ABSTRACT

Title of Dissertation: SEX DIFFERENCES IN THE FOREBRAIN DOPAMINERGIC CIRCUIT

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Several psychiatric disorders exhibit different incidence rates in men and women and are associated with dysfunctions in forebrain dopaminergic circuits. Although anatomical and functional sex differences in the brain have been studied, little is known about sex differences in the forebrain dopaminergic circuits associated with behavioral dysfunction. We hypothesized that known sex differences in forebrain dopamine circuit-associated behaviors would be the result of sex differences in forebrain dopamine circuit anatomy. As a first step to address this hypothesis, we combined a mouse transgenic driver line (tyrosine hydroxylase promoter-driven Cre recombinase) with virally encoded fluorescent reporters (FLEX-tdTomato and SynaptophysinGFP) to compare the density of midbrain dopaminergic axon projections and terminal boutons in dopamine projection target regions. Using this technique, we analyzed projections from the ventral tegmental area (VTA) to prefrontal cortex and basolateral amygdala (BLA) in male and female adult mice. Multiple analyses at 10x and 25x magnification revealed higher bouton density in BLA in males compared to females. To determine if this anatomical difference is mediated by gonadal steroid hormones, subjects were treated with a drug used to reduce gonadal steroid hormone production in clinical populations, leuprolide acetate (Lupron), before anatomical measures. Leuprolide administration resulted in a reduction in circulating

testosterone, but did not show an effect on dopamine circuit anatomy. The finding of an anatomical sex difference in the forebrain dopamine circuit provides a structural foundation for further investigation of how sex differences in brain circuits may underlie behavioral dysfunction that play roles in psychiatric illnesses.

SEX DIFFERENCES IN THE FOREBRAIN DOPAMINERGIC CIRCUIT

by

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Chapter 1: Background

Sex differences in behavior and psychiatric illness risk

Even before the advent of functional imaging studies, sex differences in active avoidance learning and extinction suggested the presence of differences in male and female brains (Beatty & Beatty, 1971; Denti & Epstein, 1972). More recent studies have shown these behavioral sex differences to extend more broadly into performance in social cognition tasks, responses to aversive and rewarding stimuli, and sensorimotor performance (Ding et al., 2017; Kumari, 2011; Tunç et al., 2016). These behavioral sex differences often correlate with sex-dependent functional activity differences detectable using non-invasive imaging techniques like functional magnetic resonance imaging (fMRI) and electroencephalography (EEG) (Ding et al., 2017; Sacher, Neumann, Okon-Singer, Gotowiec, & Villringer, 2013).

The expression of many psychiatric illnesses also differs between males and females; both the onset and phenotype of the same disease can be different between sexes (reviewed in McCarthy, Nugent, & Lenz, 2017). Major depression and anxiety disorders – including generalized anxiety, eating disorders, post-traumatic stress disorder, and obsessive-compulsive disorder – all occur more frequently in females (Alonso et al., 2004; Bogetto, Venturello, Albert, Maina, & Ravizza, 1999; Breslau, Davis, Andreski, Peterson, & Schultz, 1997; Fenton, Halliday, Mason, Bredy, & Stevenson, 2016; McCarthy et al., 2017; C. P. McLean, Asnaani, Litz, & Hofmann, 2011; Woodside et al., 2001). In contrast, men show an increased incidence of schizophrenia, and affected men and women typically present different symptoms (Aleman, Kahn, & Selten, 2003; Castle & Murray, 1991; Goldstein, 2006; McCarthy et al., 2017). Men with schizophrenia often display increased cognitive and language deficits, as well as increased

positive symptoms (e.g., hallucinations), while women more commonly show flattened affect (Aleman et al., 2003; Castle & Murray, 1991; Goldstein & Link, 1988; Goldstein, Tsuang, & Faraone, 1989; McCarthy et al., 2017). In addition, men are more likely to experience substance abuse (Becker & Hu, 2008; Lynch, Roth, & Carroll, 2002). It has also been shown that despite the lower number of women that abuse drugs, women are more likely to show a rapid progression through the stages of drug addiction (Becker & Hu, 2008; Lynch et al., 2002). The biological explanations for sex differences in psychiatric illness, especially in schizophrenia, are largely unknown, although mechanisms have been proposed ranging from chromosomal differences to differences in expression of gonadal steroid hormones (Walder et al., 2006).

Molecular genetic basis of sex differences

Levels of sexual differentiation

Sexual differentiation begins at the chromosomal level, with the presence or absence of the Y-chromosome, which contains the *Sry* gene (written *SRY* in humans) (Arnold, 2017; Jost, Vigier, Prepin, & Perchellet, 1973; A. H. Sinclair et al., 1990). *Sry* encodes for testis-determining factor (TDF), which causes the primordial gonadal tissue to develop into testes, whereas in the absence of TDF, the primordial gonad will develop into ovaries (Beach, 1971; Jost et al., 1973; A. H. Sinclair et al., 1990). While sex differences can be caused by genes present on the Y-chromosome, they are more often caused by differences in hormone expression (Dewing et al., 2006). Ovaries and testes produce gonadal steroid hormones, which act on body tissue to sexually differentiate the tissue. This is referred to as the organization action of hormones (Arnold, Wade, Grisham, Jacobs, & Campagnonih, 1996; Beach, 1971; Phoenix, Goy, Gerall, & Young, 1959). The differentiated tissue is then acted upon by circulating hormones, in what is referred to as the activating action (Arnold et al., 1996; Phoenix et al., 1959). Activation of

structures by gonadal steroid hormones is important for a number of critical behaviors, especially those related to reproduction (Beach, 1976).

The timing of tissue exposure to gonadal steroid hormones is important for both organization and activation. Variation in timing of exposure can cause sexual differentiation to diverge from the described pathway at any stage of differentiation, resulting in male or female phenotypes that do not match the expected phenotype for earlier stages (Arnold et al., 1996; McCarthy, Arnold, Ball, Blaustein, & De Vries, 2012; Phoenix et al., 1959; Wade & Arnold, 1996). For example, a person with XY chromosomes may have complete androgen sensitivity as a result of lack of androgen receptors (AR) in body tissue, and thus would have *SRY* and testes, but gonadal hormones would not act on tissue to produce a male phenotype, resulting in a phenotypical female (Hamann et al., 2014). It is also possible to have more or fewer than two sex chromosomes, which often results in differences in sexual differentiation (Ford, Jones, Polani, De Almeida, & Briggs, 1959; Smyth & Bremner, 1998).

Regulation of gonadal hormones by the hypothalamic-pituitary-gonadal axis

Gonadal steroid hormone production is mediated by the hypothalamic-pituitary-gonadal (HPG) axis. Gonadotropin releasing hormones (GNRH) are released from the hypothalamus in a pulsatile manner, and act on the anterior pituitary gland (Perrett & McArdle, 2013; Wilson, Vadakkadath Meethal, Bowen, & Atwood, 2007). The anterior pituitary gland then produces and secretes gonadotropins – luteinizing hormone (LH) and follicle-stimulating hormone (FSH) – which stimulate the gonads to release gonadal steroid hormones (Wilson et al., 2007).

GNRH pulses typically increase gonadotropin release, and subsequently stimulate gonadal steroid hormone release, whereas sustained GNRH releases decrease the response of

GNRH receptors, resulting in an inhibition of gonadotropin release and a downregulation of hormone release by the gonads (Marshall et al., 1991; Terasawa, 1995; Wilson et al., 2007)

Gonadal hormone regulation of sex differences

Gonadal steroid hormones are a class of hormones sharing cholesterol as a precursor, and are produced through a series of enzymatic steps (reviewed in McHenry, Carrier, Hull, & Kabbaj, 2014; Miller, 2013). Androgens include testosterone (T), as well as a reduced form of testosterone, dihydrotestosterone (DHT) – which has stronger androgen effects – and other further reduced androgens (Celotti, Melcangi, Negri-Cesi, & Poletti, 1991; McHenry et al., 2014; W. L. Miller, 2013). Testosterone is also a precursor to estradiol (E2), an important estrogen, and is converted to E2 by aromatase (McHenry et al., 2014; W. L. Miller, 2013). The activity of each gonadal steroid hormone is dependent on the presence of specific receptors for each hormone in the target tissue, namely the AR or estrogen receptors alpha (ERα) or beta (ERβ) (Ramirez & Zheng, 1996). Gonadal steroid hormones can have both slow genomic effects on intracellular nuclear receptors or more rapid effects via membrane-bound receptors, such as G-protein coupled estrogen receptors (GPER) (Cato, Nestl, & Mink, 2002; Lenz, Nugent, & McCarthy, 2012; Macêdo et al., 2020).

The effect of gonadal steroid hormones on brain development

Over the course of development, gonadal steroid hormone concentrations fluctuate at different rates between males and females (Hill, Wu, Kwek, & Van den Buuse, 2012). Prenatal hormone levels are regulated by the presence of alpha-fetoprotein, which binds estrogens from the mother and prevents them from reaching the fetal brain (McEwen, Plapinger, Chaptal, Gerlach, & Wallach, 1975). During this time, T reaches the brain in males and is then aromatized

into E2, while females do not have high enough levels of androgens to convert to E2 in the brain (Lieberburg & McEwen, 1975). This sensitive prenatal period is when neural circuits are masculinized and defeminized by E2 in males or feminized and demasculinized in the absence of steroid hormones in females (Arnold, 2009; Beach, 1971; Phoenix et al., 1959). These organizing effects are stable over development and lay the foundation for adolescent activational changes – temporary effects that occur in response to gonadal steroid action on established circuits (Beach, 1971; McCarthy et al., 2012; Phoenix et al., 1959).

Hormonal increases during adolescence are largely the result of maturation of the gonads during puberty (Arnold, 2009; Hill et al., 2012). Global serum T levels show a sharp increase in males between 6 and 10 postnatal weeks in mice, while serum E2 levels in females show a gradual increase over the same developmental period (Hill et al., 2012). The dramatic prenatal changes in hormone levels have been observed in specific brain regions, while the adolescent effects are weaker in the brain, but co-occur with global circulating hormone changes (Hill et al., 2012; Konkle & McCarthy, 2011). These changes occur to different degrees between males and females, and show different patterns between E2 and T, but are not a direct reflection of global gonadal steroid hormone changes, as evidenced by their persistence after gonadectomy (Konkle & McCarthy, 2011; Lenz et al., 2012; D. Sinclair, Purves-Tyson, Allen, & Weickert, 2014).

It is also during the adolescent period when both males and females show an increase in psychiatric illness onset, namely schizophrenia in males or anxiety in females (Ernst, 2014; Ernst, Pine, & Hardin, 2005; C. P. McLean et al., 2011; D. Sinclair et al., 2014). While hormone activational changes coincide with the period when psychiatric illnesses onset most frequently, this is not necessarily a guarantee that a hormonal cause of psychiatric illness would be an

activational effect. If early organization occurs atypically, the activational effect of hormones during adolescence could unmask a previously existing organizational defect.

Deviations from the typical range of gonadal steroid hormones are often associated with increased psychiatric risk. Men with decreased T levels are more likely to develop depression than males with typical T levels (Westley, Amdur, & Irwig, 2015). Meta-analysis of studies that gave T treatment to men with depression showed a reduction in depressive symptoms in hypogonadal men given T supplement (Amanatkar, Chibnall, Seo, Manepalli, & Grossberg, 2014). T treatment has also been shown to reduce measures of stress in both male and female animals (Frye & Walf, 2009; Wainwright et al., 2016). The response to treatment also varies between sexes, as tricyclic antidepressants tend to be more effective in males, while selective serotonin reuptake inhibitors (SSRIs) work better in females (Baca, Garcia-Garcia, & Porras-Chavarino, 2004). The mechanisms by which gonadal steroid hormones affect sex differences in psychiatric illness are largely unknown.

Anatomical basis of sex differences in the adult brain

Classically, sex differences in the brain are reported in neural regions that are strongly associated with reproduction and sex-specific behaviors (Gorski, Gordon, Shryne, & Southam, 1978; Wade & Arnold, 1996). The sexually dimorphic nucleus of the medial preoptic area of the hypothalamus (SDN-MPOA), which has a larger volume in males and plays a large role in copulatory behavior, has been a major focus of research on sexual dimorphisms in the brain (de Vries & Södersten, 2009; Gorski et al., 1978; Raisman & Field, 1971, 1973; Shah et al., 2004). In addition to the gross volume difference in the MPOA, cellular structure differences in the MPOA can be found between males and females. For example, males have more synapses onto

dendritic shafts, while females have more synapses onto dendritic spines (Raisman & Field, 1973).

The sex differences in MPOA appear to result from gonadal steroid effects on dopamine in the MPOA (Dominguez & Hull, 2005; Kleitz-Nelson, Dominguez, & Ball, 2010). In males, MPOA dopamine increases in response to presentation of a sexually receptive female, and copulation decreases when MPOA dopamine is blocked, suggesting that dopamine in the MPOA is responsible for male sexual motivation (Dominguez & Hull, 2005; Kleitz-Nelson et al., 2010). This idea is supported by the suggestion that projections to MPOA originate in the VTA or midbrain A14 dopaminergic cells (Dominguez & Hull, 2005; S. M. Miller & Lonstein, 2009).

Another hypothalamic area where sex differences have been studied extensively is the anteroventral periventricular nucleus (AVPV). While the MPOA is larger in males and is known to play a role in male sexual behavior, the AVPV is larger in females and is associated with maternal parenting behavior (Waters & Simerly, 2009). Of particular importance, this difference has been noted in a subset of AVPV cells expressing dopamine (Waters & Simerly, 2009). Ablation of this population of cells results in a decrease in maternal behavior, while stimulation results in increased maternal behavior and an increase in circulating oxytocin (Scott, Prigge, Yizhar, & Kimchi, 2015). Uniquely, this region has also been shown to exhibit increases in cell number during adolescence, suggesting an additional possibility for hormonal influence (Ahmed et al., 2009).

While estrogen and androgen receptor distributions overlap in some areas, like the MPOA, they show dramatically different concentrations in other areas between males and females (Simerly, Swanson, Chang, & Muramatsu, 1990). It has been suggested that sex differences in gonadal steroid hormone receptor distributions in the brain may play a larger role

in behavioral differences than differences in global circulating hormone levels. It is known that local synthesis of E2 in the brain occurs relative to the amount of local aromatase, and aromatase distribution has been shown to be different between males and females (Azcoitia, Yague, & Garcia-Segura, 2011; Konkle & McCarthy, 2011; Lenz et al., 2012). Interestingly, this pattern of gonadal steroid hormone distribution in the brain is not affected by gonadectomy in males or females, suggesting this profile is not directly linked to global circulating hormones, but dependent on a difference in local E2 synthesis (Azcoitia et al., 2011). Differences between male and female adult brain structures have also been recognized in several other regions, including the hippocampus, frontal cortex, and amygdala, and these have been observed across anatomical, molecular, and physiological measures (Gould, Westlind-Danielsson, Frankfurt, & McEwen, 1990; Johnson, Breedlove, & Jordan, 2008; Kolb & Stewart, 1991). These differences usually appear as a result of prenatal organization, but can also emerge postnatally (Beach, 1971; Bowers, Waddell, & McCarthy, 2010; de Vries & Södersten, 2009; Nuñez & McCarthy, 2007). Early sex differences include rate of neurogenesis and dendritic growth, while later-emerging differences appear in activational state of specific circuits and cellular subpopulations (Ahmed et al., 2009; Bowers et al., 2010; Nuñez & McCarthy, 2007). These later differences occur in regions that do not play a role in reproductive behavior, and often occur in regions associated with psychiatric illness risk, demonstrating the broad importance of studying the neurobiological differences that exist in them between males and females and how hormones influence these regions.

The dopaminergic system

Dopamine in behavior and psychiatric illnesses

Dopamine is an important neurotransmitter in motivated behaviors, including decision-making, goal-directed movement, and both appetitive and aversive response (Campi, Greenberg, Kapoor, Ziegler, & Trainor, 2014; J. Kim, Zhang, Muralidhar, LeBlanc, Sarah A., & Tonegawa, 2017; Lammel, Lim, & Malenka, 2014; Vitrac et al., 2014) . Due to the varied nature of dopamine function, its mechanistic role in psychiatric illness is still not completely understood, although its relevance in illnesses like schizophrenia has been well studied and documented (O. D. Howes et al., 2012; O. Howes, McCutcheon, & Stone, 2015; Purves-Tyson et al., 2014). Dopamine cannot be classified as having exclusively an excitatory or inhibitory role on postsynaptic target potential, as its effects vary by brain location, distribution of dopamine receptors in the postsynaptic cell, and concentration of dopamine (Nicola, Surmeier, & Malenka, 2000). For example, presence of D1 and D2 dopamine receptor subtypes in the caudate nucleus results in an inhibitory response at high dopamine concentrations and an excitatory response at low concentrations (Akaike, Ohno, Sasa, & Takaori, 1987).

Reward-seeking, harm-avoidance, and top-down behavioral regulation are all known to be dopamine-regulated behaviors (Wahlstrom, Collins, White, & Luciana, 2010). It is also known that regions associated with these behaviors – specifically nucleus accumbens (NAC), basolateral amygdala (BLA), and prefrontal cortex (PFC), respectively – are part of the same forebrain dopaminergic circuit, with all three receiving projections from the midbrain ventral tegmental area (VTA) (Wahlstrom et al., 2010).

It is known that a dramatic increase or decrease from baseline dopamine levels in the frontal cortex leads to cognitive deficits. Therefore, if the frontal cortex deviates from its typical balance in either direction, other forebrain regions that receive inhibitory regulation from the PFC, such as the NAC and BLA, could remain unbalanced (Arnsten, Cai, Murphy, & Goldman-

Rakic, 1994; Murphy, Arnsten, Goldman-Rakic, & Roth, 1996). This has been seen in positron emission tomography (PET) studies showing increased striatal dopamine uptake in patients with schizophrenia, which can be predicted by decreased PFC activation (O. Howes et al., 2015; Meyer-Lindenberg et al., 2002). Within a single illness phenotype like schizophrenia, there may be areas with dopamine hyperfunction and other areas with dopamine hypofunction.

In addition to dopamine's diverse roles in different brain regions, the areas that receive dopaminergic innervation from the VTA also show heterogeneity in function within subregions or subpopulations of cells. While NAC is primarily associated with reward-seeking, the degree of effect on reward-seeking is unevenly distributed between the nucleus accumbens core and shell. The medial accumbens shell receives stronger inputs from the posteromedial region of the VTA compared to the anterolateral VTA, and plays a stronger role than the core in reward-seeking and hippocampally-mediated learning (Ikemoto, 2007; Ito & Hayen, 2011). Accumbens core lesions typically show weaker associations with reward and play a stronger role in amygdala-mediated learning (Ikemoto, 2007; Ito & Hayen, 2011). The amygdala also shows heterogeneity, both between and within amygdala subregions. The amygdala is divided into several functionally distinct regions, including the medial amygdala (MEA), central amygdala (CEA), and BLA. While all are associated with emotion to a degree, the MEA is known to be critical for relaying olfactory cues in motivated sexual behavior, while CEA and BLA are more strongly associated with fear-learning and harm-avoidance (Beyeler et al., 2016; Gresham et al., 2016; Keshavarzi, Sullivan, Ianno, & Sah, 2014). Of the latter two, BLA is considered to play a role in association and cue-related learning of both rewarding and aversive stimuli, while CEA is considered to enact fear responses to previous learning (Beyeler et al., 2016). The amygdala also has very strong clinical relevance, due to the reported difference in incidence rate of depression

and anxiety between men and women (Logrip, Oleata, & Roberto, 2017; C. P. McLean et al., 2011). Within the BLA, subpopulations of cells respond to either rewarding or aversive stimuli, and these cells do not show a topographical organization (Beyeler et al., 2016; Shabel & Janak, 2009). They can, however, be defined by their projection targets; cells that encode rewarding stimuli project to the NAC, while cells encoding aversive learning project to the CEA (Beyeler et al., 2018, 2016). While fine-scale analysis of forebrain dopamine projection targets has been documented, this analysis has not taken sex into account (Aransay, Rodríguez-López, García-Amado, Clascáand, & Prensa, 2015; Walsh & Han, 2014). To date, the extent to which sex differences in forebrain dopamine projections exist has yet to be shown.

Effects of gonadal steroid hormones on the adult dopamine system

The observed differences in psychiatric illness rates between men and women are suggested to involve effects resulting from hormone changes during puberty. Evidence suggests, however, that hormone manipulation in adult animals can also result in changes in the forebrain dopaminergic circuit. Studies in male rats show both increased dopamine axon innervation to the PFC and increased circulating dopamine in the PFC after gonadectomy (M. F. F. Kritzer, 2003). This effect was prevented by providing androgen replacement after gonadectomy, but not affected by estrogen treatment after gonadectomy (M. F. F. Kritzer, 2003). Gonadectomy in male rats also decreased performance on a frontal-cortex dependent working memory task, specifically the T-maze spontaneous alternation test; this effect was also prevented with androgen replacement but not affected by estrogen treatment (M. F. Kritzer, McLaughlin, Smirlis, & Robinson, 2001). Additional evidence for the influence of sex hormones on forebrain dopamine circuits comes from a study showing that administration of E2 into the medial PFC (mPFC) increases the chance that rats will use place memory to solve a behavioral task, which is frontal

cortex-dependent, over response memory, which is dorsal striatum-dependent (Almey et al., 2014). This demonstrates an approach shift in response to E2 treatment – from a response-learning strategy to a more cognitively demanding memory strategy. This is suspected to be facilitated by increased dopamine transmission in the mPFC after E2 treatment.

Previous research has also shown differences in the MEA in measures of volume and cell number, as well as gonadal steroid hormone-dependent neurogenesis during puberty (Hines, Allen, & Gorski, 1992; McCarthy et al., 2017; Nishizuka & Arai, 1981). In addition, a study showed that the basolateral amygdala has a higher concentration of aromatase in females than in males, consistent with expectation that females would synthesize more E2 (Bender et al., 2016). Interestingly, this group also reported effects of aromatase manipulation on spine density and long-term potentiation in the BLA (Bender et al., 2016, 2017). Taken together, these studies suggest a relationship between gonadal steroid hormone levels and dopaminergic function in the forebrain. Hormone manipulation studies are critical for determining if sexually dimorphic patterns are the result of direct hormone effects or other upstream factors like genes present on sex chromosomes.

What are the unknowns to be addressed in this thesis?

It is known that some sex differences can be seen in anatomical measures, particularly in the hypothalamus, which plays a role in innate reproductive function. While most sexual dimorphisms have been found in regions related to reproductive functions, the aim of this study is to investigate sex differences in circuits strongly linked to psychiatric illnesses, particularly the forebrain dopaminergic circuit. This study proposes that sex differences that play a role in forebrain-associated psychiatric illness will be detectable in dopamine projections at the cellular anatomical level, which has not been systematically investigated before.

Sexual dimorphisms in the brain are often measured using immunostaining in the area of interest to assess volume or cell number. These sex differences in brain structure have also been assessed in terms of other anatomical measures, including fiber density, dendritic branching, and synapse number (Clarkson & Herbison, 2006; Gould et al., 1990; Kolb & Stewart, 1991; McCarthy et al., 2017). In addition, dimorphisms have been reported in distribution of specific cell types, activational state, and rate of neurogenesis (Ahmed et al., 2009; Becker & Beer, 1986; Johnson et al., 2008; McCarthy et al., 2017). Studies assessing sex differences in the amygdala have measured circulating hormone levels with microdialysis, while hormone manipulation studies in the PFC have used immunostaining (M. F. F. Kritzer, 2003; Mitsushima, Yamada, Takase, Funabashi, & Kimura, 2006). While these methods provide useful measures, sex differences in forebrain dopaminergic circuits have not been reported using these techniques. A major reason for this is that most of these methods are limited in their ability to differentiate both cellular subtype and origin of the projections into a target region, which is necessary for analysis of forebrain dopamine projections.

Chapter 2: Anatomical sex differences in VTA to BLA dopaminergic projections

Introduction

The first aim of this project is to assess anatomical forebrain dopamine circuit differences between males and females in adulthood. To ensure that measurements of dopamine circuit projection density can be comparable both between animals and across sexes, the anatomical labeling used must meet several criteria. First, as the target areas PFC, BLA, and NAC receive projections from cell types other than dopamine, the method used must differentiate between cell types. Second, to assess the density of forebrain dopamine projections relative to one another, the method used must have an internal control that allows separate target regions to be compared within a single animal. Third, to compare across sex there must also be a control for efficiency of labeling across animals to account for possible sex differences in brain volume and number of VTA projections to target regions.

To resolve these issues and assess dopaminergic projection density from VTA to PFC and BLA in the same animals and compare them across sex, we will use a molecular-genetic viral tracing technique. Molecular-genetic tracing enables molecular and anatomical specificity that has only recently become readily accessible. A tyrosine hydroxylase (TH)-Cre driver line limits expression to cells that would express TH, an enzyme required for dopamine synthesis (Gong et al., 2007). While this alone would not exclude norepinephrine (NE) cells that use the same enzyme, injection of a viral tracer into the VTA, which does not contain NE cells, prevents labeling of other cell types than dopamine and reduces the chance of cells projecting to the forebrain areas from other regions (Mastwal et al., 2014).

Dopamine neurons projecting to PFC, NAC and BLA are spatially intermingled in VTA (Lammel et al., 2008, 2014). Single viral injection into the VTA can label all VTA target regions within a single animal. Normalization to the number of labeled cells at the injection site allows comparison of projection density in different target regions of the same animal and between animals of different sexes, even if the volume of the brain changes with the size of the animal. Use of two viral constructs – one that fills the entire cell and one that acts as a reporter for synaptophysin – will allow VTA projection axons and their boutons to be visualized in different colors, enabling axons and boutons to be quantified independently. Axon labeling provides a structural readout of the projection density and location, while bouton labeling provides a functional readout of synapse number.

A challenge with this approach is the potential off-target effect depending on which TH-driver line is used; there may be expression in TH-negative neurons, depending on where the TH-Cre construct was inserted into the genome. This must be carefully monitored to ensure that if expression appears in cells that do not contain TH, these regions do not project to targets of the forebrain dopamine circuit. If no off-target Cre expression occurs, it is still important to ensure that the viral injection volume used is sufficient to cover the entire population of VTA cells projecting to PFC, BLA, and NAC to prevent variability in labeled populations. In addition, viral injection volume must be low enough to prevent spread to adjacent regions that might contain TH+ neurons sharing projection targets with the VTA.

Use of a viral injection also allows a secondary mouse line to be used to confirm dopaminergic projection labeling. A dopamine transporter (DAT)-Cre mouse line can be injected with the same viral construct to confirm that labeled projections are dopaminergic. Use of DAT-Cre mice has its own disadvantages, however, as neurons projecting from VTA to PFC have

been reported to express much less DAT compared to neurons projecting to other areas (Lammel et al., 2008).

While both boutons and axons can be labeled with tdTomato alone, the appropriate intensity thresholding required to separate axons and boutons labeled in the same color is difficult to achieve. Use of two different constructs to label axons and boutons separately allows quantification to be performed independently and reduces variability in the data.

Dopamine projection density was compared in PFC, BLA, and NAC in adult males and adult females. Both total axon length and bouton count were measured in all target areas, and cell counts were measured at the VTA injection site for normalization and to determine viral labeling efficiency. In addition, estrus cycle stage was assessed in females, as bouton density can change rapidly and may vary in response to hormonal conditions. Adult sex comparison was used to determine if sex differences exist in forebrain dopamine projections in either axon projection density or bouton density.

Design and Methods

Animals

Animals were heterozygous TH-Cre mice in the C57BL/6 strain (Mastwal et al., 2014). Animals were group housed under a 12-hour light/dark cycle and only used for experiments between postnatal days 70 and 105. Experimental protocols were approved by the National Institute of Mental Health Animal Care and Use Committee and were in accordance with the National Institutes of Health Guidelines for the Care and Use of Laboratory Animals.

Viral vectors

Two viral constructs – FLEX-tdTomato (Plasmid #28306, Addgene) and SynaptophysinGFP (AAV2/9-phSyn1(S)-FLEX-tdTomato-T2A-SynEGFP-WPRE, Boston Children's Hospital) – were used in a TH-Cre mouse line to label both axon structure and boutons along labeled axons. Viral titer for FLEX-tdtomato was 1×10^{13} gc/mL while the viral titer for SynaptophysinGFP was 4.26 x10^{13} gc/ml. The viral construct was injected into the VTA of TH-Cre mice as determined by stereotaxic coordinates, and both axon and bouton density in the BLA were normalized to the number of labeled cells at the VTA injection site (Paxinos & Franklin, 2013).

Stereotaxic Surgery

Animals were anesthetized with 3% isoflurane, and 1-1.5% isoflurane was maintained throughout the surgery. Head was fixed at 5 ° angle in stereotax. Local anesthetic (0.05 mL xylocaine) was injected under the scalp prior to incision. A cranial window was drilled 0.5 mm anterior and 0.5 mm lateral to lambda. Viruses were mixed prior to uptake in a glass injection needle – 1 µL FLEX-tdTomato-SynaptophysinGFP + 0.6 µL FLEX-tdTomato – and 1 µL of the mixture was injected at a depth of 4.6 mm below the surface of the injection site. The needle was left for 5 minutes after the injection was complete before retraction. The incision was sealed with Vetbond (3M) or surgical wound clip. Animals were placed on a heating pad until they began moving on their own, and then administered 1 mg/mL ketoprofen in sterile 0.9% saline through intraperitoneal (IP) injection (1 mL/ 25 g body weight). Ketoprofen was administered for 2 additional days post-surgery.

Vaginal Cytology

Estrus stage was determined by vaginal cytology. Female mice were anesthetized using 3% isoflurane. A pipette was used to take up 25 µL 0.9% saline. The tip of the pipette was

inserted 1-2 mm into the vaginal opening and the saline was pipetted in and aspirated back out 3-5 times (Cora, Kooistra, & Travlos, 2015). The liquid was then pipetted onto a 0.1% gelatin-subbed glass slide and smeared to prevent pooling in one spot. The liquid was allowed to dry, and then gently rinsed with deionized water to remove salt crystals. Slides were then visualized with a light microscope to determine cell types present. For each animal, it was recorded which of the following cells types were present: nucleated epithelial cells, cornified squamous epithelial cells, or leukocytes (A. C. McLean, Valenzuela, Fai, & Bennett, 2012). Proestrus was characterized by predominantly nucleated epithelial cells; estrus was characterized by clusters of cornified squamous epithelial cells; metestrus was characterized by predominantly leukocytes with some cornified squamous epithelial cells; diestrus was characterized by predominantly leukocytes, with some cornified squamous epithelial cells and some nucleated epithelial cells (A. C. McLean et al., 2012). For the purposes of quantification, proestrus and estrus were grouped together, and diestrus and metestrus were grouped together.

Immunostaining

Sections were permeabilized in 50% ethanol (EtOH) in PBS for 30 minutes then blocked for 1 hour in 0.3% TritonX-100 + 5% Normal Donkey Serum (NDS). Sections were incubated in 5% NDS with 1:5000 rabbit-anti-TH (AB152, Millipore Sigma) overnight at 4°C. Sections were washed 3 times with PBS before incubating in Alexa 488 Donkey-anti-Rabbit 1:200 (A-21206, ThermoFisher Scientific) for 1 hour. Sections were washed 3 times with PBS and stored in PBS until mounting.

Microscopy

Mice were anesthetized with 5% isoflurane and perfused with under constant isoflurane. Mice were perfused with 0.9% saline, followed by 4% paraformaldehyde (PFA) in PBS, and

post-fixed overnight in 4% PFA at 4°C. Brains were sectioned on a floating vibrotome at 100 µm and mounted on 0.1% gelatin-subbed slides. Sections were allowed to dry completely, then rehydrated and cover-slipped with Aqua-Poly/Mount (Polysciences). Two microscopes were used to obtain images: an Olympus FV1000 confocal, and a Zeiss 780 confocal. VTA images were taken with a 10x objective lens (Olympus, Zeiss), PFC images were taken with a 10x objective lens (Olympus), and BLA images were taken with both a 10x objective lens (Olympus) and a 20X objective lens zoomed to 25X magnification (Zeiss). VTA sections were selected based on stereotaxic coordinates from -3.1 bregma to -3.6 bregma, BLA sections were selected from -1.0 to -3.0 bregma, and PFC sections were selected at 2.0 and 1.1 bregma (Paxinos & Franklin, 2013). An automated stage was used to tile and stitch the 25x images covering the BLA and surrounding area across 20 sections per animal. Nine 25x BLA images were acquired per section, tiled 3x3, and Z-stacks were obtained with 10 images 1 micron depth apart.

Image Analysis

After image acquisition, all VTA, PFC, and BLA images were arranged consecutively and matched between animals to ensure that the quantification region was comparable between animals. If a matching image was not found for all animals, that location was not used in any quantification. For Z-stacks, images were projected to maximum intensity for each plane, and ROIs for quantification were drawn in ImageJ around the labeled region and separated into red axon and green bouton channels for PFC, BLA, and VTA images. Images were processed using a Matlab script (Mathworks) with three subcomponents – one for detecting axons in the red channel of BLA and PFC images, one for detecting boutons in the green channel of BLA and PFC images, and one for detecting cells in both the red and green channels of VTA images. The red channel script thresholded images acquired on the Olympus microscope to 5 standard

deviations (SD) and used Hessian line filters to detect continuous segments. These segments were then skeletonized to one pixel-width so total pixel output would represent axon length. The green channel script thresholded the 10x images acquired on the Olympus microscope to 5 SD and used round shape-detection of any object larger than one pixel. For 25x BLA images acquired on the Zeiss microscope, the same script was used, but thresholding values were changed to 7 SD for bouton and axon detection. The cell detection script thresholded images acquired on both microscopes to 3 SD and identified cells as round-objects between 49 and 200 μm^2 in size.

Statistics

Sample size required to reach statistical significance for viral labeling comparison is based on previous anatomical analysis and preliminary findings in bouton density (Mastwal et al., 2014). Anatomical comparisons between males and females were assessed by performing T-tests. A P-value below 0.05 was considered a significant difference.

Results

Viral tracer co-labels with dopaminergic marker

To determine viral injection efficacy, we used TH immunostaining to examine the extent of co-labeling between endogenous TH+ axons and viral labels in PFC, NAC, and BLA from a single VTA injection. We found that the viral labeling co-labeled extensively with the TH+ axons, indicating that the labeling was highly efficient and specific to TH+ axons (Figure 1).

TdTomato and SynaptophysinGFP distinguish between axons and boutons

To determine if boutons could be separated from axons for analysis, we acquired images using a 25x lens with 3x zoom for 75x magnification. High-magnification images in PFC

showed that boutons and axons are clearly labeled in red and green channels, respectively (Figure 2). This enabled axons and boutons to be separated for automated quantification.

VTA shows differential projection density to target regions

To determine relative density in VTA projection targets, we quantified axon and bouton density in PFC, BLA, and NAC. Our quantifications showed high labeling density in NAC, intermediate density in BLA, and sparse labeling in PFC, across both boutons and axons (Figure 3). NAC axon density appeared too high to allow segmentation of single axons in confocal images, so NAC has been omitted from the subsequent analyses. ROI intensity comparisons between PFC and BLA ($P<0.05$), BLA and NAC ($P<0.001$), and PFC and NAC ($P<0.001$) were all significant for both axons and boutons ($N=4$). These findings suggest that projections from VTA are likely to have a varied degree of effect on forebrain targets; NAC will be under stronger VTA control, while PFC will be under less control by VTA activity.

Projection density in adult PFC shows no sex difference

To observe effects of sex on forebrain projection density, we first acquired images at 10x from representative PFC slices. Sections were 100 microns thick, and two sections per animal – at 2.0 bregma and 1.1 bregma – were summed for quantification. Survey of the PFC showed no sex difference in either axon density (T-test, n (males) = 6, n (females) = 5, P= 0.15) (Figure 4A, 4C) or bouton density (T-test, n (males) = 6, n (females) = 5, P=0.25) (Figure 4B, 4D) using 10x magnification. These findings indicate that frontal cortex dopamine projections do not differ between males and females.

Projections in adult BLA show bouton sex difference, but no axon sex difference

To account for possible differences in brain region size, the BLA – which showed a distinct border outlined by dopamine axons– was imaged across 13 coronal slices and BLA volume was approximated by summing the area from 13 slices for each animal. No sex difference appeared in BLA area (T-test, n (males) = 6, n (females) = 5, P=0.85) (Figure 5A, 5B). To observe effects of sex on forebrain projection density, we summed axon and bouton density in the same 13 sections for quantification. Sections were 100 microns thick. We observed no sex difference in axon density in BLA (T-test, n (males) = 6, n (females) = 5, P=0.26) (Figure 5C). However, the density of boutons in the BLA was significantly higher in males than in females at 10x magnification (T-test, n (males) = 6, n (females) = 5, P= 0.04) (Figure 5D, 5E). Estrus stage assessment from vaginal cytology samples indicated that all 5 females were coincidentally in estrus at the time of perfusion. These findings suggest that while forebrain dopamine axon density does not differ between males and females in the BLA, a sex difference in dopamine boutons in these projections would likely result in increased dopamine release in the male BLA.

Higher magnification replicates sex difference in BLA bouton number

To further confirm the observed sex differences of dopaminergic bouton density in BLA, a second set of brains was imaged using 25x magnification to increase bouton resolution (Figure 6A-6D). As in the preliminary set, the BLA showed no sex difference in axon projection density (T-test, n = 5 animals per group P= 0.57) (Figure 6E) but showed significantly higher bouton density in males than in females (T-test, n = 5 animals per group, P=0.01) (Figure 6F). No difference was found in bouton count when divided by estrus stage (T-test, n (diestrus) = 3, n (estrus) = 4), P = 0.32). Estrus and proestrus animals were grouped together; full breakdown of stages was diestrus (n=3), proestrus (n=1) or estrus (n=3).

Conclusions

These findings indicate that a sex difference in bouton number is present in dopamine axon projections to the BLA but not in density of the axon projections themselves, and that female BLA bouton number appears to be stable across the estrus cycle. Neither axon nor bouton density differences were found in projections from VTA to PFC. This finding raises questions about the possibility of males and females showing a difference in BLA function, as well as the question of whether bouton density in the BLA is mediated differentially by gonadal steroid hormones in males and females.

Chapter 3: Anatomical effects of hormone manipulation on VTA dopamine cells and BLA dopaminergic projections

Introduction

As evidenced by the series of studies by Kritzer et al., hormone manipulation, even in adulthood, has been demonstrated to alter function in the forebrain dopamine circuit, both in males and females. E2 administration in adult females has been shown to reduce expression of the immediate early gene (IEG) *c-fos* in BLA after extinction recall assessment, although it is not known if this is associated with dopamine (Zeidan et al., 2011). The aim of this study was to determine if manipulation of gonadal steroid hormones in adult animals altered the anatomy of the VTA-BLA dopamine circuit. If the anatomy of these circuits is susceptible to hormonal influence, this would be a valuable step in determining how the anatomical sex difference we reported emerges, and could provide an avenue for intervention, should the reported sex difference be associated with sex differences in behavior or psychopathology.

For this study, multiple hormone manipulations were considered, as the conclusions that can be drawn are dependent on the specificity of the manipulations used. The advantages and disadvantages of each method are described below.

Hormone synthesis targets

Gonadectomy is a classically used method to remove gonadal steroid hormones indiscriminately, however, this comes with the disadvantage that broad removal of androgens (male) or estrogens (female) means that only broad conclusions about androgens or estrogens can be drawn (Beach, 1971; Gorski et al., 1978). In females, ovariectomy could have an effect based on loss of E2, estrone, estriol, or progesterone, while in males this effect could be related

to T or DHT (Celotti et al., 1991). This method would also prevent estrogen removal in males and androgen removal in females, and thus cannot be used alone to determine hormonal effects across sex.

A more specific method that is still effective in both males and females is to halt or reduce T synthesis. Pharmacological prevention of T synthesis can be achieved by administration of agents such as leuprolide acetate and would subsequently prevent synthesis of E2 or DHT in males or females (Sherwin, 2003; Wilson et al., 2007; Wuttke et al., 1996). Blocking androgens allows systematic manipulation of both estrogens and androgens by adding in each hormone without the presence of the others. This would enable broad conclusions to be drawn before drawing conclusions about individual hormone effects.

Another option would be to halt synthesis of downstream androgens. Pharmacological blockade of 5-α reductase (5-AR) would prevent conversion of T to DHT (Celotti et al., 1991). This manipulation can separate direct T effects from effects of T as a DHT precursor. While DHT is only present in low concentrations in females, this manipulation is possible in both males and females, which is important to precisely understand androgen effects on dopamine projections.

Similarly, synthesis of E2 can be blocked. Pharmacological blocking of aromatase will prevent aromatization of T to E2, allowing separation of direct T effects from effects of T as an E2 precursor (Wade & Arnold, 1996).

Receptor targets

There are also several manipulations that can target gonadal steroid hormone receptors, rather than directly altering hormone synthesis (Dupont et al., 2000; Ramirez & Zheng, 1996). Conditional genetic knockout can eliminate a class of hormone receptors in a cell-type dependent

manner. This could be used for either AR or ER, and can be done in both male and female mice. As there are multiple types of androgen and estrogen receptors, this manipulation enables narrower conclusions about hormone effects based on receptor type in a specific cell population.

Small interfering RNAs (siRNAs) can also block a class of hormone receptors by transiently preventing the receptors from being translated, and can be administered by injection at any stage of development (Giatti, Garcia-Segura, Barreto, & Melcangi, 2018; Perrett & McArdle, 2013). This type of manipulation enables conclusions based on receptor type and enables temporary knockdown of receptors. Temporarily halting receptor production can be used to determine precisely when during development the receptor must be activated for the hormone effect to occur normally.

For the purposes of this project, we used a single administration of leuprolide acetate (Sigma-Aldrich), also called Lupron, in order to manipulate gonadal steroid hormone levels in a clinically relevant manner. Lupron is a GNRH receptor agonist used in clinical treatments ranging from prostate cancer treatment to in vitro fertilization and endometriosis treatment (Wilson et al., 2007). Leuprolide is a synthetic compound similar in structure to GNRH, and has a stronger affinity for GNRH receptors, resulting in a sustained activity on GNRH receptors in the anterior pituitary. This results in a sustained gonadotropin release, which is normally released in pulses. This change in GNRH binding to GNRH receptors causes an inhibition of gonadotropin release. Thus, a short increase in activity is followed by a sustained decrease in secretion of hormones from the gonads (Perrett & McArdle, 2013; Wilson et al., 2007). In human clinical patients, the increase in gonadal hormones is within 3 days of treatment, followed by a suppression of gonadal hormones from 2-4 weeks (Wilson et al., 2007). A similar acute increase followed by long-term suppression of gonadal steroid hormones has also been reported

in rodents after a single dose of leuprolide (Maeng et al., 2017). In this study, we used a similar method – a single dose of leuprolide or saline vehicle 3 weeks before collection of brain tissue for anatomical analysis.

Design and Methods

Animals

Animals were heterozygous TH-Cre male mice in the C57BL/6 strain (Mastwal et al., 2014) for viral injections, or wild type C57BL/6 for hormone assays and immunostaining. Animals were group housed under a 12h light/dark cycle and only used for experiments between postnatal days 70 and 120. Experimental protocols were approved by the National Institute of Mental Health Animal Care and Use Committee and were in accordance with the National Institutes of Health Guidelines for the Care and Use of Laboratory Animals.

Viral vectors

Two viral constructs – FLEX-tdTomato (Plasmid #28306, Addgene) and SynaptophysinGFP (AAV2/9-phSyn1(S)-FLEX-tdTomato-T2A-SynEGFP-WPRE, Boston Children's Hospital) – were used in a TH-Cre mouse line to label both axon structure and boutons along labeled axons. Viral titer for FLEX-tdtomato was 1×10^{13} gc/mL while the viral titer for SynaptophysinGFP was 4.26 x1013 gc/ml. The viral construct was injected into the VTA of TH-Cre mice as determined by stereotaxic coordinates, and both axon and bouton densities in the BLA were normalized to the number of labeled cells at the VTA injection site (Paxinos & Franklin, 2013).

Leuprolide treatment

Leuprolide acetate was prepared to allow doses at 1.2 mg/kg to not exceed 200 microliters in a 30 g animal. 5 mg leuprolide acetate powder was dissolved in 25 mL 0.9% saline

for a final concentration of 157.554 micromolar to allow ~ 0.36 mg in 180 microliters for a 30 g animal. This solution was filtered and pH tested and fell within the 4.0 to 6.0 pH range as required by animal use guidelines (pH 5.0). Aliquots were stored at -80 degrees C until time of administration. Animals were weighed and injection volume calculated to reach 1.2 mg/kg dose. Lupron or 0.9% saline was injected IP without anesthesia.

Hormone measurements

In order to verify the efficacy of leuprolide treatment, level of circulating T was measured via ELISA. At the designated time point after drug administration (21 days), animals were anesthetized under 5% isoflurane as typical for perfusion and kept exposed to isoflurane during blood collection and perfusion (maximum length 10 minutes). Chest cavity was opened and clamped to expose heart. Before insertion of perfusion needle, the right atrium was cut and blood was collected from the chest cavity and moved to a heparin-coated collection tube via transfer pipette. Blood collection tube was stored on ice for no more than 4 hours before centrifugation. Whole blood was centrifuged for 15 minutes at 13000 x *g* at 4° C and serum was pipetted into an Eppendorf tube for storage at -20 ° C until ELISA. Samples and ELISA kit (Calbiotech) were placed at room temperature for 2 hours prior to assay to reach room temperature before running assay. For male animals, 25µL samples were measured in duplicate.

Stereotaxic Surgery

Animals were anesthetized with 3% isoflurane, and 1-1.5% isoflurane was maintained throughout the surgery. Head was fixed at 5 ° angle in stereotax. Local anesthetic (0.05 mL xylocaine) was injected under the scalp prior to incision. A cranial window was drilled using a robotic stage programmed to target the VTA based on skull position (NeuroStar) with coordinates set to 3.1 posterior to bregma, 0.5 mm lateral to bregma, and 4.6 mm below the

surface of the injection site (Paxinos & Franklin, 2013). For each animal, the skull position was mapped by placing the drill at bregma, lambda, and two points 2 mm left and right of the midline to adjust injection coordinates for skull tilt. Viruses were mixed prior to uptake in a syringe (Hamilton) – 1 µL FLEX-tdTomato-SynaptophysinGFP + 0.6 µL FLEX-tdTomato – and 1 µL of the mixture was injected. The needle was left for 5 minutes after the injection was complete before retraction. The incision was sealed with Vetbond (3M) or surgical wound clip. Animals were placed on a heating pad until they began moving on their own, and then administered 1 mg/mL ketoprofen in sterile saline through IP injection (1 mL/ 25 g body weight). Ketoprofen was administered for 2 additional days post-surgery.

Immunostaining

Sections were permeabilized in 50% EtOH in PBS for 30 minutes then blocked for 1 hour in 0.3% TritonX-100 + 5% NDS. Sections were incubated in 5% NDS with 1:2000 rabbit-anti-TH (AB152, Millipore Sigma) overnight at 4°C. Sections were washed 3 times with PBS before incubating in Alexa 488 Donkey-anti-Rabbit 1:200 (A-21206, ThermoFisher Scientific) for 1 hour. Sections were washed 3 times with PBS and stored in PBS until mounting. Sections were counterstained with 1:5000 Hoechst 33342 (ThermoFisher Scientific) in PBS for 10 minutes and washed with PBS.

Microscopy

Mice were anesthetized with 5% isoflurane and perfused with under constant isoflurane. Mice were perfused with 0.9% saline followed by 4% paraformaldehyde (PFA) in PBS, and post-fixed overnight in 4% PFA at 4°C. Brains were cryosectioned at 80 µm and mounted on 0.1% gelatin-subbed slides. Sections were allowed to dry completely, then rehydrated and cover-slipped with Aqua-Poly/Mount (Polysciences). Two microscopes were used to obtain images: a

Nikon A1R confocal and a Zeiss AXIO Fluorescence Slide Scanner. VTA images were taken with a 10x objective lens (Nikon, Zeiss), and BLA images were taken with a 20x objective lens zoomed to 25x magnification (Nikon, Zeiss). VTA sections were selected based on stereotaxic coordinates from -3.1 bregma to -3.6 bregma, and BLA sections were selected from -1.0 to -3.0 bregma. Images for viral-tracing data were acquired on the confocal microscope at 10x magnification or 25x magnification (Nikon). An automated stage was used to tile and stitch the 25x images covering the BLA and surrounding area across 20 sections per animal. Nine BLA images were acquired per section, tiled 3x3, and Z-stacks were obtained with 6 images 1.9 μm depth apart. Sections immunostained for TH were imaged in batches on the high-throughput fluorescence slide scanner (Zeiss). ROIs were drawn around the tissue, and the microscope was set to auto-focus on nuclear Hoechst staining.

Image Analysis

For virus-injected animals, Z-stacks were projected to maximum intensity for each plane, and ROIs for quantification were drawn in ImageJ around the labeled region and separated into red axon and green bouton channels both for BLA and VTA images. Images were processed using a Matlab script (Mathworks) with three subcomponents – one for detecting axons in the red channel of BLA images, one for detecting boutons in the green channel of BLA images, and one for detecting cells in both the red and green channels of VTA images. The red channel script thresholded images acquired on the Nikon microscope to 6 standard deviations (SD) and used Hessian line filters to detect continuous segments. These segments were then skeletonized to one pixel-width so total pixel output would represent axon length. The green channel script thresholded the images to 6 SD and used round shape-detection of any object larger than one pixel. The cell detection script thresholded images acquired on the Nikon microscope to 3 SD (2

SD for 10x images acquired one the AXIO) and identified cells as round-objects between 49 and 200 μm^2 in size. For 25x images acquired on the AXIO microscope, the same script was used, but thresholding values were changed to 5 SD for bouton and axon detection.

Statistics

Sample size required to reach statistical significance for immunostaining comparison is based on previous anatomical analysis and findings in viral labeling comparison (Mastwal et al., 2014). T level comparisons between baseline and leuprolide treatment groups were assessed by performing a Kruskal-Wallis ANOVA followed by Wilcoxon Rank Sum tests for individual comparisons. Anatomical comparisons between baseline, saline, and treatment groups were assessed by performing one-way ANOVA followed by T-tests for individual groups. A P-value below 0.05 was considered a significant difference.

Results

Leuprolide treatment reduced circulating T 21 days after treatment

Prior to collection of anatomical data, blood T levels were used to verify the effectiveness of leuprolide treatment. Animals that received leuprolide treatment showed a significant reduction in circulating T 21 days after injection compared to untreated animals [Kruskal-Wallis ANOVA, n (baseline) = 7, n (saline21) = 4, n (lupron21) = 5, P = 0.002 ; Wilcoxon rank sum test, P(L21, baseline) = 0.0016] (Figure 8D). Baseline T readings were in the expected range listed for the ELISA kit, and also matched the ranges expected for adult male mice in literature (Nelson, Latham, & Finch, 1975).

Examinations of virally labeled dopamine axon or bouton density in BLA 21 days after leuprolide administration in males

Measurement of VTA to BLA dopamine projections was limited by the number of available TH-cre mice in the colony. Of 10 available TH-Cre males, 5 were treated with IP leuprolide and 5 were treated with IP saline vehicle. One saline animal died prior to viral injection. One leuprolide brain was found to be hydrocephalic after dissection, and was removed from the study. One leuprolide brain was damaged during dissection and cryosectioning. Of the remaining brains, two saline-treated animals and one leuprolide-treated animal had off-target viral injection, resulting in a total of two successful animals per experimental group. Due to the small sample size, no conclusions can be drawn regarding the effect of leuprolide on VTA to BLA dopamine projection density (Figure 7).

Hormone blockade using leuprolide showed no effect on VTA dopamine cell number or BLA bouton count by immunostaining

Measurement of VTA cells and BLA boutons were performed separately after immunostaining for TH in the same wild type animals used for hormone analysis, with each measure normalized to the area of measurement [VTA cells ANOVA, n (baseline) = 7, n (saline21) = 4, n (lupron21) = 3, P =0.51] (Figure 8E, Figure 9F). BLA bouton counts were also plotted against T concentration (Figure 9G). While the treatment group did not show a significant effect on either VTA cell count or BLA bouton count, bouton count showed a weak negative correlation with T concentration after removal of two outliers ($r^2 = 0.17$).

Conclusions

Based on our findings that males show a higher bouton count in dopamine projections from VTA to BLA, our hypothesis was that reduction in gonadal steroid hormones via leuprolide treatment would reduce bouton count in the BLA. We did not see a decreased bouton count in Lupron conditions, despite reporting that leuprolide effectively reduced T after 21 days (Figure 8D). However, the average bouton count, though not significantly lower, was reduced compared to both saline and baseline groups. This is likely a result of variability from a small number of subjects.

We are not able to conclusively say if the sex difference we report is mediated by gonadal steroid hormones. In order to fully explain the sex differences we see in bouton number, additional experiments will be necessary. To determine if bouton concentration is mediated by gonadal steroid hormones, it will be important to measure both T and E2 in the subjects to determine if bouton number is more strongly correlated with aromatized E2 than T. In addition, it will be important to see how androgen and estrogen levels vary with bouton number in both males and females. In addition to completing the leuprolide manipulation with a larger sample size of both males and females, it may be helpful to include an earlier time point, during the GNRH agonist phase of leuprolide treatment, to determine if the effects are a result of the short-term agonist effect or long-term antagonist effect.

Chapter 4: Discussion

Ultimately, while we cannot make claims about the effect of gonadal steroid hormones on bouton density in the BLA, it is still notable that we detected a significant sex difference in dopamine projection anatomy. This finding raises several important questions about how bouton density might play a role in physiology and behavior, as well as how and when such an anatomical difference emerges. This sex difference may have an effect on dopamine release quantity in the BLA and may have further downstream effects that we have not yet been able to detect, as well as possible functional behavior implications. In addition, while we have reported a sex difference in adult animals, it is important to determine how this difference may play a role in earlier developmental stages.

Physiological effects of bouton density difference

To further understand the relevance of a sex difference in bouton density, it will be important to know if functional dopamine release differs between males and females, but also to know the identity of post-synaptic targets of VTA projections to BLA.

Anatomical difference in bouton number may result in a different functional dopamine release between males and females if other physiological parameters, such as rate of neuronal firing, are comparable. This could be quantified by either physiological measurement of dopamine neuron firing, post-synaptic neuron activity, and direct quantification of dopamine release through microdialysis in the BLA after VTA stimulation. Measurement of VTA effect on BLA could be measured indirectly through stimulation of the VTA (pharmacological or otherwise) and measurement of general activity markers in the BLA, such as immunostaining for an IEG like *c-fos* (Perrin-Terrin et al., 2016).

To understand the role of the VTA-BLA projection and how it differs between males and females, it will be important to determine which BLA cells are targeted by VTA projections. Within the BLA, subpopulations of cells respond to either rewarding or aversive stimuli, and these cells do not show a topographical organization (Beyeler et al., 2016; Shabel & Janak, 2009). They can, however, be defined by their projection targets; cells that encode rewarding stimuli project to the NAC, while cells encoding aversive learning project to the CEA (Beyeler et al., 2018, 2016) . There do not appear to be any studies that have explicitly mapped the post-synaptic targets of projections from VTA to BLA, but this could be achieved with a similar viral-tracing experiment using an anterograde trans-synaptic virus, such as AAV1 (Zingg et al., 2017). Post-synaptic targets that are labeled by the anterograde trans-synaptic virus can be further identified by immunostaining for other markers to determine if they are excitatory or inhibitory subpopulations of BLA cells.

One possible post-synaptic target for investigation could be parvalbumin (PV) neurons in the BLA. Dopamine projections have been shown to synapse onto inhibitory PV+ interneurons in the PFC and forebrain PV+ activity has been associated with synchrony in cortical areas (Carr & Sesack, 2000; T. Kim et al., 2015). In addition, sex differences have been reported in PV-positive cell number in the BLA, with females showing fewer PV+ cells than males (Blume et al., 2017). Synchronization of target areas through acute activation of PV+ interneuron populations is one possible pathway by which forebrain dopamine projections may play a role in behavioral and clinical sex differences.

Behavioral effects of bouton density difference

If the anatomical sex difference in BLA has a functional difference in activation of BLA, it will be important to determine if this translates to a behavioral sex difference. A large body of

literature focuses on sex differences in rodent subjects, including several studies showing sex differences in the BLA. Female rodents show a higher rate of correct response on active avoidance tasks that require the subject to escape a harmful stimulus (Denti & Epstein, 1972). In addition, females show a greater generalization of fear response to conditioned stimuli and a more dramatic amplitude of conditioned fear response, which are dependent on the BLA (Blume et al., 2017; Dalla & Shors, 2009; Day, Reed, & Stevenson, 2016; Maren, De Oca, & Fanselow, 1994; Pezze & Feldon, 2004).

Dopamine has been shown to play a role in acute fear conditioning through the projection of the VTA to the amygdala, specifically the BLA and CEA (de Oliveira et al., 2011). Male rodents, however, show an increased acquisition rate of fear conditioning, contrary to the expectation based on fear-conditioning in females (Maren et al., 1994). Fear potentiated-startle responses have also been used to assess direct manipulation of the dopamine projection from VTA to BLA in a manner dependent on dose of dopamine to the BLA (de Oliveira et al., 2011). Based on the quantitative difference in dopamine boutons in VTA projections to the BLA, we would expect to see a quantitative difference in startle amplitude between males and females after stimulation.

Development of the forebrain dopaminergic circuit

We reported an anatomical sex difference in adult animals, but we do not know when in development this sex difference emerges. It could be that this anatomical effect emerges during prenatal organization of the brain, or it could emerge or become more dramatic during adolescence. In addition, while we did not report an anatomical sex difference in the PFC during adulthood, it is possible that the developmental trajectory of the PFC may differ between males and females.

Adolescence and psychiatric illness risk

Adolescence is considered the physical, mental, and social transition period between childhood and adulthood in which motivated behavior matures. Adolescent humans show changes in social structure, increased emotional intensity and lability, increased impulsivity, and increases in reward-seeking behaviors, such as drug use (Ernst, 2014; Ernst et al., 2005). Adolescent shifts in social values focus less on the family and more toward peer relationships, indicating not only a change in reward-seeking, but a change in the type of stimuli that are considered rewarding (Crews, He, & Hodge, 2007; Spear, 2000). During adolescence, avoidance of harmful or uncertain situations is diminished compared to adulthood, resulting in increased risk-taking to achieve a reward that an adult might deem too small to pursue (Ernst et al., 2005). While adolescent cognitive functions, such as working memory, are not as strong as in adults, they are gradually improving toward adult levels during this time (D. Sinclair et al., 2014). Performance in behavioral inhibition tasks (Go-No Go, Stroop, etc.) show diminished performance in childhood and adolescence compared to adulthood, suggesting that the system that controls inhibition is not fully mature until after adolescence (B. J. Casey, Giedd, & Thomas, 2000; Leon-Carrion, Garcia-Orza, & Perez-Santamaria, 2004).

As a result of this characteristic pattern of high reward-seeking and low avoidance and inhibition, a large percentage of preventable deaths in adolescence are the result of elevated risk-taking, ranging from drug- and alcohol-related deaths to automobile accidents (Steinberg, 2008). In addition to trends in natural settings and behavioral tasks, functional brain imaging studies have showed increased response to rewarding stimuli and inefficient activation patterns during cognitive control tasks in adolescent subjects (Wahlstrom et al., 2010). These patterns of findings

have been integrated into the triadic model of adolescent motivated behavior, which suggests that adolescent behavioral changes are the result of differential maturation of cognitive and motivational systems. The triadic model proposes that reward-seeking systems reach adult levels before harm-avoidance and inhibitory regulation systems, leading to an increased motivational weight of reward compared to risk-avoidance during adolescence (Ernst et al., 2005).

Imbalance in the maturation of the triadic model behavior systems is suspected to play a role in several adolescent-onset psychiatric illnesses, including several that show differences in incidence rate between males and females. These illnesses are varied in manifestation – including schizophrenia, depression, drug addiction and others – but most are suspected to arise from abnormalities in the typical maturation of brain systems that occurs in adolescence (Ernst et al., 2005; Paus, Keshavan, & Giedd, 2008; D. Sinclair et al., 2014). An increased weight in reward systems with decreased weight in harm-avoidance and behavioral inhibition systems could play a role in drug use and addiction, while a disruption in reward strength coupled with an atypically strong motivation for harm-avoidance could result in anxiety or depressive pathology (B. Casey & Caudle, 2013; B. J. Casey, Jones, & Hare, 2008). The adolescent brain is more plastic than the adult brain, and often the changes that occur in adolescence – healthy or not – persist into adulthood (Mastwal et al., 2014). This makes adolescence a particularly vulnerable time for a deviation from normal development, and it is important to understand how the anatomical and functional development of the brain differs between males and females.

Maturation of the dopamine system

Dopamine innervation density from VTA to the frontal cortex, frontal cortex dopamine receptor number, and circulating dopamine concentrations in the frontal cortex have all been found to increase during adolescence (Naneix, Marchand, Di Scala, Pape, & Coutureau, 2012).

One possible anatomical explanation for imbalance in the triadic model could be that the NAC reaches adult levels of innervation before the BLA, resulting in an increased motivational weight of reward-seeking relative to harm-avoidance. If the PFC is not fully matured at this point, it could fail to balance the actions of the NAC with those of the BLA, resulting in increased risk-taking to obtain reward. In psychiatric illnesses, one or more of the triadic model regions could mature early, late or not at all, resulting in persistent aberrant behavior. It is known that dramatic increase or decrease from baseline dopamine levels in the frontal cortex leads to cognitive deficits, so if the frontal cortex deviates from its typical maturation in either direction, the NAC and BLA could remain unbalanced (Arnsten et al., 1994; Murphy et al., 1996).

It is known that the dopaminergic projection to PFC exhibits protracted maturation in the postnatal period compared to the relatively early maturation of the NAC, which could result in delayed onset in behavioral inhibition of the NAC (Kalsbeek, Voorn, Buijs, Pool, & Uylings, 1988). While little is reported on amygdala development, one study in gerbils reported amygdala maturation from P14 to P20, which would be intermediate to the hypothesized maturation of nucleus accumbens and frontal cortex in mice, consistent with the triadic model hypothesis (Brummelte & Teuchert-Noodt, 2006, 2007). While comparison of relative anatomical maturation rate between NAC and PFC have been documented, these have been done in a single sex and have not been able to determine if males and females show different maturation rates between these regions. One possibility could be that a transient sex difference emerges in the PFC during puberty when gonadal steroid hormone levels are changing most dramatically, but becomes more similar between males and females as hormone levels stabilize and circuits reach maturity (Hill et al., 2012). To date, hormone manipulation studies on dopamine innervation density have only been performed in adults, when hormone levels are already stable. To

investigate the role of hormones on dopamine circuits, it will be important to manipulate hormone levels during the adolescent period when they are naturally changing. A deviation in adolescent hormone levels may be expected to result in an even more dramatic and persistent change, or could result in a compensatory mechanism to restore typical density and behavior.

To determine the role of gonadal steroid hormones on development of the forebrain circuit, this effect can be further probed using a pharmacological hormone blockade similar to the one used in our study, but at earlier developmental stages. Systemic blockade of gonadal steroid hormones prenatally, early postnatally, and in adolescence could all have different effects on maturation of the forebrain dopamine circuit anatomy and function.

Conclusion

Our report of a sex difference in bouton density in VTA projections to BLA provides an important starting point for understanding how sex differences in dopamine-dependent behavior might emerge between males and females. This finding raises additional questions about dopamine circuit development, as well as circuit function in adulthood. These directions will all be important to study further, as they may improve our understanding of dopaminergic circuit roles in psychiatric risk, as well as provide possible targets for differential therapeutic intervention between males and females.

Figure legends

Figure 1. Labeling of dopaminergic projection axons in PFC, NAC, and BLA by AAV9-FLEX-tdTomato and TH immunostaining. (A) Bregma 2.0, 9 µm z-stack, 10x in prefrontal cortex (PFC), (B) – 2.0 bregma, at 10x zoom. Shows lateral regions including basolateral amygdala (BLA) and striatum, and (C) bregma 2.0, 9 µm z-stack, 10x in nucleus accumbens (NAC), (D) Injection site of virus (red) and TH antibody stain on contralateral side (green). VTA slice taken from bregma -3.1 at 10x zoom.

Figure 2. Images of PFC of FLEX-tdTomato-SynaptophysinGFP injected animal show clear separation of bouton and axon labeling. Sections were counterstained with DAPI.

Figure 3. VTA projection intensity in PFC (A), BLA (B), and NAC (C) normalized to NAC projection intensity (N=4) (D,E). Error bars show SEM ($P < 0.05$ *, $P < 0.01$ **, $P < 0.001$ ***). Error bars show SEM.

Figure 4. Male/female comparison shows no sex difference in adult PFC at 10x image magnification. Sample male (n=6) and female (n=5) axon (A) and bouton (B) images. Axon density shows filtered pixel count normalized to VTA red cell number [T-test, $P = 0.15$]. (C) and bouton density shows bouton count normalized to VTA green cell number [T-test, $P = 0.25$]. Box plots represent median, quartile 1, and quartile 3.

Figure 5. Male/female comparison shows a sex difference in bouton density in BLA at 10x image magnification. Representative image sections from male (n=5) and female (n=5) BLA area covering 12 coronal sections from each animal [T-test, $P = 0.85$]. (B) Axon density shows filtered pixel count normalized to VTA red cell number [T-test, $P = 0.6$]. (C). Images from matched anterior-posterior sections in males and females were binarized and filtered for boutons, then filtered images were collapsed into a single image (D). Bouton density shows bouton count normalized to VTA green cell number [T-test, $P = 0.04$]*. (E). Box plots represent median, quartile 1, and quartile 3.

Figure 6. Repeated male/female comparison shows a sex difference in bouton density in BLA at 25x image magnification. Representative images from a single 25x section showing axons (A), thresholded axons (B), boutons from the ROI in the blue box (C) and thresholded boutons from the ROI in the blue box (D). Density was summed across 20 matched coronal sections (-3.06 bregma to -1.16 bregma) from each animal in males (n=5) and females (n=5). Axon density shows axon pixel count normalized to VTA red cell number [T-test, $P = 0.57$]. (E). Bouton density shows bouton count normalized to VTA green cell number [T-test, $P = 0.014$]*. (F). Bouton density shows bouton count normalized to VTA green cell number in females separated grouped into estrus (n=4) or diestrus (n=3) [T-test, $P = 0.32$]. (G). Box plots represent median, quartile 1, and quartile 3.

Figure 7. Virally labeled dopamine axon or bouton density in BLA 21 days after leuprolide administration in males. Axon density shows filtered pixel count normalized to VTA red cell number in saline- (n=2) leuprolide-treated (n=2) animals 21 days after treatment (A). Bouton

density shows bouton count normalized to VTA green cell number (B). Box plots represent median, quartile 1, and quartile 3.

Figure 8. TH immunostaining in VTA shows no difference between baseline, saline control, and leuprolide treatments. VTA sections were summed between four sections per animal from no treatment group (n=7) (A), 21 days post-saline treatment group (n=4) (B) and 21 days post-leuprolide treatment group (n=3) (C). T measurements from blood collection at the time of perfusion in baseline (n=7) , saline control (n=4) , and leuprolide treatment (n=5) groups [Kruskal-Wallis ANOVA, P = 0.002; Wilcoxon rank sum test , P(L21, baseline) = 0.0016] (D). VTA cell counts were normalized to summed ROI area pixels for each animal (ANOVA , P =0.51) (E). Box plots represent median, quartile 1, and quartile 3.

Figure 9. TH immunostaining in BLA shows no difference between baseline, saline control, and leuprolide treatments. BLA bouton counts were summed between seven sections per animal from no treatment group (n=7) (A), 21 days post-saline treatment group (n=4) (B) and 21 days post-leuprolide treatment group (n=3) (C). Representative images from a single 10X section of VTA showing TH immunostaining and thresholded VTA cells (D), and 25X section of BLA showing TH immunostaining and thresholded boutons (E). Bouton counts were normalized to summed ROI area pixels for each animal [ANOVA, P = 0.36](F). Bouton counts were plotted against T concentration (N=12) (r^2 = 0.17) (G). Box plots represent median, quartile 1, and quartile 3.

Figures

Figure 1.

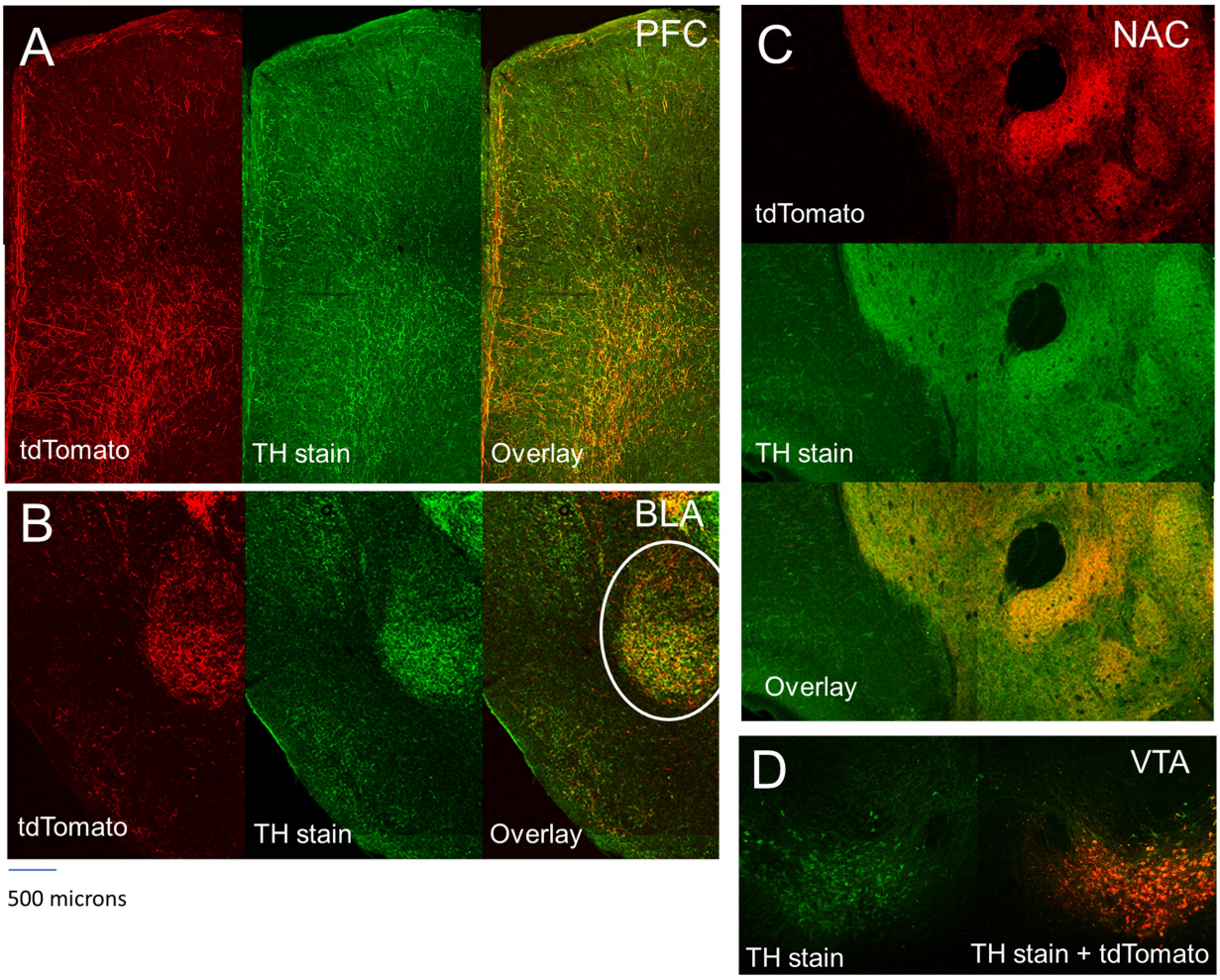


Figure 2.

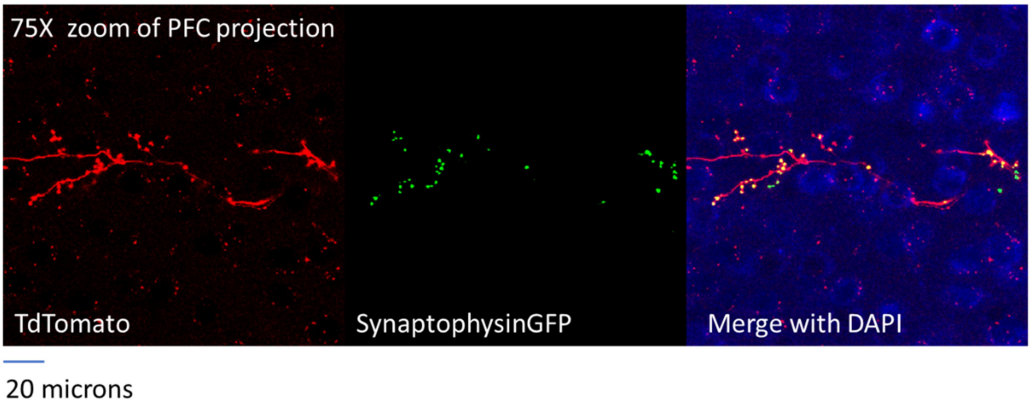
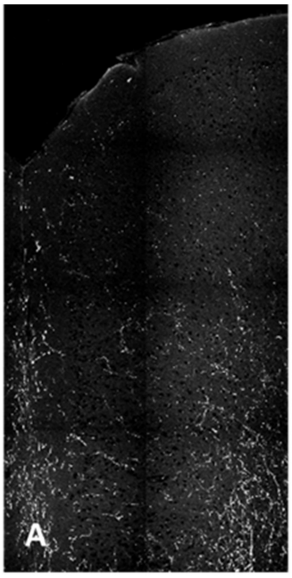
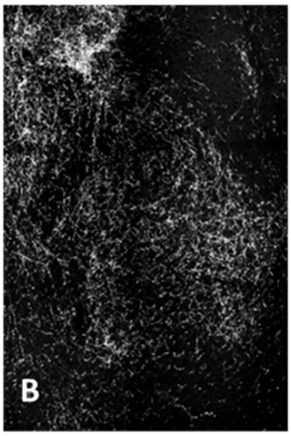
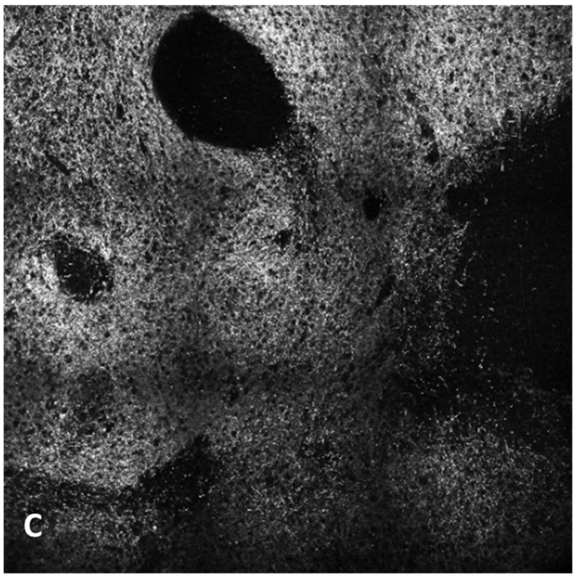

75X zoom of PFC projection
TdTomato
SynaptophysinGFP
Merge with DAPI
20 microns

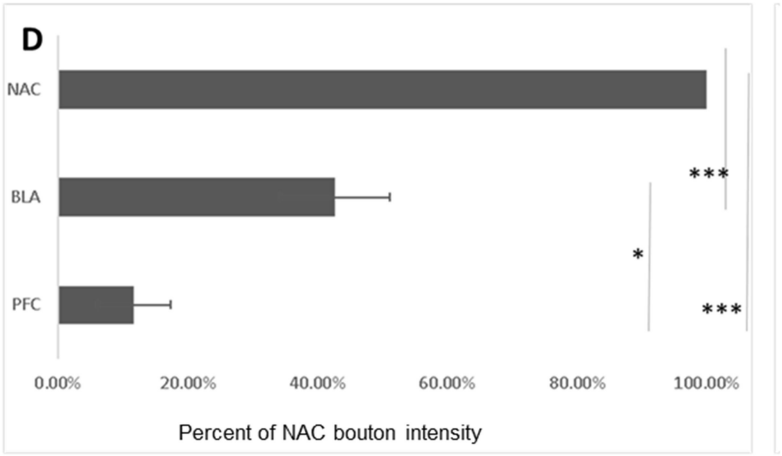
Figure 3.







500 microns



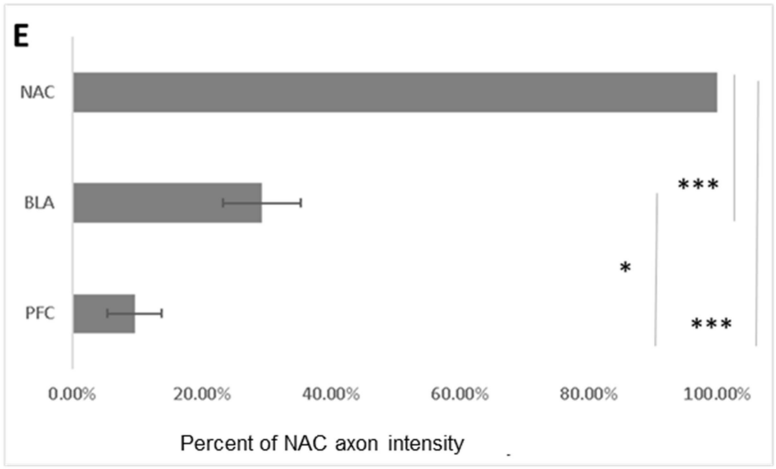


Figure 4.

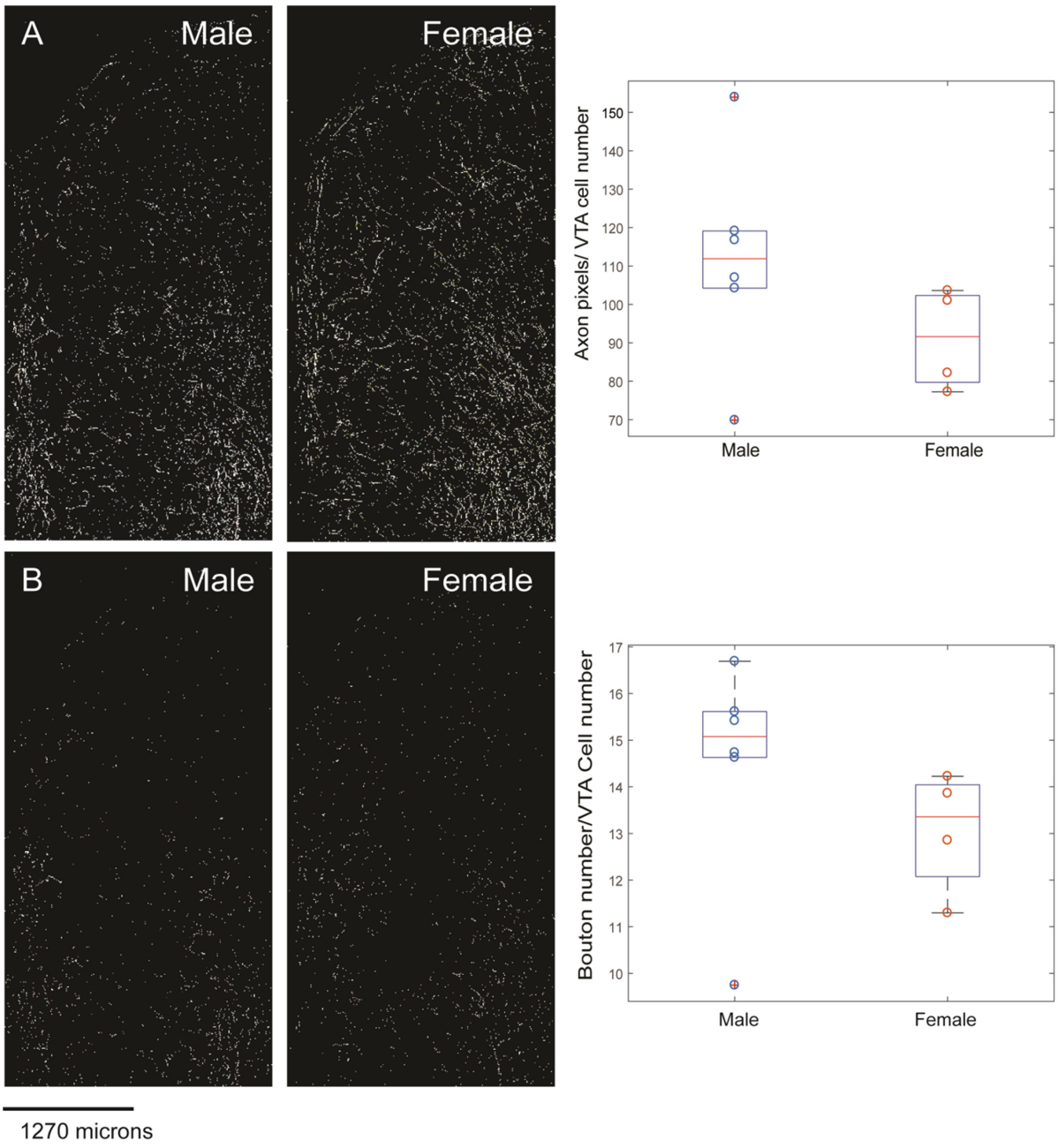

A
Male
Female
B
Male
Female
1270 microns
Axon pixels/ VTA cell number
150
140
130
120
110
100
90
80
70
Male
Female
Bouton number/VTA Cell number
17
16
15
14
13
12
11
10
Male
Female

Figure 5.

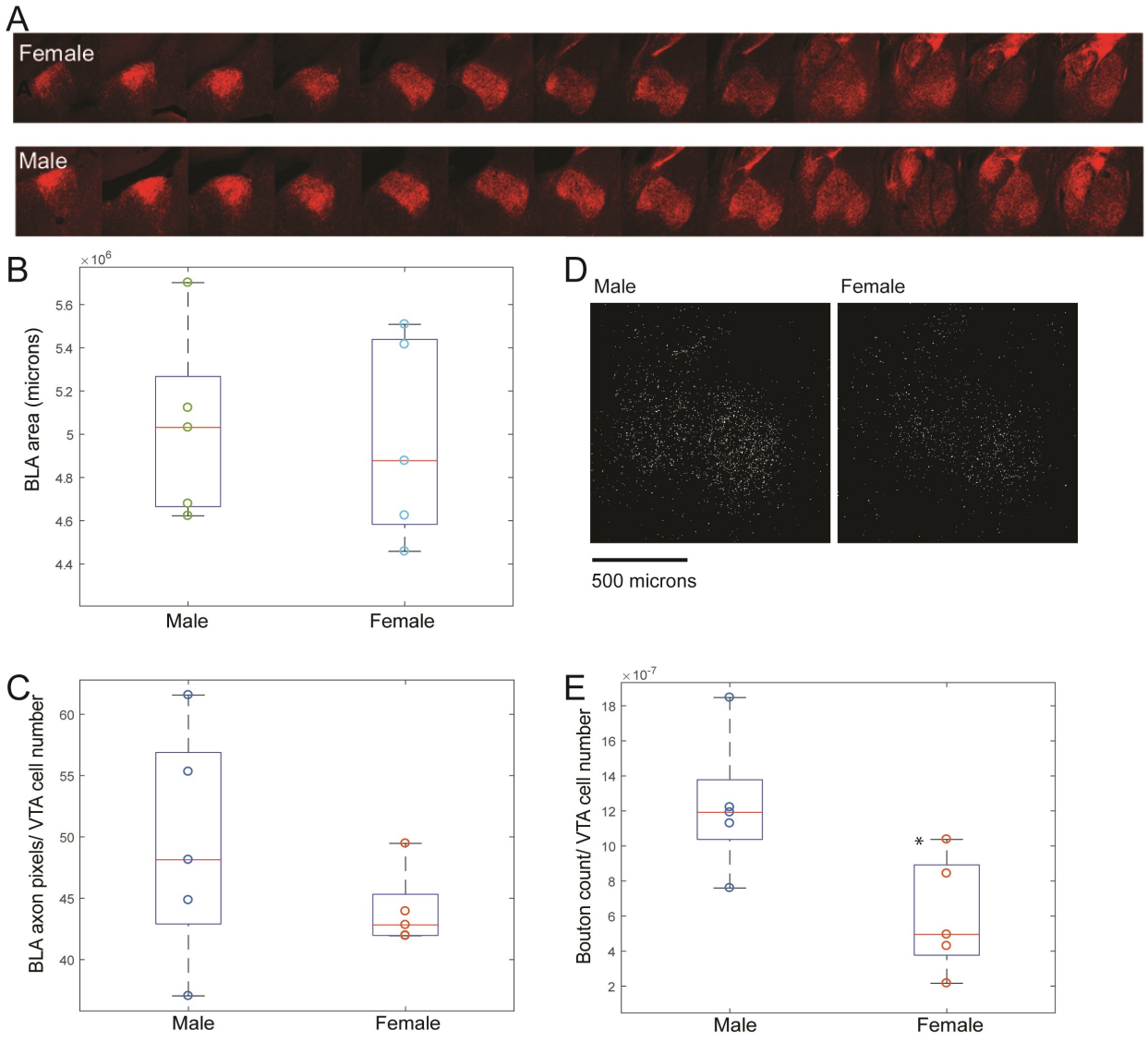

A
Female
Male
B
×10^6
BLA area (microns)
5.6
5.4
5.2
5
4.8
4.6
4.4
Male
Female
D
Male
Female
500 microns
C
BLA axon pixels/ VTA cell number
60
55
50
45
40
Male
Female
E
×10^-7
Bouton count/ VTA cell number
18
16
14
12
10
8
6
4
2
*
Male
Female

Figure 6.

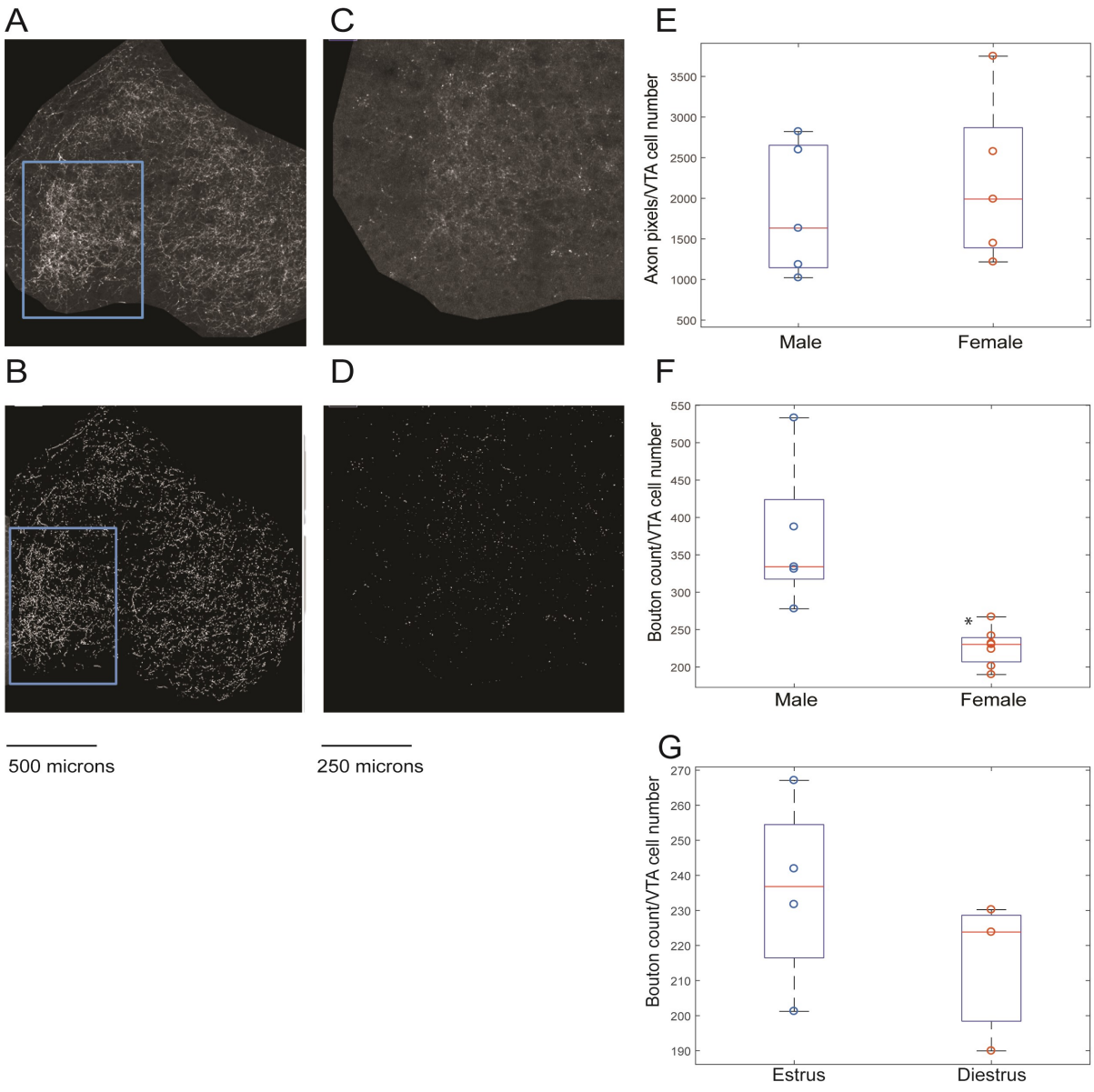

A
C
E
B
D
F
G
500 microns
250 microns
Axon pixels/VTA cell number
3500
3000
2500
2000
1500
1000
500
Male
Female
Bouton count/VTA cell number
550
500
450
400
350
300
250
200
*
Male
Female
Bouton count/VTA cell number
270
260
250
240
230
220
210
200
190
Estrus
Diestrus

Figure 7.

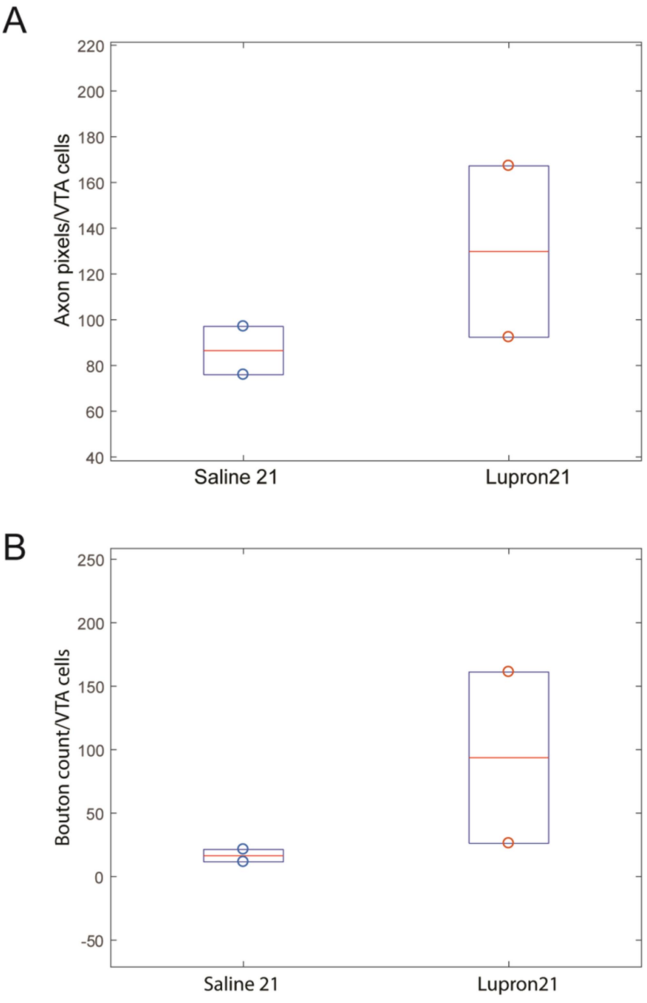

A
Axon pixels/VTA cells
220
200
180
160
140
120
100
80
60
40
Saline 21
Lupron21
B
Bouton count/VTA cells
250
200
150
100
50
0
-50
Saline 21
Lupron21

Figure 8.

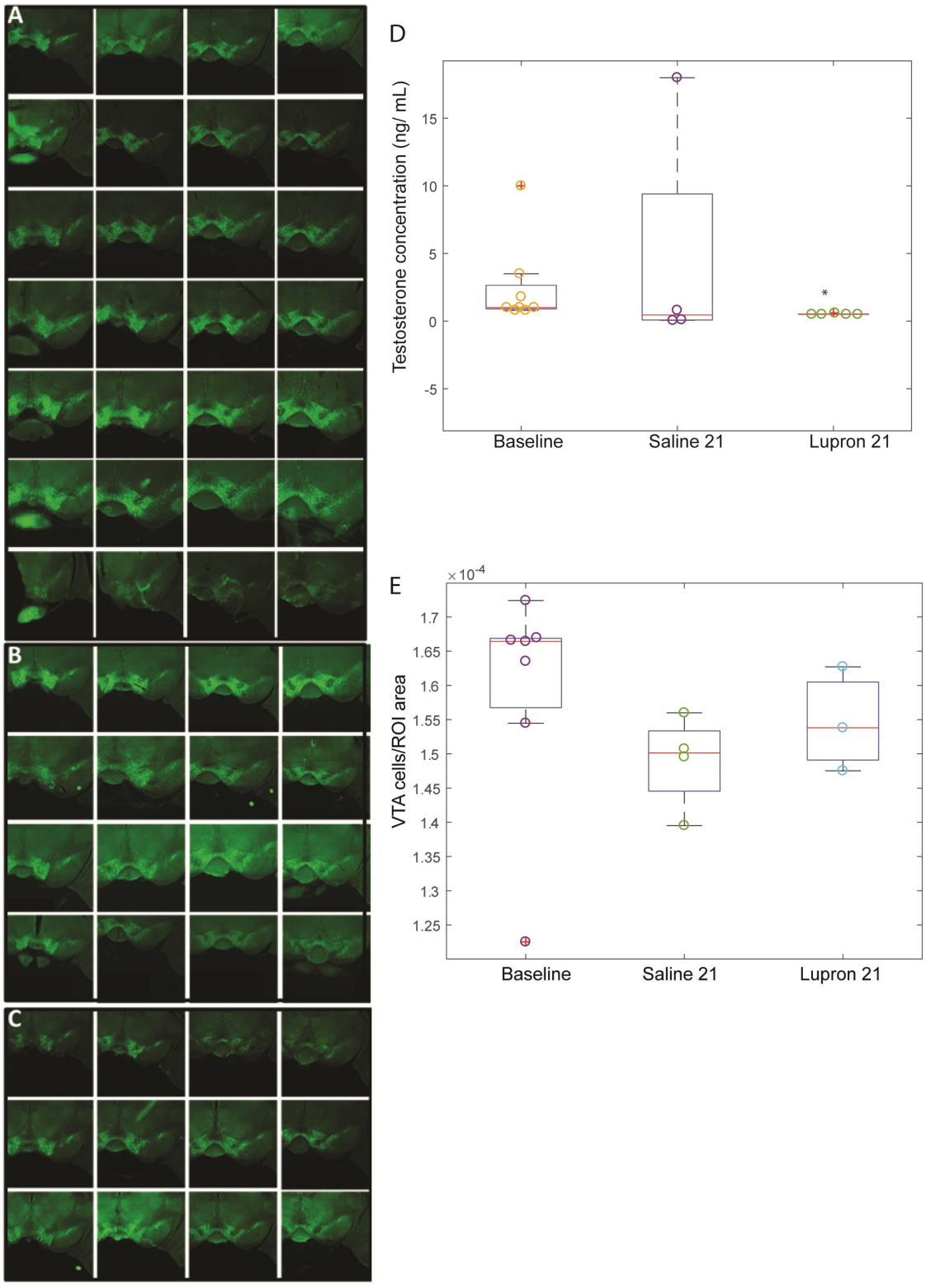
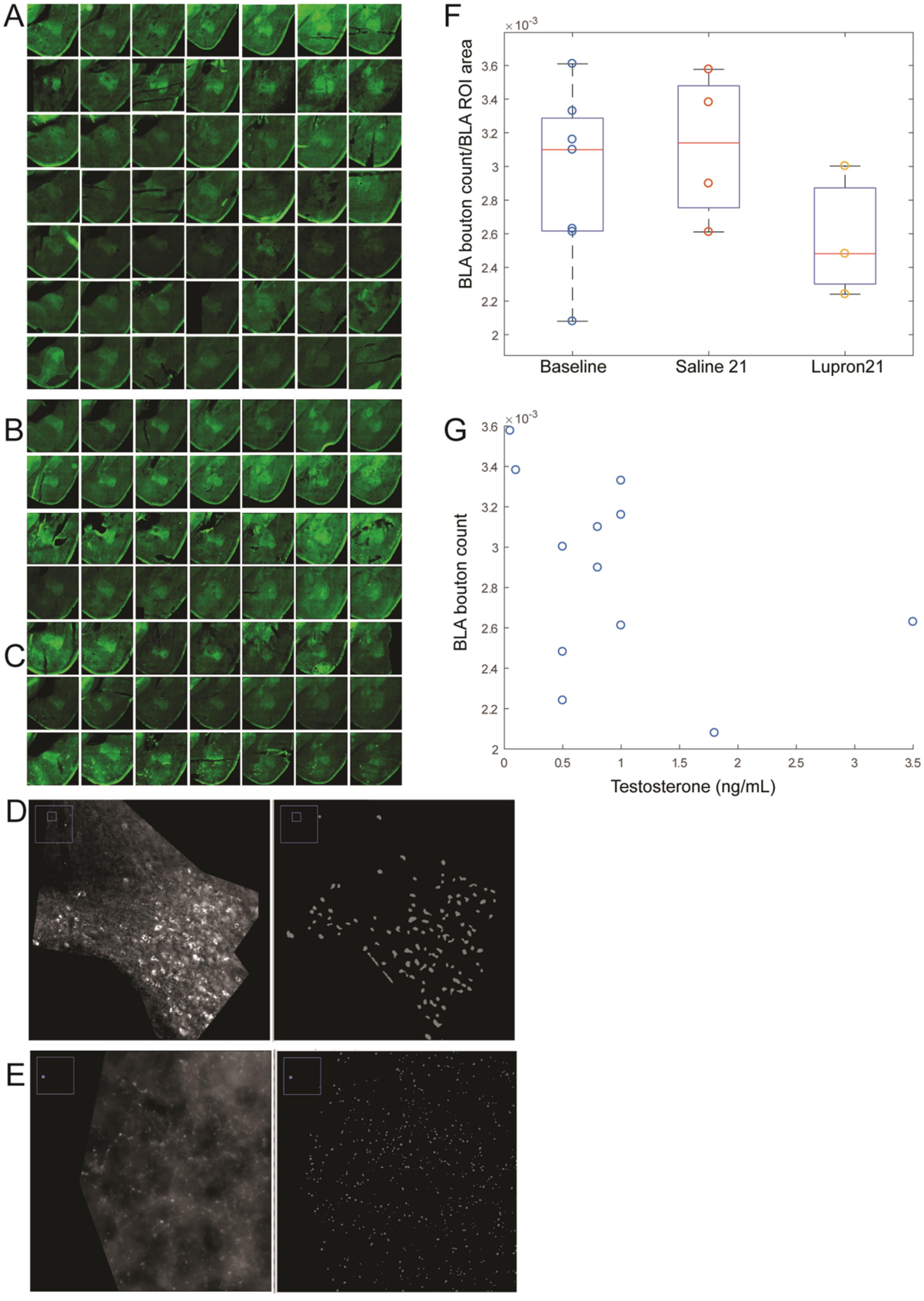

A
B
C
D
Testosterone concentration (ng/ mL)
15
10
5
0
-5
*
Baseline
Saline 21
Lupron 21
E
×10^-4
VTA cells/ROI area
1.7
1.65
1.6
1.55
1.5
1.45
1.4
1.35
1.3
1.25
Baseline
Saline 21
Lupron 21

Figure 9.


A
B
C
D
E
F
×10-3
BLA bouton count/BLA ROI area
3.6
3.4
3.2
3
2.8
2.6
2.4
2.2
2
Baseline
Saline 21
Lupron21
G
×10-3
BLA bouton count
3.6
3.4
3.2
3
2.8
2.6
2.4
2.2
2
0
0.5
1
1.5
2
2.5
3
3.5
Testosterone (ng/mL)

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