

Sixty-four male and female Sprague Dawley rats were used for these experiments. All rats were bred in-house in order to minimize stressful experiences such as shipping prior to testing. All rats were pair-housed, given *ad libitum* access to food and water and were kept on a 12/12 hr. light/dark cycle. All procedures were approved by UC Berkeley's Animal Care and Use Committee.

Stress

Each cage of rats was randomly assigned to either a stress or control condition. For animals in the stress condition (n=32), at postnatal day 28 (p28), juvenile male and female Sprague Dawley rats were exposed to three hours of severe stress (restraint stress with exposure to a predator odor; Figure 1A). Specifically, rats were quickly weighed, restrained in plastic Decapicone bags (Braintree Scientific, Inc, Braintree, MA) and placed in a clean cage inside a fume hood. Inside that cage was a cotton ball infused with 1mL of synthetic fox urine (Red Fox Urine, Trap Shack Company, Arcadia, WI) taped approximately 1 inch from the animal's nose. Cagemates were placed side by side in the cage for the extent of the stressor. Blood samples from the tail were also taken at three time points (see details below). All stress testing was conducted between the hours of 8am and noon. At the end of the three-hour stressor, both cagemates were returned to a clean cage and allowed to self-groom. Pair-housed animals in the stress condition were kept in a separate housing room for three days prior to being returned to their normal housing room, in order to minimize stress transmission to other rats. The light cycle of this separate room was identical to the original housing room. Animals in the control condition (n=32) were similarly weighed at p28 but otherwise remained in their home cage undisturbed. On the day stress animals were returned to their original housing room (three days post-stress exposure), rats in all conditions were weighed again.

Serum sampling for corticosterone analysis

Tail vein blood was collected from each rat at 0 minutes, 3 minutes and at the end (3 hours) into the acute traumatic stressor. Specifically, a sterile scalpel was used to remove a small segment

at the end of the tail, and approx. 0.5mL of blood was collected at each time point. Samples were kept on ice throughout the stressor. Blood clots were then removed, samples were centrifuged at 9,391 g for 20 minutes at 4°C, serum was extracted and stored in clean tubes and stored at -80°C. Samples were assayed using a Corticosterone EIA kit (Arbor Assays, Ann Arbor, MI), with 2 replicates per sample. An area under the curve (A.U.C) was calculated for each animal using the three time points.

Behavioral Assays

All animals underwent extensive behavioral profiling of fear- and anxiety-like behavior either one week following the stressor (at p35), or in adulthood (at p90), to test for short- or long-term effects respectively (Figure 1A). Six different behavioral tests were conducted: an open field test (OFT) in a brightly lit environment and in a dim light environment (OFT Light and Dim respectively), a light/dark box test (LD), elevated plus maze (EPM) in both a light and dim light environment (EPM Light and Dim respectively), and a fear conditioning test. At the end of each test, the number of feces were recorded as an additional measure of stress. All tests are described fully below. Behavioral testing occurred over five days, and tests were performed in the same sequence for each animal: Day 1 – OFT Light, EPM Light, LD, Day 2 – OFT Dim, EPM Dim. Days 3-5 – Fear Conditioning. On each day, animals were brought into the testing room and were provided 10-30 minutes to acclimate prior to the start of testing. Between each test, animals were returned to their home cage and allowed to rest with their cagemate for 10 minutes prior to starting the next assay. All animals were weighed prior to beginning the OFT Light assay on Day 1, and prior to perfusions on Day 5.

Open Field Test (OFT)

Each animal was placed in an open plastic square box (50 l x 50 w x 58 cm h) and was allowed 10 minutes to freely explore the arena. All animals were placed in the center of the box at the start of the test. Behavior was recorded with cameras positioned directly above the center of the arena and was acquired using Geovision software (Geovision, Irvine, CA). The OFT Light was conducted under approximately 280 lux, and the OFT Dim was conducted under approximately 28 lux. Videos for each animal were saved and compressed prior to analysis. EthoVision software (Noldus, Leesburg, VA) was used to analyze several measures of behavior, including latency to, frequency and total amount of time spent in the center (inner 50%) of the box. In addition, measures such as total distance travelled, and average velocity were analyzed. At the end of testing, the arena was cleaned with 1% acetic acid, followed by Formula 409 All Purpose Cleaner, in order to eliminate smells in between animals.

Elevated Plus Maze (EPM)

Each animal was placed in an elevated plus maze apparatus and was allowed 10 minutes to freely explore (height: 50cm of the ground, arms: 10cm wide, 60cm length). Two of the arms were enclosed with high walls (51 cm high). All animals were placed in the center of the EPM facing an open arm at the start of the test. In the EPM Light, open arms were approximately 210 lux, while closed arms were approximately 120 lux. In the EPM Dim, open arms were approximately 17 lux, while closed arms were approximately 10 lux. Behavior was recorded using

a Logitech c270 Webcam (Logitech, Newark CA) mounted directly above the center of the arena. Videos for each animal were scored by hand by experimenters blind to animal condition. Latency to, frequency and total amount of time spent in the open arm was analyzed. Animals were considered to be in the open arm as soon as more than 50% of their body and two paws were placed in the open arm. If an animal fell off the apparatus, they were given at least 30 minutes to recover and then re-tested for 10 minutes. Between each animal, the maze was cleaned with 1% Process NPD Disinfectant (STERIS Life Sciences).

Light-Dark Box (LD)

Each animal was placed in a light-dark box apparatus and was allowed 10 minutes to freely explore both the light and dark halves of the box (each 15 w x 15 l x 8 in h), which were separated by a small door. All animals were placed facing the entrance to the dark half of the box at the start of the test. The LD box test was conducted under approximately 300 lux lighting. Behavior was recorded using a Logitech c270 Webcam (Logitech, Newark CA mounted directly above the apparatus. Because the dark half of the box was enclosed, rats were only visible when they moved through the door to the light side. Videos for each animal were scored by hand by experimenters blind to animal condition. Latency to, frequency and total amount of time spent in the light half of the box was analyzed. Animals were considered to be in the light half as soon as more than 50% of their body and two paws passed beyond the door. Between each animal, the maze was cleaned with 70% Ethanol.

Fear Conditioning

On the first day of fear conditioning (Day 3 of behavioral testing), animals were placed in a Coulbourn sound-attenuating fear conditioning chamber with electrified grid floors (12 w x 10 l x 12 in h). Following 5 minutes of acclimation, rats received 10 un-signaled 0.8 mA, 1 sec duration shocks. The interstimulus interval was 15-120 sec between shocks and was pseudo randomly determined. Rats were left in the chamber for 3 minutes after the last shock and then were returned to their home cage. On the second day of fear conditioning, rats were placed back into the same fear context 5 times, with each trial lasting 10 minutes each. No shocks were given, as the goal was to test for fear extinction. As before, between each trial, animals were returned to their home cage and allowed to rest with their cagemate for ~10 minutes prior to starting the next trial. Extinction was quantified as area under the curve (A.U.C) from the 5 extinction trials. On the third day of fear conditioning, animals were again placed in the fear context for one 10-minute trial, in order to assess the retention of fear extinction. On all days of fear conditioning, videos were recorded by cameras inside each conditioning chamber. Videos were converted to AVI file format and analyzed by hand by experimenters blind to animal condition. The time spent freezing was quantified for each trial across the three days. Rats were considered to be ‘frozen’ when they had stopped moving, all paws were still, and their back muscles were tense for a minimum of 1 second. In addition, on the day of shocks, whether or not the animal attempted to escape each shock or instead remain frozen was quantified. Between each animal, the boxes were cleaned with 70% Ethanol.

Composite Scoring

For each parameter of the behavioral tasks, a behavioral cutoff was defined by the 20th percentile of the control distribution. Rats falling below the 20th percentile were classified as "Affected" and received a score of 1; rats that were "Unaffected" received a score of 0. A quantitative measure of anxiety called the "Rat Anxiety Score" (RAS) was calculated for each rat by tallying the number of Affected scores across all tasks and the three primary measures (latency, frequency and total time) for a total possible score of 15. A similar measure, the "Rat Fear Score" (RFS) was calculated for fear behavior, with a total possible score of 7. This method was based on a Cutoff Behavioral Criteria developed by Cohen and Zohar and was used previously in the lab (H. Cohen & Zohar, 2004; Long et al., *under review*) Figure S1).

Perfusions and Brain Extractions

Rats were deeply anesthetized with sodium pentobarbital 200 mg/kg (Euthasol ®, Vibrac AH Inc.) and transcardially perfused with ice-cold 0.9% saline followed by freshly made 4% paraformaldehyde (PFA) in 0.1 M PBS. Brains were extracted, post-fixed for 24 hours at 4°C in 4% PFA and sunk in 30% sucrose in 0.1 M PBS over several days. Brains were then stored at -80°C until they were ready to be sliced.

Histology and Immunohistochemistry

Frozen brains were cryosectioned at 40 µm on an NX70 CryoStar Cryostat (Thermofischer Scientific) Free-floating sections were stored in 12 tubes with antifreeze, with every 12th slice placed in the same tube. Samples were stored at -20°C prior to staining.

Immunohistochemical (IHC) staining was conducted in order to quantify oligodendrocyte and myelin markers. Specifically, IHC was used to detect myelin basic protein (MBP), one of the essential proteins in the myelin sheath (Hamano, Iwasaki, Takeya, & Takita, 1996) and glutathione s-transferase pi (GSTpi), a marker for immature to mature oligodendrocytes (Tansey & Cammer, 1991). Tissue slices from one vial of tissue (every 12th slice) were stained. Slices were first washed in tris-buffered saline (TBS), and blocked with 3% normal donkey serum (NDS) in TBS with 0.3% Triton-X100 for one hour at room temperature. Slices were then incubated overnight at 4°C with the following primary antibodies: rat anti-MBP (1:500, Abcam ab7349) and rabbit anti-GSTpi (1:5000, MBL 311). All antibodies were diluted in 0.3% TritonX-TBS containing 1% NDS. On day two, following three rinses in TBS (3 x 5 min), sections were incubated for two hours with the following secondary antibodies: AlexaFluor 488 donkey anti-rabbit and Cy3 donkey anti-rat (711-545-152 and 712-165-153 respectively, Jackson ImmunoResearch Labs Inc). Sections were then rinsed in TBS (3 x 5 min) and incubated for 10 minutes with DAPI (1:40,000 in 1xPBS), followed by three more rinses in TBS. Lastly, sections were mounted on glass slides and cover slipped using 1,4 diazabicyclo[2.2.2]octane (DABCO). A list of antibodies and their dilutions can be found in Table S1.

Fluorescent Microscopy and Image Analysis

All tissue was imaged on an AxioScan Slide Scanner at 20x (AxioScan.Z1, Zeiss, Oberkochen, Germany). Using ZEN blue imaging software (Zeiss), regions of interest were chosen

and exported as TIFFs. For analysis of the prefrontal cortex, five to seven PFC sections from AP 4.2 - AP 1.2 were used for analysis. Four regions of the PFC were quantified: anterior cingulate cortex (ACC), prelimbic cortex (PL), infralimbic cortex (IL) and orbitofrontal cortex (OFC). Each of the four regions of interest (ROIs) were hand-drawn in Fiji (Schindelin et al., 2012), and saved as an ROI set zip file (Figure S2).

For all regions, myelin content was measured by the integrated fluorescence intensity of MBP expression, normalized by area in μm^2. Oligodendrocytes (GSTpi+ cells) were quantified using a custom written Fiji script, which included background subtraction, automated thresholding and particle analysis. The same parameters were used for all animals and all tissue regions. In addition, parameters were chosen such that the script counted within 10% of counts obtained by human experimenters. Oligodendrocyte cell density is presented as the number of cells within a given ROI per mm^2. In addition, cell density was calculated as a % of all DAPI+ cells; these data corresponded well with cell density by area, so all data presented are shown as cell density/area. Measures for each ROI were averaged across the anterior-posterior extent of sections and from both hemispheres.

Statistical Tests

All data are presented as mean ± the standard error of the mean (SEM). Two-way ANOVAs were used to compare male and female rats in the stress and control conditions at each age time point. Bonferroni post-hoc analyses were used to test for specific comparisons. Unpaired independent sample student's t-tests were used to directly compare changes between control and stressed animals within each sex, when appropriate. Pearson correlations were used to compare the relationship between oligodendrocyte and myelin markers, serum corticosterone and behavioral measures. In all tests, the alpha value was set at 0.05. All analyses were performed with GraphPad Prism version 8.4 (GraphPad Software, San Diego, California USA) and RStudio (RStudio Team, 2018).

Results

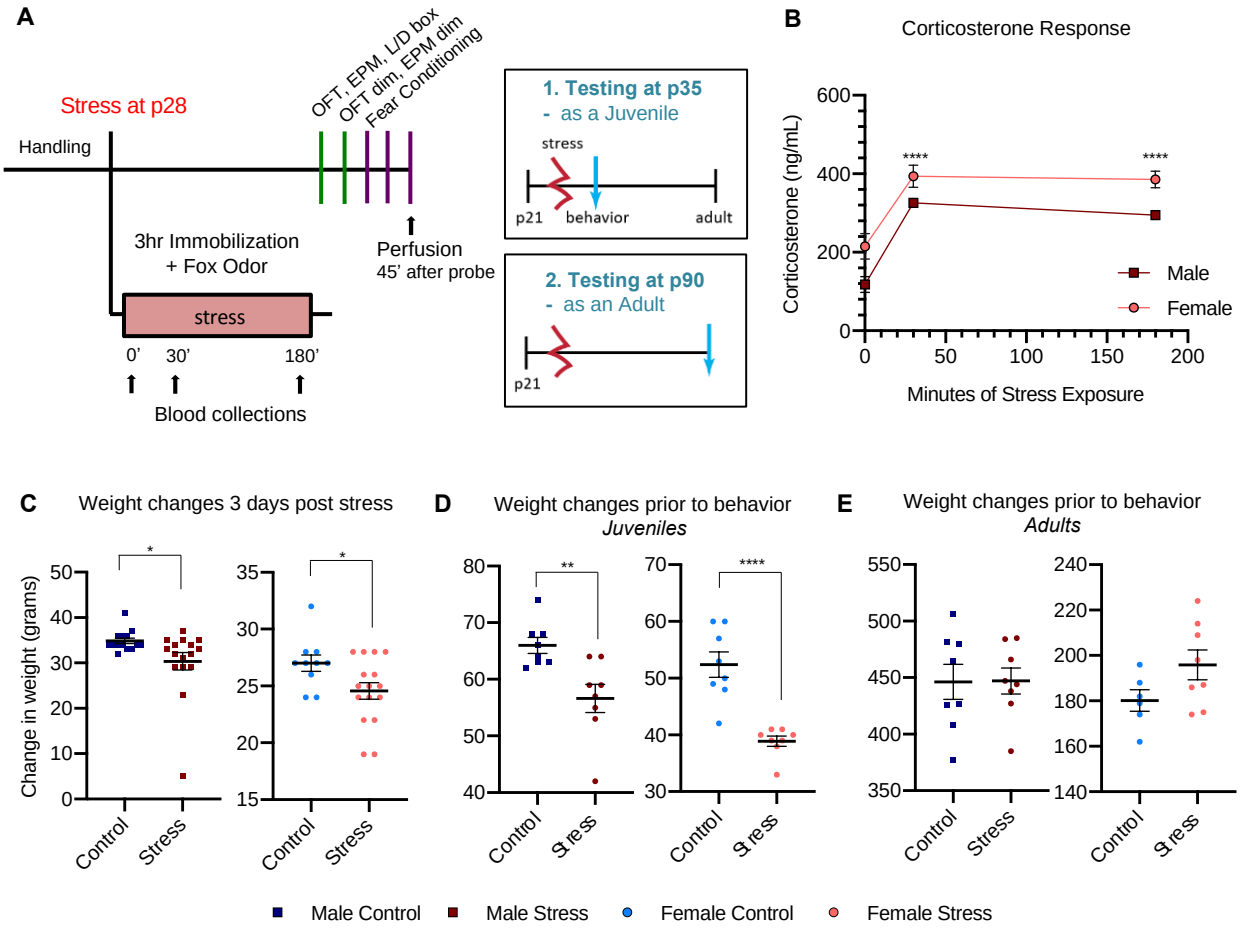
Male and female juvenile rats exhibit a robust physiological response following exposure to acute, severe stress

In order to model acute, severe trauma, male and female Sprague Dawley rats underwent 3 hours of immobilization stress while being exposed to a predator odor (n=16 males, 16 females; Figure 1A). This stressor occurred at postnatal day 28 (p28), prior to the onset of puberty (Eiland & Romeo, 2013; Piekarski et al., 2017). Blood was collected at 0, 30 and 180 minutes into the stressor for later assessment of corticosterone, the rodent stress hormone (Bruce S. McEwen, Weiss, & Schwartz, 1968; Zenker & Bernstein, 1958). A separate group of rats remained in their home cage as a control (n=16 males, n=16 females). First, we examined the physiological response of male and female juveniles to the stressor, in order to confirm our paradigm indeed was a potent stressor at this age. Previously, our lab and others have shown that predator scent coupled with immobilization produces a large corticosterone response in adult rats (Long et al., *under review*.; Morrow, Redmond, Roth, & Elsworth, 2000; Muroy, Long, Kaufer, & Kirby, 2016; Zoladz & Diamond, 2016). As expected, here, acute severe stress produced robust increases in serum corticosterone over baseline levels, in both male and female juveniles (Figure 1B, S3A). A 2-way

repeated measures ANOVA yielded a main effect of time ($F(1.346, 40.39) = 90.49$, $p<0.0001$), and a main effect of sex ($F(1, 30) = 11.49$, $p<0.002$), with no statistically significant interaction between the two. A Sidak's post-hoc test identified robust differences between corticosterone levels at baseline compared to 30 and 180 minutes for both males and females ($p<0.0001$). Male rats also demonstrated lower levels of corticosterone relative to females all time points, including at baseline (Figure 1B, males: mean = 84.079 ± 12.191 ng/mL, females: mean = 267.054 ± 52.657 ng/mL). Furthermore, an unpaired t-test comparing the area under the curve of corticosterone levels identified a statistically significant difference between the two sexes ($t(14)=5.063$, $p=0.0002$) consistent with known literature that female rats, at least as adults, can exhibit higher baseline corticosterone levels (Kalil, Leite, Carvalho-Lima, & Anselmo-Franci, 2013; Mitsushima, Masuda, & Kimura, 2003). Overall, both female and male juvenile rats experienced physiological increases in corticosterone that persisted throughout the duration of the stressor.

In order to identify both short-term and long-term effects of stress, one group of animals was tested one week following stress (at p35), while another was tested almost two months later at an adult age (at p90) (Figure 1A). Changes in body weight are another physiological indicator of stress (R. B. S. Harris et al., 1998; Pulliam, Dawaghreh, Alema-Mensah, & Plotsky, 2010). Here, rats were weighed at p28, prior to stress for those in the stress condition, three days following stress, at the start of behavior (either p35 or p90) and at the termination of behavior prior to euthanasia. Three days after stress, both males and female juveniles gained less weight relative to controls (males, $t(28)=2.133$, $p=0.0419$;females, $t(24)=2.246$, $p=0.0342$); Figure 1C, S3B). For animals tested in behavior one week later, once again, stressed males and females gained less weight relative to controls (males, $t(14)=3.271$, $p=0.0056$; females, $t(14)=5.572$, $p<0.0001$; Figure 1D, S3C). However, animals tested as adults demonstrated no differences in weight gain across groups (Figure 1E, S3D), indicating that acute severe stress only leads to short term changes in weight. Collectively, these data suggest that acute, severe stress elicits a rapid physiological stress response in juvenile rats and leads to short term changes in weight gain.

Figure 4-1. Physiological responses to acute traumatic stress.
(BELOW) A) Experimental Timeline. 32 Sprague Dawley rats underwent 3 hours of immobilization stress with exposure to fox urine at post-natal day 28 (p28). (n= 16 males, n=16 females). An additional 32 animals (n=16 males, n=16 females) remained in their home cage. Blood was collected at three timepoints: just prior to stress, 30 minutes into the stress, and at the end of stress at 180 minutes. Following a delay, animals underwent five days of behavioral profiling. Animals began behavioral testing at one of two timepoints: either one week later (at p35), or when they were adults (at p90). The first two days consisted of approach-avoidance tasks including: the Open Field Test (OFT) and Elevated Plus Maze (EPM) tests under bright and dim conditions, and a Light-Dark box (L/D box) test. On the last three days of testing, animals underwent a contextual Fear Conditioning paradigm. **B)** Corticosterone responses to acute traumatic stress. Corticosterone levels (ng/mL) robustly increased during exposure to the stressor for both male and females, with higher corticosterone at 30 and 180 minutes over baseline values. **C)** Weight changes three days post stress. **D)** Weight changes prior to behavior for animals tested 1 week following stress (day 7 - day 0). **E)** Weight changes prior to behavior for animals tested ~ 2 months following stress (day 90– day 0). Statistically significant differences are marked with asterisks (*$p<0.05$, ** $p<0.01$, ***$p<0.001$, ****$p<0.0001$).



Exposure to acute, severe stress as a juvenile yields a spectrum of fear and anxiety-like behavior in male and female rats, with no significant group differences

Following exposure to an acute traumatic stressor, animals were tested in a battery of fear and anxiety-like tests either one week later or as adults, in order to assess if there are persistent behavioral changes following stress (H. Cohen, Kozlovsky, Alona, Matar, & Joseph, 2012; H. Cohen et al., 2004; H. Cohen & Zohar, 2004). This stress paradigm, three hours of immobilization combined with exposure to a predator scent, has been previously validated in our lab and others and leads to persistent behavioral changes in a subset of adult male rats (Long et al., *under review*; Muroy, Long, Kaufer, & Kirby, 2016; Zoladz & Diamond, 2016). Thus, we hypothesized we would see a spectrum of anxiety-like and fear behavior in the current group of juveniles, with many recovering to control levels of behavior. Specifically, to characterize the extent of behavioral changes, we conducted three days of approach-avoidance testing; this included open field (OFT) and elevated plus maze (EPM) tests in both bright and dim conditions, and a light-dark box (LD) assay. This was followed by three days of contextual fear conditioning including an initial day pairing footshock to a specific context, one day of extinction trials, and a final day to test extinction retention. For each approach/avoidance assay, we quantified frequency, duration, and latency of visits to anxiogenic and anxiolytic zones. For fear extinction assays, we quantified the total time frozen during each trial.

For animals tested one week later and those tested as adults, few differences were observed in individual parameters of avoidance and fear behaviors between control and stress-exposed animals (Figures S4-S7). This was not unexpected, as we anticipated only a subset of animals would show persistent anxiety-like and fear behavior. In order to assess animal behavior in a more wholistic way, we utilized a composite scoring system to take all avoidance and fear measures into account. This method is modeled off of the human CAPS score for PTSD and has been used previously in our lab. Specifically, the model closely follows the Cutoff Behavioral Criteria method developed by Cohen (and colleagues), where animals are considered "affected" by stress if they fall above the 20^{th} percentile of the unexposed control group. This behavioral cutoff provides a binary method of quantifying behavior that is unaffected by extreme values that might otherwise skew distributions. We employed this behavioral cutoff method for both the avoidance scores and fear scores separately, as they are distinct domains of behavior. Specifically, to create a composite 'Rat Avoidance Score' (RAS) latency, frequency and duration measures from the five OFT, EPM and LD tests were given binary scores and then summed, for a maximum score of 15. Similarly, to create a composite 'Rat Fear Score'(RFS), the percent of shocks frozen for, and the time spent freezing across the 5 extinction trials and 1 probe trial were given binary scores and then summed, for a maximum score of 7. (Figure S1). High scores on these scales therefore reflect that an animal consistently displayed more avoidance or fear behavior compared to control animal distributions.

Using this method, we found that exposure to acute traumatic stress as a juvenile did not significantly alter composite measures of either fear or anxiety-like behavior in male or female rats compared to controls (Figure 2A&B). However, stress-exposed males tested one week later showed higher mean scores and more variability compared to control males, indicating that indeed, some animals displayed higher levels of fear and anxiety-like behavior while yet others were resilient to stress (RAS: control: 3.25 ± 0.65 stress: 4.375 ± 1.24; RFS: control: 1.50 ± 0.46 stress: 3.0 ± 0.85; Figure 2A&B). Stress-exposed females however did not show any differences in variability relative to controls (RAS: control: 2.63 ± 0.84 stress: 4.13 ± 0.67: RFS: control: 1.25 ± 0.62 stress: 1.25 ± 0.45). For animals tested as adults, animals in both sexes and conditions demonstrated highly variable behavior, and little differences in mean scores relative to controls. (Figure 3A&B). On the whole, acute stress did not significantly alter composite behavioral scores in either the short or long term.

Despite the lack of difference in mean composite scores on the group level, there was a statistically significant difference in fear behavior in juvenile males tested at p35. Specifically, a repeated measures ANOVA identified a main effect of time ($F(2.946,39.48)=36.18$, $p<0.0001$), and condition ($F(1,14)=4.625$, $p=0.0459$), indicating that stress-exposed males maintained high levels of freezing throughout the extinction testing and extinction retention (Figure 2C). Interestingly, a two-way ANOVA comparing the Area under the Curve (AUC) of time frozen across all fear trials also identified a main effect of sex ($F(1,28)=5.6$, $p=0.0249$) and a statistically significant interaction ($F(1,28)=4.7$, $p=0.0380$) whereby female stress-exposed animals exhibited less time freezing relative to their controls and males exhibit greater time freezing relative to their controls (Figure S8A). Thus, stressed males were unable to successfully extinguish fear as well as the controls (Figure 2C). This effect was not observed in the females tested at p35, or in either sex tested at p90. (Figure S8B).

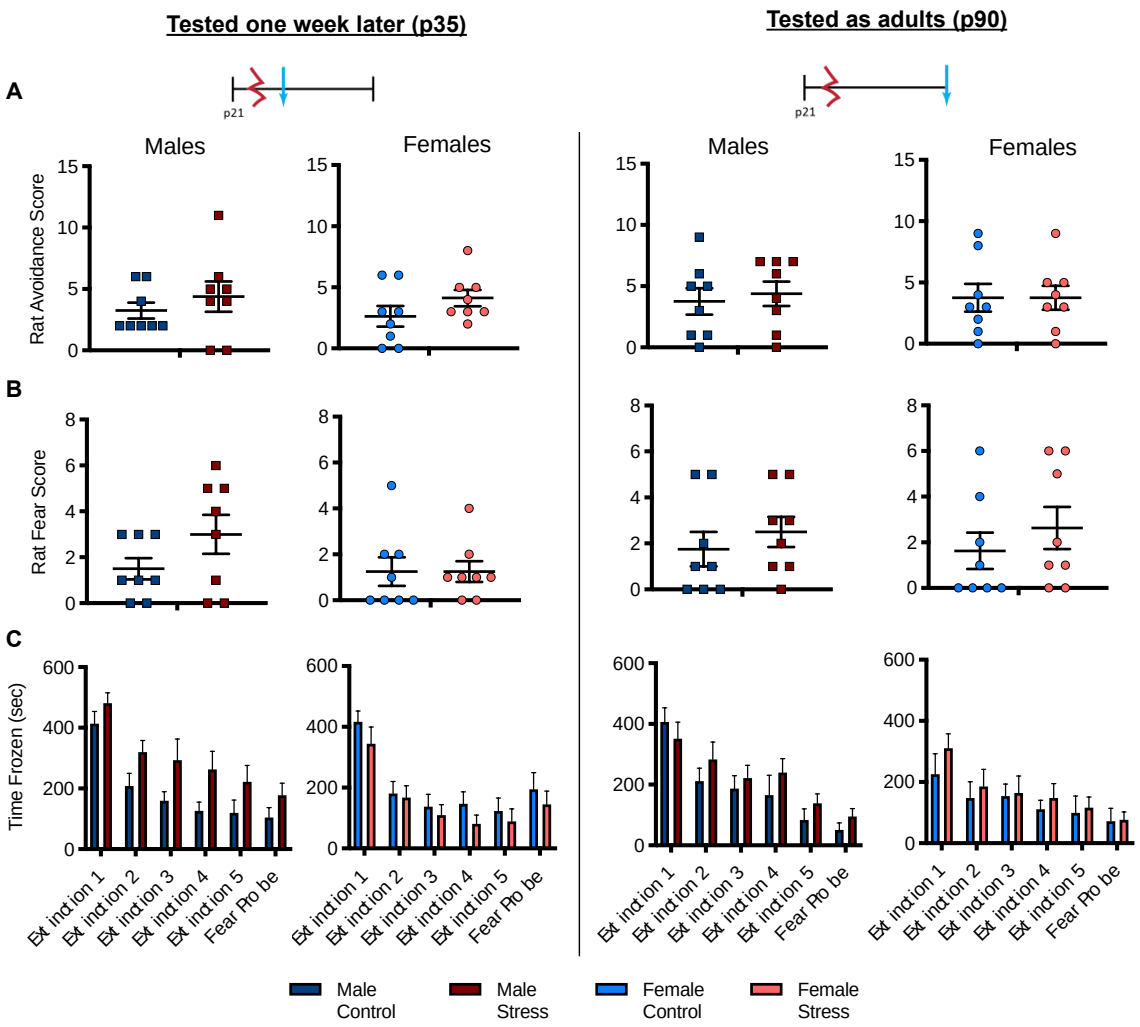


Figure 4-2. Behavioral scores following acute traumatic stress.
Assessed either one week later (left) or as an adult (right). **A)** Composite approach-avoidance scores. **B)** Composite fear scores. **C)** Fear behavior (time frozen) across all extinction sessions.

Myelin and Oligodendrocytes are altered in the PFC following acute stress

Our lab has previously shown that rats exposed to chronic stress or one week of corticosterone injections have increased oligodendrogenesis in the hippocampus (Chetty et al., 2014). In addition, we recently showed that in adult male rats exposed to the same acute, severe stress used here, there is increased oligodendrocyte and myelin content in the dentate gyrus, specifically in stress-reactive animals that exhibit high composite scores of avoidance behavior. On a group level, stress did not alter hippocampal myelin, however myelin levels positively correlated with behavioral changes following the stressor (Long et al., *under review*) Given these previous findings, we sought to address whether stress can affect grey matter myelin and oligodendrocytes within the PFC. Specifically, given past literature suggesting there is a critical period where myelin is sensitive to stress (Makinodan et al., 2012), we administered an acute, severe stress during the juvenile period. We stained prefrontal cortex tissue for two makers: GSTpi, a marker of immature to mature oligodendrocytes and myelin basic protein (MBP) as a marker of

myelination (Figure 3A &B). We analyzed four subregions of the prefrontal cortex: the anterior cingulate cortex (ACC), the prelimbic (PL) and infralimbic (IL) cortices, and the orbitofrontal cortex (OFC). All data was averaged across representative sections covering the anterior-posterior extent of PFC and these subregions (Figure S2).

We first looked at changes in the prefrontal cortex as a whole, to see if there was an overall effect on the PFC. For all analyses, we first ran 2-way ANOVAs in order to assess main effects of sex and condition (stress or control). For prefrontal cortex MBP in animals tested one week after stress, there were no main effects of sex or condition, and no interaction between them. Similarly, student's t-tests directly comparing stressed males and females to their respective controls found no differences in MBP fluorescence intensity in males, yet found a trend towards an increase in MBP in stress-exposed females (Control = 25.8 ± 1.9 fluorescence/± μm^2, Stress = 30.8 ± 1.8 fluorescence/μm2, $t(14)=1.874$, p=0.08). When looking at GST-pi in the PFC as a whole, once again, there were no main effects of sex or condition, yet there was a statistically significant interaction between them ($F(1,27)=5.3$, p=0.0292). Interestingly, for the females, a student's t-test revealed that stress-exposed females had lower levels of GST-pi averaged across the PFC (Control = 265 ± 7.1 cells/mm^2, Stress = 222 ± 8.4 cells/mm^2, $t(13)=3.842$, p=0.002). This suggests that for females, acute, severe stress as a juvenile globally decreases oligodendrocyte cell density in the PFC in the short term (Figure 3C).

For animals tested almost two months following stress exposure, a 2-way ANOVA identified no main effects of sex or condition for either MBP or GST-pi, however there was a statistically significant interaction between sex and condition for MBP levels across the whole PFC ($F(1,28)=4.4$, p=0.0436). When each sex was looked at separately, stress-exposed females were found to have lower levels of MBP than their control counterparts (Control = 72.0 ± 6.5 fluorescence/± μm^2, Stress = 54.2 ± 3.8 fluorescence/μm^2, $t(11)=2.378$, p=0.036). No change was observed in GST-pi across conditions. This suggests that for females, acute, severe stress as a juvenile globally decreases PFC grey matter myelin in the long term (Figure 3D).

Next, we looked at how acute severe stress might change myelin and oligodendrocyte content in specific subregions of the PFC. For animals analyzed one week later at p35, individual subregions of the PFC demonstrated variations in both MBP and GST-pi levels, showing main effects of brain region in both males and females (Males MBP: $F(3,52)=8.65$, p<0.0001, Females MBP: $F(3,56)=14.93$, p<0.0001, Males GST-pi: $F(3,55)=3.425$, p=0.0233, Females GST-pi: $F(3,52)=4.427$, p=0.0076). Most interestingly, female-stress exposed animals had higher levels of MBP in all PFC subregions compared to their respective controls, demonstrating a strong main effect of stress ($F(1,52)=24.72$, p<0.0001). This effect did not appear to be driven by any one PFC subregion; a multiple comparisons test identified significant differences in the IL and OFC regions (p=0.0318 and p=0.0378 respectively), with trends in the ACC and PL (p=0.08 and p=0.149, respectively). Exposure to stress had the opposite effect on GST-pi levels for females; here, stress-exposed females were found to have lower levels of oligodendrocytes compared to controls ($F(1,56)=11.04$, p=0.0016). Once again, this effect did not appear to be driven by any single PFC subregion. Thus, overall, acute traumatic stress affected juvenile females but not males, leading to increases in PFC myelin and decreases in oligodendrocytes across all subregions. (Figure 3E).

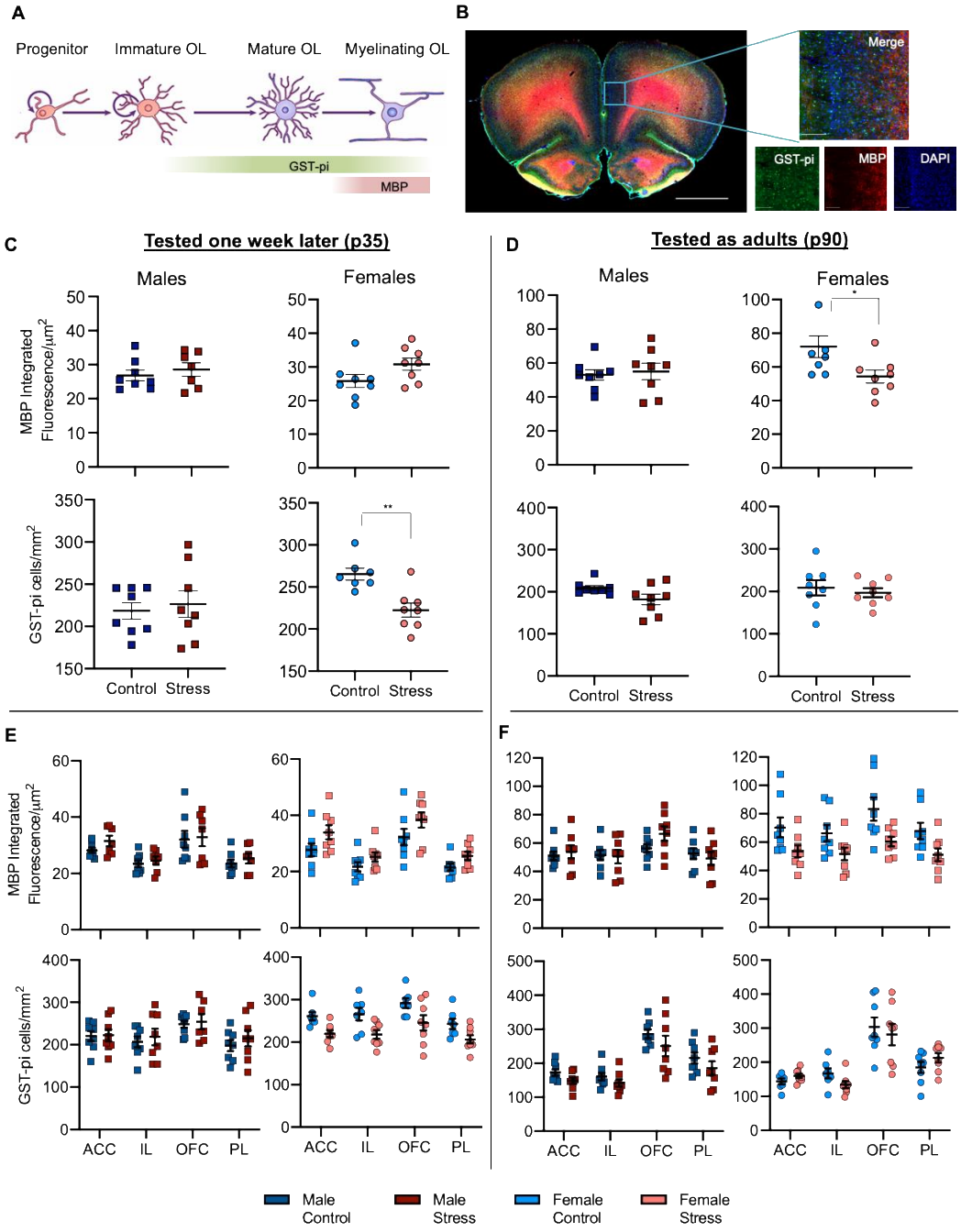


Figure 4-3. Effects of acute traumatic stress on myelin and oligodendrocytes.
A) Schematic of oligodendrocyte (OL) development and myelin, and their associated markers: Glutathione S-transferase pi (GST-pi), and myelin basic protein (MBP). **B)** On left: Example prefrontal cortex coronal section. Scale bar 2mm. On right: Representative staining of GST-pi and MBP in the prelimbic cortex. Scale bar: 200μm. **C, D)** MBP and GST-pi levels averaged across the whole PFC, for animals tested one week following stress (C) or tested as adults (D). **E, F)** MBP and GST-pi levels within each PFC subregion, for animals tested one week following stress (E) or tested as adults (F). *p<0.05; **p<0.01

For animals tested as adults, individual subregions of the PFC again demonstrated variations in MBP and GST-pi, with the highest levels in the OFC. For MBP, stress exposed males showed no changes compared to controls, in all PFC subregions. Stress-exposed females however, showed a decrease in MBP compared to controls, demonstrating a main effect of stress ($F(1,48)=11.13$, $p=0.0016$). This effect was not driven by one particular PFC subregion; all subregions showed a mean decrease in MBP fluorescence intensity compared to control animals. Although there was an effect on MBP, stress-exposed and control females had similar levels of GST-pi in all PFC subregions (Figure 3F). Lastly, stress-exposed males had decreased levels of GST-pi relative to controls, showing a main effect of stress ($F(1,56)=5.4$, $p=0.0234$), that was not driven by any particular subregion.

Overall, the effect of stress on prefrontal cortex myelin and oligodendrocytes appeared to primarily affect female rats. Interestingly, the effect on MBP switched depending on how long the animal had to recover following stress; MBP levels increased in the short term but decreased in the long-term. The more short-term decrease in GST-pi+ oligodendrocytes for females also did not persist into adulthood.

Changes in myelin and oligodendrocytes in the PFC do not correlate with behavior

In line with the hippocampal findings from adult males (Long et al., *under review*), we hypothesized that animals with greater changes in behavior following stress would also demonstrate greater changes in PFC myelin, suggesting a possible functional role for myelin in the prefrontal cortex. To test this, we assessed whether there were correlations with myelin markers and behavior following stress exposure. In animals tested one week following stress exposure, composite avoidance scores were positively correlated with prefrontal regions in control male and females (Figure 4A, male: $r = 0.72$, $p=0.041$; female: $r = 0.88$, $p=0.009$), however these correlations were not seen in stressed animals, nor were they statistically significant when correcting for multiple comparisons (Bonferroni correction, alpha value: 0.005 with 10 tests/group). Further, for all groups, there were no statistically significant correlations with composite fear scores. Animals tested as adults overall showed a similar lack of correlation between myelin and oligodendrocytes and composite behavioral scores (Figure 4B). Composite avoidance and fear scores did not correlate with myelin or oligodendrocytes for any of the groups. Overall, we did not observe consistent correlations between behavioral scores and prefrontal cortex myelin and oligodendrocytes.

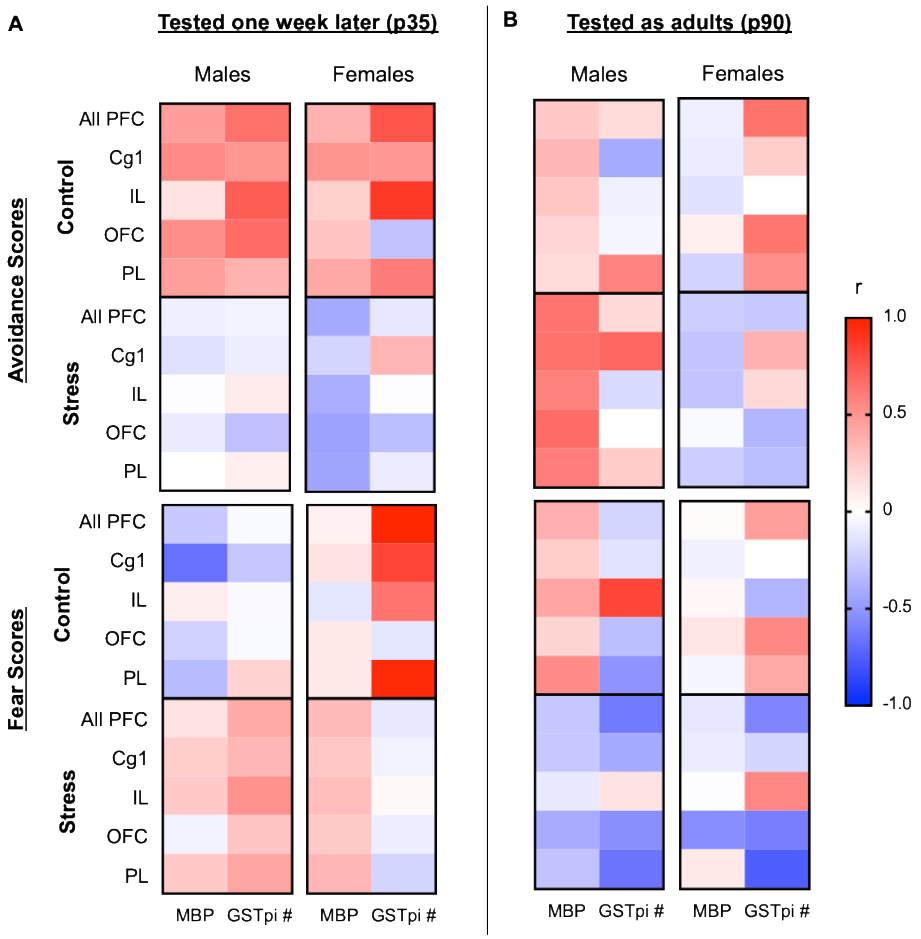


Figure 4-4. Correlations of PFC myelin and OLs with composite behavioral scores.
A) Correlations for animals tested one week later, as juveniles B) Correlations for animals tested as adults.

Discussion

Summary of findings

Changes in myelin and oligodendrocytes are beginning to be recognized as a novel mechanism that may contribute towards stress-induced pathologies. Here, we sought to fill a gap in the literature surrounding myelin and oligodendrocyte plasticity following early life trauma. In particular we focused on changes in the prefrontal cortex, a region that is highly plastic and sensitive to stress during the peri-adolescent period. Furthermore, we sought to address if PFC myelin and OLs relate to individual differences following trauma, and whether sex differences are observed.

In this study, we exposed male and female juvenile rats to an acute traumatic stressor. First, we confirmed that stress exposure increased serum corticosterone levels and reduced weight gain over time relative to controls, in both males and females. Next, we tested whether exposure to acute severe stress produced subsequent changes in behavior, with the expectation that some animals would be resilient while others would be vulnerable to developing persistent fear and anxiety-like behaviors. We demonstrated that in the short term, fear behavior was increased for

male but not female rats following stress exposure. Mean avoidance scores were also increased for both male and female rats, however this effect was not statistically significant at the group level. As expected, we saw a wide range of individual variability in behavior following stress. In the long term, stress-exposed and control animals of both sexes demonstrated similar avoidance and fear behavior, indicating that the effects of our specific stressor on behavior were limited to a shorter time frame. Most interestingly, stress exposure was associated with changes in prefrontal cortex myelin and oligodendrocytes in a sex specific manner. In females, increased myelination was observed one week following stress-exposure, yet decreased myelination was observed in the long-term. Decreases in prefrontal cortex oligodendrocytes were also seen in the short-term, yet there were no differences relative to controls in the long-term. Thus, a single stressor as a juvenile may provoke long-term changes in PFC myelin in a sex-specific manner. Lastly, we assessed whether these changes in myelination and OL cell count corresponded with behavioral measures. Contrary to our hypothesis, neither PFC myelin nor OLs corresponded with either avoidance or fear behaviors following stress exposure. Altogether, we conclude that experiencing acute severe stress as a juvenile may alter PFC myelination and OLs yet these changes are not associated with the development of subsequent avoidance or fear behavior.

Interpretation of Findings

Here, we found that exposure to acute traumatic stress as a juvenile did not lead to group-wide changes in avoidance or fear behavior when tested one week later. This finding was not entirely unexpected, as we anticipated to see variability in individuals' susceptibility to stress, as was previously observed in adult animals tested one week after the same stress paradigm (Long et al., *under review*). Indeed, we found that stress-exposed males and females had higher mean avoidance scores relative to controls, indicating that a subset of animals was susceptible to juvenile stress. Interestingly, males but not females, displayed greater fear behavior one week following trauma. Thus, juvenile females may be susceptible to changes in one domain of behavior (avoidance), while being simultaneously resilient in another (fear). This sex difference has been similarly observed in adults; adult females rats demonstrate resilience to this particular stressor, while males are more affected (Long et al., *under review*).

These changes in behavior were only observed in the short-term; we did not observe any mean changes in avoidance or fear behavior when tested as an adult, for either sex. This is in contrast to prior studies that have observed changes in behavior that persist into adulthood following juvenile trauma. For example, in one study, juvenile stressed animals displayed reduced exploration and poor avoidance learning when tested as adults (Tsoory & Richter-Levin, 2006). There are several possible explanations for why we did not observe significant behavioral changes in adulthood. First, prior studies of acute trauma have utilized a paradigm with three different stressors across three days. In contrast, our stressor was a single session of 3 hours of restraint stress combined with exposure to a predator scent. Three days of variable trauma, although still brief, may be more severe than the stressor we used here, and may explain why others have observed persistent behavioral changes yet we did not. Secondly, the exact behaviors that we assayed here were different from prior work on juvenile stress. Specifically, others have identified changes in avoidance learning using a two-way shuttle test; this assay also identifies increases in learned helplessness behavior following juvenile trauma (Tsoory & Richter-Levin, 2006). Here, we used more standard measures of avoidance such as the elevated plus maze and light-dark box assays. It is possible that were we to add additional behavioral tests, we may have observed similar

findings. In addition, it is possible that any changes in behavior as an adult would be un-masked following a stress challenge. In prior work, responses to a second stressor as an adult were altered in animals with a history of juvenile stress (Avital & Richter-Levin, 2005). The current study did not directly address whether stress-coping is altered and would be an interesting area for future investigation. Lastly, prior work of juvenile stress has primarily focused on male animals (Avital & Richter-Levin, 2005; Tsoory & Richter-Levin, 2006). Ours was one of the first studies to test for sex differences.

Most intriguingly, here we found that exposure to an acute traumatic stressor as a juvenile produces both short and long-term changes in prefrontal cortex myelin and oligodendrocyte cell density. This suggests that PFC myelin and oligodendrocytes are sensitive to acute stress during this critical time period of development. Further, we found these effects were primarily in females, suggesting sex-differences in stress-induced plasticity. Specifically, in the short-term, females displayed increased levels of MBP across the prefrontal cortex. This finding supports the possibility that stress drives early maturation of circuits (Bath, Manzano-Nieves, & Goodwill, 2016; Callaghan & Richardson, 2011; Gee, Gabard-Durnam, et al., 2013; Thomas, Caporale, Wu, & Wilbrecht, 2016). For example, early life stress due to fragmented maternal care is known to drive an earlier rise in MBP in the hippocampus (Bath et al., 2016). Alternatively, rather than driving early maturation to adult levels, stress may be altering developmental trajectories in the brain in a more transient manner (Thomas et al., 2020). To our knowledge, ours is the first study to examine changes in myelination in the PFC after a single, acute stressor during a critical developmental window. In the PFC, myelination acts to inhibit axonal sprouting and spine turnover, thereby acting as a brake on plasticity (R. D. Fields, 2008). Therefore, early myelination of PFC circuits, whether via stress or otherwise, could lead to detrimental functioning of the PFC at a time when plasticity is critical for behaviors such as cognitive flexibility and decision making (Thomas et al., 2016).

Although PFC myelination increased in females one week following stress, oligodendrocyte cell density decreased, suggesting stress may impair oligodendrocyte proliferation or survival in the PFC. Interestingly, when tested as adults, females had reductions in PFC myelination. Thus, the observed short-term decrease in oligodendrocytes might explain the longer-term decrease in MBP; less immature and mature oligodendrocytes at p35 might lead to fewer mature, myelinating oligodendrocytes by p90, as OLs undergo maturation over time. In our dataset, the same animals could not be examined at both time points (p35 and p90) due to the nature of our method, however it would be interesting for future studies to try to analyze myelin in vivo in a longitudinal manner, perhaps with MRI imaging. Similar to a longitudinal method, adding additional timepoints would add resolution to the dataset, allowing one to better map a curve of both GST-pi and MBP trajectories following stress.

A reduction in later PFC myelination is also in line with prior studies of juvenile stress. For example, in the chronic stress literature, social isolation stress as a juvenile (from p21 to p35) led to reductions in PFC myelination, alterations in OL morphology and changes in mPFC-mediated behaviors when tested at postnatal day 65 (Makinodan et al., 2012). Social isolation outside of this critical time window did not lead to such changes. Here, we also found no changes in adult PFC oligodendrocyte cell number following juvenile stress. This is in line with a number of studies with stressors both in adolescence and adulthood that demonstrate changes in PFC myelin levels but not oligodendrocytes (Lehmann et al., 2017; J. Liu et al., 2012; Makinodan et al., 2012). A key difference however is that in all of these prior studies, changes in PFC myelination were observed in male, but not female animals, while here we observe the opposite.

For example, one study found that social isolation (p30-p35) reduced MBP levels, quantified by western blots, in male but not female rats (Leussis & Andersen, 2008). Here, in contrast, in both the short and long-term, we found that the effects of traumatic stress on myelination and OLs was limited to females. Different stressor types (social isolation vs. physical restraint stress), the timing of stress (whether before or after puberty onset) or the method of MBP assessment (whether through immunohistochemistry or western blot) could all contribute to these observed dissimilarities. Further, the majority of studies focused only on male rodents, and there remains a great need for side by side comparison of male and female animals using the same paradigms.

Counter to our hypothesis, PFC myelin and oligodendrocytes did not correspond with avoidance behavior and fear learning following stress. One possibility is that there is truly no association of PFC myelin and OLs with avoidance and fear behaviors. Here, we did not see large changes in behavior following stress. Therefore, an additional possibility is that the altered PFC myelin observed is advantageous. Perhaps, for females, increased myelination in the short term is adaptive and contributed to their resilience in fear behavior relative to the males. Alternatively, PFC myelin and OLs may correlate with behaviors that we did not measure in the present study. For example, plasticity in the juvenile PFC is known to be associated with measures of cognitive flexibility, including rule learning and rule reversal (C. Johnson & Wilbrecht, 2011). Further, social interaction as well as depressive-like behaviors have been a focus of prior studies measuring stress-induced changes in PFC myelin (Birey et al., 2015; Lehmann et al., 2017; Leussis & Andersen, 2008; J. Liu et al., 2012, 2016). For example, loss of oligodendrocyte precursor cells (OPCs) in the PFC was sufficient to phenocopy depressive-like behaviors driven by chronic social stress (Birey et al., 2015). In addition, clemastine rescued PFC myelination deficits following chronic social defeat stress, and further rescued social interaction behaviors (J. Liu et al., 2016). Thus, it is possible that the effects of acute stress on PFC myelin that we observed here could correlate with social behavior or cognitive flexibility assays. Future studies should explore these possibilities and test a wider range of behavioral domains.

The prefrontal cortex is also a highly diverse brain region, with many subregions and local microcircuits across cortical layers (Dalley et al., 2004; B. Kolb, 1984). Although we looked at several specific subregions of the PFC each known to be involved in different aspects of behavior, we did not observe correlations with any given region. For example, we'd hypothesized that myelin and OLs in the infralimbic cortex might be more strongly correlated with fear extinction behavior, given prior literature (Do-Monte et al., 2015; Sierra-Mercado et al., 2011; Vidal-Gonzalez et al., 2006b). However, we did not look at specific layers of the PFC, nor could we distinguish myelin within a given circuit. Prior literature has also focused on only deep layers of cortex (layers 5+6) where there are primarily pyramidal projection neurons (K. D. Harris & Mrsic-Flogel, 2013; Makinodan et al., 2012). This is yet another difference between our study and others. Future work should aim to look at layer specific changes in the PFC, as well as try to identify changes in myelination within specific circuits (for example, IL projections to the amygdala).

Limitations and Future Directions

While many limitations of the current study have already been discussed, there are several additional important considerations. In the current study, the sample size for each group was low, with only 8 rats per group. In particular, we expected, and indeed see, variability in the response to acute traumatic stress. Due to this high variability, more animals would better allow us to take advantage of individual variation and to better assess the relationship of PFC myelin and

oligodendrocytes to behavior. With only 8 animals per condition, correlations should be interpreted with caution, and only as a starting point for further investigation.

In addition to more animals, the present study focused only on the prefrontal cortex. During the juvenile period, other brain regions that are critical for emotional responses, including the hippocampus and amygdala, are also undergoing plasticity (Spear, 2000). Thus, investigating changes in myelin and OLs in additional brain regions will be critical. In particular, previous work from our lab found that, in adults, immobilization stress redirected the developmental fate of neural progenitor cells in the hippocampus toward becoming oligodendrocytes (Chetty et al., 2014). More recently, our lab identified associations of hippocampal myelin with avoidance behavior following acute trauma (Long et al., *under review*). In addition to the hippocampus, the amygdala is a key region in the development of fear memories (Ledoux, 2000; Roozendaal et al., 2009), and our recent work identified a positive correlation of fear learning with amygdala myelin in adult rats (Long et al., *under review*). Furthermore, both of these regions are known to play a role in PTSD and should be investigated further using rodent models of acute stress (Fenster, Lebois, Ressler, & Suh, 2018; Shalev et al., 2017; L. M. Shin, Rauch, & Pitman, 2006; VanElzakker, Dahlgren, Davis, Dubois, & Shin, 2008).

The findings described here should also be considered within a broader circuit context. The prefrontal cortex has crucial connections to other limbic regions essential for emotional regulation, including to the amygdala and hippocampus (Ishikawa & Nakamura, 2003; Roozendaal et al., 2009). Myelination of axons corresponds with conduction velocity and synchronization across these brain regions (Monje, 2018; Pajevic & Basser, 2013). Thus, future studies should try to identify changes in myelination within a specific circuit, focusing on specific axonal projections between brain regions. Understanding how and if particular PFC axonal projections are preferentially myelinated following stress will further our knowledge of circuit plasticity and its connection to behavior. A recent study found that pharmacological stimulation of neurons led to increases in myelination in an axon specific manner. In addition, and relevant to the current study, they found that juveniles showed higher sensitivity to stimulation than adults (Mitew et al., 2018). This suggests that neural activity, whether driven by stress or otherwise, may drive circuit specific modulation of myelin. Lastly, a critical area for future study will be to go beyond correlation into causation, and to manipulate myelin and oligodendrocytes directly within a brain region or circuit in order to assess their functional contribution to stress pathology and behaviors.

Importance and Conclusions

The findings presented here have important implications for understanding stress-sensitive developmental periods. There is increasing evidence that stress during late childhood and early adolescence may confer vulnerability for developing psychiatric disorders later in life (C. P. Carr et al., 2013; Heim & Nemeroff, 2001; Nemeroff, 2004; Ventriglio et al., 2015). Exposing rats to peri-adolescent stress can be used to model the detrimental effects of childhood and early-adolescent trauma in humans (Tsoory, Cohen, & Richter-Levin, 2007; Tsoory & Richter-Levin, 2006). The majority of prior studies have tested chronic stressors during the peri-adolescent time period; however, many traumatic experiences are often acute in nature and can lead to long-lasting changes to the brain and behavior (Carrion & Wong, 2012; Nemeroff et al., 2006; Tsoory et al., 2007; Tsoory & Richter-Levin, 2006). Further, understanding acute trauma provides us detailed knowledge about vulnerable windows during development when the brain is most sensitive to stress. Many studies have suggested that peri-adolescence is a sensitive period of development in

which there is significant remodeling of limbic regions following stress, including in the prefrontal cortex (Spear, 2000). Our work here adds to the growing literature demonstrating that myelin and OLs are sensitive to stress early in life, providing an additional mechanism by which stress remodels the brain (Bath et al., 2016; Leussis & Andersen, 2008; Makinodan et al., 2012). Many psychiatric disorders, including schizophrenia, depression and PTSD, are characterized by alterations in PFC myelination (R. D. Fields, 2008; P. Lee & Fields, 2009; Regenold et al., 2007), however whether changes in myelin contributes to vulnerability to these disorders, or whether they are simply a biomarker remains to be determined. Understanding stress-induced plasticity of prefrontal cortex myelin and oligodendrocytes may contribute to our understanding of these psychiatric disorders, as well as the vulnerability to developing pathology following early life stress. Overall, findings in rodents could inform our knowledge of how traumatic stressors may impact human prefrontal cortex development and mental health. Ultimately, the goal is to boost resilience to stress and ensure normal, healthy development of the human brain.

Supplemental Materials

Primary Antibody Name	Primary Antibody Dilution	Secondary Antibody Name	Secondary Antibody Dilution
Rat anti-MBP	1:500	Cy3 donkey anti-rat	1:500
Rabbit anti-GSTpi	1:5000	AF488 donkey anti-rabbit	1:500

Table 4-S-1. Antibodies used for Immunohistochemistry.

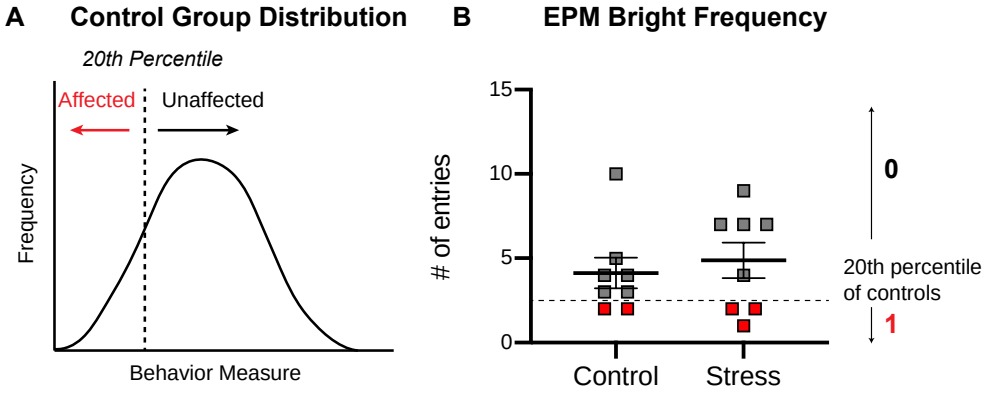


Figure 4-S-1. Composite Score for Avoidance and Fear Behavior.
A) Distribution demonstrating the 20th percentile cutoff criteria. For each behavioral measure (x axis), values are compared to the control group distribution. Values falling below the 20th percentile are categorized as "affected" and given a score of 1. Values above this threshold are considered "unaffected" and given a score of 0. **B)** An example of one avoidance measure, frequency of entries into the open arm, for one of the five avoidance assays, the EPM under bright conditions. Values falling below the 20th percentile of the control distribution are colored in red and are given a value of 1.

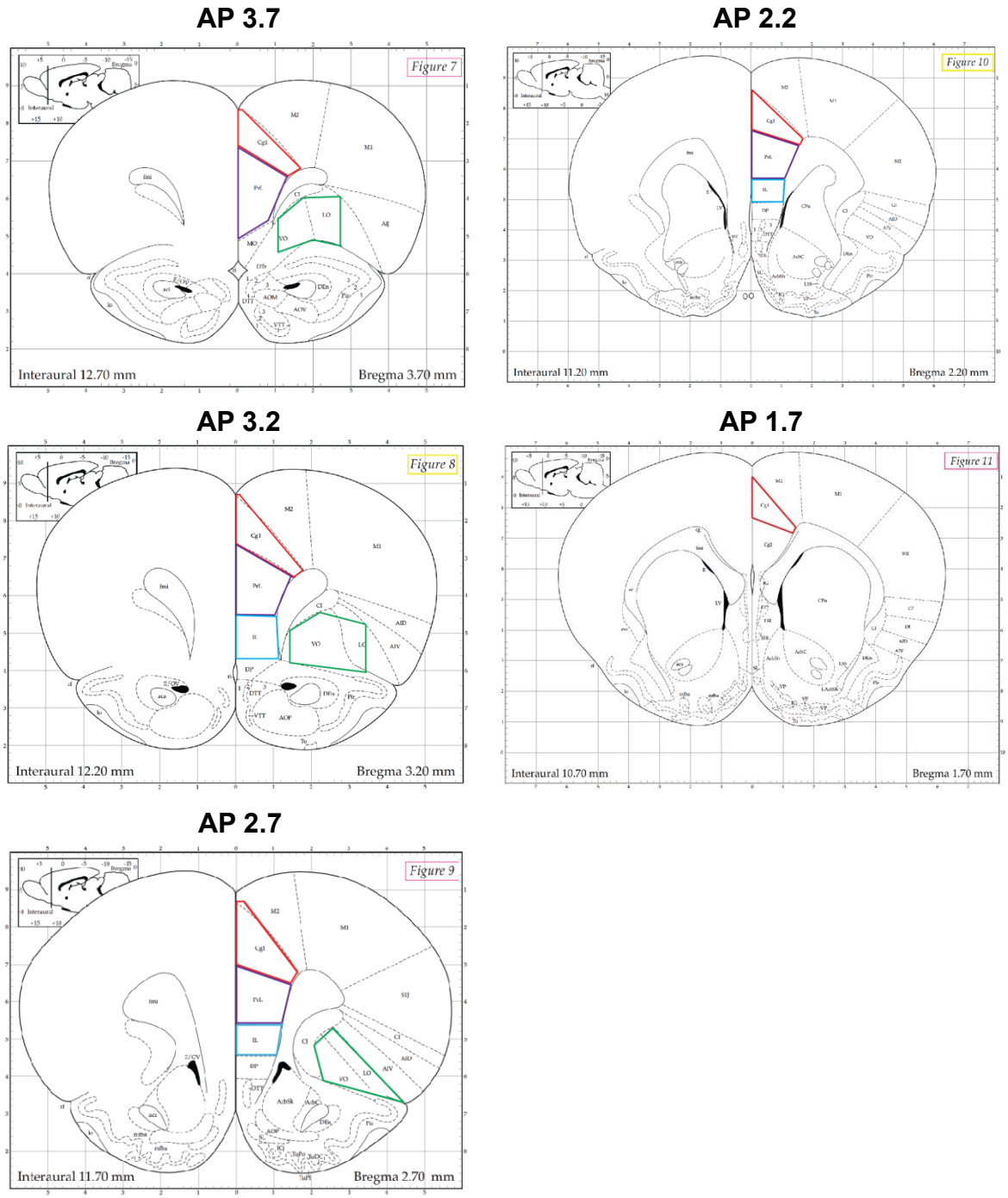


Figure 4-S-2. Atlas images of the five representative PFC regions used for immunohistochemistry. ROIs were hand drawn in ImageJ approximately as shown above. Red = Cg1, Purple = PrL, Blue = IL, Green = OFC. Coordinates are anterior-posterior from bregma (Paxinos & Watson. The Rat Brain Atlas. 1998).

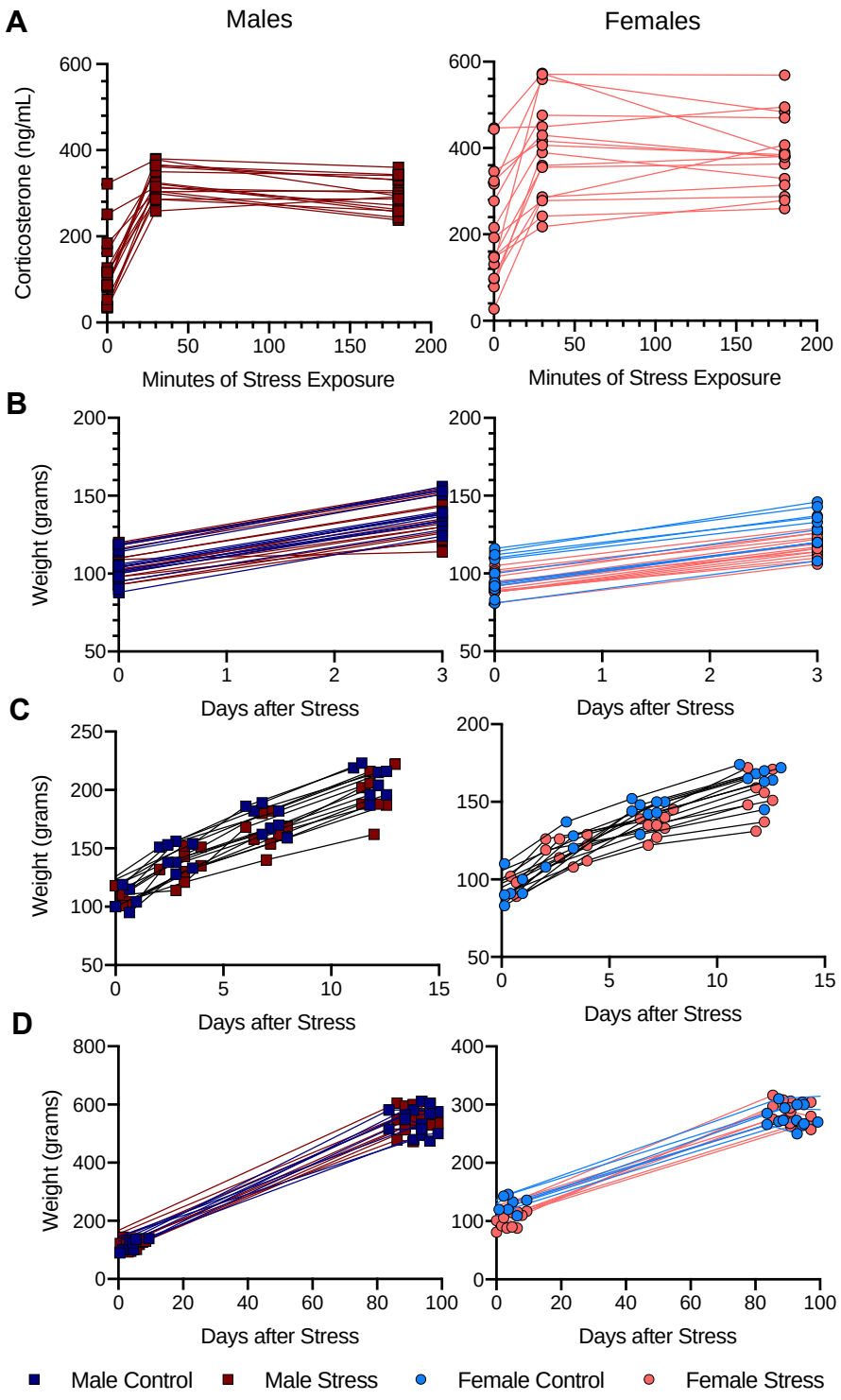


Figure 4-S-3. Individual changes in physiological responses to acute traumatic stress.
A) Corticosterone responses for individual male and female rats. **B)** Individual weight changes three days post stress for all 64 animals. **C)** Individual weight changes for the 32 male and female animals that were tested in behavior one week following acute severe stress. **D)** Individual weight changes for the 32 male and female animals that were tested in behavior as adults.

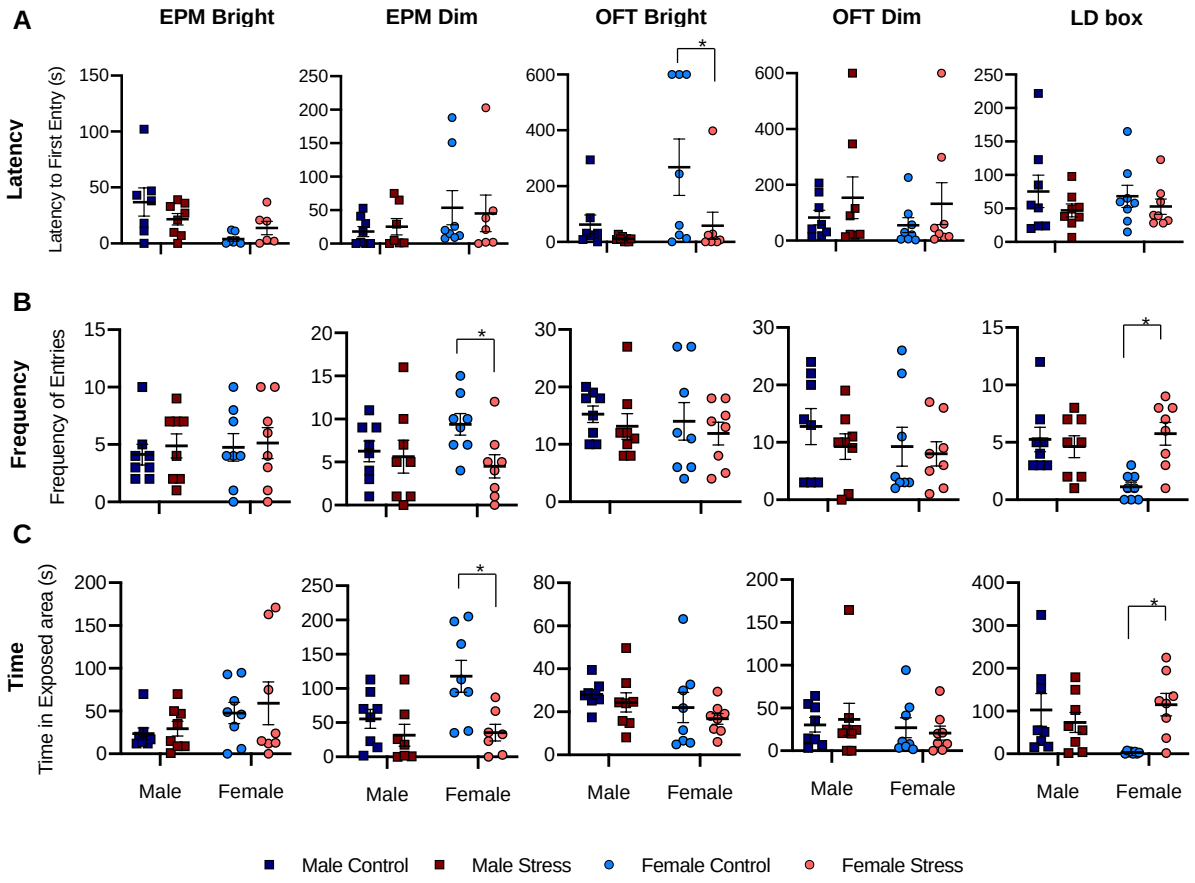


Figure 4-S-4. Approach-avoidance behaviors for animals tested one week prior to stress.
For each of the five tests, **A)** the latency to the first entry **B)** the frequency of entries and **C)** the time spent in the anxiogenic zone, was measured.

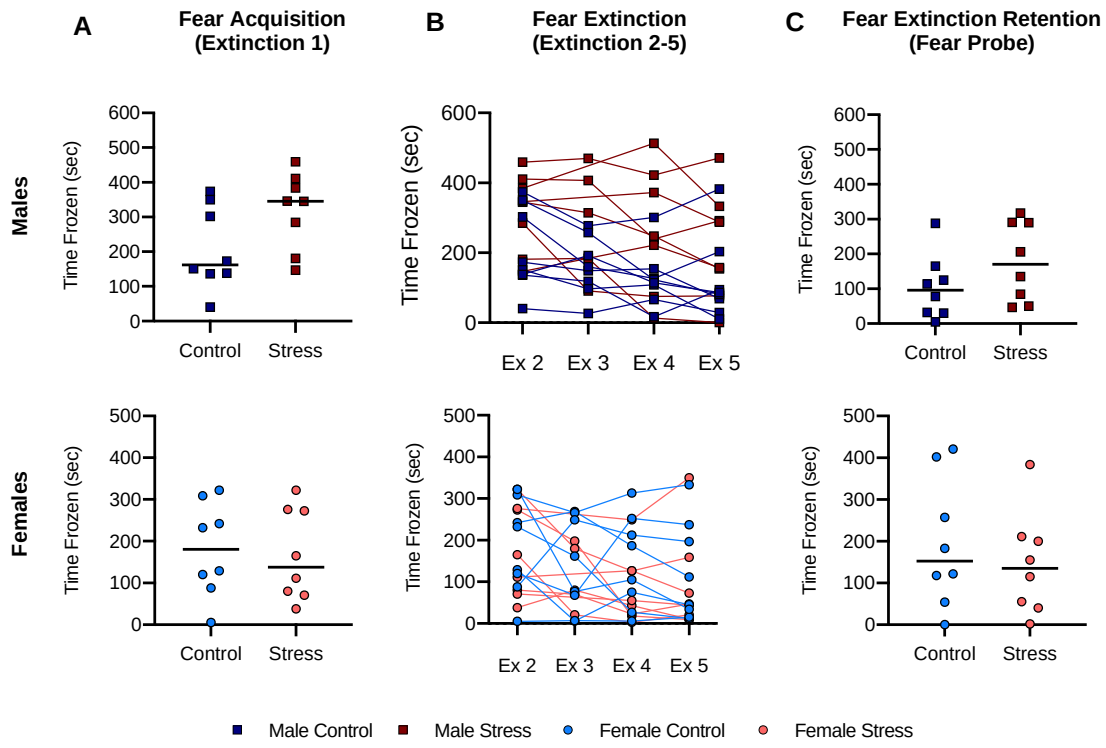


Figure 4-S-5. Fear behaviors for animals tested one week prior to stress.
A) Fear acquisition was measured by the time spent freezing on the first extinction trial. **B)** Fear extinction was measured as the time spent freezing across the four subsequent extinction trials. Individual differences are shown. **C)** Retention of fear extinction was measured on the next day as the time spent freezing on a single probe trial.

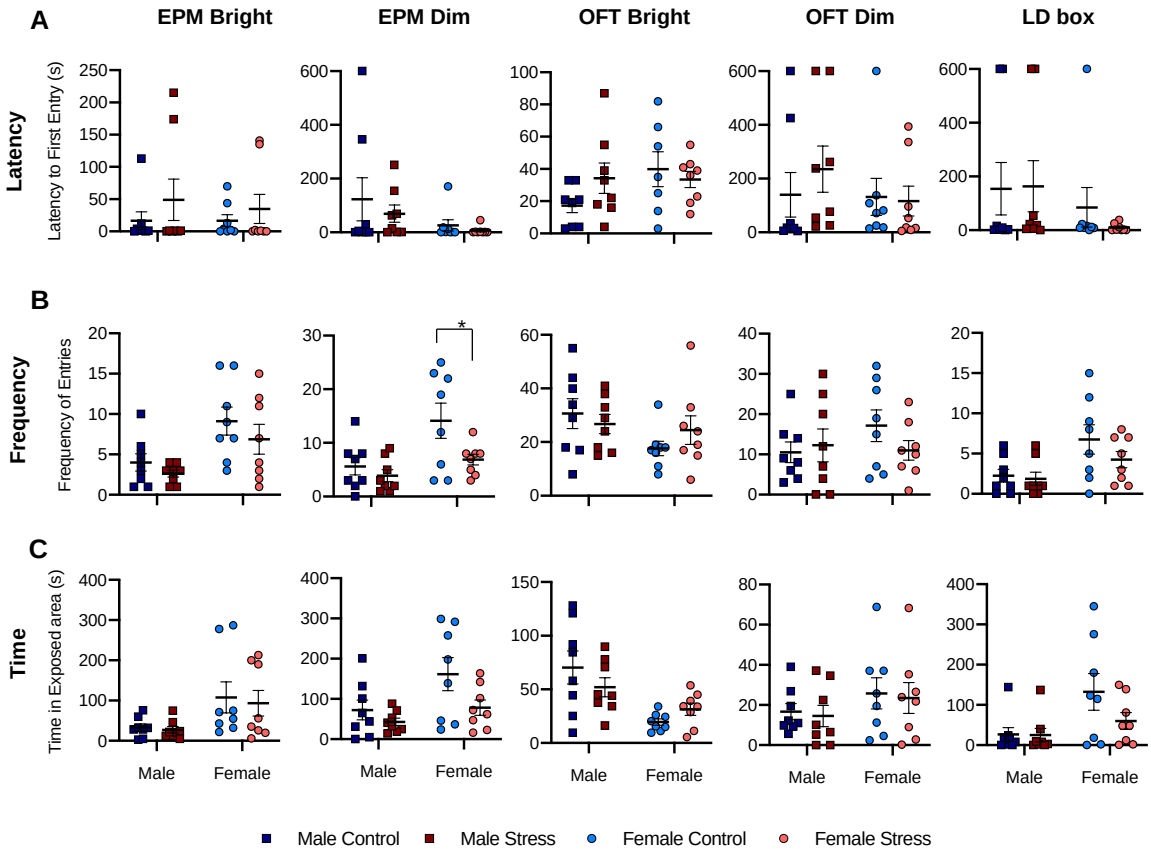


Figure 4-S-6. Approach-avoidance behaviors for animals tested as adults.
For each of the five tests, **A)** the latency to the first entry **B)** the frequency of entries and **C)** the time spent in the anxiogenic zone, was measured.

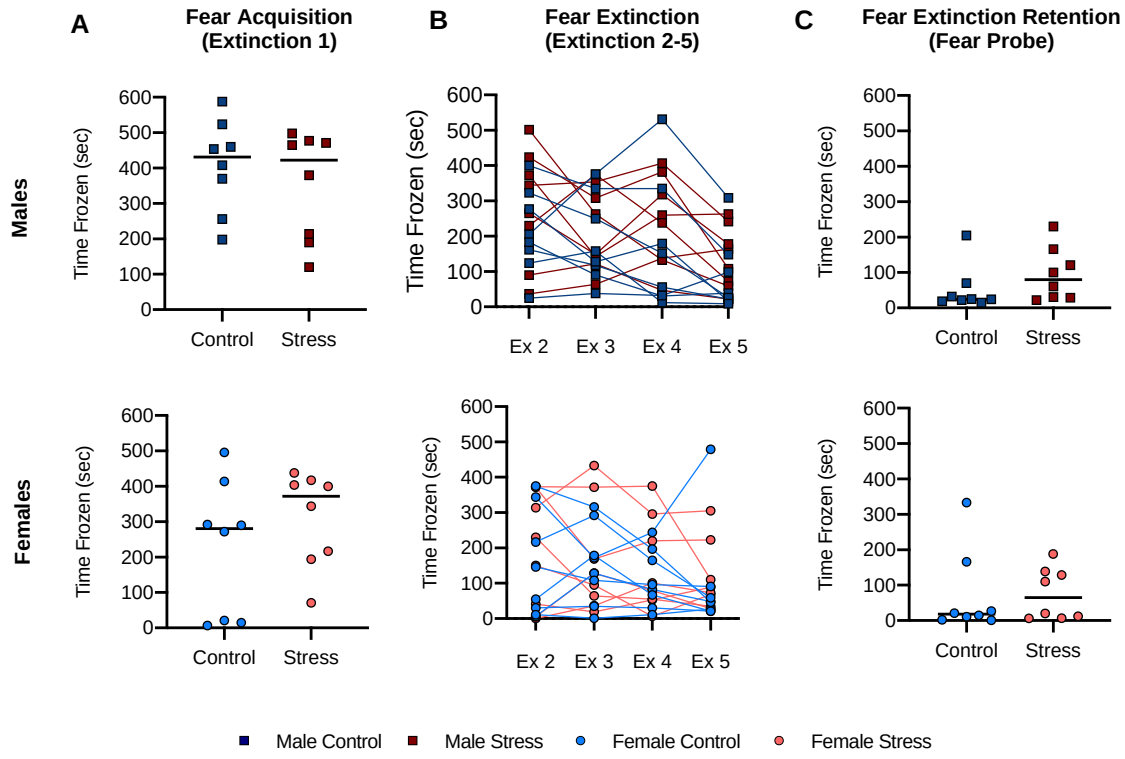


Figure 4-S-7. Fear behaviors for animals tested as adults.
A) Fear acquisition was measured by the time spent freezing on the first extinction trial. **B)** Fear extinction was measured as the time spent freezing across the four subsequent extinction trials. Individual differences are shown. **C)** Retention of fear extinction was measured on the next day as the time spent freezing on a single probe trial.

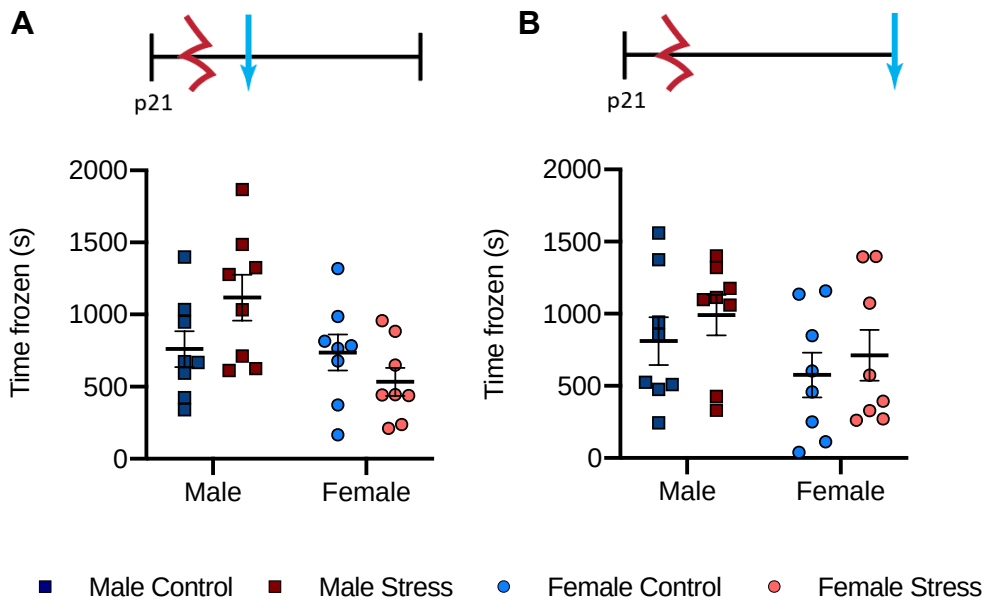


Figure 4-S-8. Area Under the Curve (AUC) of the time frozen across all extinction trials. **A)** AUC for animals tested one week later, and **B)** for those tested as adults

Chapter 5: Conclusions

The prefrontal cortex is a critical region of overlap between stress and motivation circuits in the brain. Both stress and reward modulate PFC activity and plasticity (Arnsten, 2015; Wallis & Kennerley, 2010); the PFC is especially sensitive to such experiences early in life as it continues to develop (Carrion & Wong, 2012; Tottenham & Galván, 2016). Importantly, the PFC receives and integrates input from regions involved in emotion and motivation such as the amygdala, hippocampus and VTA. Further, it sends projections back to these same regions as well as to the striatum in order to regulate stress and emotion and guide behavior (Passingham & Wise, 2012; S. P. Wise, 2008). Understanding prefrontal cortex circuits, how they change in response to stress, and ultimately how they contribute towards complex behaviors are all central questions in the field of neuroscience. This thesis aims to provide insight into these questions, contributing to the growing literature on stress and motivation.

Summary of Chapters

The VTA is a heterogeneous structure in the midbrain that is involved in both stress and reward (Howard L. Fields et al., 2007). In chapter two, we set out to systematically characterize and compare VTA dopaminergic and GABAergic projections to ten target nuclei, including to the anterior cingulate, prelimbic and infralimbic cortices. Overall, we found that VTA efferents are primarily *non*-dopaminergic. We also found that VTA cell bodies retrogradely labeled from the various target brain regions had distinct distribution patterns within the VTA. For example, PL-projecting neurons were more densely located in the ventral VTA, while a greater percentage of IL-projecting neurons were located in the more dorsal VTA. The distribution patterns and localizations of DAergic and GABAergic VTA neurons were also distinct between projections; however, each target region received inputs from neurons within every ipsilateral sampling region, indicating that VTA neurons in near proximity to one another can project to different targets. Together, these observations indicate an organized but intermixed structure to the VTA. Overall, this anatomical information will aid in both the interpretation and the guidance of behavioral studies, including those exploring VTA projections to prefrontal cortex subregions.

Stress comes in many shapes and forms. We are sensitive to direct personal stress as well as to the stress of others; both humans and animals experience vicarious stress upon observing another in distress (Batson, Fultz, & Schoenrade, 1987; Jeon et al., 2010; Meyza et al., 2017). Importantly, vicarious stress can be a motivator for prosocial behaviors (Eisenberg, 2003). Yet, pro-social behavior, in particular helping others in need, is biased towards members of the same group across species (Bartal et al., 2014; Cikara et al., 2011). In chapter three, we utilized a rodent model to explore the development and neural mechanisms that promote selective pro-sociality towards ingroup members. Specifically, we used a rat helping behavior test to explore the neural activity of ingroup bias for pro-social behavior in juvenile and adult rats. We found that adult rats helped ingroup, but not outgroup members, by releasing them from a restrainer. In contrast, juvenile rats helped both ingroup and outgroup members, demonstrating no group biases. Brain-wide neural activity, indexed by expression of the early-immediate gene c-Fos, identified one neural pattern associated with group identity, and another pattern associated with age. Interestingly, c-Fos activity was observed in the prelimbic and orbitofrontal cortices in all

conditions regardless of helping, evidence that these regions participate in processing the distress of others but are not necessarily predictive of pro-social behavior. In contrast, other brain regions, including the reward system, were distinctly activated only in the ingroup conditions. In particular, the nucleus accumbens emerged as a central region for the ingroup condition. *In vivo* calcium imaging in adults further revealed increased Nac population activity for ingroup, but not outgroup members. Lastly, retrograde tracing identified activation of a sub-population of cells projecting from the anterior cingulate cortex to the Nac that correlated with helping. Together, these findings demonstrate that biases for pro-social behavior develop with age and that pro-social intent toward ingroup members recruits distinct neural circuits. This research adds to a growing literature exploring how vicarious stress can lead to motivated prosocial behaviors.

Stress, especially stress early in life, can have lasting impacts on neural development and behavior. In particular, during peri-adolescence, the prefrontal cortex is still developing and is highly sensitive to stress (Eiland & Romeo, 2013). In chapter four, I examined the effects of an acute traumatic stressor during peri-adolescence on maturation of the PFC. I focused on stress effects on oligodendrocytes and myelination, a topic that is beginning to be explored as a novel and underappreciated mechanism contributing towards stress pathology (Gibson, Geraghty, & Monje, 2018). Using a rodent model, I found that juvenile acute traumatic stress increased myelination in PFC regions one week later in female but not male rats. In the long-term however, stress-exposed females showed reduced PFC myelination, while male animals had no change, indicating that stress may also have long-lasting effects on PFC development. This suggests that a single stressor as a juvenile may provoke alteration of PFC myelination in a sex-specific manner. Counter to our expectations, PFC oligodendrocytes and myelin were not associated with avoidance and fear behaviors following stress. Thus, the functional implication of these stress-induced changes remains to be determined.

Future Directions

The prefrontal cortex does not function on its own to affect stress and motivation-related behaviors; rather, it sits within a broader circuit, with connections to the VTA (as described in chapter 2) as well as to other crucial limbic regions such as the amygdala and hippocampus (Ishikawa & Nakamura, 2003; Roozendaal et al., 2009). Future research should therefore focus on pathway specific effects. For example, in chapter 3 we describe a specific projection from the anterior cingulate cortex to the nucleus accumbens that may play a role in helping behavior. Future work can expand upon this, testing for changes in plasticity within particular pathways, or examining physiological changes within the circuit during prosocial behavior. Effects of stress can also be examined at the pathway level (Lowery-Gionta et al., 2018; Rocher, Spedding, Munoz, & Jay, 2004). For instance, rather than looking at changes within a whole brain region as was done in chapter 4, future work can look at changes in myelination within a particular projection. Understanding how and if particular PFC axonal projections are preferentially myelinated following stress will further our knowledge of circuit plasticity and its connection to behavior.

In addition, an exciting avenue will be to use newly developed tools to interrogate the causal role of PFC circuits and specific projections in stress and motivated behaviors (Boyden, 2015; Lammel, Tye, & Warden, 2014; Roth, 2016). For example, optogenetics or DREADDS could be used to manipulate the subpopulation of Nac-projecting ACC neurons in order to test their functional role in helping behavior. Furthermore, the field is moving beyond manipulation of neurons and is expanding to include manipulations of glia, including oligodendrocytes (Birey et

al., 2015; J. Liu et al., 2016). For example, two recent studies found that arresting oligodendrogenesis impaired fear consolidation, while induction of new myelin formation improved fear memory, suggesting that new oligodendrocytes and the myelin they produce have a causal role in fear behavior (R. D. Fields & Bukalo, 2020; Pan, Mayoral, Choi, Chan, & Kheirbek, 2020; Steadman et al., 2020). The manipulation of neurons and glia within a brain region or circuit will allow us to assess their functional contribution to stress pathology and behaviors. Overall, these are just a few of the many exciting directions for future work.

Implications for human disorders

The work presented in this thesis has important implications for understanding psychiatric disorders, including disorders such as PTSD, social and empathic disorders, and substance use disorders. Importantly, experiencing stress early in life is associated with vulnerability for developing such psychiatric disorders (C. P. Carr et al., 2013; Heim & Nemeroff, 2001; Nemeroff, 2004; Sinha, 2008; Ventriglio et al., 2015). Furthermore, there is high co-morbidity between disorders such as PTSD and substances use disorders (R C Kessler et al., 1996), suggestive of an overlapping mechanism of vulnerability. Importantly, the PFC is a likely candidate, as PFC dysfunction is common in all of these disorders (Duval et al., 2015; Goldstein & Volkow, 2011; L. M. Shin & Liberzon, 2010; Sugranyes, Kyriakopoulos, Corrigall, Taylor, & Frangou, 2011). Furthermore, PFC dysfunction may be initiated or exacerbated by stress (Arnsten, 2015). For example, stress exposure weakens PFC driven self-control, which contributes to substance use (Sinha & Li, 2007). Rodent studies, such as those described here, allow us to explore PFC circuitry and stress effects in a more detailed way. For example, we can characterize anatomical projections of the mesocortical pathway (chapter 2). These mesocortical projections are activated by stress, and indeed both dopaminergic and non-dopaminergic projections from the VTA to the PFC have implications for psychiatric disorders (Moghaddam, 2002). Furthermore, behavioral studies in rodents allow us to analyze the neural circuitry of pro-social behaviors (chapter 3). Understanding these neural circuits provides insight into human disorders such as autism spectrum disorder and schizophrenia, where there are disruptions in social motivation (Blanchard, Park, Catalano, & Bennett, 2015; Chevallier et al., 2012; Dubey, Ropar, & de C Hamilton, 2015; Fervaha, Foussias, Agid, & Remington, 2015). Lastly, rodents can be used to model disorders such as PTSD (chapter 4) (Schöner, Heinz, Endres, Gertz, & Kronenberg, 2017), allowing us to manipulate the timing of stress exposure and better understand PFC alterations following trauma. Overall, the work presented in this thesis will add to the growing literature linking stress and motivation within PFC circuits and will help inform our understanding of human disorders and mental health.

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