Developmental plasticity and circuit mechanisms of dopamine-modulated aggression

Darshini Mahadevia

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ABSTRACT

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Aggression and violence pose a significant public health concern to society. Aggression is a highly conserved behavior that shares common biological correlates across species. While aggression developed as an evolutionary adaptation to competition, its untimely and uncontrolled expression is maladaptive and presents itself in a number of neuropsychiatric disorders. A mechanistic hypothesis for pathological aggression links aberrant behavior with heightened dopamine function. However, while dopamine hyper-activity is a neural correlate of aggression, the developmental aspects and circuit level contributions of dopaminergic signaling have not been elucidated. In this dissertation, I aim to address these questions regarding the specifics of dopamine function in a murine model of aggressive behavior.

In **chapter I**, I provide a review of the literature that describes the current state of research on aggression. I describe the background elements that lay the foundation for experimental questions and original data presented in later chapters. I introduce, in detail, published studies that describe the clinical manifestation and epidemiological spread, the dominant categories, the anatomy and physiology, and the pharmacology of aggression, with a particular emphasis on the dopaminergic system. Finally, I describe instances of genetic and environmental risk factors impacting aggression, concluding with studies revealing an important role for interactions among genetics, environmental factors, and age in the development of aggression.

In **chapter II**, I investigate the developmental origins of aggression by examining sensitive periods during which perturbations to the dopaminergic system impact adult aggressive behavior. Previous work in our laboratory has concluded that periadolescent (postnatal days 22-41) elevation in dopamine, via transient dopamine transporter blockade, leads to increased adult aggression and heightened response to amphetamine. I expanded on these findings by temporally refining the opening and closing of this window of sensitivity, specifically to postnatal days 32 to 41, during which increases in dopaminergic tone increase adult aggression and behavioral sensitivity to psychostimulants. The potentiated response to amphetamine indicated to us a state of altered dopaminergic physiology. We next validated this hypothesis and found increased firing rate (*in vitro*), and increased bursting and population activity (*in vivo*) at baseline. These data indicate that elevated periadolescent dopamine impacts maturation of the dopamine system, leading to a hyper-active dopaminergic and aggressive predisposition. *In conclusion, this chapter introduces a developmental component to the hyper-dopaminergic model of aggression.*

In **chapter III**, I report a series of experiments exploring the direct and causal involvement of dopamine in driving aggression. While dopamine hyper-activity is a neural correlate of aggression, the precise brain circuits involved have not been elucidated. Using optogenetics, I established a causal role for the ventral tegmental area (a key source of dopamine) in aggression modulation. I further advanced this finding by demonstrating that the modulatory role of dopamine, is population- and projection-specific. I found that activity of ventral tegmental area, but not substantia nigra, dopamine neurons promotes aggression. Furthermore, controlled stimulation of ventral tegmental area dopaminergic terminals in the lateral septum, but not the nucleus accumbens, mediates increased aggression. I selectively traced connectivity between the lateral septum and the ventral tegmental area using a Cre-driven, population-specific viral vector.

I used this virus to show that anatomically distinct clusters of ventral tegmental area dopamine cells send projections to the lateral septum and the nucleus accumbens, thereby dissociating the two target sites both behaviorally and anatomically. Furthermore, I found that while local dopamine release in the lateral septum increases aggression, it has no bearing on reward behaviors thus indicating a stronger association with impulsive, and not motivated, aggression. *In conclusion, this chapter offers causal evidence for dopamine's role in modulating impulsive aggression by identifying a distinct pathway from the ventral tegmental area to the lateral septum that controls aggression.*

In the work described in **chapter IV,** my aim was to determine the mechanism underlying ventral tegmental area to lateral septum dopamine-mediated aggression. I first characterized the expression of dopamine receptors in the lateral septum and found that D2 receptors heavily colocalize with the dominant population of neurons in the lateral septum, i.e. GABAergic cells. Moreover, the D2 receptors are perfectly aligned with incoming dopamine afferents. Next we investigated, in acute brain slices, how D2 signaling affects lateral septum function. We revealed that activating D2 receptors hyperpolarizes D2-expressing lateral septum neurons. This effect was abolished with bath application of the D2 receptor antagonist, sulpiride. We validated the functional involvement of post-synaptic D2 signaling in a behavioral test, and found that the aggression induced by direct terminal release of dopamine at the lateral septum is reversed by acutely blocking local D2 receptor signaling. *In conclusion, this chapter demonstrates that the ventral tegmental area to lateral septum dopamine pathway, via D2-mediated inhibition of GABAergic lateral septum neurons, is necessary to drive ventral tegmental area-triggered aggression.*

In **chapter V**, I engage in a general discussion addressing how the findings from each chapter can be linked to provide a more comprehensive outlook on environmental and genetic risk factors that can modulate ventral tegmental area-triggered aggression. I discuss possible pre- and post-synaptic mechanisms that could impact the functionality of the identified dopaminergic ventral tegmental area to lateral septum pathway. Moreover, in distinguishing this specific dopamine circuit and lateral septum D2 signaling as an underlying correlate of violent pathology, this dissertation aims to evoke deeper understanding of the mechanism of current antipsychotics used to manage aggression. I end this dissertation by proposing new empirical questions, techniques and lines of research that could further develop my findings as well strengthen the links between dominant models of aggression that exist in the field today.

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DEDICATION

"I'm a woman
Phenomenally.
Phenomenal woman,
That's me."

Maya Angelou

I dedicate this thesis to my late grandmother, Usha Mahadevia.

You embodied power, resilience and a unique command over life.

I will forever aspire to be like you.

CHAPTER I: Aggression and Violence

a. Epidemiology, clinical presentation and management

Aggression, in its non-pathological form, is a behavior that manifests in many species, including humans. It is a complex social behavior, which is defined as an overt act that can occur when the interest of two or more individuals conflict (Nelson and Trainor, 2007). Functionally, it evolved across species as a beneficial phenomenon, to aid in dealing with competition for food, mates and space (Georgiev et al., 2013). However, while aggression has its survival advantages, in an escalated form, the costs could outweigh the benefits. When there is an increase in the intensity, persistence and duration of aggression, it can be maladaptive and inflict physical, psychological and social damage to the individual(s) involved.

In fact, violence and aggression are pervasive global issues, and represent a significant burden to society and public health. The World Health Organization (WHO) has reported a 1 year world-wide estimate of 1.3 million people dying from either self-inflicted, interpersonal or collective violence, with a much larger number of nonfatal victims too (Brown et al., 2007). The fatal cases account for 2.5% of global mortality. In fact, violence is the fourth leading cause of death for people between the ages 15-44 worldwide (GBD 2013 Mortality and Causes of Death Collaborators, 2015). Moreover, men are more aggressive than women and this difference is more pronounced for physical than psychological aggression (Eagly and Steffen, 1986). For example, homicide victims and perpetrators are disproportionately male (79% of victims and roughly 95% of perpetrators) and young (almost half of homicide victims are aged 15-29 years) (GBD 2013 Mortality and Causes of Death Collaborators, 2015). Between the years 2000 and 2014, about 6 million people globally were killed in acts of interpersonal violence, making homicide a more frequent cause of death than all wars combined during this period (GBD 2013 Mortality and

Causes of Death Collaborators, 2015). One quarter of all men and approximately half as many women report acts of physical aggression after age 18 (Robins and Regier, 1991). Victims of aggression suffer serious, lifelong health and social consequences and so it is important to understand how, and when, such violence most commonly surfaces.

Pathological aggression presents itself in many different ways and towards different targets (Nelson and Trainor, 2007). But the common symptom that unifies the sub categories of aggression is an explosive behavioral outburst, which causes harm, and is disproportionate in nature. These inappropriate expressions of aggression often occur as a positive symptom in neuropsychiatric and neurodegenerative disorders (Steiner et al., 2003; Haller and Kruk, 2006). Individuals suffering from mental disorders are at greater risk for violence in comparison to the general population (Otto, 1992). In fact, one of the primary reasons for admission to inpatient psychiatric facilities is aggression (Goldsmith et al., 1993). About 20% of psychiatric emergency room (ER) patients reveal a history of aggressive behavior (Steadman et al., 1994). Furthermore, patients discharged from psychiatric institutes are significantly more aggressive than controls from the community (Rabkin, 1979). The *Diagnostic and Statistical Manual of Mental Disorders*, *4th edition, Text Revision* (American Psychiatric Association, 2000) identifies aggression comorbidity with psychiatric disorders like drug abuse (Beck et al., 2014; Coccaro et al., 2016), schizophrenia (Hoptman, 2015), depression (Dolenc et al., 2014), autism spectrum disorders (Fitzpatrick et al., 2016), personality disorders (Anderson and Kiehl, 2014) and post-traumatic stress disorder (Miles et al., 2015), among others. This relationship between mental illness and aggression is well supported by evidence from criminal records, twin studies, ER patients, psychiatric inpatients and outpatients, or the general population. The precipitants of aggression, in such groups, are often blunted emotional processing, autonomic imbalances, cognitive distortions (Mattson, 2003),

frustration (frustration-aggression hypothesis) (Berkowitz, 1989), anger, irritation, fear, and in some cases, pleasure (Nelson, 2005).

Neurological degeneration and chemical alterations, caused by cell death, in conditions including dementia, traumatic brain injury and stroke are also strongly associated with aggression (Mattson, 2003). For example, a study on patients with Alzheimer's disease, reported that 44% (31 out of 70) patients were aggressive (Gormley et al., 1998). Patients in this clinical category reveal many structural differences in regions important for aggression control. The increasing incidence of aggression with different mental disorders warrants that interventions be specifically tailored to the patient's vulnerabilities and mental illness diagnosis.

Current aggression management includes a few lines of treatment. Pharmacotherapy, using neuroleptic drugs is amongst the most common approaches used by clinicians to manage violence in people suffering from mental disorders. First generation antipsychotics work primarily by sedation and have negative side effects such as tardive dyskinesia (Correll et al., 2004). Given the side effects, these antipsychotics are less appealing for cases of chronic aggressive behavior. Nonetheless, first generation antipsychotics like chlorpromazine and haloperidol are still used as a benchmark for the evaluation of new compounds developed for reducing violent behavior (Connor et al., 2003). The more current generation ('atypical' antipsychotics) of medications is less sedating, however they still produce negative side effects when administered chronically (Swann, 2003). For example, risperidone is effective for aggressive patients with autism spectrum disorder. However, its usage is associated with an increased risk of body-mass gain and other metabolic side effects (Simon et al., 2009). Mood stabilizers including lithium (Jones et al., 2011) and anticonvulsants like carbamazepine (Stanford et al., 2005) and oxcarbazepine (Mattes, 2005) are also utilized for aggression control, albeit less frequently. Although less efficacious, selective

serotonin reuptake inhibitors (SSRIs) are also used for the treatment of aggression. Fluoxetine is the most widely studied SSRI used for aggression. Use of fluoxetine results in decreased anger (Salzman et al., 1995) and verbal aggression (Coccaro, 1997) in patients with personality disorders. Finally, pharmacological strategies are often complemented with psychotherapies, either psychodynamic (transference-based therapy) or behavioral (dialectical behavioral therapy), which aid in the inhibition of aggressive responses (Siever, 2008).

Despite the existence of treatment strategies, aggression is still very prevalent as there are many shortcomings in the patient recovery process (Barlow et al., 2000). These include side effects (especially in cases of chronic aggression), lack of treatment opportunity or medical non-compliance (Mattson, 2003). It's worthy of noting that most of these reasons apply only to documented cases of patient perpetrators in official healthcare settings. However, at large, there are many undeclared cases of maladaptive aggression that remain under the radar and that present a significant public health concern. The alarming prevalence of documented, as well as undeclared psychiatric disorders globally, together with the high probability of aggression comorbidity, raises alarming cause for concern. This necessitates a deeper understanding of pathological aggression, which can aid in the development of better diagnosis and therapeutic interventions.

b. Subtypes of aggression

Aggression is a complex social construct. Its heterogeneous nature often complicates efforts to dissect its underlying neurobiology. This variability makes it imperative to identify the main categories of aggression, as well as associated traits and factors that might characterize each form. While there are many modes of classification, two dominant forms that largely encapsulate aggression are: (1) proactive or "cold-blooded" aggression that is more premeditated, planned and

directly motivated by a drive for appetitive reward and (2) reactive or "hot-blooded" aggression which occurs more impulsively in response to perceived external threat (Barratt and Felthous, 2003; Gollan et al., 2005; Meloy, 2006).

Proactive aggression is explicitly initiated in order to obtain something of value, such as an object, power, status, or social dominance (Rosell and Siever, 2015). It is closely related to the concept of predatory aggression. Individuals who belong to this category are more purposefully aggressive and are less likely to be volatile in affect (Vitiello and Stoff, 1997). For example, antisocial subjects of this subtype compared with normal controls show lower heart rate and skin conductance, both indicating a low-arousal state (Loeb and Mednick, 1977; Wadsworth, 1976). In fact, individuals who are diagnosed with antisocial personality disorder show unusually low autonomic responsiveness (Viding et al., 2007). This could blunt typical emotional responses and lead one to calculated expressions of aggression, where the assaultive behavior is rationalized as leading to a 'favorable' outcome, or positive outcome expectancy (Smithmyer et al., 2000; Walters, 2007). High profile incidents including mass killings, genocides or assassinations, may be driven by this type of aggression. This controlled subtype of aggression is regulated by higher cortical systems and is less dependent on the innate hypothalamic and limbic systems that mediate reactive aggression (Nelson and Trainor, 2007).

In contrast, reactive aggression is a more impulsive manifestation of pathological aggression. It is often a response to a perceived provocation or threat and is accompanied by anger, rage, or hostility and at times can also be self-directed (Vitiello and Stoff, 1997). It's abrupt and retaliatory nature is largely motivated by anger and fear, and more fundamentally, by the purpose of dispelling any unpleasant affect states (Rosell and Siever, 2015). Reactive aggression is explosive and associated with negative emotionality and hostile attribution bias i.e. a tendency to

over-attribute hostile intent to social cues (Crick and Dodge, 1996; Arsenio et al., 2009). This subtype of aggression results in sudden, heightened, enduring or inappropriate aggressive responses (Nelson and Trainor, 2007). Mental disorders that are correlated with increased autonomic arousal, including intermittent explosive disorder, post-traumatic stress disorder and depression, are all linked to reactive aggression. In considering the underlying basis, there is growing evidence that connects reactive aggression with specific neurotransmitter abnormalities (Knutson, 1999). In fact, subjects with reactive/impulsive aggression have decreased levels of serotonin metabolites in the CSF (Brown et al., 1979; Linnoila et al., 1983) Neurologically, reactive aggression has been reported in cases of specific insults involving the temporal or frontal lobes, that can eventually affect one's temperament and personality (Stein et al., 1995).

In clinical populations with severe pathological aggression, both subtypes are prominent, to varying degrees. Clearly, both high- and low-arousal states can lead to exaggeratedly inappropriate responses, with different neural substrates contributing in each context. In the next section of this chapter, I detail the anatomies in which these behaviors are rooted.

c. Neuroanatomy of aggression

The comorbidity of aggression with mental disorders is unsurprising given that the brain regions and circuits that modulate aggression are also implicated in other disorders. This overlap in anatomy makes it challenging to understand the independent biological basis of aggression alone. That said, given that aggression is largely related to affect processing, impulse control, and emotional decision making, these traits provide insight into what regions might be involved. Indeed, several brain structures that contribute to these processes are also involved in the regulation of aggression. Brain lesion and brain imaging studies in humans suggest possible aggression neural

substrates to focus on. These include parts of the limbic system and of the frontal cortex, which have also been modeled in animals to test their direct role in aggression (Kringelbach, 2005). Perturbations to these regions whether due to trauma, tumors, or even metabolic disruptions, can impact emotional function.

Hypothalamus:

The hypothalamus is a key attack node. It is classically discussed in light of the innate “fight or flight” response and it typically remains under the tonic inhibition of other structures in the brain (Wong et al., 2016). Many neural substrates of aggression including the septum, the amygdala and the frontal cortex interface with the hypothalamus to mediate aggression. Given that it’s a key aggression center, several studies have attempted to assess the role of the hypothalamus using diverse surgical, pharmacological, and genetic methods (Falkner and Lin, 2014). The first demonstration of the indispensability of the hypothalamus in rage expression was presented by Walter Hess in cats. He demonstrated that electrical stimulation of discrete parts of the hypothalamus, in awake and freely moving cats, led to a rage response and attack behavior (Hess, 1928). Later Bard and colleagues found that the physical dissociation of the forebrain area in cats (leaving the hypothalamus and its outgoing projections intact), led to extreme aggression in the form of hissing and paw striking, a phenomenon referred to as “sham rage” (Bard, 1928). In a different group of cats, the hypothalamus itself was surgically lesioned, and this abolished any signs of the rage response (Bard, 1928). These data pioneered the idea that an optimally functioning hypothalamus is essential for display of aggressive behaviors.

In the human, Sano and colleagues were the first group to lesion parts of posteromedial and lateral hypothalamus and reduce or abolish aggression in violent patients (Sano et al., 1970) – a procedure referred to as a “hypothalamotomy” that was successfully replicated by other groups

too (Dieckmann et al., 1988; Ramamurthi, 1988; Laitinen, 2001). Conversely, electrical stimulation of the same area induced aggression in humans, in a manner similar to that seen in laboratory animals (Bejjani et al., 2002). In a clinical setting, the hypothalamic hamartoma is a noteworthy example of hypothalamic aggression control. A subgroup of patients developing this malformation show increased aggression. In contrast, removing the growth abolishes this phenotype (Weissenberger et al., 2001; De Almeida et al., 2008). Finally, positron emission tomography (PET) studies in domestic violence perpetrators show that hypothalamic activation is associated with aggression (George et al., 2004).

In addition to work exploring the hypothalamic rage response in cats and humans, researchers extended their investigation of this topic to include work on chickens (Putkonen, 1966) cichlid fish (Demski, 1973), guinea pig (Martin, 1976), lizards (Sugerman and Demski, 1978) monkeys (Lipp, 1978) and rats (Panksepp, 1971; Olivier, 1977; Kruk, 1991). Most recently technical innovation has advanced the understanding of hypothalamic circuitry of aggression. Optogenetic stimulation of neurons in the ventrolateral subdivision of the ventromedial hypothalamus (VMHvl), a region referred to as the "hypothalamic attack area", causes male mice to initiate attacks, while pharmacogenetic silencing of the VMHvl reversibly inhibits inter-male aggression (Lin et al., 2011). These data provide a clear picture of how the hypothalamus is a key aggression node, which is modulated by more than one upstream region, and is evolutionarily conserved across many species (Haller, 2013).

<u>Septum:</u>

The septum projects to the hypothalamus and plays a strong role in regulating hypothalamic aggression (Hess, 1928; Spiegel et al., 1940; Maeda, 1978; Wong et al., 2016). Human patients with septal forebrain tumors experience changes in emotional behavior, measured by an elevation

in irritability and interpersonal aggression (Zeman and King, 1958). Individuals who suffered ischemic damage of the septal area display extreme rage behavior (Felten, 2010). While this phenomenon is observed in some humans, it has been most successfully documented in cats and rodents (Gotsick and Marshall, 1972). The ‘‘septal rage syndrome’’, first demonstrated in rats with septal lesions, is a robust phenomenon characterized by violent hyper-emotionality, hyper-irritability and hyper-reactivity (Brady and Nauta, 1953; Maeda, 1978). Specifically, the lesioned rats were described as “presenting a picture of striking alertness with limbs rigidly extended and eyes intently following the movements of the observer approaching the cage […] Rapidly approaching objects were attacked immediately with vigorous biting […] Attempts to capture or handle the animal were responded to by fierce attacks on the experimenter” (Brady and Nauta, 1953).

Within the septal area, it is the lateral septum (LS) that exerts a downstream break on medial hypothalamic function (Wong et al., 2016). Therefore, lesions disrupt downstream inhibition and result in exaggerated or inappropriate reactions to nonthreatening environmental stimuli (Albert and Chew, 1980; Wong et al., 2016). For example, lesions enhance the startle reaction to auditory stimuli (Sparks and LeDoux, 2000) and also lead to exaggerated reactions to normally non-noxious stimuli (Olton and Gage, 1976). Moreover, permanent lesions or pharmacological inactivation of the LS, dramatically drives an increase in the number of attacks toward conspecifics (Slotnick and McMullen, 1972; Potegal et al., 1981a). Lesions to the rostral LS cause hyper-defensiveness/hyper-irritability (Sodetz and Bunnell, 1970). In contrast, electrical stimulation of the LS suppressed aggression in cats and hamsters (Siegel and Skog, 1970; Potegal et al., 1981b; Siegel and Shaikh, 1997). Immediate early gene mapping reveals a negative correlation between LS activity and aggressive behaviors (Goodson et al., 2005). The LS is

dominantly GABAergic, and it exerts a top down inhibition on the hypothalamus. Indeed, optogenetic activation of these projection neurons, directly terminates attack behavior, through a shift in the balance between 'attack-excited' and 'attack-inhibited' cells in the medial hypothalamus (Wong et al., 2016). Taken together, these data demonstrate that the LS acts as an effective modulator of aggressive behavior.

Amygdala:

The limbic system consists of many components that interact with each other and project to areas of autonomic and somatomotor emotional regulation (Cardinal et al., 2002). A key part of this system is the amygdala, which is highly implicated in aggression control. The amygdala is functionally involved in processing emotionally salient events and in controlling fear responses, defensive reactions and emotional learning (Cardinal et al., 2002). The amygdala interacts with other regions like in the frontal cortex to control behavior (Davidson et al., 2000). Functional connectivity between the amygdala and ventral PFC, as indicated by resting-state fMRI, is significantly lower in aggressive schizophrenia patients as compared to healthy controls (Hoptman et al., 2010). Similarly, the coupling between the amygdala and medial OFC, that typically occurs while healthy participants view angry faces, is lacking in patients with intermittent explosive disorder. In this study, amygdala activation for both groups was positively correlated with scores on the Lifetime History of Aggression (LHA) scale, indicating a role for enhanced amygdala responsivity in aggression (Coccaro et al., 2007). Astonishingly, in the mid-twentieth century, electrolytic lesions of the amygdala were used as a treatment option for those who were deemed excessively aggressive (Heimburger et al., 1966). In rats, small lesions in the cortical amygdaloid nucleus reduced or eliminated attacks and signs of dominance in fights (Miczek et al., 1974). More recently, elegant work from the laboratory of Nirao Shah identified a genetically-specified group

of cells within the amygdala that play a strong role in aggression regulation. Specifically, chemogenetic silencing of a subset of aromatase+ medial amygdala neurons reduces both male aggression and maternal aggression (Unger et al., 2015). These studies highlight that aberrant function of the amygdala, even down to a small subset of neurons, is strongly associated with changes in behavioral disinhibition and violent behavior.

Cortex:

Finally, the frontal cortex is also important in the consideration of aggression etiology. A prominent example is the case of Phineas Gage, a railroad worker who was injured by a tamping rod that penetrated his skull and severely damaged his orbital frontal cortex (OFC). Following his injury he became angry, irritable and aggressive, all traits that were previously absent (Damásio et al., 2013). In fact, empirical work in humans supports a link between damage to the frontal cortex and increased aggressive behavior (Anderson et al., 1999) . Like Gage, patients who had suffered penetrating head injuries during their service in Vietnam (Grafman et al., 1996; Pardini et al., 2011) and patients with brain lesions in the OFC have higher aggression scores compared to patients with lesions in other brain regions and to normal controls (Grafman et al., 1996). Damage to the ventro-medial prefrontal cortex (PFC), is strongly associated with sociopathic tendencies and, reduced autonomic skin conductance responses to emotional visual stimuli (Damasio, 1996). Findings linking physical insult of frontal cortical areas to aggression are consistent with reports that show overall low baseline activity in the frontal cortex of individuals who rank highly on measures of reactive aggression (Hodgins, 2008). PET studies in subjects with impulsive personality disorders, or psychiatric patients with a history of violence, show relative hypo-metabolism in areas of the frontal and temporal cortex, compared with healthy control subjects (Goyer et al., 1994; Volkow et al., 1995; Soloff et al., 2003). Alzheimer's patients with behavioral disinhibition show decreased

metabolic activity in the OFC, PFC, and anterior cingulate cortex (ACC) – changes that are not present in dementia patients with intact impulse control (Kumar et al., 1990). Antisocial individuals who committed unplanned impulsive murders had lower PFC metabolic activity compared to controls (Yang et al., 2008). Imaging studies further support a role of the mPFC and OFC in modulating aggressive behavior. For example, patients with borderline personality disorder or antisocial personality disorder display reductions in mPFC and OFC volumes (Raine et al., 2000; Hazlett et al., 2005; Narayan et al., 2007). The PFC and the OFC exert top-down control of regions, including the hypothalamus and the amygdala, that promote aggression (Davidson et al., 2000).

The importance of this top-down control is also demonstrated in animal lesion work that shows deliberate insult to the OFC results in higher aggression in male rats (De Bruin et al., 1983). Electrical stimulation of the PFC in cats delayed the onset of predatory attack induced by hypothalamic stimulation, thus supporting the idea of inhibition from higher cortical networks (Siegel et al., 1974, 1975). More targeted optogenetic work in mice concluded that normal medial PFC activity is important for the initiation and execution of aggressive behavior. More specifically, optogenetic silencing of mPFC activity drives an increase in the number of attack bites during aggressive bursts (Takahashi et al., 2014). These examples highlight how loss-of-function states in frontal cortical regions could drive aggression by affecting processes in downstream subcortical regions, including the limbic system.

The neuroanatomy of aggression is extensive and complex. Figure 1.1 summarizes the roles of the regions and their connectivity in aggression modulation. In considering this network, it is safe to conclude that dysfunction may pose a problem for regulation of aggressive behavior. Therefore, optimal function of, and communication between, these brain regions is important in

protecting one from violent predispositions. In the next section of this chapter, I will discuss the role of neurotransmitter signaling in aggression etiology.

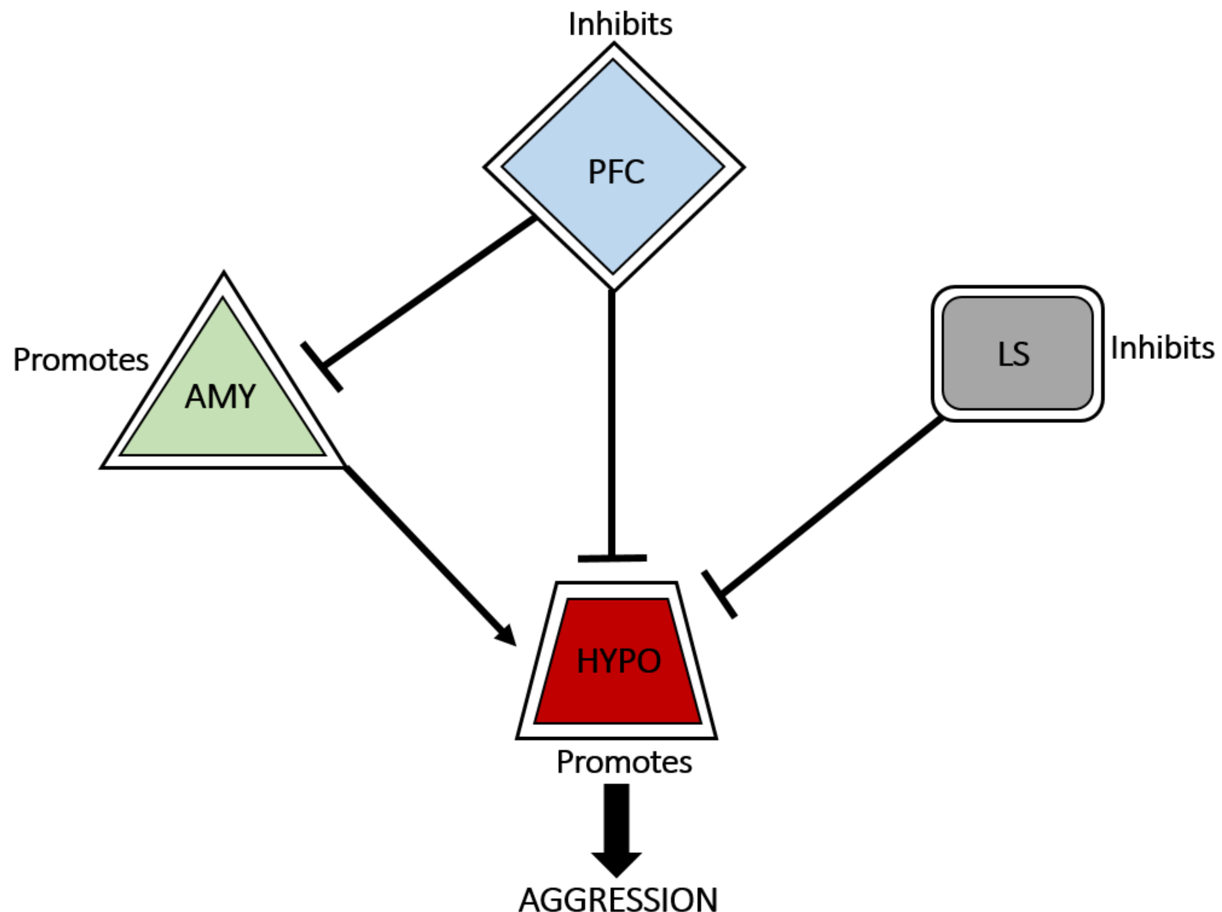

Figure 1.1: Model of the neuroanatomy of aggression. Key regions that either inhibit or promote aggressive behaviors. HYPO, hypothalamus; LS, lateral septum; AMY, amygdala; PFC, prefrontal cortex.

d. Neuromodulators and aggression

Like many behaviors, human aggression is a multi-determined act. Research into the etiologic determinants of aggression have focused on various environmental and biological factors. Among the biologic factors are abnormalities in the functioning of central neuromodulatory systems. Previously discussed aggression 'nodes' are innervated by, and communicate with each

other through, these signaling molecules. While there are many neuropeptides and neurotransmitters that have been implicated in regulating aggression, this section will focus on the ones that have been most notably linked to aggression, including γ-Aminobutyric Acid (GABA), serotonin (5-HT) and dopamine (DA).

GABA:

In mammalians, GABA is the main inhibitory neurotransmitter and exerts powerful control on many mechanisms of the brain (Narvaes and de Almeida, 2014). Thus, altered GABAergic activity may directly impact behavior, including aggression. For example, GABA reuptake inhibitors, like tiagabine (Lieving et al., 2008), diaminobutyric acid or nipecotic acid amide, decrease aggression (Allegra et al., 1979; Puglisi-Allegra and Mandel, 1980; Kršiak et al., 1981; Rodgers and Depaulis, 1982). The anti-aggressive effects of anticonvulsant agents (including carbamezapine, phenytoin and valproate) which act on GABAergic neurons, further underscore GABA's inhibitory control of aggression. In fact, these agents are often used as pharmacotherapies for patients with many diagnoses that co-occur with aggression (Barratt, 1993; Pabis and Stanislav, 1996). For example, carbamezepine is effective in managing aggression in adult schizophrenia (Neppe, 1988) and dementia patients (Tariot et al., 1998). Phenytoin decreased aggressive outbursts in prisoners with a history of impulsive aggression (Barratt et al., 1997) and in men with either obsessive-compulsive, anti-social or narcissistic personality disorders (Stanford et al., 2001). While anticonvulsants may directly reduce aggression, the action of allosteric modulators of GABAA receptors, including benzodiazepines, barbiturates and alcohol, is more paradoxical. Moderate doses evoke aggression, but low or high doses reduce aggressive behavior, thus following an inverted U-shaped dose–response curve, presumably through a more complex interaction with the serotonergic system (Narvaes and de Almeida, 2014).

Pre-clinical research implicating a role for GABA in aggression has been more prominent than human studies. Once again, for the most part, animal research has identified an inverse relationship between GABA activity and aggressive behavior (Miczek, Fish, & De Bold, 2003). For example, low levels of GABA and glutamic acid decarboxylase (GAD - the enzyme that catalyzes GABA production), were found in the brains of mice and rats that recently engaged in a bout of aggression (Clement et al., 1987). Pharmacological increases in GABAergic signaling reduces aggressive behavior in isolated mice (DaVanzo and Sydow, 1979; Puglisi-Allegra and Mandel, 1980; Kršiak et al., 1981; Poshivalov, 1981) and in mice that are aroused by electric tail shocks (Allegra et al., 1979). Zolpidem, a compound that has greatest selectivity for GABAA/α1 receptors, effectively sedates aggressive mice, and does not increase aggressive behavior at low doses like other benzodiazepines such as midazolam, diazepam and chlordiazepoxide (Miczek and O'Donnell, 1980; Rodgers and Waters, 1985; Gourley et al., 2005). In rats, shock induced defensive aggression (Rodgers and Depaulis, 1982) and muricide (Molina et al., 1986) decreases with an increase in GABA activity. In cats, the hypothalamically evoked defensive "rage" reaction is decreased by the GABA agonist muscimol (Luo et al., 1998). Using optogenetic approaches that are more targeted than traditional pharmacology, Dayu Lin's group found that activation of GABA inputs from the LS to the medial hypothalamus inhibited 'attack-excited cells' and increased activity of 'attack-inhibited cells' thus terminating ongoing attacks immediately (Wong et al., 2016). These data highlight the inhibitory role of GABA on aggression.

Serotonin:

In general, hypo-activity of the 5-HT system is correlated with increased impulsive aggression. In humans, low cerebrospinal fluid (CSF) concentration of the 5-HT metabolite 5-hydroxyindoleacetic acid (5-HIAA) is associated with impulsivity and aggression (Brown et al.,

1979; Linnoila et al., 1983; Kruesi et al., 1992; Virkkunen et al., 1994, 1995). Several studies also report a blunted neuroendocrine and central metabolic response to a pharmacological 5-HT challenge using fenfluramine in individuals with high aggression (Coccaro et al., 1996, 1997b; Siever et al., 1999). Importantly, the endocrine response to fenfluramine challenge also inversely correlates with self-rated aggression and impulsivity in a group of healthy controls (Manuck et al., 1998). Conversely, SSRIs reduce impulsive aggression (Coccaro, 1997; Berman et al., 2009) and signaling through the 5-HT2A and 5-HT2C receptors exerts opposing effects on impulsive behavior, with 5-HT2A antagonists reducing and 5-HT2C antagonists increasing impulsivity (Winstanley et al., 2004; Krakowski et al., 2006). Furthermore, orbitofrontal 5-HT2A receptor availability is increased in physically aggressive personality disorder patients (Soloff et al., 2007; Rosell et al., 2010). Interestingly, 5-HT depletion is implicated in decreased learning of cooperation and diminished perception of trustworthiness (Wood et al., 2006). 5-HT is also important for normal prefrontal cortical suppression of aggressive behaviors. Thus, deficiencies in 5-HTergic innervation of these frontal regions could be expected to cause aggression upon provocation (Siever, 2008). Human genetic-association studies have also linked the 5-HT system to aggression. Allelic variants in the *5-HTT* and *TPH1* are associated with aggression in some studies (Patkar et al., 2002; Davidge et al., 2004; Volavka et al., 2004; Winstanley et al., 2004), and a *TPH2* haplotype is associated with suicidal/parasuicidal behavior and aggression scores (Mercedes Perez-Rodriguez et al., 2010). Furthermore, the *Tyr452* allele of the *HTR2A* has been associated with childhood onset aggression (Mik et al., 2007).

Animal models in rhesus and macaque monkeys further support a correlation between aggression and lower 5-HTergic activity in freely moving primates (Dee Higley et al., 1992). In rhesus macaques and vervet monkeys, levels of 5-HIAA in the CSF are negatively correlated with

aggression (Dee Higley et al., 1992; Higley et al., 1996a, 1996c), risk taking (Higley et al., 1996b), and impulsivity (Mehlman et al., 1994; Fairbanks et al., 2004). Silver foxes displaying low levels of defensive aggressive behavior, express much higher 5-HT levels in specific brain regions than their more aggressive counterparts (Popova, 2004). In rats, prefrontal extracellular 5-HT declines to 80% of baseline levels during aggressive encounters (van Erp and Miczek, 2000). Furthermore, manipulations that lower 5-HTergic signaling, such as PCPA injections, increase impulsivity and aggression, whereas increasing 5-HT signaling using 5-HT precursors or SSRIs can reduce aggressive behavior in rodents (Koe and Weissman, 1966; Di Chiara et al., 1971; Chiavegatto et al., 2001; Miczek et al., 2001). The link between 5-HT and aggression has been further established using genetically modified mouse models. Pet1 knockout mice, which have an 80% reduction in the number of 5-HTergic neurons, exhibit increased aggression (Hendricks et al., 2003). Likewise, life-long 5-HT depletion resulting from *TPH2* deletion (Alenina et al., 2009; Angoa-Pérez et al., 2012; Mosienko et al., 2012) or *TPH2* hypo-function (Beaulieu et al., 2008) increases adult aggression and impulsivity. Conversely, in mice lacking the 5-HTT, increased extracellular 5-HT is associated with reduced aggression and social approach behavior (Bengel et al., 1998; Holmes et al., 2002; Mathews et al., 2004; Kim et al., 2005; Page et al., 2009). Supporting the model in which 5-HT1B receptor signaling exerts inhibitory control over aggressive behavior, male mice that lack 5-HT1B receptors exhibit increased aggressive behavior (Saudou et al., 1994; Brunner and Hen, 1997; Zhuang et al., 1999). More specifically, forebrain 5-HT1B heteroreceptors participate in the development of neural systems that underlie aggression (Nautiyal et al., 2015). Activation of 5-HT1A receptors reduces aggressive behavior (Miczek et al., 1989; Olivier et al., 1995; De Boer and Koolhaas, 2005; Bannai et al., 2007; Centenaro et al., 2008). Decreasing 5-HTergic activity during adulthood, using a pharmacogenetic approach, increases territorial

isolation-induced aggression using the resident–intruder assay (Audero et al., 2013). Finally, recent work identified a subpopulation of 5-HTergic neurons in the dorsal raphe that modulate aggression via interaction with the dopaminergic system (Niederkofler et al., 2016). Considered together, these studies support the 5-HT hypothesis of aggression, which states that 5-HTergic hypo-function increases aggression.

Dopamine:

In addition to 5-HT, multilevel analyses have implicated dopamine (DA) neurotransmission in aggression. More specifically, hyper-activity of the DA system is associated with increased impulsive aggression. In humans, levels of DA metabolites in the CSF of violent offenders positively correlate with psychopathy (Soderstrom et al., 2001). Patients suffering from schizophrenia, who display high levels of aggression, show an upregulation of the D_2 receptor in the striatum (Hirvonen et al., 2005). In clinical practice, particularly with schizophrenic patients, antipsychotic drugs primarily acting on DA receptors are used in the management of psychotic outbursts that include aggressive and violent behavior (Citrome and Volavka, 1997a, 1997b). Typical and atypical antipsychotic agents that antagonize the D2 receptor attenuate pathological aggression (Brizer, 1988; Lenox et al., 1992; Chengappa et al., 1999; Dorevitch et al., 1999; Schulz et al., 1999; Rocca et al., 2002). For example, the use of risperidone is common in autism and schizophrenia patients who are also aggressive (Soyka et al., 2007; Bronsard et al., 2010). Chronic clozapine significantly reduces the frequency of aggressive acts, including a decrease in violent assaults on staff (Glazer and Dickson, 1998; Ian Hector, 1998; Volavka, 1999). However, low levels of D2/D3 receptors in the rodent nucleus accumbens (NAc) and decreased D2/D3receptor binding in the midbrain of the human, are likewise correlated with impulsive behavior (Dalley et

al., 2007; Buckholtz et al., 2010), still highlighting the DA system but possibly suggesting compensatory adjustment.

Human genetic-association studies have also linked the DA system to aggression. Mutations in the gene coding for catechol-O-methyltransferase (*COMT*), responsible for the degradation of catecholamines including DA (Meyer-Lindenberg et al., 2005), have been linked to aggression (Hosak, 2007). Among people with a diagnosis of axis II personality disorders, the low-expressing G allele of the *COMT* rs165599 SNP is associated with self-reported aggression (Flory et al., 2007). Likewise, among individuals diagnosed with schizophrenia, the low-activity Met allele of the *COMT* Val158Met polymorphism is associated with high aggression (Lachman et al., 1998; Kotler et al., 1999; Strous et al., 2003; Han et al., 2004; Volavka et al., 2004; Hong et al., 2008; Gu et al., 2009; Bhakta et al., 2012; Koh et al., 2012; Singh et al., 2012). DA receptor gene variants (*Drd2* and *Drd4* mutations) interact to predict adolescent conduct disorder and adult antisocial behavior (Beaver et al., 2007), as well as dysfunctional impulsivity (Colzato et al., 2010). Differences in inhibitory control are associated with the *Drd4* VNTR polymorphism (Congdon et al., 2008). Collectively, these human studies support the DA hypothesis of aggression, which states that DAergic hyper-function increases aggression (De Almeida et al., 2005; Seo et al., 2008).

Results from preclinical animal models are congruent with human work. For example, *in vivo* microdialysis showed that NAc DA release increases in anticipation of aggressive episodes (Malison et al., 1998; Ferrari et al., 2003), and NAc and PFC DA release increases during and following aggressive encounters in rats (Tidey and Miczek, 1996; van Erp and Miczek, 2000). Early laboratory research on the influence of DA on aggressive behavior found that apomorphine or amphetamine increases aggressive behavior in isolated rats and mice (Crowley, 1972;

Hasselager et al., 1972; Ray et al., 1983). Systemic administration of methamphetamine, or the DA receptor agonist apomorphine, decreases the threshold for defensive attack behavior elicited by electrical stimulation of the ventromedial hypothalamic (VMH) nucleus in cats (Maeda et al., 1985; Maeda and Maki, 1986). More specifically, local microinjections of apomorphine or D_2 receptor agonist into the medial preoptic anterior hypothalamus (mPO-AH) facilitates feline affective defense behavior elicited by electric stimulation of the VMH (Sweidan et al., 1991). Conversely, D_2 receptor antagonist infusion into the AH of an aggressive Syrian hamster model dose dependently suppresses aggressive behavior (Schwartzer and Melloni, 2010). Systemic administration of the D1/D2 receptor antagonist risperidone (Rodríguez-Arias et al., 1998), the D2 receptor antagonist raclopride (Aguilar et al., 1994), and the D1 antagonist SCH23390 (Rodríguez-Arias et al., 1998) all reduce aggression. Furthermore, blockade of D1 or D2 receptors in the NAc attenuates aggression in mice (Couppis and Kennedy, 2008). Mice lacking the dopamine transporter (DAT) exhibit a hyper-DAergic tone, which correlates with hyper locomotion (Giros et al., 1996), and increased reactive aggression following mild social contact (Rodriguiz et al., 2004). Adult aggressive behavior is sensitive to periadolescent (postnatal days: P22-41) DA manipulations. Specifically, transient DAT blockade during periadolescence mimics the adult hyper-aggressive phenotype found in MAOA-deficient mice (Yu et al., 2014). Moreover, cocaine, which blocks the DAT, significantly escalates aggression when administered during adolescence (Harrison et al., 2000; DeLeon et al., 2002), and methamphetamine significantly increases aggression in male mice when administered chronically (Sokolov et al., 2004; Sokolov and Cadet, 2006). Heterozygous catechol-o-methyl transferase (COMT)-deficient male mice exhibit increased frontal cortex DA levels and increased aggression (Gogos et al., 1998). Thus, there is strong evidence of DAergic modulation of aggressive behavior, specifically involving the

D2 receptor type. This unique role for DA in modulating aggression is the focus of my dissertation and will be discussed in greater detail in the following chapters.

In conclusion, the GABA, 5-HT and DA systems show substantial changes associated with the aggressive phenotype. Biogenetic changes or environmental perturbations in these systems might produce profound changes in circuitry, structure and function of the brain, all of which could contribute to abnormal aggressive behaviors. Consequently, it becomes important to understand how such internal and external factors may impact aggression.

e. Genetic and environmental factors of aggression

Aggression is influenced by both genetic and environmental factors which shape how it is expressed. The influence of genetics on aggression, especially the impulsive subtype, has been demonstrated by a number of twin and adoption studies (Coccaro et al., 1993, 1997a). In humans, heritability estimates for aggressive behavior in people are worthy of note. For example, data from a study of 3-10 year old Dutch twins ranged from 51 to 72%, higher than the average heritability range for most human behaviors (Hudziak et al., 2003). More broadly, a meta-analysis study found that impulsive aggression has a 44%-72% heritability rate (Miles and Carey, 1997).

The most salient empirical evidence rooting aggression in human genetics was from a study done by Brunner and colleagues on a large Dutch family (Brunner et al., 1993). Many men in this family were classified as suffering from borderline mental retardation and violent aggression, most prominently of the impulsive and reactive type. Their pathological tendencies also included arson, attempted rape, and exhibitionism. In examining the underlying commonalities between all affected individuals, researchers found a loss-of-function mutation in the monoamine oxidase A gene (*MAOA*). MAOA catalyses the oxidative deamination of monoamines (Shih et al., 1999).

This study was followed up by others that implicated this gene in aggression etiology. Subjects with low activity of the *MAOA* gene display more aggression and exhibit significant volume reductions in regions involved in aggression including the amygdala, ACC, and subgenual ACC (Meyer-Lindenberg et al., 2006). A low-expressing polymorphism in the *MAOA* gene, the *MAOA* VNTR, is also associated with aggressive behavior (Manuck et al., 2000; Zalsman et al., 2005; Jabbi et al., 2007). The discovery that monoamines play a critical role, genetically and pharmacologically, in modulating aggressive behavior directed significant attention to the role of 5-HTergic and DAergic genetic variants in aggression research.

In the 5-HTergic system, several polymorphisms have been associated with aggression (Suri et al., 2015). College students, who have reduced 5-HT1B receptor expression, indicated a greater history of aggressive behaviors than controls (Takahashi et al., 2012). Alleles in the tryptophan hydroxylase-1 (*TPH1*) (Nielsen et al., 1994; New et al., 1998) tryptophan hydroxylase-2 (*TPH2*) genes (Zhang et al., 2004; Zhou et al., 2005), which impact 5-HT biosynthesis, have not only been associated with aggression in personality disorder patients, but are also related to affective instability in healthy controls (Gutknecht et al., 2007).

Moreover, exploration into the DAergic system has yielded other candidate genes too. Mutations in the gene coding for catechol-O-methyltransferase (*COMT*), responsible for the degradation of catecholamines including DA (Meyer-Lindenberg et al., 2005), have been linked to aggression (Hosak, 2007). For example, the low-expressing G allele of the *COMT* rs165599 single nucleotide polymorphism (Flory et al., 2007) and low-activity of the *COMT* (V158M) allele (Rujescu et al., 2003) are associated with aggressive personality traits. Genetic variations in *Drd1* and *Drd3* genes predispose Alzheimer's patients to psychotic and aggressive outbursts (Sweet et al., 1998). Gene variants in other receptors from the DA family, *Drd3* and *Drd4*, interact

to predict adolescent conduct disorder and adolescent antisocial behavior (Beaver et al., 2007; Congdon et al., 2008). Carriers of the 10-repeat allele of DA transporter gene (*DAT1*), responsible for the reuptake of DA, are predisposed to aggressive and antisocial dispositions (Vaughn et al., 2009). Finally, pathological violence is positively correlated with *Drd2* and *DAT1* polymorphisms in adolescents (Chen et al., 2005). These examples strongly suggest a genetic contribution to pathological aggressive behavior in humans.

Preclinically, evidence for the genetic roots of aggression extends across species. High aggression heritability estimates have been reported in monkeys (Fairbanks et al., 2004), dogs (Saetre et al., 2006), birds (Drent et al., 2003) and mice (van Oortmerssen and Bakker, 1981). Interestingly, when inbred strains of mice were first evaluated for variance, aggression was one of the primary behaviors that appeared different among the strains (Scott, 1942). In fact, mice are sometimes bred to select for either increased or decreased aggressive behavior (Anholt and Mackay, 2012). For example, strains are bred to display short attack latency or long attack latency (Sluyter et al., 1995). Furthermore, many of the specific genes linked to human aggression including *MAOA*, *5-HT1B* and *DAT1*, have been successfully modeled in animals as well. To elaborate, genetic deletion of MAOA in mice increases aggression and neophobia (Cases et al., 1995; Godar et al., 2011). Conversely, restoration of MAOA expression from postnatal day 1 in MAOA knockout mice normalizes levels of monoamines and eliminates the aggressive phenotype (Chen et al., 2007). Increased aggression and decreased impulse control is successfully modeled in male mice lacking the 5-HT1B receptor subtype, suggesting an inhibitory role for signaling through this receptor in wild-type mice (Saudou et al., 1994; Zhuang et al., 1999; Bouwknecht et al., 2001). DAT knockout mice display a similar aggressive phenotype. When compared to controls they exhibit a hyper-DAergic tone, which correlates with increased aggression following

mild social contact (Rodriguiz et al., 2004). These models directly demonstrate how genetic factors can impact the likelihood of developing aggression.

It is clear that aggression could be rooted in multiple susceptibility genes. However, since aggression doesn't manifest itself in a vacuum, it is vital to consider it in the broader context of the environment as well. In fact, important progress has been made in identifying environmental risk factors that can impact the manifestation of aggression (Anholt and Mackay, 2012). Factors including socio-economic circumstances, stress and childhood maltreatment interact with different genetic variants to predispose one to aggression (Moffitt, 2005; Craig, 2007). As previously described, deficits in activity of the *MAOA* gene are associated with increased aggression, both in humans and animal models. Advancing this finding, Caspi and colleagues demonstrated that the environment interacts with repeat length polymorphisms in the *MAOA* gene to predispose an individual to be more violent (Caspi et al., 2002). Specifically, they found that if an abused child carried the short form of the *MAOA* promoter gene, which causes decreased MAOA activity, the effect of abuse on behavior would be significantly stronger. In fact, this group of children was more antisocial, was more likely to develop conduct disorder and had a higher probability of committing violent offences than abused children with high MAOA activity. A subsequent meta-analysis conducted by Kim-Cohen and group concluded that low MAOA activity, combined with parental maltreatment, was again associated with high aggression (Kim-Cohen et al., 2006). Beyond these seminal reports, many groups have continued to document gene–environment interactions and their impact on young and adult aggression (Foley et al., 2004; Haberstick et al., 2005; Huizinga et al., 2006; Fergusson et al., 2011).

The effects of gene–environment interactions can be seen when environmental factors interact with genes regulating separate monoaminergic systems as well. In the 5-HTergic system,

the short allele of the *5-HTT* gene is associated with reduced expression of the protein. This in turn results in inefficient reuptake of 5-HT from the synapse and is linked to exaggerated stress responses (Greenberg et al., 1999; Barr et al., 2004). Men, but not women, who were homozygous for the short allele of the *5-HTT* gene were more likely to administer shocks to an experimenter in a stressful condition (Verona et al., 2006). In the DAergic system, children exposed to the combined risk of the *Drd4* 7-repeat polymorphism and insensitive care were at much greater risk for developing aggressive outbursts than children exposed to either or none of the risks (Bakermans-Kranenburg and Van Ijzendoorn, 2006).

Laboratory animal studies have also probed the impact of gene-environment interactions on aggression. Work in rhesus monkeys showed that monkeys that were reared under adverse social conditions (small social groups versus large interactive groups) were more aggressive, especially when they carried the low activity-associated *MAOA* allele (Karere et al., 2009). In rodents, isolating 5-HT1B knockout male mice (rather than group housing them) increased isolation-induced aggressive behavior to a greater degree than in singly housed wild-type mice (Saudou et al., 1994; Bouwknecht et al., 2001). Most recently, research shows that progesterone receptor-expressing neurons in the VMH can drive aggression in solitary, but not socially housed, males unless their pheromone-sensing is disabled (Yang et al., 2017). Together, these examples demonstrate how various risk factors interact to control the development of aggression.

Aggressive behavior is dynamic and multi-dimensional. The research detailed above highlights how genes, the environment, and both together can predispose one to develop pathologically aggressive tendencies. In evaluating this interaction, it is important to consider that an individual's development passes through sensitive periods, guided by both internal and external factors. While allowing for adaptation, such sensitive periods also serve as vulnerability windows

during which genetic blueprints and/or the environmental context could confer risk of developing disorders by derailing otherwise resilient developmental programs (Suri et al., 2015). In the context of aggression development, such sensitive periods could shape the formation of specific neurocircuits that mediate and control aggression. Therefore, an analysis of the relationship between predisposing biological factors, life experiences, and timing would lead to a clearer understanding of the etiology of aggression. This gene x environment x time interaction, as it relates to aggression, is detailed in the following chapter.

CHAPTER II: Periadolescent dopamine transporter blockade is associated with altered adult behavior and physiology

a. Introduction

Sensitive periods of development

The influence of changing experiences on the brain is not the same at every stage of development. Individuals go through developmental phases referred to as "sensitive periods", during which the effects of experiences on neural circuits, and subsequently behavior, are remarkably strong for a limited window of time (Knudsen, 2004). These sensitive periods encompass mechanisms of plasticity that dynamically guide the brain to adapt its structure and function in response to environmental experiences and physiological and genetic alterations (Pascual-Leone et al., 2005). This concept has been investigated at different ages, including infancy (Kuhl et al., 2003; Hannon and Trehub, 2005; Bosseler et al., 2013), early childhood (Bailey and Penhune, 2012; Putkinen et al., 2013), adolescence (Conway et al., 2005; Murre et al., 2013) and even adulthood (Finn et al., 2013). From these examples, it is evident that neuronal activity during sensitive periods can determine the 'wiring' of the mammalian brain, through mechanisms which allow complex behaviors to develop normally (Suri et al., 2015).

While sensitive periods are mainly adaptive from an evolutionary perspective, heightened plasticity during these phases also allow environmental and genetic factors to shift ontogenetic pathways and confer risk for disorders. The best-studied example that demonstrates this, is the sensitive period for the formation of ocular dominance columns, where retinal activity is necessary for the development of regular columnar size (Hensch, 2005; Espinosa and Stryker, 2012). Other seminal work done in this system links early monoaminergic signaling to normal barrel field formation, as evidenced by the disruptions in monoamine oxidase A (MAOA) and serotonin

transporter (5-HTT) knockout mice (Cases et al., 1996; Salichon et al., 2001). While developmental aberrations are most commonly recorded in sensory systems, there are other pertinent studies that highlight the importance of sensitive periods in affective and cognitive psychiatric disorders.

There is a wealth of evidence that presents a case for sensitive time windows in the development of circuits underlying depression and pathological anxiety (Gross et al., 2002; Gingrich et al., 2003; Ansorge et al., 2007). Specifically, 5-HTT knockout mice display behavioral alterations including increased novelty-suppressed feeding, latency to escape shock, immobility in the tail suspension test and immobility in the forced-swim test. Surprisingly, these are in direct contrast to the actions of selective serotonin reuptake inhibitors (SSRIs) in adult mice (Lira et al., 2003). Furthermore, many aspects of this behavioral phenotype are replicated in mice treated with SSRI, fluoxetine, from postnatal days (P) 4-21, but not in adulthood (P56–70 or P90–107) (Ansorge et al., 2004, 2008). This SSRI-sensitive period has been further refined to P2–11, with additional behavioral deficits including impaired hippocampal-dependent spatial learning and contextual fear learning, as well as diminished amygdala and prefrontal cortex (PFC)-dependent fear extinction and extinction recall (Rebello et al., 2014). Collectively, these data confirm that the effects of 5-HTT knockout are particularly due to its absence during this sensitive developmental window.

In autism, while there is a strong genetic component, external perturbation can wreak damage too. Supporting this claim, Stromland and colleagues (Strömland et al., 1994) found a strong correlation between autism and thalidomide exposure between gestational days 20 and 24. This period also corresponds with closure of the neural tube and production of the first neurons that form the motor nuclei of the cranial nerves. Moreover, these individuals had ear deformities

and associated hearing deficits, providing further evidence for the grave consequences of this specific temporal disturbance on behaviors that are related to autism.

Finally, in schizophrenia, developmental dysregulations of the dopaminergic (DAergic) system have been linked to the classic symptoms of the disease (Baumeister and Francis, 2002; Simpson et al., 2010; Grace, 2016). Compelling evidence of the same was drawn out by Kellendonk and colleagues who reversibly increased levels of dopamine receptor D2 (D2R) only in the striatum of mice (Kellendonk et al., 2006). These mice displayed specific working memory deficits, which persisted even after turning off the D2 overexpression, indicating that developmental, not constitutive, overexpression of D2Rs underlies schizophrenia's cognitive etiology. Furthermore, alterations in dopamine (DA) levels, rates of DA turnover, and activation of DA receptor D1 (D1R) receptors in the PFC were also reported in the D2 overexpressing mice (Kellendonk et al., 2006). This study is a strong example of how sensitive period disturbances can lead to aberrant development.

Adolescence as a sensitive period of development

Much of the research on sensitive periods has focused on early childhood. However, plasticity in later developmental periods, such as adolescence, has received less attention. It's only recently that adolescence is beginning to be viewed as a second wave of heightened malleability (Selemon, 2013; Blakemore and Mills, 2014; Ebert, 2015). The World Health Organization (WHO) has defined human adolescence as being between the ages of 10 and 19 i.e. the period of life that encompasses puberty (Sacks et al., 2003). During this time there are marked changes in brain structure and function. For example, adolescent white and grey matter development is accompanied by changes in cognitive function. These include improvements in intelligence quotient (IQ) (Schmithorst et al., 2005), working memory (Østby et al., 2011; Tamnes et al., 2013)

and problem solving (Squeglia et al., 2013). Social cognition also undergoes pronounced changes during this period of life, including significant improvements in perspective development (Dumontheil et al., 2010) and face processing (Cohen Kadosh et al., 2013). Emotional development, including the processing of autobiographical memories, most strongly commences following age 10 (Rubin and Schulkind, 1997).

In addition to normal developmental milestones, the genesis of many mental disorders is also during adolescence (Kessler et al., 2005, 2007). A statistical report of adults with mental disorders showed that roughly 74% were diagnosed before age 18 and 50% before age 15 (Kim-Cohen et al., 2003). It is believed that psychiatric disorders may partly be triggered by stress exposure in adolescence (Andersen and Teicher, 2008). Social stress is particularly impactful during this period (Andersen and Teicher, 2008). Indeed, human adolescents exhibit greater stress reactivity than non-adolescents (Dahl and Gunnar, 2009) predisposing individuals to outcomes such as depression and anxiety (Sirin et al., 2013). Chronic stress during adolescence affects the development of the PFC (Hoftman and Lewis, 2011) and is associated with poorer cognitive performance in adulthood, both in humans (Casey et al., 2010; Rahdar and Galván, 2014) and rodents (Lukkes et al., 2009). Rat social isolation during P25-45 has enduring effects on exploratory behavior, but only when isolated during the said period, not before or after (Einon and Morgan, 1977). While this social deprivation window of sensitivity has not been directly translated to humans, evidence shows that adolescents are more anxious following social exclusion, than adults (Sebastian et al., 2010, 2011). For a cross species comparison of adolescence time points refer to Figure 2.1.

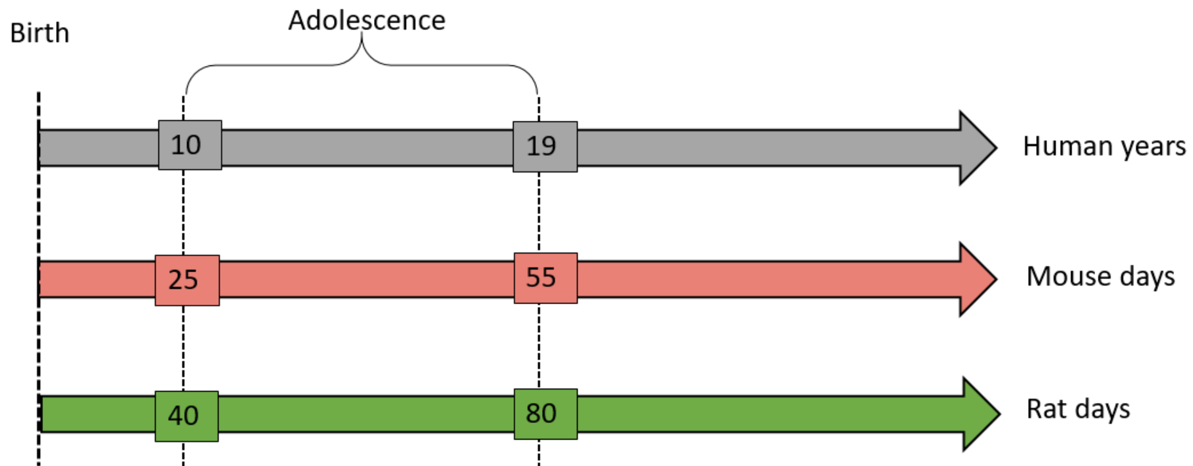

Figure 2.1: The putative adolescence age span for humans (years) and rodent (postnatal days) development (Sacks et al., 2003; Schneider, 2013; Fuhrmann et al., 2015).

Adolescence and dopamine system development

Interestingly, adolescence is the time in development during which the DA system undergoes extensive restructuring (Figure 2.2). While midbrain DA neurons and fibers appear during early gestation (E12 and E15 in rodents), the DA system development continues to P90 (Olson and Seiger, 1972). For example, the development of cortical DA innervation increases until P60, after which the density and topography of DAergic afferents remain constant (Kalsbeek et al., 1988). DA transporter (DAT) density in the striatum increases from P25 through P50, but then decreases continuously until P90 (Tarazi et al., 1998a; Moll et al., 2000). While DA receptors are first expressed during gestation in the mouse, their expression continues during postnatal development (Jung and Bennett, 1996; Araki et al., 2007). Striatal DA receptor-binding capacity continues to gradually increase until P28–P40, after which it diminishes again to reach stable levels at around P60 (Giorgi et al., 1987; Teicher et al., 1995; Tarazi et al., 1998b). Likewise, DAergic cell activity in mice is low at weaning, then increases to a peak at P45, after which it declines once again (McCutcheon and Marinelli, 2009). In the ventral tegmental area (VTA), this transient

increase in DAergic activity is characterized by increased non-bursting activity and longer burst duration (McCutcheon et al., 2012). Lastly, tissue DA levels peak between P25 and P40 (Noisin and Thomas, 1988). Figure 2.2 below summarizes how several measures of DAergic system maturation transiently peak during adolescence which is roughly between P25-55 (Schneider, 2013; Yu et al., 2014; Suri et al., 2015).

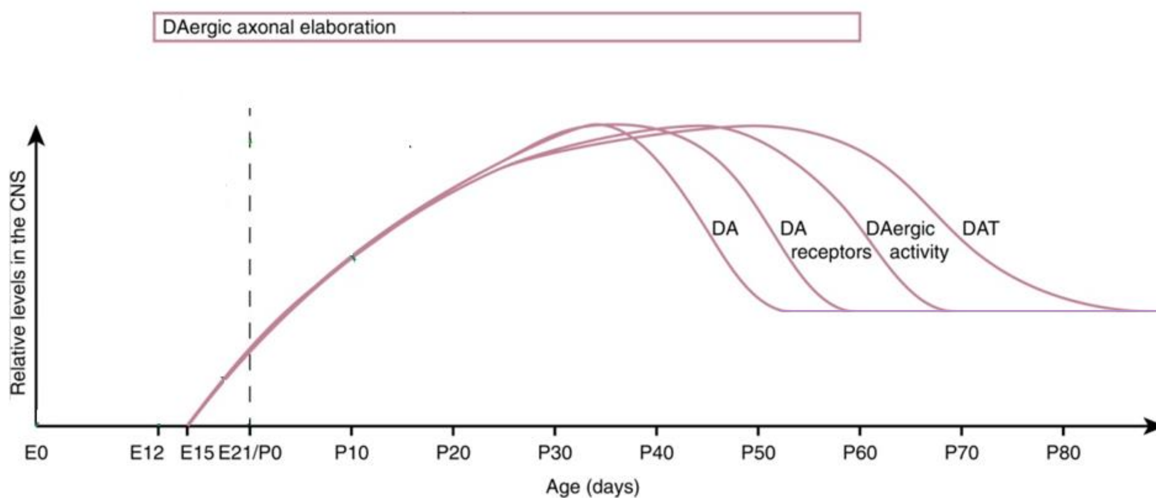

Figure 2.2: Transient peaks in dopamine system development. The graph displays relative levels DAergic measures in the CNS across rat and mouse development: DA tissue concentration (DA), DA receptor binding (DA receptors), DAergic firing frequency (DAergic activity) and DAT binging (DAT). The dashed line separates embryonic (left) from postnatal (right) development. CNS, cerebrospinal fluid; DA, dopamine. Adapted from (Suri et al., 2015).

<u>Adolescent perturbation to dopamine system development</u>

Given the many changes taking place in the DA system during periadolescence, it is unsurprising that developmental perturbations to this system, during that time, can exert significant effects on anatomy, physiology, and behavior. For example, exposure to social defeat stress during adolescence, and not adulthood, reduces basal extracellular levels of DA in the medial PFC (Watt

et al., 2009) and increases behavioral response to an amphetamine challenge (Burke et al., 2013). Similarly, periadolescent DAergic manipulations also alter the behavioral response to psychostimulants like amphetamine in adulthood, with transient DAT blockade potentiating locomotor activity after an amphetamine challenge (Yu et al., 2014). Post-weaning stress through isolation rearing decreases DA turnover in the medial PFC (Heidbreder et al., 2000), but enduringly increases both basal and stimulant-induced DA levels in NAc (Jones et al., 1992; Hall et al., 1998).

While studies have manipulated the DAergic system to probe its developmental role in various adult phenotypes, examples that have received particular attention have been in the study of aggressive behaviors. Taking a step back to monoamines in general, a seminal report by Brunner and colleagues revealed that constitutive loss-of-function mutations of *MAOA* result in a syndrome characterized by antisocial/aggressive behavior in humans (Brunner et al., 1993), an outcome that is largely opposite to the typical anxiolytic effects of pharmacologic MAOA inhibition in adulthood. Consistently, mice with genetic inactivation of *MAOA* exhibit not only neophobia but also heightened levels of aggression (Cases et al., 1995; Scott et al., 2008; Godar et al., 2011). The divergent effects of genetic (life-long) mutations versus pharmacologic inhibition (during adulthood) suggest that perturbed monoamine signaling, during sensitive periods of brain maturation, differentially modulates adult aggression. This hypothesis was further refined, to specifically implicate the DAergic system in modulating aggression. One study of particular importance to my dissertation is the work of Yu et al (2014) conducted previously in our laboratory (Yu et al., 2014). Yu and colleagues found that adult aggressive behavior is sensitive to periadolescent (P22–41) monoaminergic manipulations. Specifically, they found that periadolescent MAOA blockade with clorgyline (CLO) phenocopied the adult aggression found

in MAOA-deficient mice (Figure 2.3A). Furthermore, transient postnatal (P2–21) or adult (P180–201) MAOA blockade did not impact adult aggressive behavior (Figure 2.3B). This effect was attributed to the DAergic system, given that only DAT blockade with GBR12909 increased aggression (Figure 2.3C), and manipulations of other monoamines either decreased (5-HTT blockade with fluoxetine; FLX) or had no effect (Norepinephrine transporter blockade with desiprimine; DMI) on adult aggression (Figure 2.3D). The aggressive behaviors in CLO and GBR treated mice were accompanied by concurrent changes in responsivity to amphetamine, indicating a change in underlying DAergic function (Figure 2.4). These data help establish the existence of a DA-sensitive developmental period and can aid in explaining the increased aggression seen in constitutive *MAOA*, *DAT*, and *COMT* loss-of-function mouse lines (Cases et al., 1995; Gogos et al., 1998; Rodriguiz et al., 2004; Scott et al., 2008).

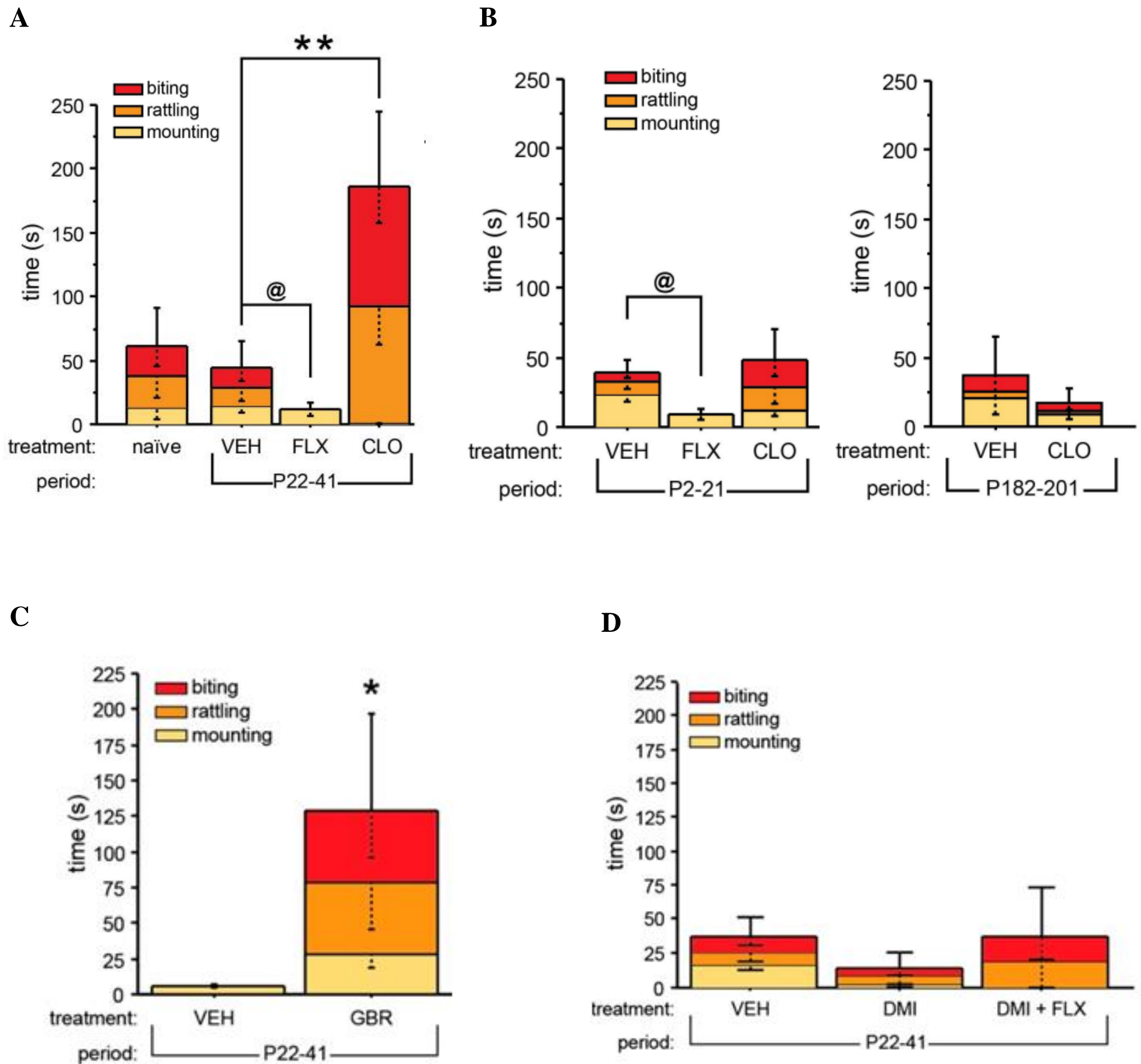

Figure 2.3: Altered aggression after developmental MAOA, 5-HTT, NET and DAT blockade. (**A**) Aggressive behavior was increased in mice treated with CLO from P22–41 when compared with control mice treated with VEH from P22–41. Mice treated with FLX displayed reduced aggression when compared with VEH treated control mice. (**B**) Aggressive behavior was unaffected by transient CLO treatment from P2–21 and P182-201. (**C**) Aggressive behavior was increased only in mice treated with GBR treatment and (**D**) not in mice treated with DMI or DMI+FLX from P22–41 when compared with VEH treated control mice. ([@]indicates main effect of treatment: VEH, vehicle; FLX, fluoxetine; CLO, clorgyline; GBR, GBR12909; DMI, desiprimine. *, $p<0.05$; **, $p<0.01$; n =5–15 per group. Adapted from (Yu et al., 2014).

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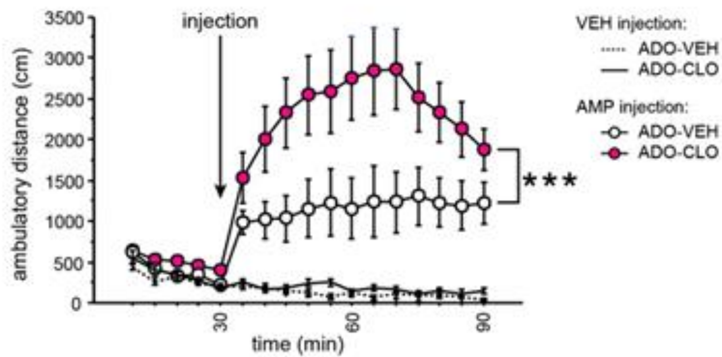

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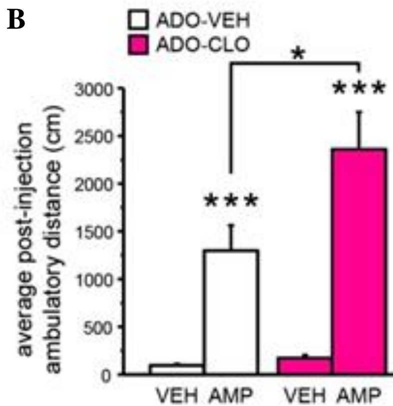

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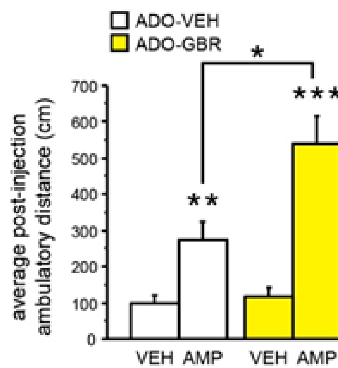


Figure 2.4: Behavioral amphetamine response was assessed using the open field. (**A**) Amphetamine injection (AMP, 3 mg/kg, i.p.) induced locomotor hyperactivity in adolescent VEH and adolescent CLO treated mice. PA CLO-treatment altered the response to amphetamine (indicated significance refers to peri-adolescent treatment x time interaction). (**B**) Post-injection activity demonstrates increased behavioral response to amphetamine in adolescent CLO mice when compared to adolescent VEH mice. (**C**) Adolescent GBR treatment increased the behavioral response to amphetamine challenge. *, $p < 0.05$, ***, $p < 0.001$; n = 5-15 per group. Adapted from (Yu et al., 2014).

Based on this previous work done in our laboratory it is evident that periadolescent DA has a critically involved role in modulating adult aggression. In this chapter I will describe, in detail, our efforts to expand on past findings by testing the following aims:

1. First, we aim to refine this DA sensitive period that affects aggression:
   Specifically, we hypothesize that adolescence contains a narrow window during which DAT blockade impacts adult aggression.

2. Next, we aim to probe the consequences of developmental DAT blockade on DA system function in awake behaving animals:
   Specifically, we hypothesize that periadolescent DAT blockade, during the narrow sensitive period, will significantly potentiate adult sensitivity to amphetamine.

3. Finally, we aim to probe the electrophysiological consequences of developmental DAT blockade on DA system function in anesthetized animals and slice preparations:
   Specifically, we hypothesize that periadolescent DAT blockade, during the narrow sensitive period, will be associated with changes in adult DA physiology. **

   **Note: The above aim was executed by Dr. Deepika Suri (*in vivo*) from the Ansorge lab and Dr. Nao Chuhma (slice), a collaborating electrophysiologist from Dr. Stephen Rayport's lab.

b. Materials and methods

Animals

Wild type male mice were cross bred at Columbia Psychiatry, New York State Psychiatric Institute. F1 mixed background (C57BL/6J x 129S2/129SvEv/Tac) were studied. Given that female mice do not display territorial aggression, only male mice were investigated. Mice used for experiments were born to litters containing 4–6 pups. Mice were separated by sex and weaned into groups of five mice per cage at P21. Mice were treated postnatally from P22-31, P32-41 and P42-51. Treatment consisted of the following intraperitoneal (i.p.) drug injections: vehicle (VEH, 0.9% NaCl, 5 ml/kg) and GBR12909 (GBR, 20 mg/kg). GBR12909 is a DAT blocker. Mice were injected in random order, and an entire cage was injected within 3 minutes. After injections, mice were placed back into their home cage and were observed for any abnormalities in behavior. Their weights were recorded throughout the treatment period to track any abnormal fluctuations. Injections were administered daily between 3pm and 5 pm. Mice were housed under a 12:12-hour light:dark cycle (lights on at 7am) in a temperature controlled environment and provided with food and water *ad libitum*. Animal testing was conducted under protocols approved by Columbia University and New York State Psychiatric Institute Institutional Animal Care and Use Committees.

Drugs

For the studies involving drug administrations, GBR 12909 (ANAVA Trading SA) was dissolved in 0.9% NaCl to achieve 4mg/ml. GBR12909 has a very high binding selectivity and long half-life (Andersen, 1989; Ingwersen et al., 1993). The dose for the GBR treatment was chosen based on previous experiments: GBR at 20 mg/kg is a standard dose to achieve functional DAT blockade in mice (Hirate 1991). D-Amphetamine (Sigma A5880) was dissolved

in sterile saline (0.9% NaCl) and administered via intraperitoneal injection at a dose of 0.5 mg/kg. Solutions were prepared fresh every day/on the same day of the experiment.

Behavioral testing

All animals were exposed to the same behavioral paradigms and timeline, starting at 4 months of age. Mice first underwent the isolation induced aggression test followed by the amphetamine challenge with at least 3 weeks between the two behaviors. All behavioral testing took place during the light cycle between 12:00pm and 7:00 pm. To eliminate odor cues, each apparatus was thoroughly cleaned after each animal.

Isolation-induced aggression test

In order to assess aggressive behavior, the isolation-induced aggression paradigm was used (Takahashi et al., 2012). The home cage was divided in half by a perforated partition made of clear plastic. A pair of mice with the same treatment was placed in the cage, one in each compartment. The mice were able to see, hear and smell each other through the holes in the plastic divider, but physical interaction was blocked. Mice were housed for 10 days before the test experiment was performed. On the test day, dividers were taken out and mice were allowed to freely interact for 15 minutes. The time spent fighting was scored as a sum of the time spent biting, tail rattling and mounting. Given that behavioral data was collected across more than one cohort, all data was normalized. All fights were video recorded to allow for any additional post-hoc analysis. The experimental design is detailed in a schematic on the next page in Figure 2.5.

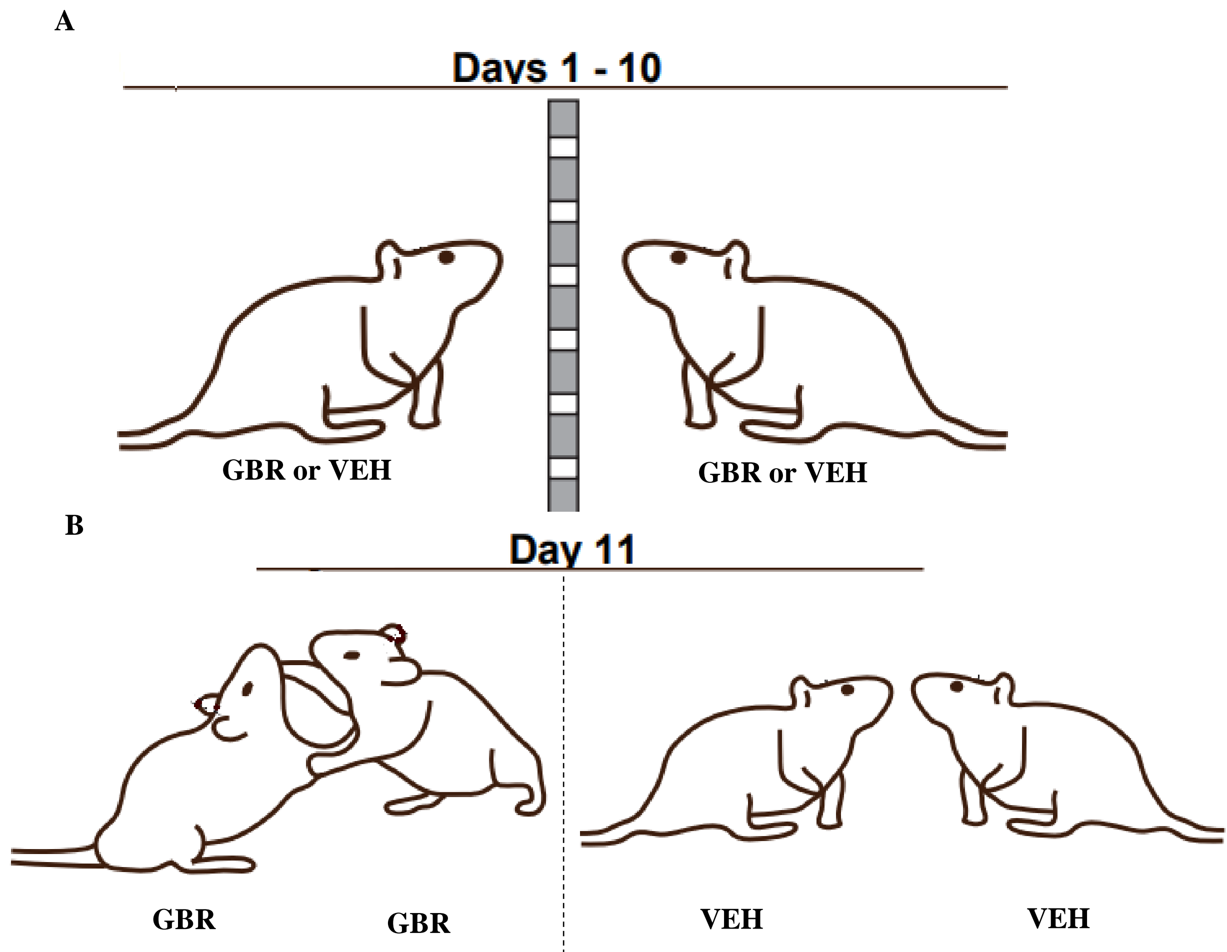


Figure 2.5: Isolation-induced aggression paradigm. Schematic of the isolation-induced aggression experimental design between males with the same periadolescent treatment. (**A**) After separation for 10 days, mice underwent the (**B**) experimental fight on Day 11. GBR, GBR12909; VEH, vehicle.

Open field amphetamine challenge

Locomotor activity was assessed using the open field test. Mice were allowed to explore brightly lit (800-900 lux). Plexiglass activity chambers (43.2 × 43.2 × 30.5 cm, length × width × height; model ENV-520; Med Associates, Georgia, VT) equipped with infrared beams located 1.5cm above the chamber floor and spaced 2.5cm apart as well as beams 6cm above the chamber floor, spaced 2.5cm apart. Beams were positioned such that both horizontal and vertical activity was detectable. Mice were placed into the center of the open field and activity was recorded for 30min to assess baseline locomotion. To assess amphetamine-induced locomotion, the system

was programmed to halt recording 30 min after starting a session, at which point mice were injected with amphetamine (0.5 mg/kg, i.p.) or saline (SAL, 0.9% NaCl) and placed back in the open field. Recording of locomotor activity was resumed for another 60 min immediately after injections. Testing took place under bright ambient light conditions. Total distance travelled over the 90mins was measured.

Data and statistical analyses

Statistical analysis for behavior was performed using StatView 5.0 software (SAS Institute, Cary, NC, USA) and Microsoft Excel. Data were analyzed using Student's t-test, 2-way and repeated measures ANOVAs. The criterion for significance for all analyses was *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$. Results from data analyses are expressed as mean±s.e.m.

c. Results

The main experimental goals of this chapter were to:

1. Temporally define the periadolescent DAergic sensitive period which impacts adult aggressive behavior.
2. Probe the consequences of developmental DAT blockade on DA system function in awake behaving animals.
3. Probe the electrophysiological consequences of developmental DAT blockade on DA system function in anesthetized animals and slice preparations. (Deepika Suri from the Ansorge lab conducted the *in vivo* recordings and Nao Chuhma from the Rayport lab conducted the slice recordings).

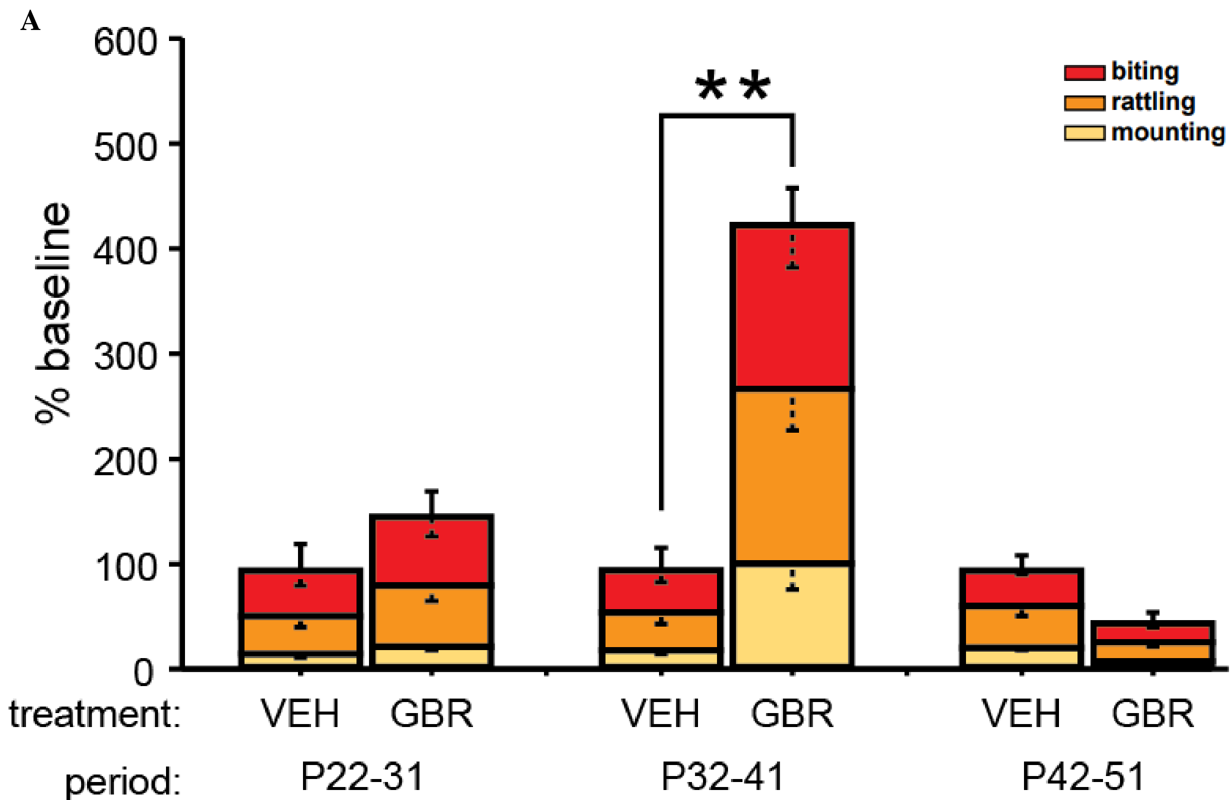
Using the paradigms discussed in the methods section, we conducted experiments to test the above and found the following:

1. **Increased periadolescent DA from P32-41, and not P22-31 and P42-51, increases adult aggression:**

Periadolescent DAT blockade from P22-41 increases aggressive behavior in adult mice (Yu et al., 2014). Interestingly, the DAergic system undergoes dramatic changes during the same time period. During circuit-maturation, plasticity is often high, allowing for activity-dependent refinements, genetic and/or environmental. Our treatment period, P22-41, encompasses a large proportion of this behavioral and circuitry maturing phase. This aim enables us to better asses the specific temporal dynamics of when this window opens and closes. We hypothesized that a narrower period exists, (P22-P31 or P31-P41), during which DA disruption has the same impact on adult behavior. Furthermore, we predicted that this window of sensitivity closes at P41.

To test these hypotheses, 3 separate cohorts of male mice were injected with GBR (20 mg/kg per day) to block DAT, or VEH from P22-31, P32-41 and P42-51. Mice were later tested for aggression in adulthood (>P120). The time pairs spent in aggressive behaviors was measured as a sum of the time spent biting, rattling and mounting.

GBR treatment from P32-41, but not P22-31 or P42-51, increased aggressive behavior, when compared to VEH controls (effect of periadolescent treatment: $F_{(1, 71)} = 1.219$, $p = 0.2733$; effect of developmental timing: $F_{(2, 71)} = 3.969$, $p = 0.0232$; treatment x developmental timing interaction: $F_{(2,71)} = 6.545$, $p = 0.0025$) (Figure 2. 6). Aggressive behavior between VEH treated mice and naïve littermates did not differ in tests previously conducted by Yu et al.



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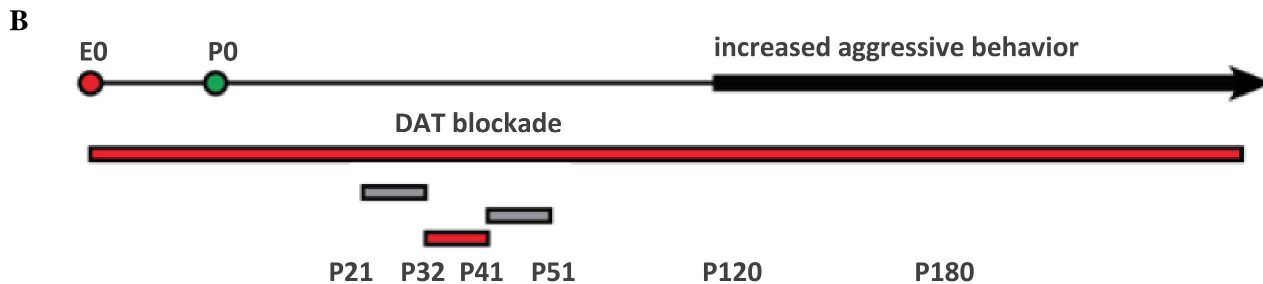

Figure 2.6: A DA-sensitive periadolescent period impacting adult aggression. Isolation induced aggressive behavior was assessed in mice by scoring the time spent mounting, tail rattling, or biting during a 15 minute encounter. (**A**) Aggressive behavior was increased in mice treated with GBR (20 mg/kg/day, i.p.) from P32-P41 when compared to control mice treated with VEH from P32-P41. In contrast, GBR treatment from P22-31 or P42-51 had no effect on aggression when compared to respective VEH treated controls. (**B**) Summary of sensitive period mapping experiments, with red indicating aggression-increasing consequences, while grey indicates no effect on adult aggressive behavior. **, $p < 0.01$ compared with their respective controls; mean ± SEM; n = 9–20 pairs per group.

2. **Increased periadolescent DA from P32-41, and not P22-31 and P42-51, increases adult response to amphetamine:**

In order to further investigate the hypothesis that dysfunction of the DAergic system might underlie the altered aggression in Figure 2.6A, behavioral response to a dose of amphetamine was evaluated as a proxy for DAergic function. The response to amphetamine (0.5 mg/kg, i.p.) and SAL challenge in adult mice (P150), which had received VEH or GBR during P22-31, P32-41, and P42-51 was analyzed. Strengthening the hypothesis of a narrower window of DA perturbation rendering a sensitized/hyper-active DA system, we found that only P32–P41 GBR treated mice displayed an exaggerated response to adult amphetamine challenge when compared with VEH treated mice of the same developmental time group (effect periadolescent treatment x adult amphetamine challenge interaction: $F_{(1,646)} = 3.948$, $p < 0.0001$) (Figure 2.7B). There were no significant differences between the VEH and GBR groups, which were treated between P22-31 group (effect periadolescent treatment x adult amphetamine challenge interaction: $F_{(1,629)} = 1.056$, $p=3.948$) (Figure 2.7A) and P42-51 group (effect periadolescent treatment x adult amphetamine

challenge interaction: $F_{(1,629)} = 1.072$, $p=3.781$) (Figure 2.7C), in response to amphetamine. Adult SAL injection did not alter behavior in any age group of VEH or GBR-treated animals.

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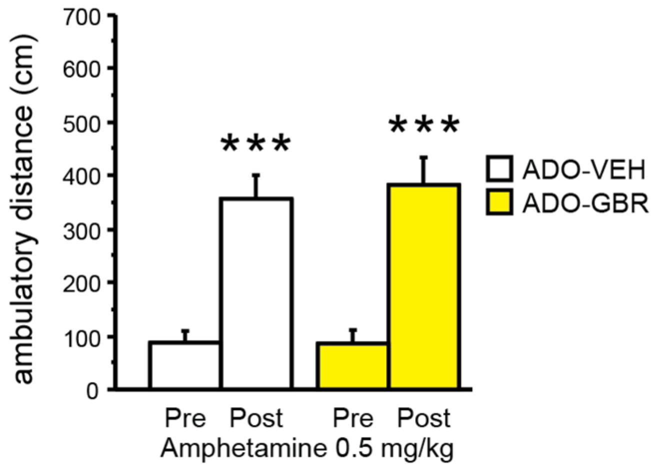

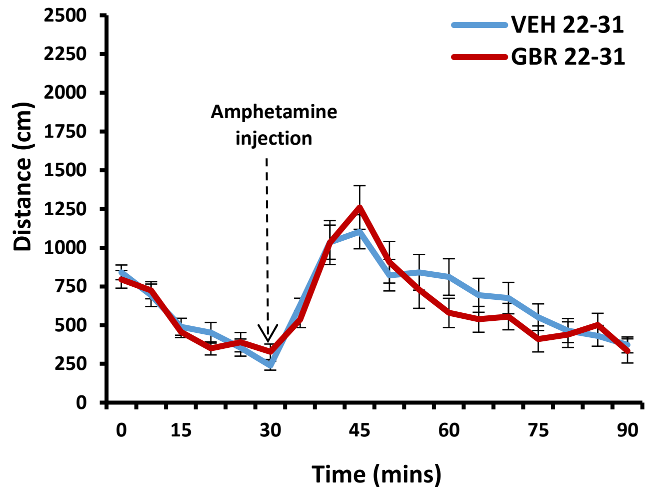

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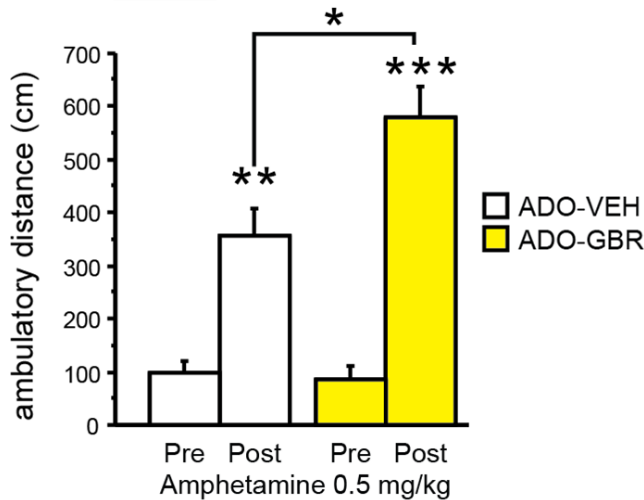

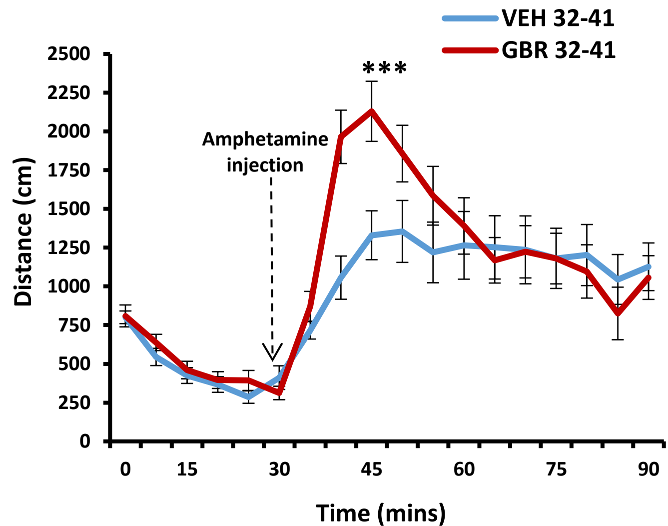

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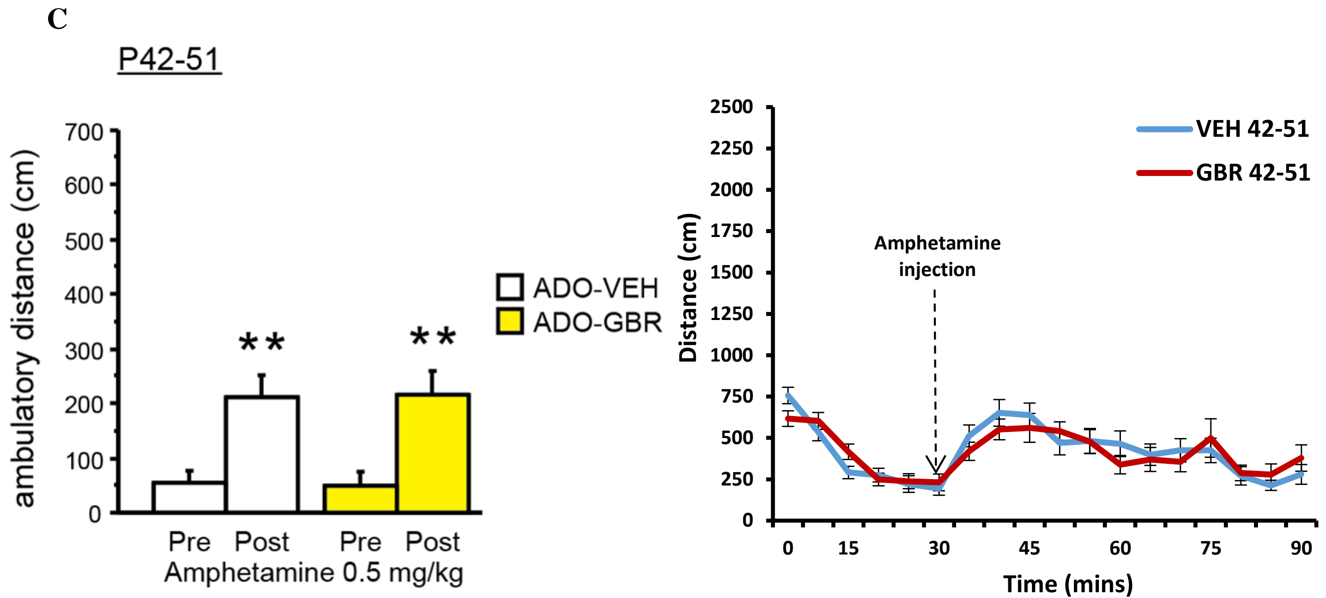


Figure 2.7: A DA-sensitive periadolescent period impacting adult amphetamine response. Behavioral amphetamine response was assessed using the open field. Amphetamine injection (0.5 mg/kg, i.p.) induced locomotor hyperactivity in VEH and periadolescent GBR treated mice. (**B**) For P32-41 we detected a significant periadolescent treatment x amphetamine treatment interaction, with increased behavioral response to amphetamine after periadolescent GBR when compared to periadolescent VEH. (**A**, **C**) No different was observed in response to amphetamine between VEH and GBR treated groups of P22-31 and P42-51. *, $p < 0.05$, **, $p < 0.01$, ***, $p < 0.001$ compared with their respective controls; mean ± SEM; n = 8-12 per group

3. Increased PA DA from P32-41, and not P22-31 and P42-51, alters underlying DA physiology:

Given the behavioral changes noticed, we hypothesized that P32-41 DAT blockade would also produce underlying physiological changes in the DAergic system. This idea was tested by Deepika Suri (*in vivo* in anesthetized mice) from the Ansorge lab and Nao Chuhma (*in vitro* in acute brain slices) from the Rayport lab, in animals either treated with GBR or VEH from P32-41. Specifically, they assessed firing properties of ventral tegmental area (VTA) and substantia nigra pars compacta (SNc) DAergic neurons following P32-41 DAT blockade and found changes in DAergic activity that could correlate with the increased aggression and sensitivity to amphetamine seen in Figure 2.8. Specifically, recordings in anesthetized mice treated with GBR during P32-41,

showed a greater percentage of spikes appearing in bursts in DAergic neurons when recorded in the VTA and SNc (Figure 2.8C). Population activity was only different between GBR and VEH treated mice in the VTA (Figure 2.8D) and not in the SNc (Figure 2.8E), thereby indicating a possible dissociation between the two populations of DAergic cells that could underlie the behavioral phenotype. In the slice recordings, this region-specific dissociation was once again reported in an assessment of firing rates following P32-41 DAT blockade, with a difference in the VTA (Figure 2.8G), but not the SNc (Figure 2.8I).

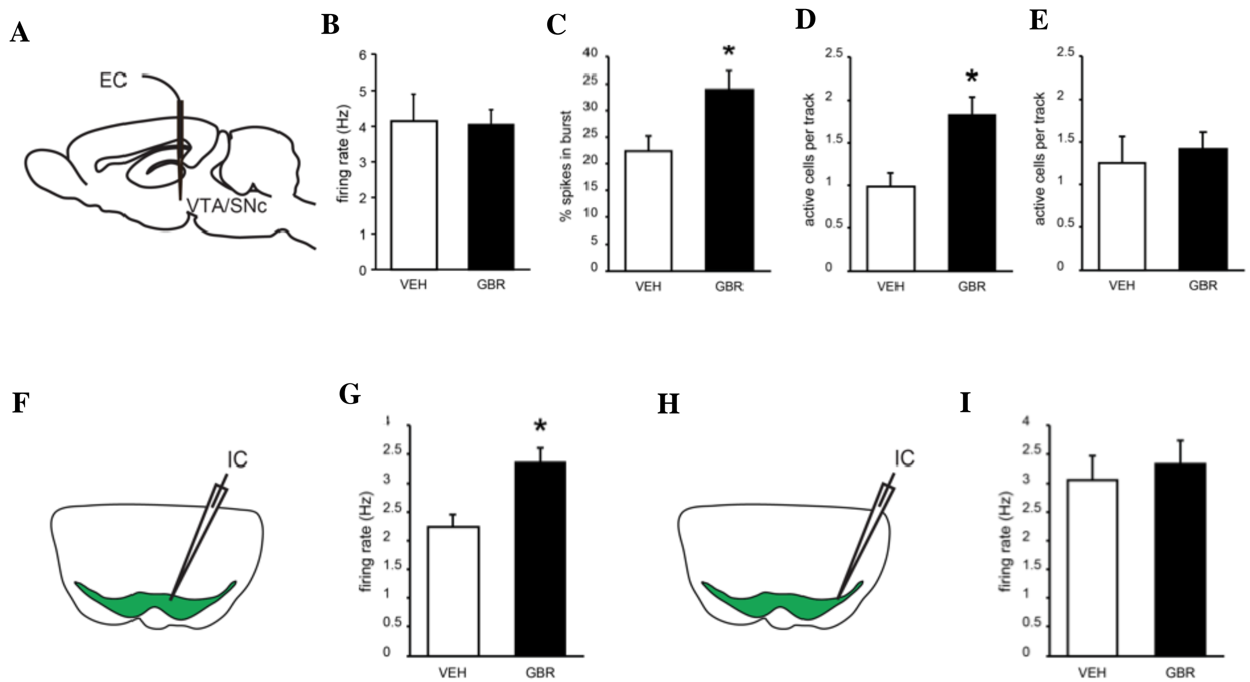


Figure 2.8: Increased DAergic activity in the VTA after periadolescent DAT blockade. We assessed firing properties of DAergic neurons *in vivo* in anesthetized mice (**A-E**) and in acute brain slices (**F-I**). *In vivo* extracellular (EC) single unit recordings of putative DAergic neurons revealed (**C**) increased % spikes in bursts and (**D**) increased number of spontaneously active neurons per track in the VTA after DAT blockade in periadolescent GBR mice when compared to periadolescent VEH controls. (**B, E**) Overall firing rates and active putative DAergic neurons per track in the SNc were not affected by treatment. Intracellular (IC) whole cell patch recordings in acute brain slices revealed (**F, G**) increased spontaneous activity of VTA DAergic neurons, but (**H, I**) not SNc DAergic neurons. *, $p < 0.05$ compared with their respective controls; n = 4 -7 per group.

Considered together, these data demonstrate that P32-41 is the narrow window during which DAT blockade increases adult aggression and sensitivity of the DA system. This window of sensitivity opens at P32 and closes at P41, as confirmed by the lack of behavioral differences between VEH and GBR treated mice from the P22-31 and P42-51 groups.

d. Discussion

In summary, the current chapter establishes that elevated DA levels, via transient DAT blockade from P32-41, impacts adult aggression, sensitivity to amphetamine and DAergic physiology. First, we identified that the broader window of P22-41 encompasses a smaller one, P32-41, during which DAT blockade has the same impact on adult aggression. Second, we behaviorally demonstrated that P32-41 DAT blockade impacts the functional baseline of the DAergic system, as seen in the heightened response to amphetamine in adulthood. Finally, we revealed that the behavioral changes are accompanied by underlying changes in DAergic physiology. These include increased DAergic burst firing and greater VTA DAergic population activity *in vivo*, and increased firing rates recorded from the VTA of acute slice preparations. Together, these data implicate an overactive DA system underlying the behavioral changes observed in our experiments (Moore et al., 2001; Chang and Grace, 2013; Zimmerman and Grace, 2016).

The drug used in our experiments, GBR12909, has similar mechanistic action to psychostimulants (Izenwasser et al., 1990). This is particularly relevant as drug-related interruptions to the DAergic system are very common in society today. For example, many psychostimulants, including methylphenidate (a DAT blocker that is used as first line treatment for attention deficit hyper-activity disorder, ADHD) (Faraone and Glatt, 2010; Shanks et al., 2015) as well as amphetamine and cocaine are widely prescribed and/or abused (Di Chiara and Imperato, 1988; Koob and Bloom, 1988; Torregrossa and Kalivas, 2008). Moreover, the use of these drugs has been commonly linked to aggression (Rodrígez-Arias et al., 2006). Chronic stimulant exposure increases aggressive behavior in rodents, non-human primates (Martin et al., 1990; Sokolov et al., 2004) and humans (Dawe et al., 2009), even in abstinent stimulant users (Sekine et al., 2006). In a

study reporting violent behavior by cocaine addicts, aggression ranged from minor psychological insults to major acts that included rape and murder (Miller et al., 1991). A more recent analysis revealed that partner and non-partner violence is common among veterans seeking cocaine use treatment (Anderson et al., 2017). Amphetamines have a similar outcome. In a study investigating the relationship between amphetamine and aggression, 47% of the sample admitted to having committed a violent crime, half of whom associated the aggressive behavior with their use of amphetamine. Moreover, 62% reported continuing aggression related to their amphetamine use (Wright and Klee, 2001). In rodents, chronic administration of methamphetamine to isolated mice increased fight behaviors (Sokolov et al., 2004; Sokolov and Cadet, 2006). These examples demonstrate that psychostimulants are closely linked to aggression. Our data, nuances this established association by offering temporal insight on how sensitive periods of development, like adolescence, interact with pharmacological changes to impact aggressive behavior. Specifically, our data indicate that psychostimulant use during adolescence might lead to a long-lasting aggression predisposition.

Adolescence encompasses our DA-sensitive period and is a time of high stimulant exposure (Kristjansson et al., 2013; Squeglia and Cservenka, 2017). For example, Ritalin (brand name for methylphenidate) is very commonly prescribed to treat ADHD. Nonetheless, the concern about the long-term repercussions of chronic stimulant exposure in children with ADHD is prevalent among doctors and parents, alike (Batstra et al., 2014). Beyond its use in the medical setting, it is increasingly abused by adolescents seeking cognitive enhancement in academic performance, work productivity, memory focus and wakefulness (Williams et al., 2004; Rob Goodman, 2010; Franke et al., 2011). Hapless risk taking has also been linked to adolescence and proposed as a contributing factor to the peak in substance use during this time in development.

One theory suggests that there could be an "imbalance" in the adolescent brain, where emotion and reward systems develop before cognitive control systems, thus predisposing the youth to be more vulnerable and engage in risk-taking behaviors like substance use (Casey et al., 2008; Mills et al., 2014). Our data suggest that adolescence in humans might constitute a sensitive period during which stimulant exposure predisposes one to adult aggression. However, it is also a period during which stimulant exposure is particularly high. In that regard, it would be interesting to evaluate the impact of diversifying our pharmacological interference during P32-41 to include commercially available psychoactive drugs including amphetamine, cocaine, and methylphenidate.

Our hypothesis linking P32-41 stimulant exposure to long-term outcomes, is advanced further by others in the Ansorge laboratory who find that P32-41 DAT blockade is not only correlated with increased aggression and sensitivity to amphetamine, but is also strongly associated with heightened motor and decision impulsivity in adulthood (Deepika Suri, unpublished data). Interestingly, supporting phylogenetic conservation, like our aggressive-impulsive mice, human individuals with aggressive and impulsive traits show mesolimbic DA hyper-sensitivity to amphetamine too (Buckholtz et al., 2010). In fact, reactive aggression is a dominant form of pathological aggression that is rooted in impulsivity (Vitiello and Stoff, 1997; Rosell and Siever, 2015). In light of the fact that impulsivity and aggression often co-occur in many psychiatric disorders (Moeller et al., 2001; Seo et al., 2008), and have both been linked to DA dysfunction, our data support the notion of developmental DAT blockade contributing to adult reactive/impulsive aggression.

Moreover, our collective findings beg the broader question about what other disorders might have their roots in, or be further impacted by, DAergic disturbances during our established period of sensitivity. This is a particularly relevant question, since dysregulated DA is implicated

in a series of neuropsychiatric disorders. The *DAT1* gene variant has been implicated in ADHD (Todd et al., 2005; Bédard et al., 2010), schizophrenia (Sáiz et al., 2010), bipolar disorders (Pinsonneault et al., 2011), and cocaine abuse (Guindalini et al., 2006). Additionally, many of the mentioned psychiatric disorders involve inappropriate social interactions, including extreme aggression. Our study directly shows that DAT blockade during adolescence leads to increased aggressive behavior in adult mice, but it would be interesting to further explore this aggressive phenotype in established models of other psychiatric disorders too.

Our sensitive period data further indicate a developmental mechanism for vulnerabilities conferred by functional genetic polymorphisms (including *MAOA*, *DAT*, and *COMT* loss-of-function mutations) that impact the development of the DAergic system (Cases et al., 1995; Gogos et al., 1998; Volavka et al., 2004; Guo et al., 2007; Scott et al., 2008). For example, in humans, the loss-of-function and low-expressing *MAOA* alleles (Brunner et al., 1993; Caspi et al., 2002), the 10R variant of *DAT1* (Guo et al., 2007; Bédard et al., 2010), and the low activity met allele of the *COMT* gene (Volavka et al., 2004) have all been associated with aggressive behavior. In rodents, mice lacking DAT are hyper-active (Giros et al., 1996) and aggressive following mild social contact (Rodriguiz et al., 2004). Our data suggest that these DAergic alleles partially confer risk by activity during adolescence, predisposing one to high aggression.

In addition to increased aggression and psychostimulant responsivity, following brief adolescent DAT blockade, we find higher *in vivo* burst firing of VTA DAergic neurons in adulthood. In fact, burst firing is associated with a much larger DA release than when these neurons fire in a slow, irregular single spike mode (Gonon, 1988; Bean and Roth, 1991; Arbuthnott and Wickens, 2007) due to decreased time intervals between action pulses allowing less time for reuptake (Venton et al., 2003). The balance between phasic and tonic firing of VTA DA neurons

is determined by the actions of pedunculopontine tegmental nucleus (PPTg), ventral pallidum (VP), and rostromedial tegmental nucleus (RMTg) afferents because they constitute the dominant glutamatergic and GABAergic input regulating VTA DAergic activity (Grace et al., 2007). Because individual afferents to the VTA can selectively alter the activity state of DA neurons, these pathways converge at an afferent 'gate' and function in concert to regulate the net output of the DA system, and therefore the state of burst firing. The altered state of firing seen in our treated animals could be due to an alteration in the balance of afferent input or a change in the responsivity of VTA DAergic neurons to excitatory and inhibitory input following periadolescent DAT blockade.

In fact the same has been found in cases of chronic psychostimulant use. White and colleagues revealed that after repeated administration of cocaine or amphetamine, VTA DA neurons were significantly more sensitive to glutamate as compared to DA neurons in control rats (White et al., 1995). Creed and colleagues confirmed the same when they found a cocaine-induced potentiation of excitatory inputs onto VTA DA neurons (Creed et al., 2016). The transition from irregular single-spike firing to a burst-firing pattern aligns well with the conclusion of a sensitized state, deducted from our amphetamine challenge.

The presence of a sensitized system is further supported by our finding of greater population activity of VTA DAergic neurons in GBR treated animals. The state of spontaneous activity of VTA DA neurons is influenced by the ventral subiculum (vSub) in the hippocampus. Stimulation of the vSub doubles DA neuron population activity and this is correlated with increased extracellular DA in the nucleus accumbens (Blaha et al., 1997; Brudzynski and Gibson, 1997; Legault and Wise, 1999; Floresco et al., 2001). The increase in the number of spontaneously active DA neurons, following vSub stimulation, is due to a multi-synaptic pathway involving the

ventral pallidum, a key GABAergic afferent to the VTA (Floresco et al., 2001). Activation of the vSub eventually inhibits ventral pallidal GABAergic afferents to the VTA, releasing DA neurons from a tonic inhibitory influence (Floresco et al., 2001). For example, local vSub infusions of NMDA increases the number of spontaneously active DA neurons in the VTA. Perhaps, periadolescent DAT blockade alters the vSub's net influence on VTA DA neurons, thus driving the higher population activity seen in our data.

Finally, DAT serves as a primary cellular target for mediating locomotor responsiveness to psychostimulants like amphetamine (Zhu and Reith, 2008). Given that DAT was the key target of our experiments, a possible explanation for our increased amphetamine response in P32-41 GBR treated mice, could be differences in functional adult DAT expression, following our adolescent manipulation.

Sensitive periods are windows of vulnerability during which neurochemical systems, and subsequently behavior, are most susceptible to modulations (Knudsen, 2004; Hensch, 2005; Crews et al., 2007). In fact, P32-41 falls in the period of DAergic system maturation during which it is in a state of flux and perturbations could alter the typical course of DA system development. While we may have some preliminary data and hypotheses highlighting changes in underlying DA functionality, many questions remain untested. Thus, in interpreting our data, future studies will help evaluate how long-lasting adaptations in DA function, following P32-41 DAT blockade, are contributing to the potentiation seen in the amphetamine challenge as well as the aggressive phenotype.

Concluding remarks

To return to the original hypothesis, this chapter underscores how a transient increase in adolescent DA, caused by P32-41 DAT blockade, perturbs the developmental trajectory of the DA system. In doing so, it predisposes an individual to high aggression, increased sensitivity to amphetamine and altered underlying DAergic physiology. While we may have only scraped the surface of what the underlying mechanisms may be, in narrowing the window of sensitivity, we set ourselves up to hone in on specific processes that might be driving the changes in behavior. Although the current study suggests that VTA DA hyper-activity might be the mechanism underlying this aggressive phenotype, many questions remain unanswered. From this point on, given the strong evidence of the involvement of the DAergic system in this phenotype, it would be worthwhile to precisely explore the specific regions and DA cell populations involved in modulating aggression. Optogenetics could be used to establish causality, as channelrhodopsin (ChR2) can be Cre-dependently expressed in DAergic neurons. This approach would help establish whether stimulating DAergic cells, in temporally and spatially controlled way, could causally link a specific DAergic pathway to aggression. The following chapter of my dissertation will empirically test these points of investigation.

CHAPTER III: Ventral tegmental area dopamine directly modulates aggression through the lateral septum

a. Introduction

Hyper-dopaminergic model of aggression

In chapter II we concluded that our developmental manipulation of dopamine transporter (DAT) function, during a sensitive period, produces aggression and a hyper-dopaminergic (DAergic) system. Here I will elaborate on prominent examples in the literature that form the basis of the general hyper-DAergic model of aggression (De Almeida et al., 2005).

Increased psychopathic behaviors are positively correlated with high levels of dopamine (DA) metabolites in the cerebrospinal fluid (CSF) of violent criminals (Soderstrom et al., 2001). Furthermore, DAT polymorphisms, involved in heightened DAergic neurotransmission, are associated with antisocial behavioral traits and personalities (Guo et al., 2007; Fergusson et al., 2011; Janssens et al., 2015). DAT is responsible for regulating normal levels of synaptic DA. However, polymorphisms such as the 9R and 10R *DAT1* variable tandem number repeats (VNTR) affect DAT expression resulting in a hyper-DAergic state (Vandenbergh et al., 1992; Doucette-Stamm et al., 1995; Bannon et al., 2001). Moreover, there is a strong association between individuals with these polymorphisms and violent behavior. For example, in a group of violent murderers in Pakistan, the 9R allele was associated with criminal tendencies (Qadeer et al., 2017). In a group of children, the 9R variant was a major risk allele for externalizing behavior (Young et al., 2002). Furthermore, the *DAT1* 10/10 and 10/9 genotypes were twice as common in a group of violently delinquent young adults when compared to controls (Guo et al., 2007; Burt and Mikolajewski, 2008; Barnes and Jacobs, 2013). Another gene associated with aggression is the gene encoding catechol-O-methyltransferase (*COMT*). COMT is a major enzyme responsible

for DA degradation and so it influences DA flux (Meyer-Lindenberg et al., 2005). Individuals with a single nucleotide polymorphism (SNP) (V158M) in the *COMT* gene display low activity of this enzyme, increased levels of DA in the brain, and aggressive personality traits (Rujescu et al., 2003). Another *COMT* mutation, which leads to reduced enzymatic activity is associated with self-reported aggression among people with a diagnosis of axis II personality disorders (Flory et al., 2007). Finally, preclinical work in mice lacking DAT, reveals a hyper-DAergic tone correlating with hyper locomotion (Giros et al., 1996), and increased reactive aggression following mild social contact (Rodriguiz et al., 2004).

Besides endogenous, genetically-driven elevations in DAergic activity that are linked to antisocial dispositions, several studies have investigated the DA-aggression relationship more directly. For example, dating back to 1973, work by Redmond and colleagues demonstrated that catecholamine depletion through local intraventricular infusions of 6-Hydroxydopamine in free ranging macaques reduced threat and attack initiation as compared to sham treated and field controls (Redmond et al., 1973). In rodents, low doses of DA infusions into the lateral ventricles of chronically cannulated male rats increased the number of attacks when compared to animals infused with norepinephrine or saline (Geyer and Segal, 1974). Another study found reliable increases in tyrosine hydroxylase (TH) in the brains of stressed and aggressive rats. They suggest that increased TH, which in turn leads to heightened catecholamine synthesis, could be a mechanism underlying the increased aggressive behavior (Lamprecht et al., 1972). More recent work aligns with traditionally laid out hypotheses linking heightened DA and aggression. Chronic administration of methamphetamine escalates aggression in male mice (Sokolov et al., 2004; Sokolov and Cadet, 2006). Cocaine, a drug that blocks the DAT, significantly increases aggression in adolescent rats (Harrison et al., 2000; DeLeon et al., 2002). These manipulations, which have

the net effect of increased DA signaling (regardless of the mode of action), are all associated with heightened aggression.

Dopaminergic circuit of aggression

Chapter II reveals a positive correlation between levels of aggression, behavioral sensitivity to amphetamine and heightened DAergic physiological activity. In doing so, we present support for the hyper-DAergic hypothesis of aggression. However, a direct and causal relationship between DAergic neuronal activity and aggression had not been demonstrated. In one of my first experiments, that laid the foundation for my thesis, I tested the role of direct and specific optogenetic activation of DAergic neuronal activity in aggression (Yu et al., 2014). I found increased aggression in mice following photo stimulation of ventral tegmental area (VTA) DA neurons (Figure 3.1). The altered DAergic physiology observed in chapter II, which suggests strong involvement of the VTA DAergic population, is well aligned with the optogenetic data. Advancing this important finding, the current chapter explores DAergic population specificity in aggression control. Furthermore, this chapter characterizes VTA DA innervation patterns at possible aggression target sites, and then investigates the role of identified projection regions in modulating behavior.

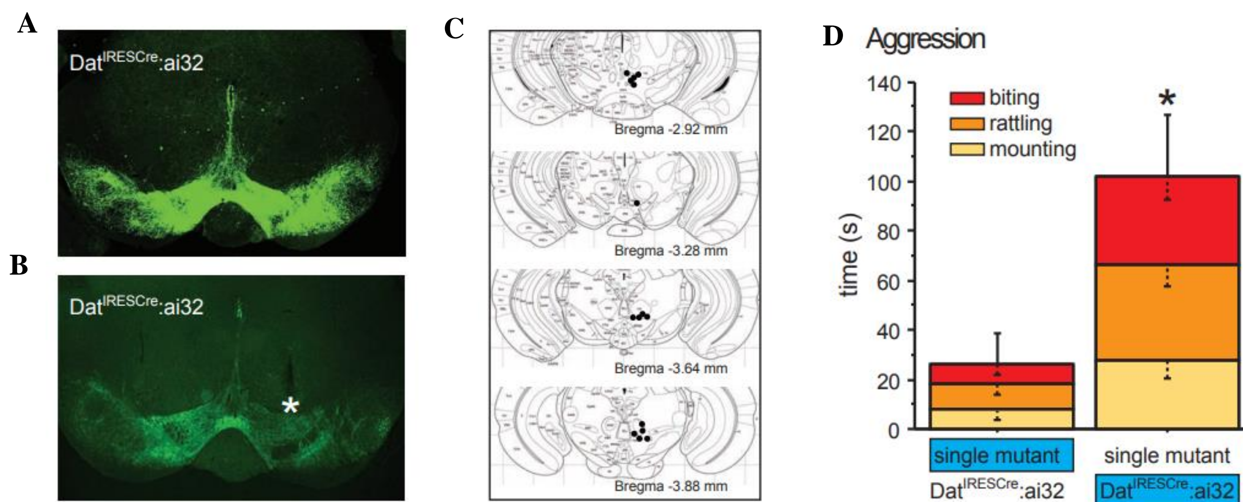


Figure 3.1: Optogenetic stimulation of DAergic VTA neurons increases aggression. Dat^{IRESCre};ai32 double mutant mice expressing ChR2-EYFP in VTA neurons as visualized by immunohistochemistry against (**A**) eYFP and (**B**) autofluorescence. A fiberoptic cable track demarks the position of an implant with its tip just dorsal of the VTA (indicated by a star, **B**). (**C**) Tip locations were assessed histologically after behavioral experiments were concluded. (**D**) Dat^{IRESCre};ai32 double transgenic mice and single mutant controls were co-housed in mixed pairs and isolation induced aggressive behavior was assessed by scoring the time spent mounting, tail rattling, or biting during a 10 minute encounter Only one mouse in a pair was stimulated (blue). Aggressive behavior was increased in pairs when Dat^{IRESCre}:ai32 double mutant mice were stimulated when compared to pairs where the single mutant controls were stimulated. *, $p < 0.05$; n = 11-18 per group. (Yu et al., 2014).

The ventrolateral subdivision of the ventromedial hypothalamus (VMHvl) and the lateral septum (LS) are two prime nodes that have been explored as direct regulators of aggression. Electrical stimulation of the VMH nucleus increases attack behavior elicited in cats (Maeda et al., 1985; Maeda and Maki, 1986). Direct optogenetic activation of the VMHvl increases aggression towards male and female mice as well as inanimate objects (Lin et al., 2011). The LS, in turn, exerts a top-down break on the medial hypothalamus (Wong et al., 2016). This downstream inhibition of the hypothalamic attack area is interrupted via lesions of the LS, resulting in a phenomenon known as “septal rage”, whereby damage to the LS leads to a significant increase in attacks (Brady and Nauta, 1953; Sodetz and Bunnell, 1970; Slotnick and McMullen, 1972; Albert

and Chew, 1980). Conversely, LS electrical stimulation (Potegal et al, 1980), and more recently LS optogenetic activation (Wong et al., 2016), decreases aggression, presumably by strengthening the LS's downstream control of the medial hypothalamus.

Beyond the traditional aggression centers, a third region of interest in this chapter is the nucleus accumbens (NAc). Although this region is not described in detail as a conventional aggression node in chapter I, the strong correlation of NAc DA levels with aggression warrants further investigation of it in the current chapter. In fact, in vivo microdialysis shows that DA release, in the NAc, increases in anticipation of (Malison et al., 1998; Ferrari et al., 2003), during and following rodent aggressive encounters (Tidey and Miczek, 1996; van Erp and Miczek, 2000; Van Erp and Miczek, 2007). Moreover, Couppis and Kennedy directly suppressed DAergic activity in the NAc and found that mice no longer engaged in instrumental behavior to gain access to aggression, thus implicating NAc DA in the rewarding properties of aggression (Couppis and Kennedy, 2008). Low anxiety rats who exhibit heightened aggression, have a strongly activated NAc (as measured by increased c-fos and zif268 mRNA expression) and higher local DA release in response to the resident-intruder test (Beiderbeck et al., 2012). Finally, increased DA turnover has been reported in the NAc of resident mice who have threatened and attacked an intruder (Haney et al., 1990). Given these findings, we questioned whether VTA DA produced its effect via direct modulation of traditional aggression centers including the VMHvl and/or the LS, or through the NAc, a region where VTA DA release is linked with the aggression experience.

In light of the hyper-DAergic hypothesis of aggression, together with initial circuit data from our lab identifying a role for VTA DA in driving aggression, in this chapter I will describe, in detail, our efforts to expand on past findings by testing the following aims:

1. First, we aim to explore DAergic population specificity in driving aggressive behavior: *Specifically, we hypothesize that DAergic aggression is driven by a VTA-specific projection circuit and not a substantia nigra pars compacta (SNc) projection, given the SNc's prominent role in motor control.*

2. Second, we aim to characterize DAergic innervation patterns in the VMHvl, LS and NAc: *Specifically, we hypothesize that the target population of cells driving the aggressive behavior receives DAergic innervation which originates in the VTA, not SNc.*

3. Third, we aim to behaviorally evaluate the sufficiency of the DAergic innervation of immunohistologically screened nodes in aggression and related behaviors:
   Specifically, we hypothesize that terminal DA release at one or more of the characterized nodes will specifically drive aggression, leaving related behaviors unaffected.

b. Materials and methods

Animals

Dat^{IRESCre} (Bäckman et al., 2006) mice and ROSA26-floxedSTOP channelrhodopsin-2-enhanced yellow fluorescent protein (ChR2-EYFP) mice (referred to as 'Ai32' mice; RCL-ChR2(H134R)/EYFP) (Madisen et al., 2012) were crossed to produce experimental cohorts consisting of Dat$^{IRESCre;}$Ai32 double transgenic and single mutant controls on a mixed F1 background (C57BL/6J x 129S2/129SvEv/Tac). For all experiments, controls were littermates that were positive for the Ai32 transgene but negative for the Cre transgene. This Ai line allows for expression of the fusion construct, only following exposure to Cre recombinase. Therefore, driven by the DAT promoter, these mice conditionally express ChR2 (fused to EYFP for fluorescent visualization) in DAergic cells only (Boyden et al., 2005). Crossed with Dat^{IRESCre} mice, we captured > 95% of DAergic neurons, based on immunohistochemistry (Mingote et al., 2017). Thus, this strategy allowed us to interrogate ~all DAergic input to target regions without variability. Given that female mice do not display territorial aggression, only male mice >4 months of age were investigated. Mice were housed under a 12:12-hour light:dark cycle (lights on at 7am) in a temperature controlled environment and provided with food and water *ad libitum*. Animal testing was conducted under protocols approved by Columbia University and New York State Psychiatric Institute Institutional Animal Care and Use Committees.

Construction of fiber optics

The fiberoptic ferrules used for optogenetic photo stimulation experiments were constructed in house by interfacing an optical fiber (OD 0.2mm, Thor labs, Newton, NJ, USA) with a 1.25-mm OD multimode ceramic zirconia ferrule stick (Bore size: 230µm) (Precision fiber products). The fiber extended beyond the end of the ferrule, to a length determined by the

dorsal/ventral coordinates of the region being investigated. Fibers were attached with epoxy resin into the ferrules, and subsequently cut with a diamond pen and polished. Mono fiberoptic patch cords were used (Doric Lenses).

Viral Construct

Cre-Dependent CAV2 retrograde virus, conditionally expressing Zs green (Sanford et al., 2017) was injected into the LS and NAc of $Dat^{IRESCre}$ mutant mice following the protocol detailed below. 4 weeks later mice were sacrificed and Zsgreen labelled neurons were imaged in the VTA. Because ZsGreen diffuses freely within axonal arbors of recombined neurons, we also qualitatively assessed ZsGreen positive axons at the sites of injection in the LS and NAc.

Stereotaxic fiberoptic implantations and viral injections

General surgery protocol: Surgeries were conducted on mice who were at least 2 months in age. Mice were anesthetized with a cocktail of ketamine (10 mg/kg body weight) and xylazine (50 mg/kg) solution via i.p. injections. Animals were then placed in a stereotaxic apparatus and core body temperature was maintained at 37 ˚C with a heating pad. Sterile ophthalmic ointment was applied to the animals' eyes to prevent corneal drying during the procedure. Depth of anesthesia was monitored by toe pinch and corneal reflex before starting, and throughout, the procedure at 10-minute intervals. If necessary, a booster anesthetic, 30mg/kg ketamine alone (30% of initial dose), was administered. The fur on the head was shaved and the area was disinfected with betadine and then alcohol. Once the mouse was fixed, a small incision (approximately 5-10mm) was made in the skin covering the cranium. All stereotaxic coordinates were measured relative to bregma.

Fiberoptic surgeries: Surgery commenced following all steps stated above. All behavioral mice were implanted with optical fiber ferrules at the sites of interest. Once the cranial coordinates were located, small openings (< 0.5 mm) were drilled at the selected sites. The optical fiber ferrules were implanted over the VTA (AP: -3.5; ML: ±0.5; DV: -4.2), the SNc (AP: -3.5; ML: ±1.6; DV: -4.4), the LS (AP: 0.6; ML: ±0.4; DV: -2.8) and the NAc (AP: 1.6; ML: ±1.4; DV: -3.8). The implants were secured in the skull using vet bond and dental cement (Dental cement kit; Stoelting).

Viral surgeries: Surgery commenced following all steps stated above. Virus microinjection was performed using Nanoject II (Drummond Scientific Nanoject II, Fisher Scientific, USA) attached to the guiding arm of the stereotaxic system. The virus was delivered using glass pipettes (tip opening 25 µm). Given that viral surgeries were only conducted for microscopy, the virus was injected unilaterally into the LS and NAc. The schematic describing the injection protocol is in Figure 3.2 below.

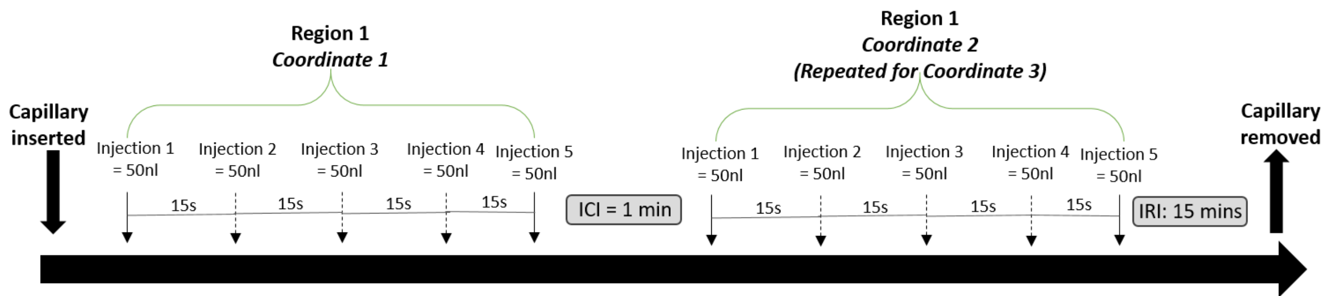


Figure 3.2: Viral surgery temporal schematic. For each region (LS and NAc), the virus was delivered at 3 DV coordinates in the LS (AP: 0.6; ML: ±0.4; DV: -2.8; 2.7; 2.6) and the NAc (AP: 1.6; ML: ±1.4; DV: -3.8; 3.7; 3.6). Each coordinate was pressure-injected 5 times with deposits of 50nl/each and a 15 second gap between each injection. At the end of injecting at each coordinate and region a 1 minute and a 15 minute gap was given, respectively. A volume of 0.25µl was delivered into each coordinate totaling to 0.75µl in volume/region. ICI = inter-coordinate interval; IRI = inter-region interval.

Drug treatment

SNc implanted mice were treated for 3 weeks with Fluoxetine (FLX; ANAWA Trading) before the rotarod test (described later in this section). The FLX was dissolved in 0.9% NaCl to achieve 10 mg/kg/day dosage when administered through drinking water. For the first 3 days they were observed for any abnormalities in behavior following the switch from regular drinking water to FLX water.

In vivo optical stimulation protocol

Before beginning any behavior, mice with fiberoptic implants were briefly immobilized in order to carefully attach the fiberoptic patch cords. These patch cords were in turn connected to a laser (473 nm) adjusted to give an output of 7-10 mw light intensity. The laser output was regulated by a programmable master-8 pulse stimulator through which the pulsing was set to occur at 20-Hz with a 10 ms pulse duration. The stimulator also allowed us to manually control the on and off switch of the laser. Once the patch cord was connected and mice were ready to commence the behavioral assay the laser was turned on.

Behavior

All animals were exposed to the same behavioral paradigms and timeline, starting at 4 months of age. Mice first underwent the open field locomotion assay, followed by real-time place preference and finally the isolation induced aggression. The SNc cohort was the only cohort to undergo the rotarod motor test at the very end. Given the number of assays, we allowed a 2 week gap between each behavior. All behavioral testing took place during the light cycle between 12:00pm and 7:00 pm. To eliminate odor cues, each apparatus was thoroughly cleaned after each animal.

Open field locomotion test

Locomotor activity in an open field, in response to optogenetic activation, was assessed in plexiglass activity chambers as previously described in detail chapter II. The open field is a standard test for locomotor behavior. It consists of a simple square enclosure that is equipped with infrared detectors to track animal movement in the horizontal and vertical planes. Measures of total distance covered during locomotion were used as an index of activity. Implanted mice were placed into the center of the open field and activity was recorded for 12 min in 3 min bouts of alternating light-off and light-on conditions. The "on" condition involved the blue light pulse protocol described above. In the "off" condition, mice remained connected to the patch cord without any stimulation. The master-8 stimulator allowed us to program the said stimulation protocol. Testing took place under bright ambient light conditions and total distance travelled was measured.

Real-time place preference test

Reward- based preference, as a function of stimulation, was evaluated in the real-time place preference test (RTPP). Mice implanted in the NAc and the LS were placed in a custom-made behavioral arena (18" x 10" x 8") for 20 min. The arena was divided into two chambers, each with different visual and tactile cues. The stimulation sides were randomly assigned such that half the cohort was stimulated on one side and the other half on the other side of the arena. Counterbalancing for genotype and stimulation side, each mouse was placed in the non-stimulation side at the onset of the experiment and delivered a 20-Hz pulse stimulation each time the mouse entered the assigned stimulation side. The stimulation was terminated the second the mouse crossed back into the non-stimulation side. Time spent in each chamber (stimulation vs. non-stimulation) was recorded using the ANY- maze video tracking software (Stoelting Co.).

Isolation-induced aggression test

In order to assess aggressive behavior, we used the isolation-induced aggression paradigm described in chapter II with modifications to include optogenetic stimulation. As detailed before, the home cage was divided in half by a perforated partition made of clear plastic. In the optogenetic assay however, $Dat^{IRESCre}$;Ai32 mutant mice were paired with controls. Mice were housed for 10 days before the experiment was performed. On test day, dividers were taken out and a mouse was stimulated in the region being investigated, in a cross-over design counterbalancing for genotype. Only one mouse of the pair was stimulated during each 15 min encounter. The time spent fighting was scored as a sum of the time spent biting, tail rattling and mounting. All fights were video recorded to allow for any additional post-hoc analysis. The experimental design is detailed in a schematic in Figure 3.3.

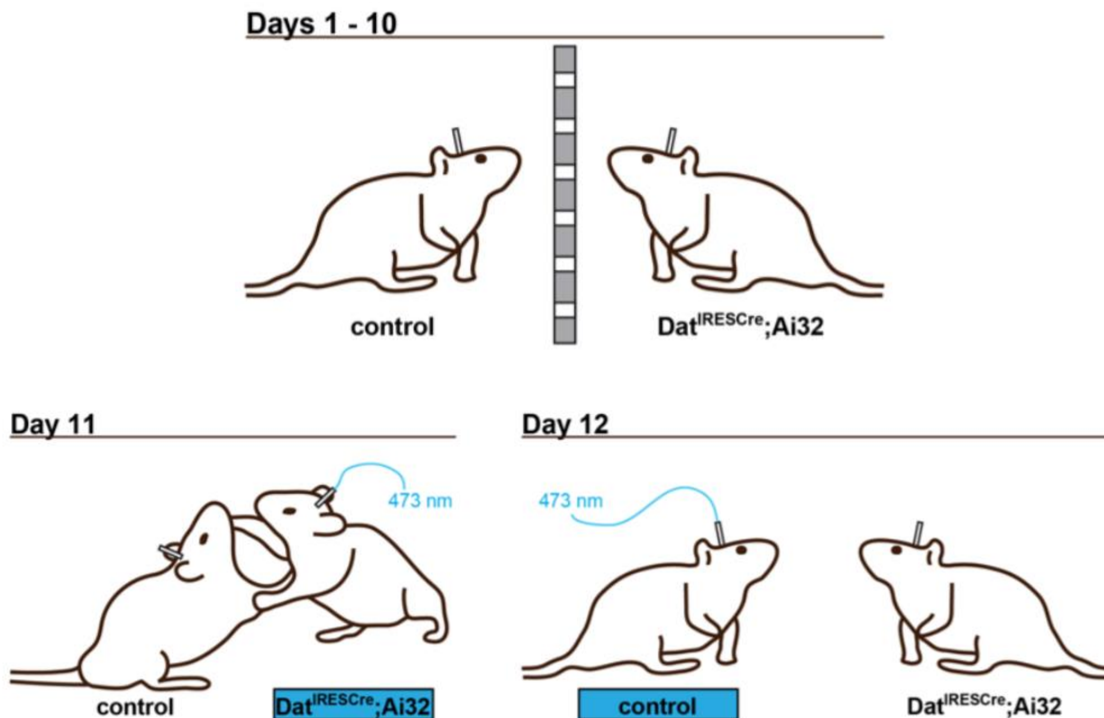


Figure 3.3: Isolation-induced aggression paradigm using optogenetics. Schematic of the isolation induced aggression experimental design between males. After separation for 10 days, mice underwent a stimulation protocol, counterbalanced for genotype, on Day 11 and 12.

In the assay against females, either control or $Dat^{IRESCre}$;Ai32 male mice were isolated in their home cage, as previously described. However, instead of co-housing with a male conspecific a female mouse occupied the other half of the cage (Figure 3.4). All females were screened using vaginal smears in a cytology protocol outlined by Cora and colleagues (Cora et al., 2015). Only mice who were not in the estrous stage of cycling were selected. On test day the male was stimulated and aggression toward the females was scored.

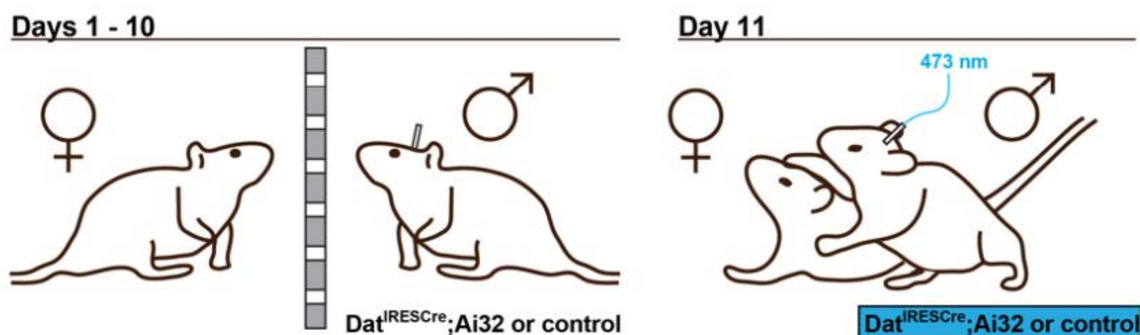


Figure 3.4: Isolation-induced aggression against females. Schematic of the isolation induced aggression experimental design against females. After isolation for 10 days, either $Dat^{IRESCre}$;Ai32 or control mice were stimulated (blue) in the VTA during an encounter with a female on Day 11.

<u>Rotarod test</u>

A rotarod apparatus (accelerating model; Ugo Basile) was used to measure motor coordination and balance. Mice were placed individually on the rotating rotarod system and trained for 6 consecutive days, first in accelerating speed mode at 0–20, 0–30, and 0–40 rpm, and then in constant speed mode at 20, 30, and 40 rpm. In all trials with constant speed, animals were individually placed on the rotarod while the rotarod was already rotating at the specified speed. Mice received three trials per day. During training period, all mice attained a stable baseline level of performance. Following training, mice were subjected to chronic FLX treatment for a period of

three weeks, after which, testing was performed at 20 and 30 rpm in constant speed mode. Overall rotarod performance was expressed as latency to fall. During testing, each mouse was bilaterally stimulated using the described blue light pulse protocol. Testing took place over two consecutive days, counterbalancing the stimulation order by genotype.

Histology

After behavioral tests were completed, mice were euthanized, and genotypes and fiber placements were confirmed. Animals were deeply anesthetized with ketamine/xylazine and transcardially perfused with phosphate-buffered saline (PBS) followed by 4% paraformaldehyde in PBS. Brains were then removed and post-fixed overnight (4% paraformaldehyde in PBS), and subsequently cryoprotected in 30% sucrose and frozen. Coronal sections (50 μm thick) were cut with a cryostat and fluorescence immunohistochemistry was performed on free-floating sections.

Immunohistochemistry and microscopy

Sections were first rinsed with PBS containing 0.2% TritonX-100 and then blocked with buffer (PBS, 2% bovine serum albumin and 0.2% Triton X-100). This was followed by overnight incubation with primary antibody and eventually conjugated to a fluorophore with the appropriate secondary antibody. The sections were incubated in primary antibody for 24 h at 4 °C, washed with PBS, and then incubated with secondary antibody for 1 h at room temperature. After washing 3x with PBS the sections were incubated with Hoechst 33258, Thermo Fisher Scientific, in a final concentration of 1μg/ml and transferred into PBS solution with 0.2% TritonX-100. Finally, the sections were mounted with Vectashield mounting medium (Vector Labs, Burlingame, CA, USA).

For GFP, immunohistochemistry was performed using a rabbit primary antibody against GFP diluted in blocking solution 1:1000; Life Technologies, Grand Island, NY, USA. As a

secondary antibody we used Cy3 donkey anti-rabbit, diluted in blocking solution 1:350; Jackson Immunoresearch, West Grove, PA, USA. For TH, immunohistochemistry was performed using rabbit primary antibody against TH, diluted in blocking solution 1:1000; Millipore Sigma, Darmstadt, Germany. As a secondary antibody we used Cy3 donkey anti-rabbit, diluted in blocking solution 1:500; Jackson Immunoresearch, West Grove, PA, USA.

For analysis of endogenous and immune-labelled EYFP proteins, images were acquired at 2x using fluorescence microscopy (Zeiss Axioplan2). CAV2 retrogradely labelled DAergic neurons in the VTA were co-stained for TH and Hoechst and imaged. For analysis of co-expression of virally infected cells and immune-labelled proteins, images were acquired at 40x using confocal microscopy (Leica TCS SP8). All post processing of images was done on ImageJ software (National Institutes of Health).

Data and statistical analyses

Statistical analysis for all behavior in the current study was performed using StatView 5.0 software (SAS Institute, Cary, NC, USA) and Microsoft Excel. For the fights and RTPP, data were analyzed using a paired sample Student's t-test (male-male interactions) or an independent samples Student's t-test (male-female interactions). For locomotor activity and the rotarod, one-way or two-way analyses of variance (ANOVA) were conducted followed by post-hoc testing as indicated. The criterion for significance for all analyses was *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$.Results from data analyses are expressed as mean±s.e.m.

c. Results

The main experimental goals of this chapter were to:

1. Test DAergic population specificity in driving aggressive behavior.
2. Characterize DAergic innervation patterns in the aggression nodes under consideration: the VMHvl, LS and NAc.
3. Evaluate behaviorally, the functional sufficiency of VTA DAergic innervation of the immunohistologically screened nodes in aggression and related behaviors.

Using the paradigms discussed in the methods section, we conducted experiments to test the above and found the following:

1. ChR2-based optogenetic activation of DAergic VTA, but not SNc, neurons increases aggression:

In Chapter II we demonstrated how transient enhancement in DA levels, via P32-41 DAT blockade, impacts adult aggressive behavior and amphetamine response, implying that altered DAergic activity is governing changes in aggression. This hypothesis is supported by the establishment of a direct relationship between DAergic neuronal activity and aggressive behavior, using optogenetics. I found that photostimulation of VTA DAergic neurons causally increases aggressive behavior (Figure 3.1) (Yu et al., 2014).

In this aim, we hypothesize that the DAergic aggression phenotype is specifically driven by VTA, and not SNc, DA neurons given the SNc's prominent role in motor control. To elucidate which specific population of DAergic neurons exert a pro-aggressive influence over behavior, we optogenetically stimulated DAergic cells in both the VTA and SNc and compared the effect on aggressive behavior. For this experiment, we used $Dat^{IRESCre}$;Ai32 mice, which conditionally

express ChR2-EYFP driven by the DAT promoter (Bäckman et al., 2006; Madisen et al., 2012). At 2 months, male mice were implanted with fiberoptic ferrules in the VTA or SNc. At 4 months of age the mice stimulated and tested in the isolation-induced aggression test as described in the methods section of this chapter. We assessed aggression behavior in pairs of male mice where one mouse expressed ChR2 (carrying the $Dat^{IRESCre}$ and the Ai32 allele), while the other mouse did not (carrying only the Ai32 allele).

We found that optogenetic stimulation of VTA DAergic neurons significantly increased aggression and decreased latency to attack, as demonstrated by an effect of genotype on time spent fighting ($t_{(15)} = -2.89$, $p = 0.0112$) (Figure 3.5) and latency to bite ($t_{(15)} = 2.29$, $p = 0.0369$), tail-rattle ($t_{(15)} = 2.087$, $p = 0.0544$), and mount ($t_{(15)} = 2.946$, $p = 0.01$) (Figure 3.6). In order to test the idea of displaced aggression towards an inappropriate target (Anderson, 2012), male mice were stimulated in encounters with females. Dramatically, although male mice typically don't initiate aggressive behavior towards females, similar aggression patterns were observed against female mice as demonstrated by an effect of genotype on time spent fighting ($t_{(15)} = 3.012$, $p = 0.0087$) (Figure 3.7A) and latency to bite ($t_{(15)} = -3.719$, $p = 0.0021$), tail-rattle ($t_{(15)} = -4.066$, $p = 0.0010$), and mount ($t_{(15)} = -2.17$, $p = 0.0461$) (Figure 3.7). The modified isolation induced assay is detailed in the schematic (Figure 3.4). Finally, optogenetic SNc DAergic neuron stimulation did not impact time ($t_{(9)} = -1.358$, $p = 0.2077$) (Figure 3.8) or latency (biting: $t_{(9)} = 2.010$, $p = 0.0753$; rattling: $t_{(9)} = 1.983$, $p = 0.0787$; mounting: $t_{(9)} = 2.013$, $p = 0.0749$) (Figure 3.9) measures of aggression.

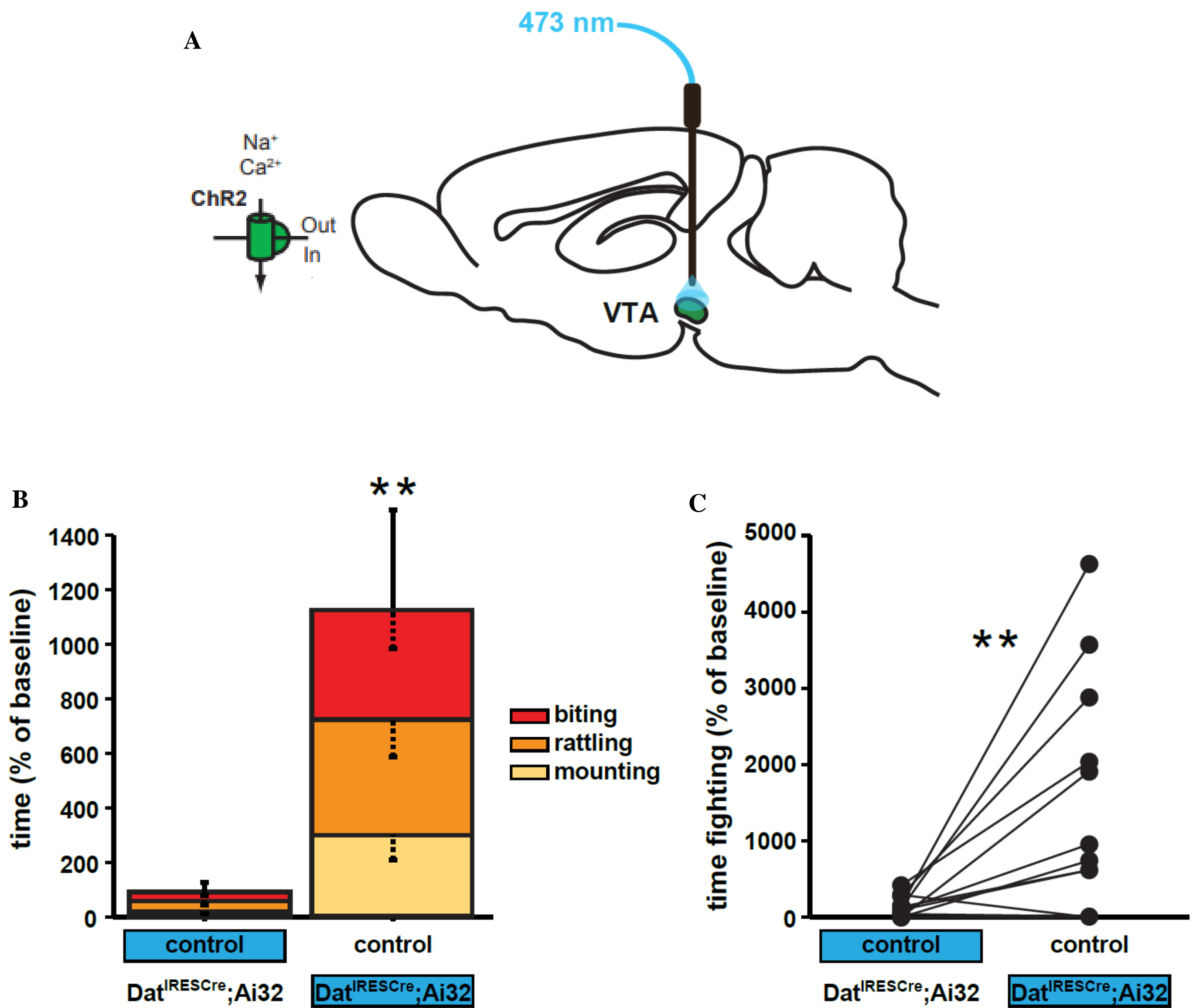


Figure 3.5: ChR2-based optogenetic activation of DAergic VTA neurons increases aggression. (**A**) Schematic diagram for stimulating VTA DA release *in vivo*. (**B, C**) Heightened aggressive behavior, measured as increased time fighting, was observed in pairs when $Dat^{IRESCre}$;Ai32 mutant mice were stimulated (blue). *, $p < 0.05$; **, < 0.01 compared with their respective controls; mean ± SEM; n = 16 per group.

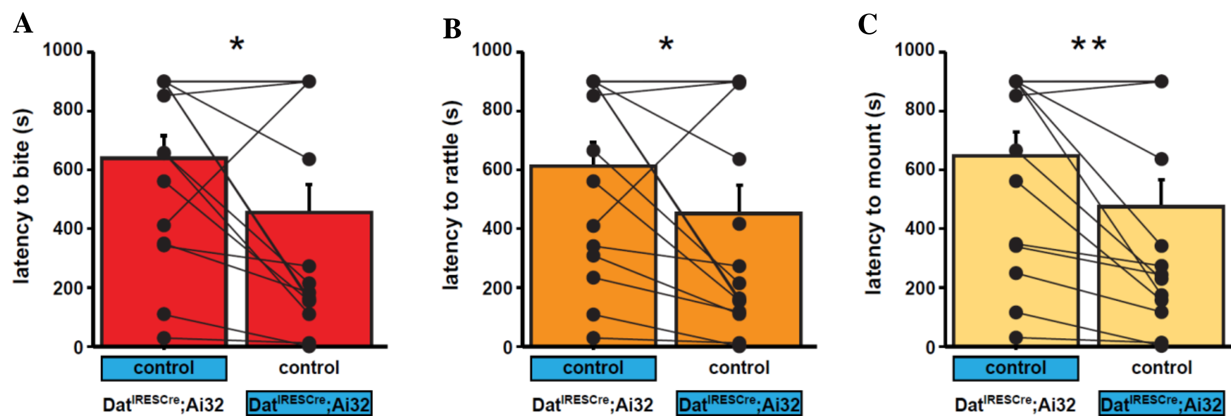


Figure 3.6: ChR2-based optogenetic stimulation of DAergic VTA neurons decreases the latency to attack. Heightened aggressive behavior, measured as decreased latency to (**A**) bite (**B**) rattle and (**C**) mount was observed in pairs when Dat^{IRESCre};Ai32 mutant mice were stimulated (blue) in the VTA. *, $p < 0.05$; **, $p < 0.01$ compared with their respective controls; mean ± SEM; n = 16 per group.

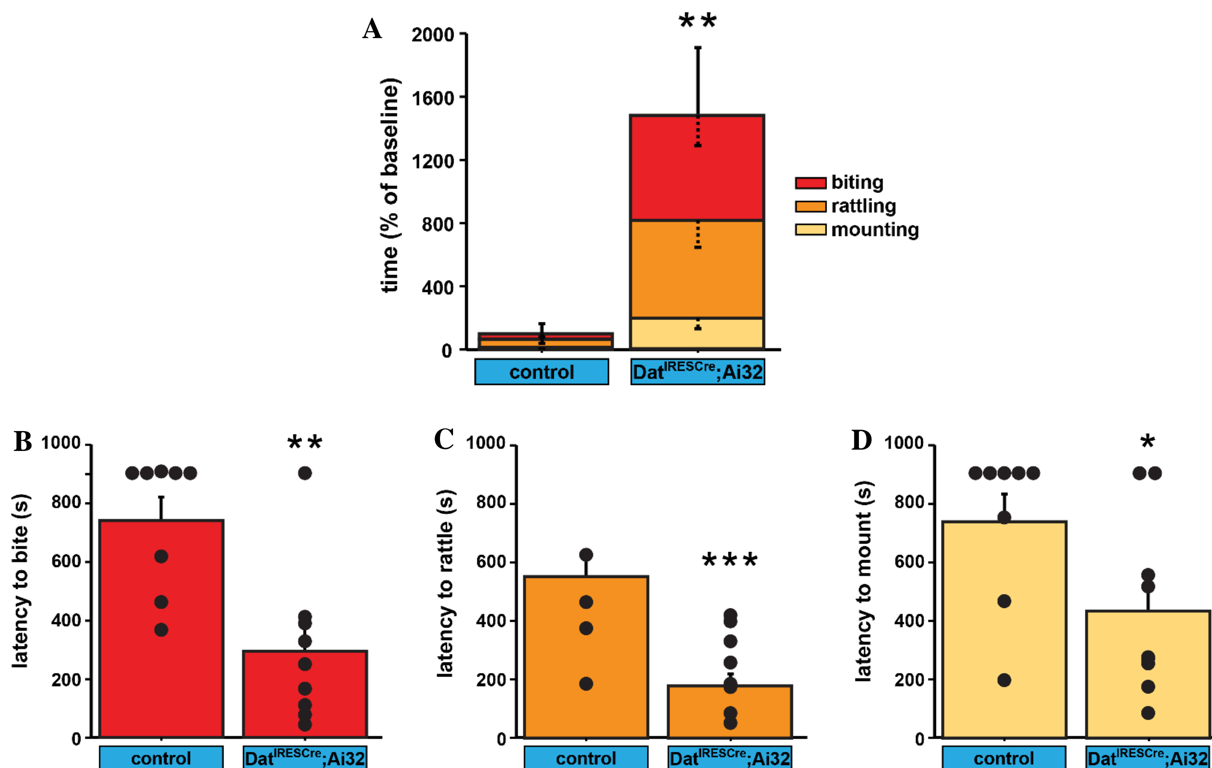


Figure 3.7: ChR2-based optogenetic stimulation of DAergic VTA neurons increases aggression against females. Heightened aggressive behavior against females including (**A**) increased time spent fighting and decreased latency to (**B**) bite (**C**) rattle and (**D**) mount was observed when Dat^{IRESCre};Ai32 mutant mice were stimulated in the VTA in comparison to when the controls were stimulated. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$ compared with their respective controls; mean ± SEM; n = 8-9 per group.

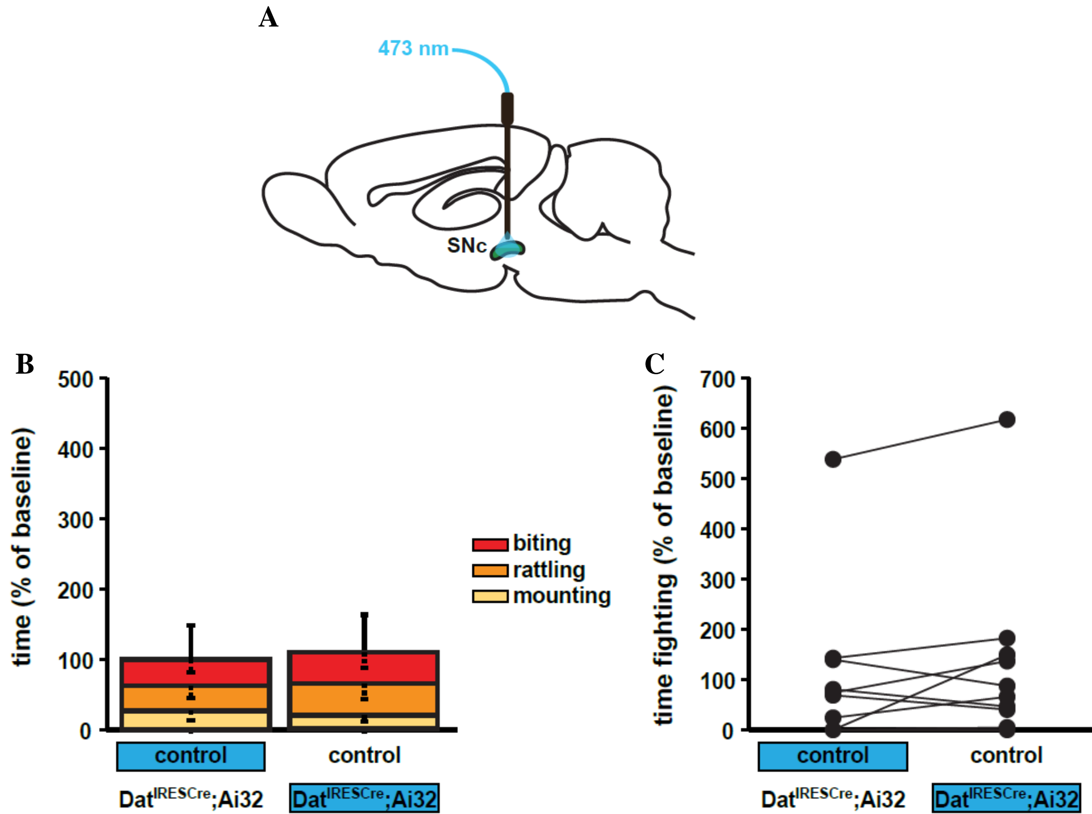


Figure 3.8: ChR2-based optogenetic activation of DAergic SNc neurons does not increase aggression. (**A**) Schematic diagram for stimulating SNc DA release *in vivo*. (**B**, **C**) No effect of SNc DA stimulation was detected. *, $p < 0.05$; **, $p < 0.01$ compared with their respective controls; mean ± SEM; n = 10 per group.

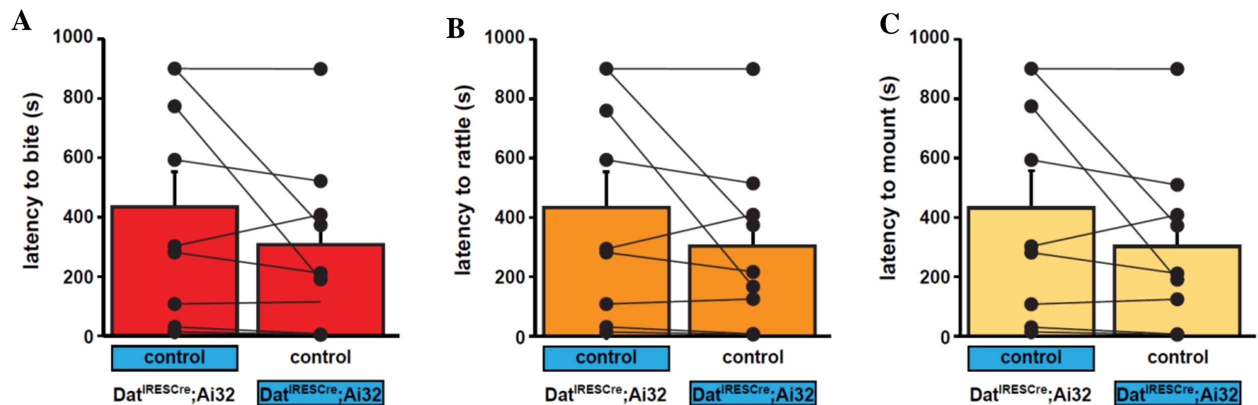


Figure 3.9: ChR2-based optogenetic stimulation of DAergic SNc neurons does not decrease the latency to attack. No effect of stimulation on latency to (**A**) bite (**B**) rattle and (**C**) mount was detected during SNc stimulation latency. *, $p < 0.05$; **, $p < 0.01$ compared with their respective controls; mean ± SEM; n = 10 per group.

In order to validate the efficacy of SNc stimulation we tested the cohort on a positive control, loss-of-function motor task using the rotarod. Our lab had previously shown that L-DOPA rescues chronic FLX induced motor deficits (Morelli et al., 2011). This finding indicates that the motor deficits are related to DA hypo-function brought on by chronic FLX exposure. We therefore hypothesized that increasing SNc DAergic activity would reverse the motor deficits elicited by chronic serotonin transporter blockade. We tested this hypothesis by optogenetically driving SNc DAergic neuronal activity in treated animals during rotarod testing, to evaluate if SNc stimulation could produce some change in behavior, if not aggression. After running the rotarod test, we detected an interaction between genotype and light stimulation (20 rpm: $F_{(1,12)} = 5.870$, $p = 0.0321$; 30 rpm: $F_{(1,17)} = 4.959$, $p = 0.0459$) with post-hoc analyses demonstrating significant improvement of motor performance with optogenetic stimulation in ChR2 expressing mice (Figure 3.10).

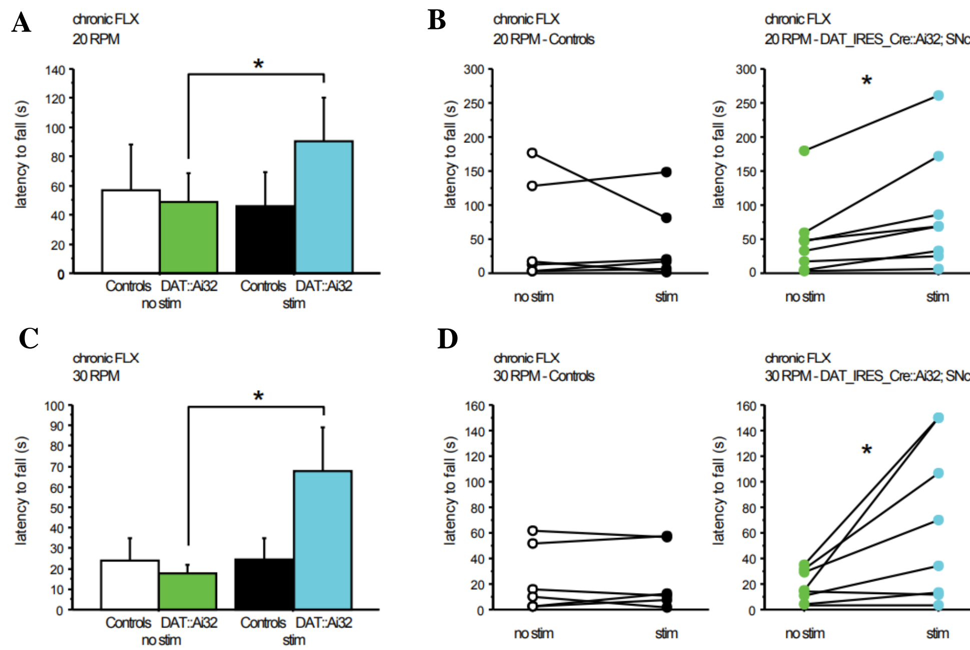


Figure 3.10: High frequency activity of SNc DAergic neurons rescues motor deficits elicited by chronic 5-HTT blockade. Motor behavior was evaluated using rotarod test. Optical stimulation (473 nm, 20 Hz, 10 ms pulse duration, 8 mW) increased the latency to fall at (**A**, **B**) 20 rpm and at (**C**, **D**) 30 rpm in $Dat^{IRESCre}$;Ai32 mice but not in control mice. *, $p < 0.05$; n = 6 - 8 mice per group.

Based on this data, I concluded that while SNc DAergic stimulation is efficacious in rescuing a hypo-DAergic motor deficit, this cell population plays no role in driving aggressive behavior. Histological confirmation of placements is in Figure 3.11.

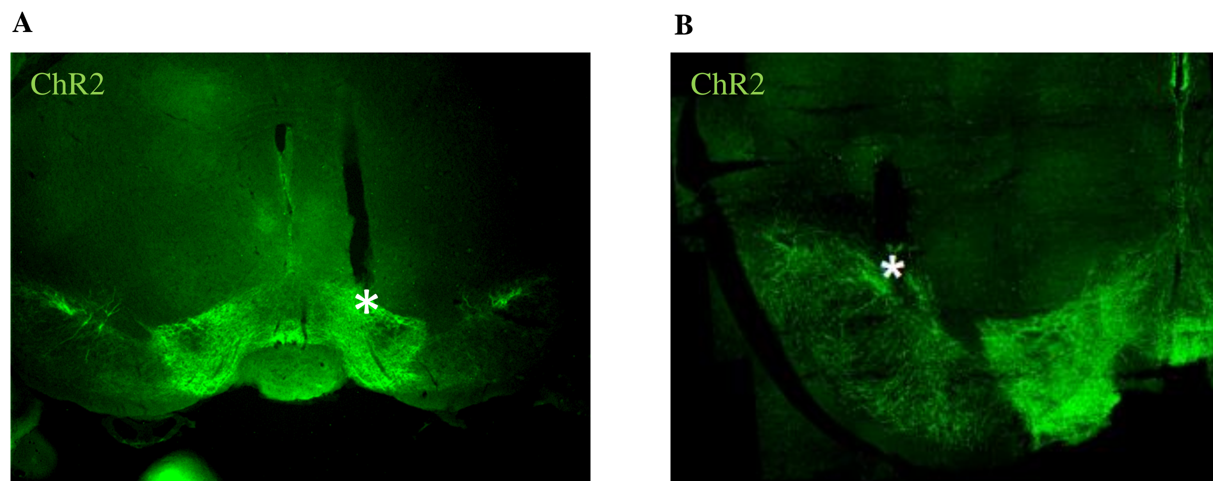


Figure 3.11: Histological confirmation of fiberoptic placement as assessed by EYFP distribution in Dat^{IRESCre};Ai32 mice. Exemplary fiberoptic placement tracks in behavioral cohorts of Dat^{IRESCre};Ai32 mutant mice expressing ChR2-eYFP in the (**A**) VTA (and (**B**) SNc. Asterisks indicate ventral tip of implant location.

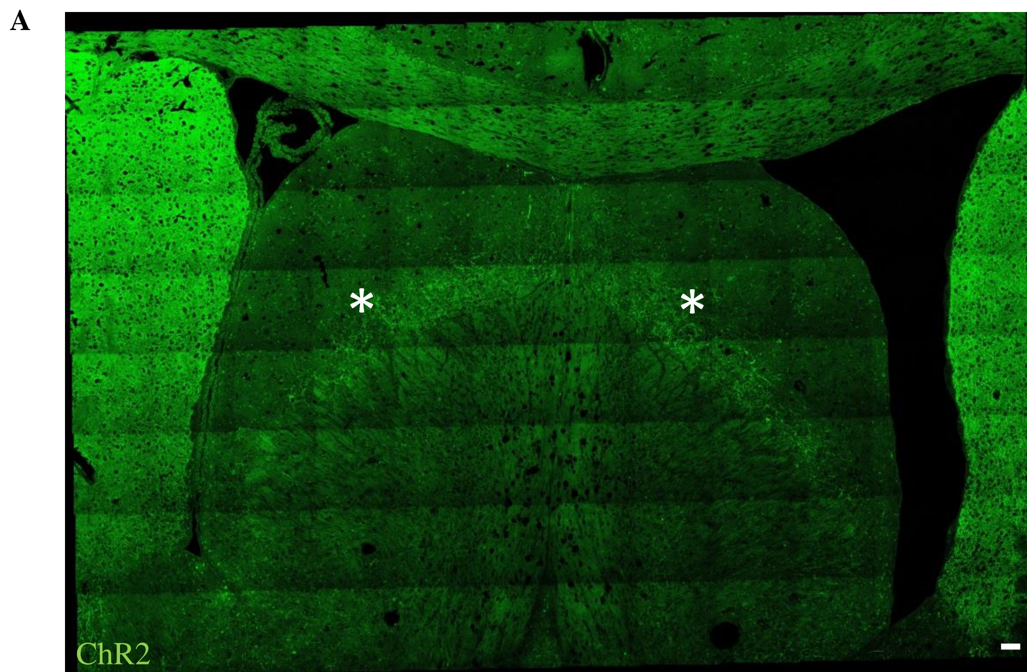
Considered together, this data therefore demonstrates a selective role for VTA DAergic neurons in driving aggression.

2. **VTA DAergic neurons innervate aggression nodes including the LS and NAc, but not the VMHvl:**

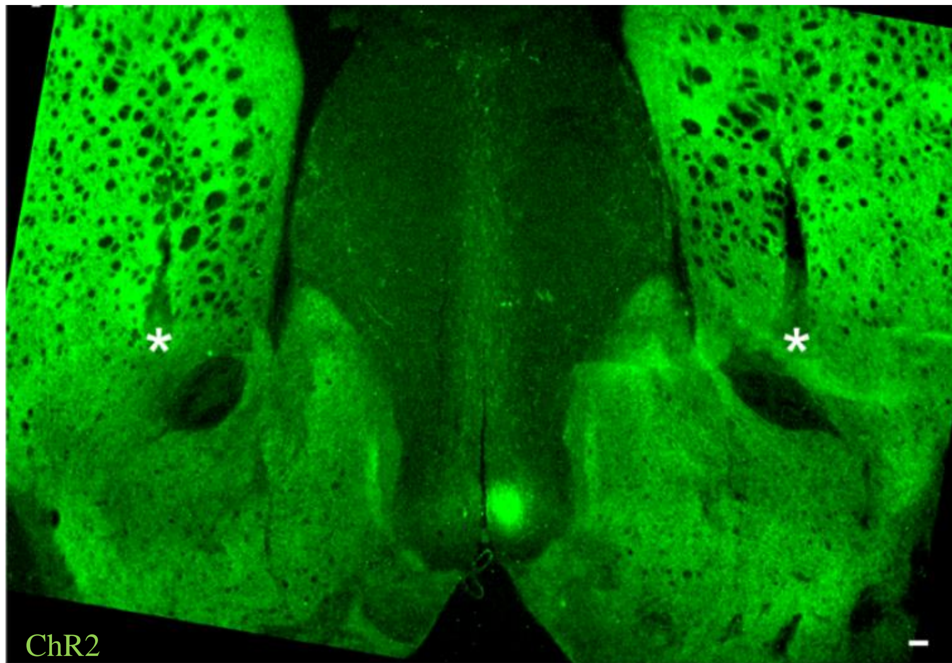
The previous aim established an exclusive role for VTA DA in driving aggression. However, the next question we asked is: where does this pathway project to, to produce this effect? In evaluating key target sites we explored three aggression nodes – VMHvl, LS, and the NAc. Our rationale for selecting these sites is discussed in detail in the introduction of this chapter. In this aim, we hypothesize that the population of cells driving the aggressive behaviors receives DAergic innervation. Furthermore, these fibers originate in the VTA, not SNc.

As a first step, we characterized DA fiber patterns in the regions of interest, the VMHvl, the LS and NAc, in Dat^{IRESCre};Ai32 mutant mice. Our histological analysis revealed DA expression in the LS and NAc (Figures 3.12A and B), but not in the VMHvl (Figure 3.12C). In the LS, previous immunocytochemistry exposed a strong pattern of DAergic marker Tyrosine Hydroxylase (TH)-positive axons distributed in the region. These axons were located along the perimeter of the septofimbrial nucleus forming a distinct diagonal band of TH immunoreactivity (Fallon and Moore, 1978; Gall and Moore, 1984; Gaspar et al., 1985). Corroborating this pattern we saw a similar band of ChrR2-EYFP fibers in LS (Figure 3.12A). For the NAc, we found prominent DAergic input, aligned with the literature that supports this histological characterization (Fallon and Moore, 1978; Gray et al., 1999). In light of our goal to establish a DA driven aggression circuit, the lack of DA expression in the VMHvl prompted us to eliminate this region from consideration as a direct site of action.

A



B



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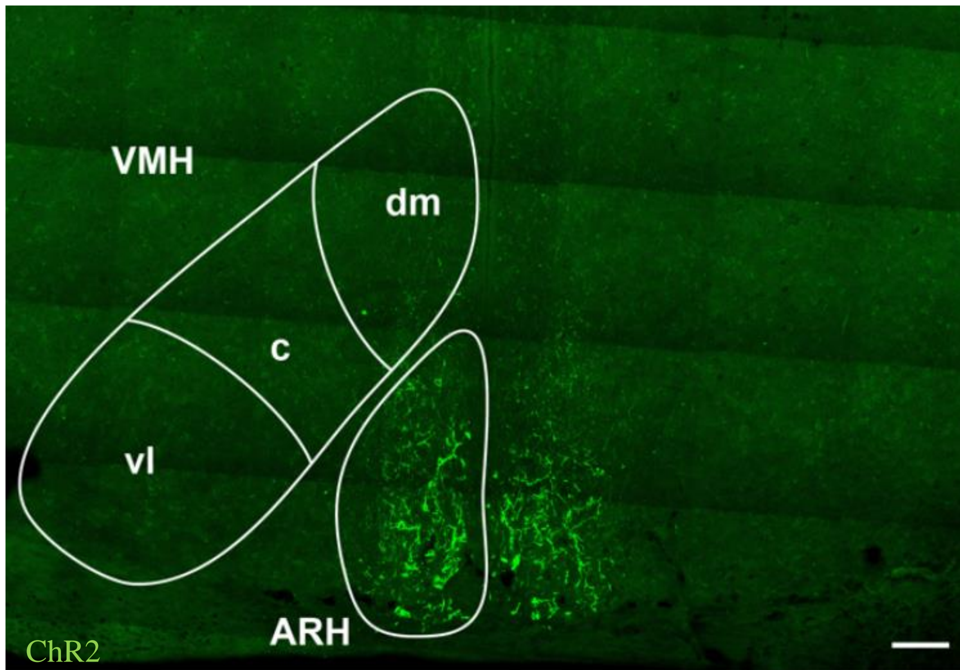
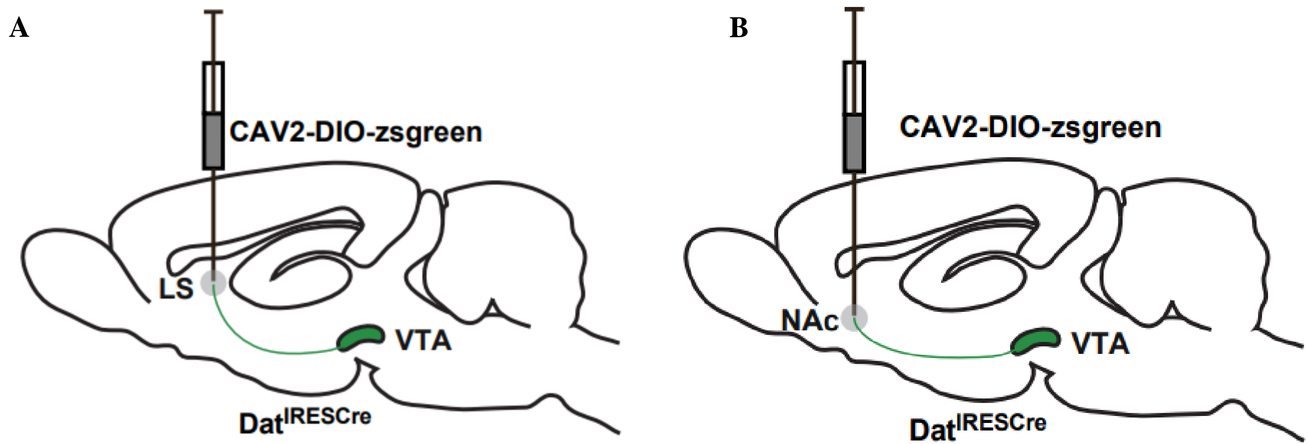
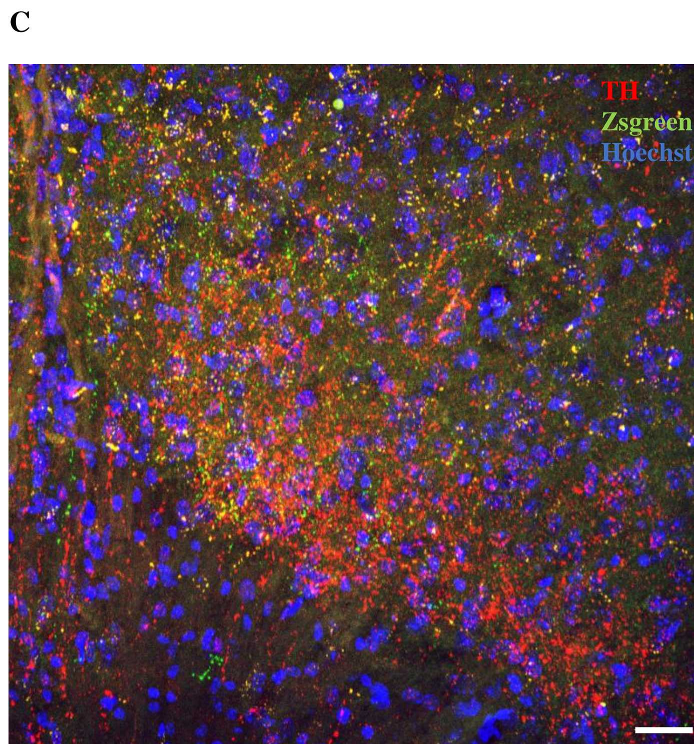
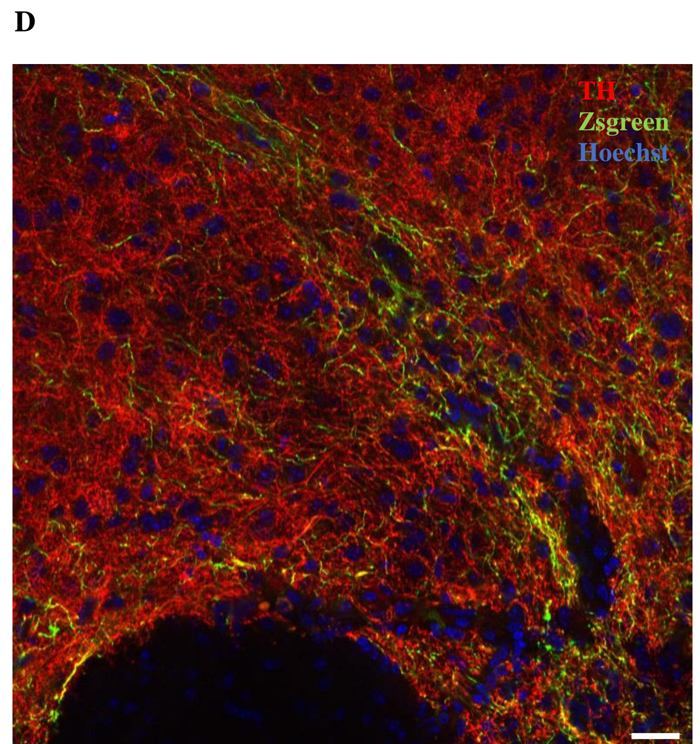


Figure 3.12: DAergic innervation as assessed by EYFP distribution in Dat^{IRESCre};Ai32 mice. Histological confirmation of prominent DAergic input into the (**A**) LS and (**B**) NAc but not the (**C**) VMHvl Scale bars: 100 µm. Asterisks indicate area of investigation.

Next, to investigate whether these DA afferents originate from the VTA, we injected a retrograde Cre- dependent CAV2 virus conditionally expressing ZsGreen (Sanford et al., 2017) into the LS (Figure 3.13A) and NAc (Figure 3.13B), followed by TH immunolabeling (Figures 3.13C and D).

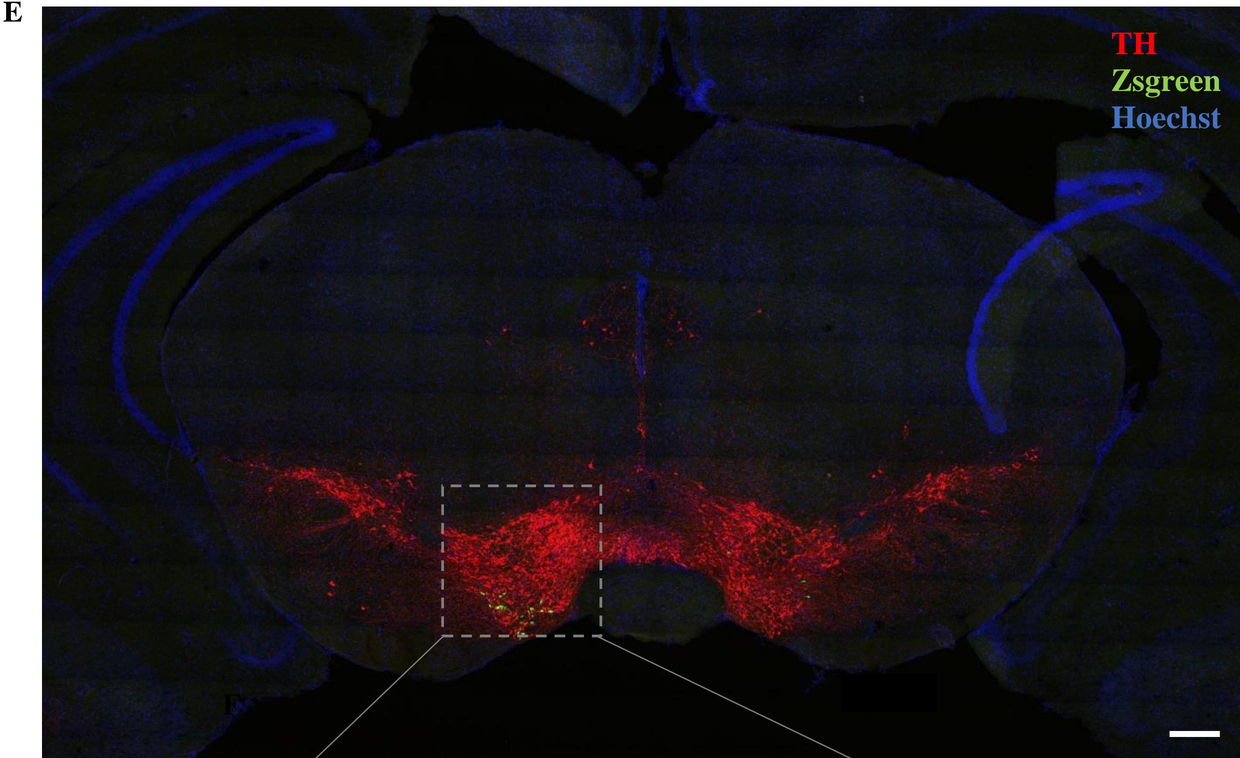




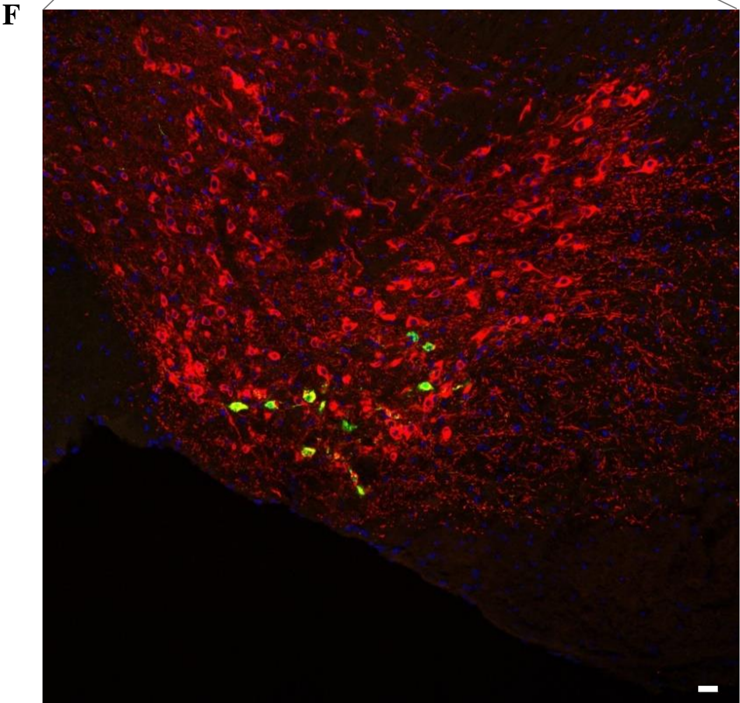


We found anatomically distinct clusters of DAergic cells projecting from the VTA to the LS (Figures 3.13E and F) and NAc (Figures 3.13G and H). These clusters were largely segregated, with LS projecting neurons notably aggregating in the most ventral tip of the VTA.

E

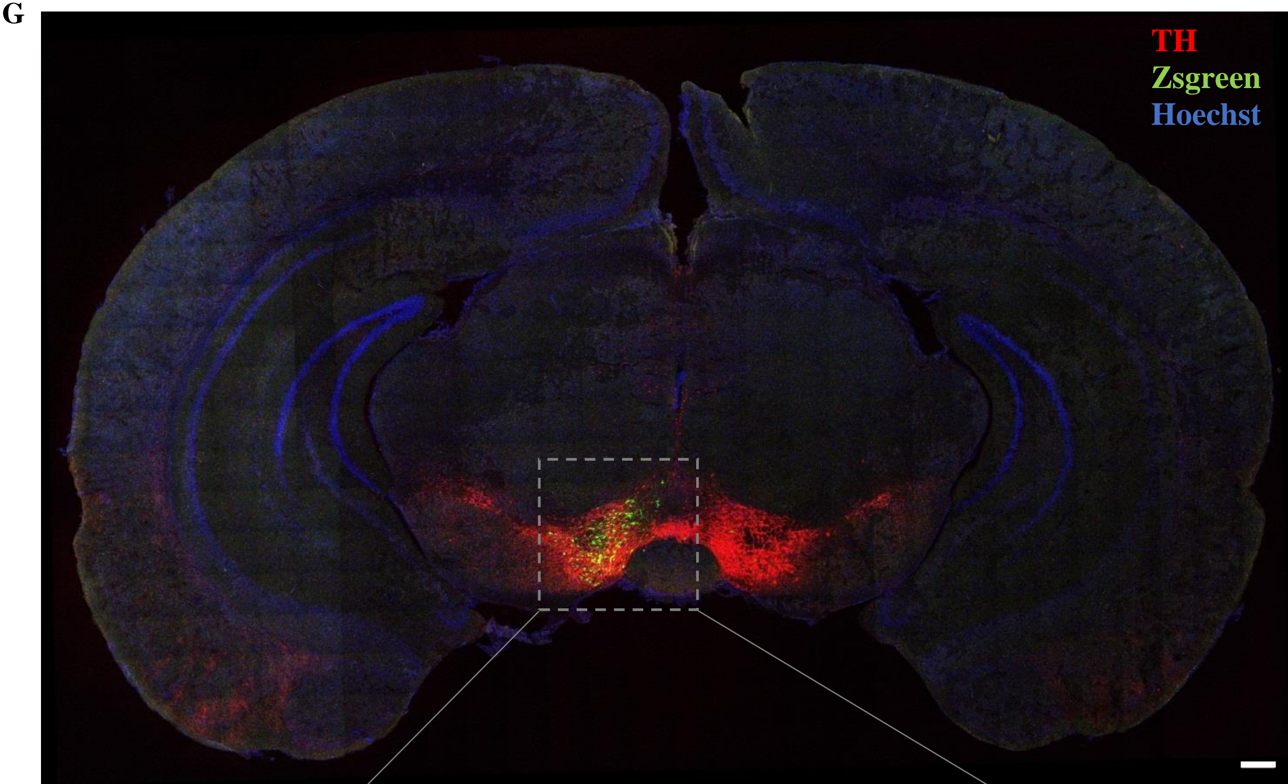


F

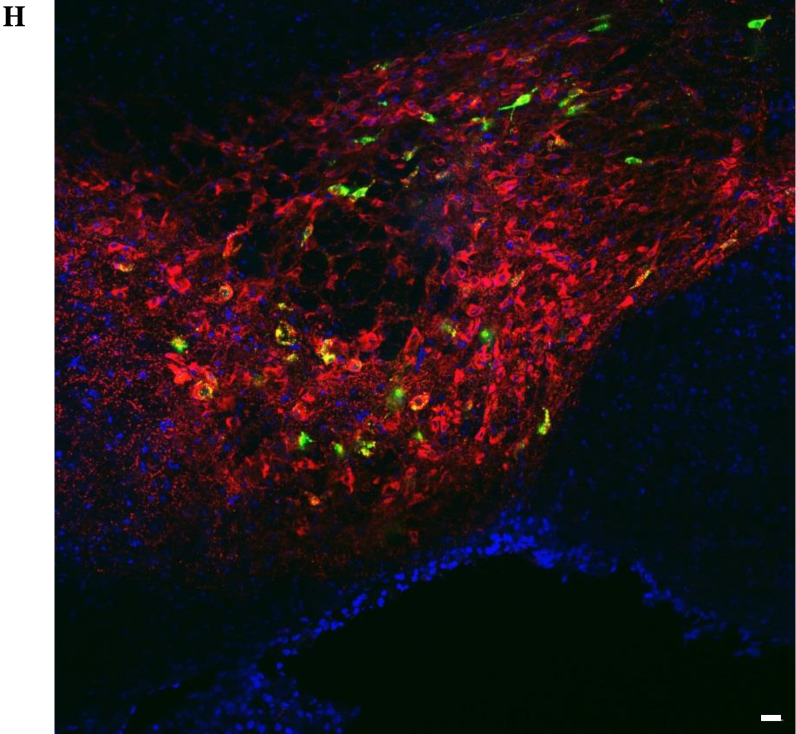


The NAc projecting cells were however distributed in both, the dorsal and ventral regions of the VTA and were relatively more anterior than the LS projecting cells.

G



H



Because ZsGreen diffuses freely within axonal arbors of recombined neurons, we qualitatively assessed collateralization. We did not detect ZsGreen positive axons in the NAc of LS injected mice (Figure 3.13I), nor in the LS of NAc injected mice (Figure 3.13J), indicating that NAc and LS projecting DAergic neuronal populations are largely non-overlapping.

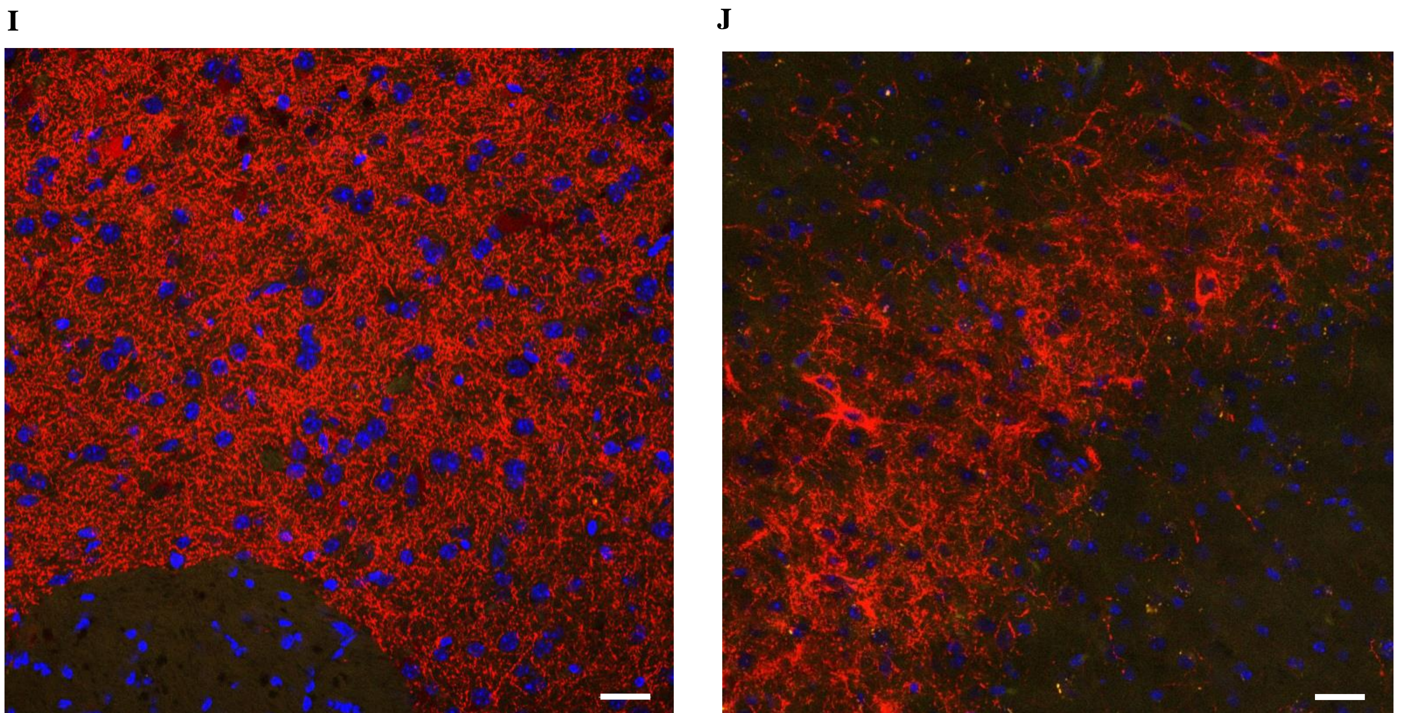
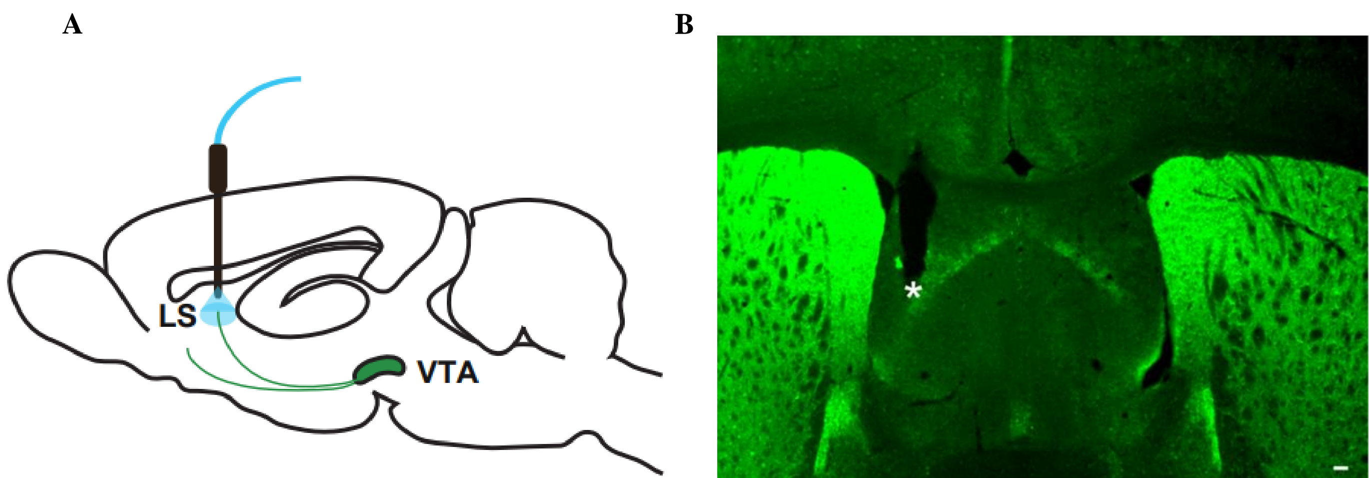


Figure 3.13: DAergic projections to the LS and NAc originate in the VTA and not the SNc. Schematic diagrams of CAV2-DIO-zsgreen injections in the (**A**) LS and (**B**) NAc of Dat^{IRESCre} mice. (**C-J**) Confocal images from CAV2-DIO-zsgreen injected Dat^{IRESCre} mice; Zsgreen (green), TH (red), Hoechst, (blue). Zsgreen positive and TH positive axons colocalizing at the (**C**) LS and (**D**) NAc injection sites. (**E**) Whole section image and (**F**) zoomed in confocal images of retrogradely labeled neurons in the VTA, in LS injected mice. (**G**) Whole section image and (**H**) zoomed in confocal images of retrogradely labeled neurons in the VTA, in NAc injected mice. Absence of Zsgreen labeled neurons in the (**I**) NAc of LS injected mice and in the (**J**) LS of NAc injected mice, respectively. Scale bars: 300 µm (**E, G**) or 30 µm (**C, D, F, H, I and J**)

3. DAergic projections from the VTA to the LS, not NAc, promote aggression:

Following histological screening of DA innervated aggression nodes in the previous aim, we hypothesized a specific and causal involvement of DAergic projections to the LS and/or the NAc, in modulating aggression. We tested this hypothesis using $Dat^{IRESCre}$;Ai32 mice with fiberoptic ferrules implanted in the LS or NAc (Figures 3.14). Following stimulation of the diagonal DA band in the LS of ChR2 expressing mice (Figure 3.14B), we found a significant increase in aggression and decrease in latency to attack, as demonstrated by an effect of genotype on time spent fighting ($t_{(15)} = -2.836$, $p = 0.0125$) (Figure 3.15) and latency to bite ($t_{(15)} = 1.787$, $p = 0.0942$), tail-rattle ($t_{(15)} = 2.48$, $p = 0.0255$), and mount ($t_{(15)} = 2.509$, $p = 0.0241$) (Figure 3.16). Once again, similar aggression patterns were observed against female mice as demonstrated by an effect of genotype on time spent fighting ($t_{(12)} = 3.120$, $p = 0.0088$) (Figure 3.17A) and latency to bite ($t_{(12)} = -3.847$, $p = 0.0023$), tail-rattle ($t_{(12)} = -3.844$, $p = 0.0023$), and mount ($t_{(12)} = -3.516$, $p = 0.0043$) (Figure 3.17B-D).



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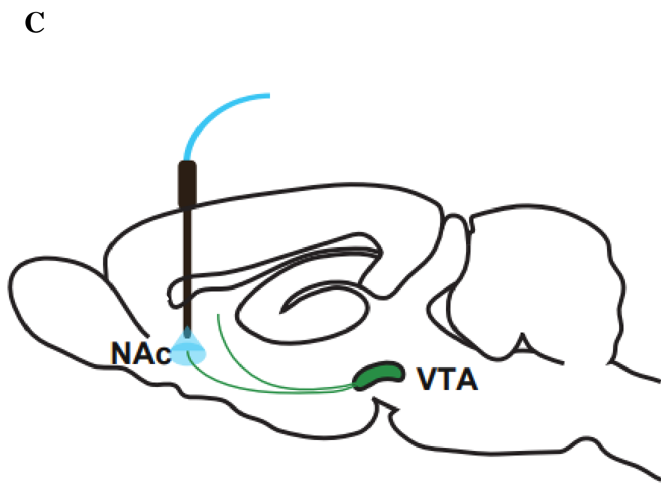

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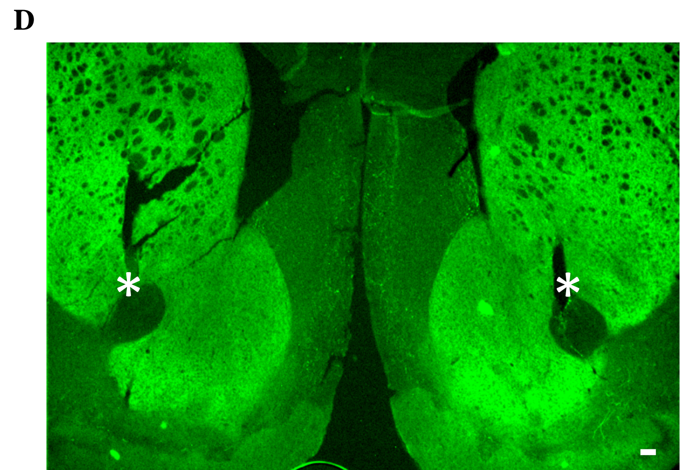


Figure 3.14: Terminal DA stimulation *in vivo* in the LS and the NAc. Schematic diagrams for stimulating DA release *in vivo* in the (**A**) LS and (**C**) NAc. Exemplary fiberoptic placement tracks in behavioral cohorts of Dat^{IRESCre};Ai32 mutant mice expressing ChR2-EYFP in the (**B**) LS and (**D**) NAc. Scale bars: 100 µm. Asterisks indicate ventral tip of implant location.

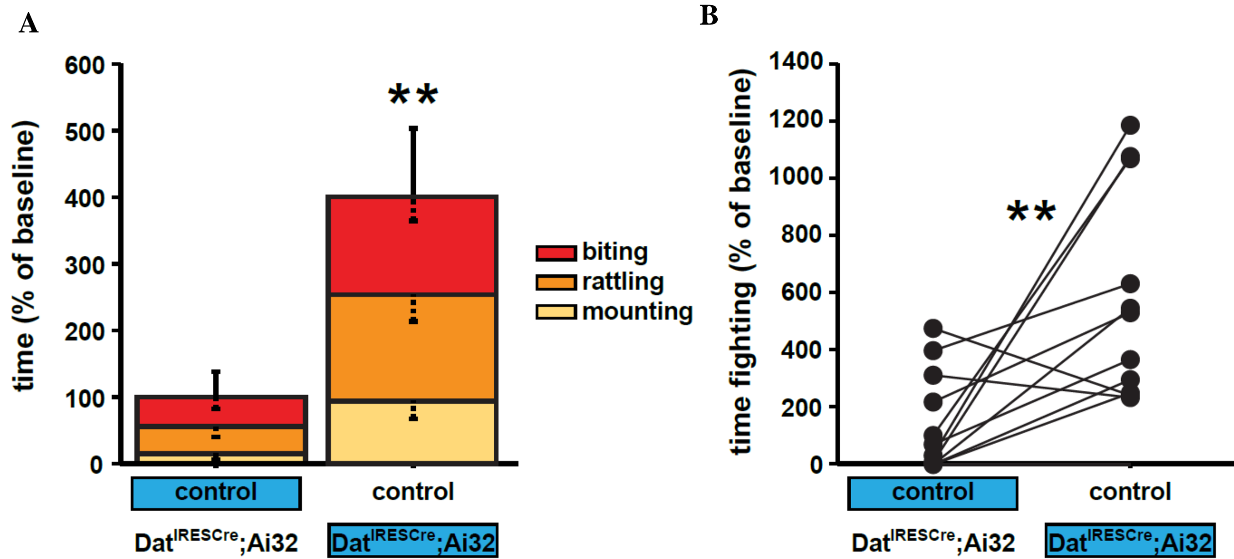


Figure 3.15: DAergic projections from the VTA to the LS promote aggression. (**A, B**) Heightened aggressive behavior, measured as increased time fighting, was observed in pairs when Dat^{IRESCre};Ai32 mutant mice were stimulated (blue) in the LS. *, $p < 0.05$; **, $p < 0.01$ compared with their respective controls; mean ± SEM; n = 16 per group.

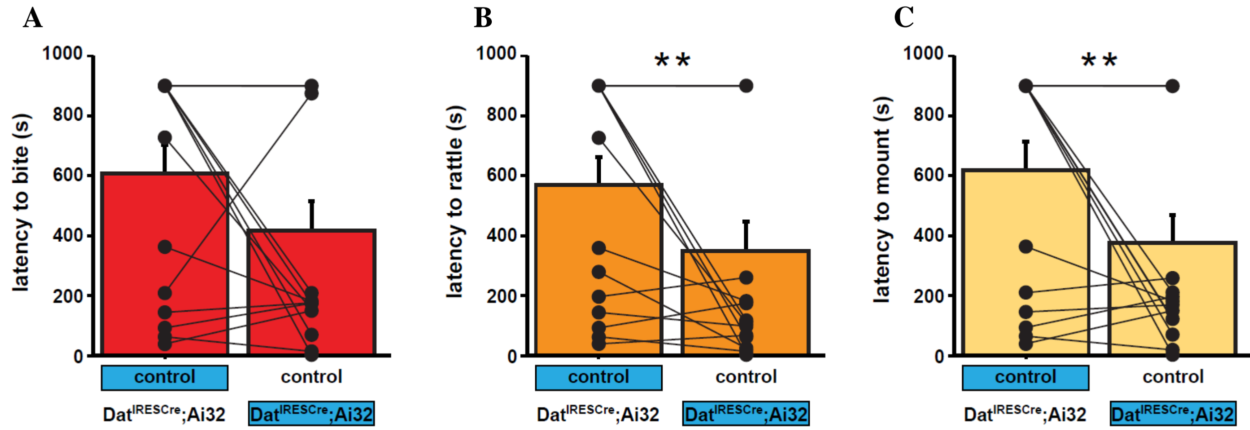


Figure 3.16: DAergic projections from the VTA to the LS decrease the latency to attack. Heightened aggressive behavior, measured as decreased latency to (**A**) bite (**B**) rattle and (**C**) mount was observed in pairs when Dat^{IRESCre};Ai32 mutant mice were stimulated (blue) in the LS. **, $p < 0.01$ compared with their respective controls; mean ± SEM; n = 16 per group.

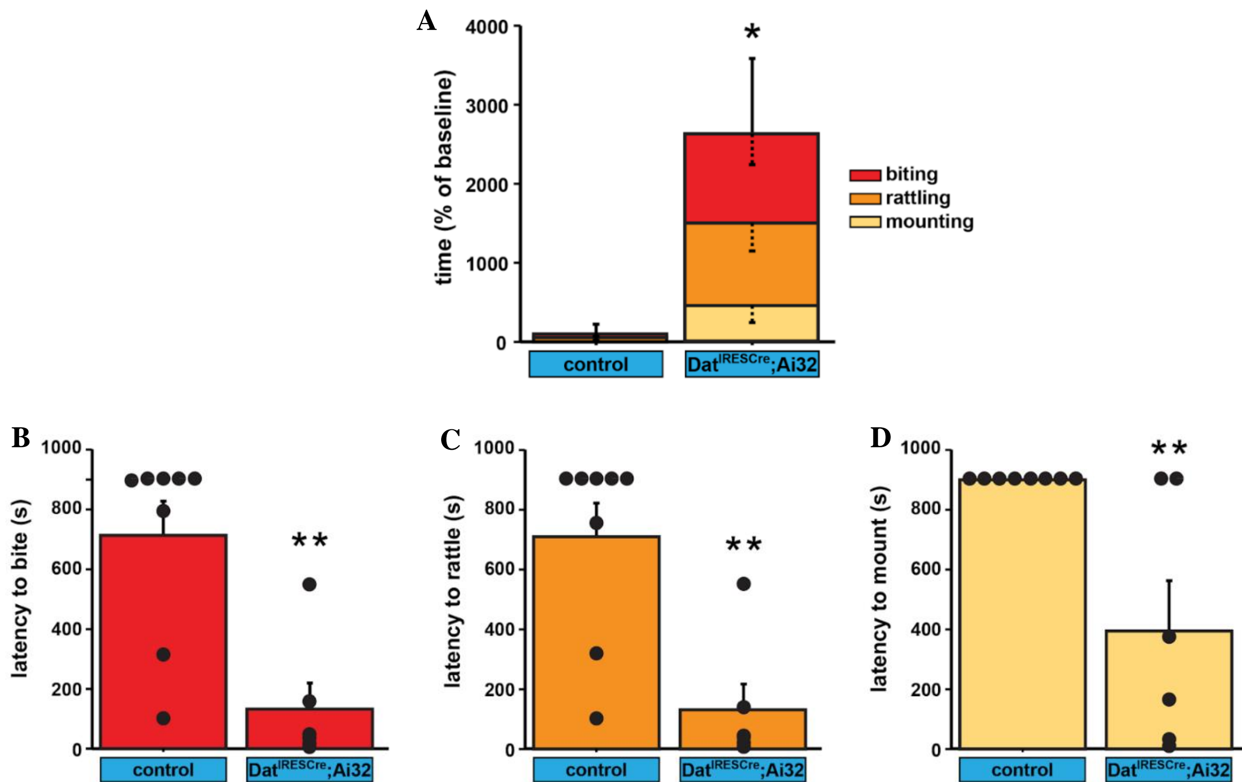


Figure 3.17: ChR2-based optogenetic stimulation of DAergic projections to the LS increases aggression against females. Heightened aggressive behavior against females including (**A**) increased time spent fighting and decreased latency to (**B**) bite (**C**) rattle and (**D**) mount was observed when Dat^{IRESCr}e;Ai32 mutant mice were stimulated (blue) in the LS in comparison to when the controls were stimulated. *, $p < 0.05$; **, $p < 0.01$ compared with their respective controls; mean ± SEM; n = 6-8 per group.

Activation of the VTA-NAc pathway produced no significant difference in time spent attacking ($t_{(19)} = 1.507$, $p = 0.1483$) (Figure 3.18) or the latency to attack (biting: $t_{(19)} = -1.581$, $p = 0.1304$; rattling: $t_{(19)} = -1.596$, $p = 0.1269$; mounting: $t_{(19)} = 1.903$, $p = 0.0724$) (Figure 3.19).

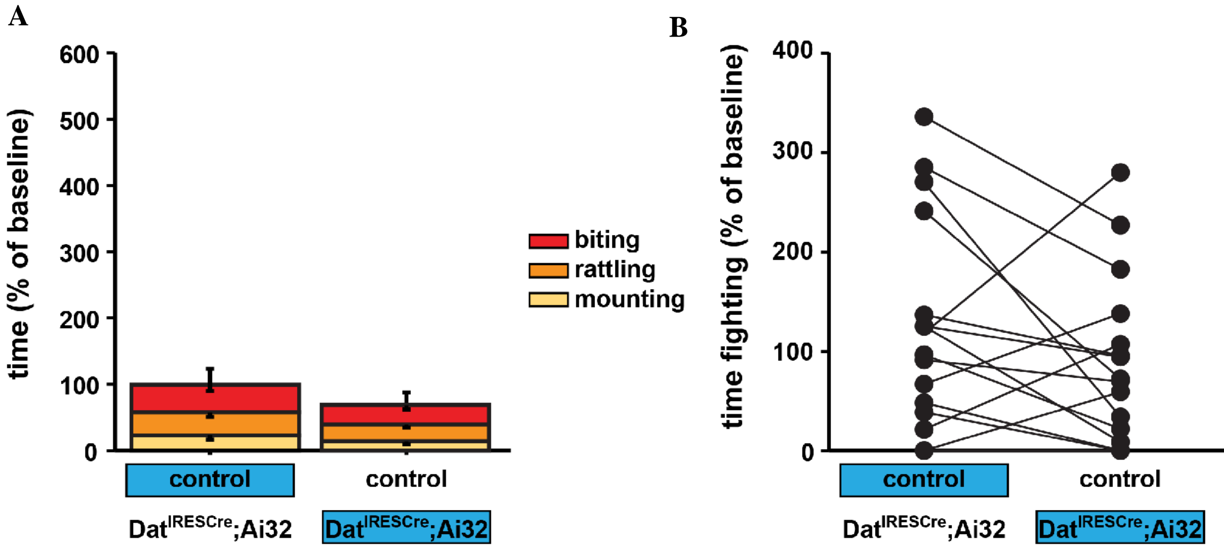


Figure 3.18: ChR2-based optogenetic activation of DAergic projections to the NAc does not increase aggression. (**A**, **B**) No effect of NAc DA stimulation was detected. *, $p < 0.05$; **, $p < 0.01$ compared with their respective controls; mean ± SEM; n = 20 per group.

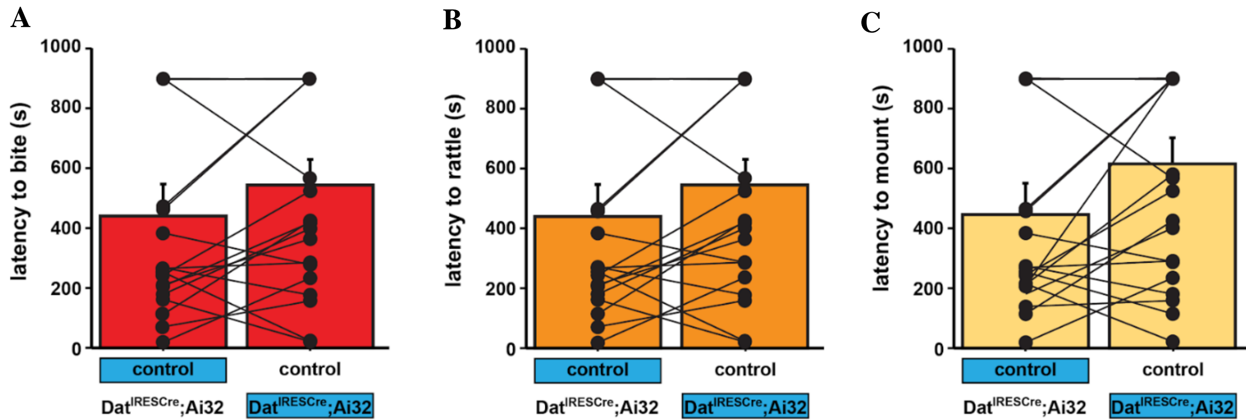


Figure 3.19: ChR2-based optogenetic stimulation of DAergic projections to the NAc does not decrease the latency to attack. No effect of stimulation on latency to (**A**) bite (**B**) rattle and (**C**) mount was detected during NAc DA stimulation. *, $p < 0.05$; **, $p < 0.01$ compared with their respective controls; mean ± SEM; n = 20 per group.

To investigate general motor activity, we stimulated both sets of mice in the open field and saw no locomotor differences between genotypes in the LS animals ($F_{(3, 45)} = 1.785$; $p = 0.1635$) (Figure 3.20A). The NAc cohort, however, was more hyperactive with a significant interaction between stimulation and genotype ($F_{(3, 54)} = 10.643$; $p<0.0001$) (Figure 3.20B).

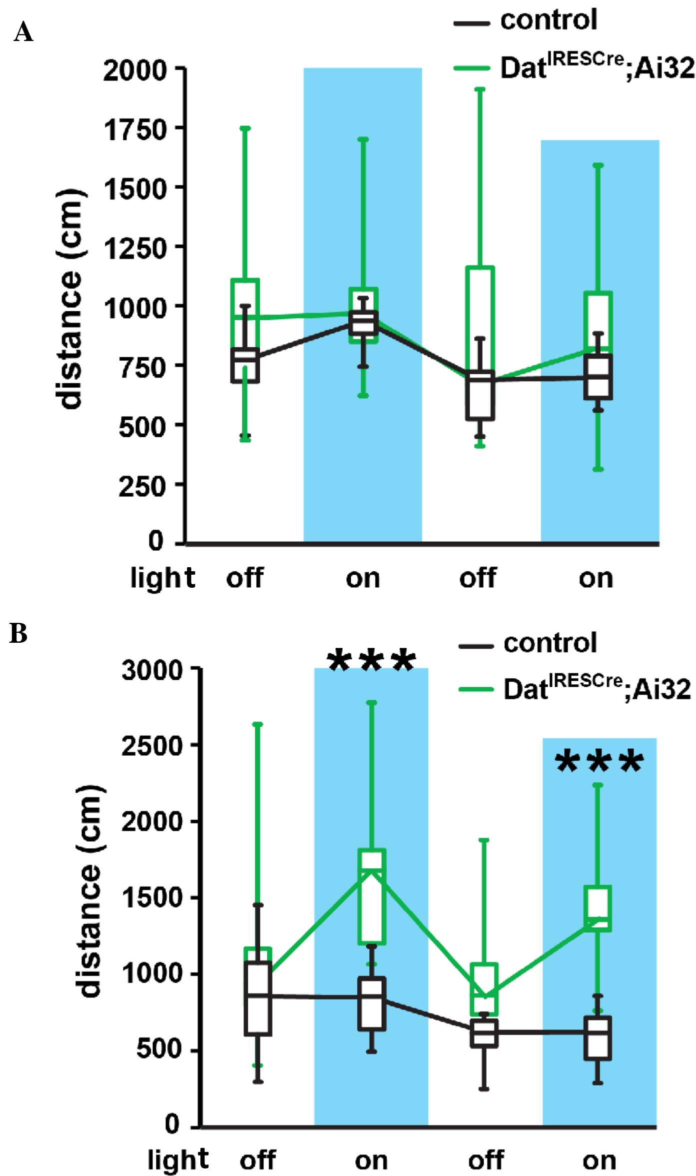


Figure 3.20: The effect of terminal DA stimulation on locomotion. (**A**) No effect of LS stimulation was detected in locomotor activity in the open field. (**B**) NAc stimulation increased locomotor activity in the open field, during alternating 3- minute off and on bouts of 20 Hz stimulation only in $Dat^{IRESCre}$;Ai32 mutant mice. *, $p < 0.05$; **, $p < 0.01$ compared with their respective controls; mean ± SEM; n = 16-19 per group.

Because the NAc plays a prominent role in motivation, we tested animals in real-time place preference. While the LS cohort showed no difference in preference ($t_{(14)} = 1.589$, $p = 0.1345$) (Figure 3.21A), the NAc cohort showed a significant preference for side associated with stimulation ($t_{(18)} = 3.575$, $p = 0.0022$) (Figure 3.21B).

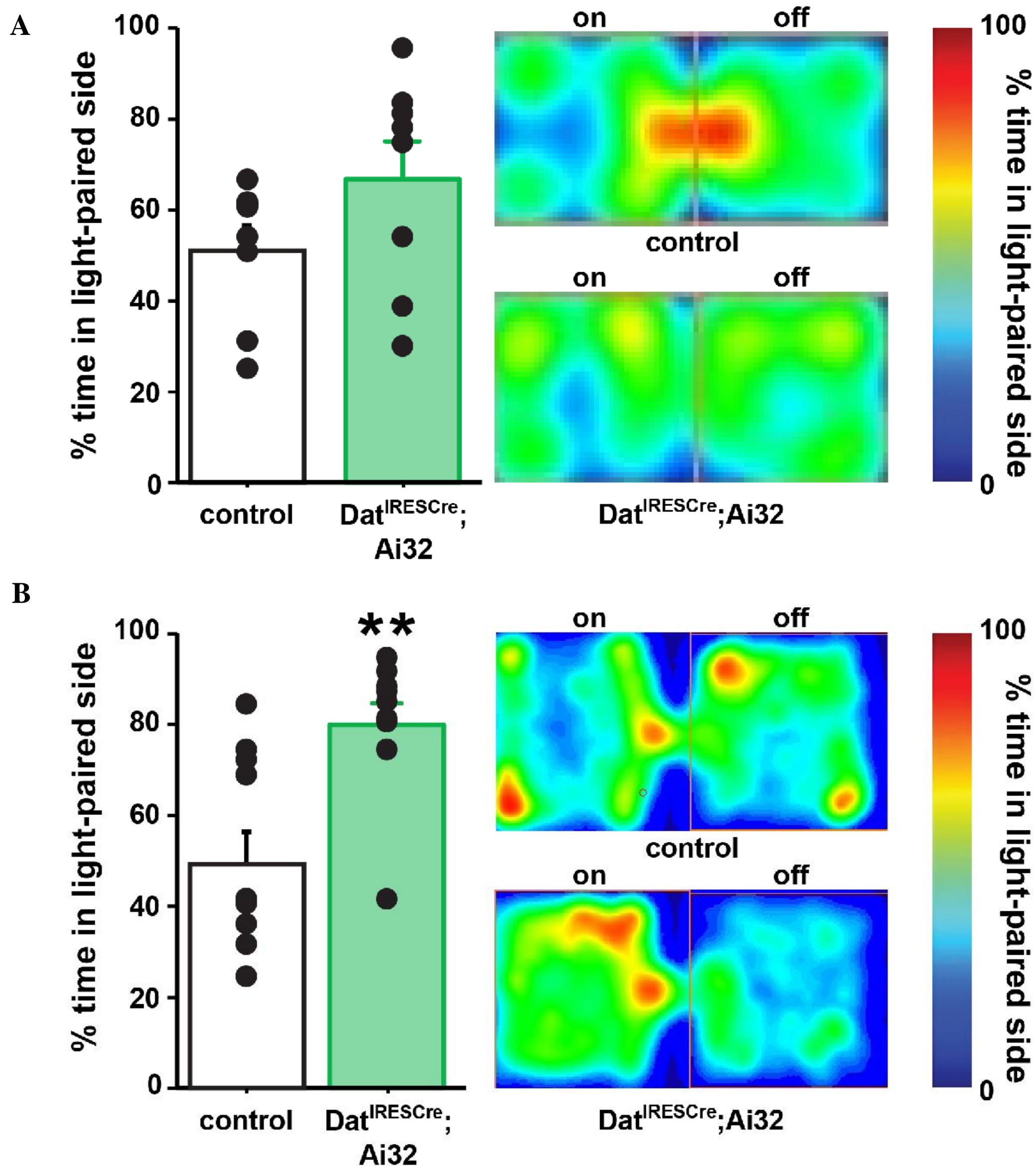


Figure 3.21: The effect of terminal DA stimulation on real-time place preference. (**A**) No effect of LS stimulation was detected in real-time place preference. (**B**) Percentage of time, over a 20-min session, spent in the stimulated zone during RTPP was significantly increased following NAc stimulation in mutants. Also shown: representative heat maps of the time spent in the stimulated zone of the chamber during RTPP.*, $p < 0.05$; **, $p < 0.01$ compared with their respective controls; mean ± SEM; n = 15-19 per group.

Finally, to test for off-target effects, generated by light traveling from the LS to the DS, we activated DAergic input into the DS directly (Figure 3.22) and saw no effect on time ($t_{(18)} = -0.880$, $p = 0.3907$) (Figure 3.23) or latency (biting: $t_{(18)} = 0.249$, $p = 0.8060$; rattling : $t_{(18)} = -0.424$, $p = 0.6767$; mounting $t_{(18)} = -0.250$, $p = 0.8054$) (Figures. 3.24) measures of aggression.

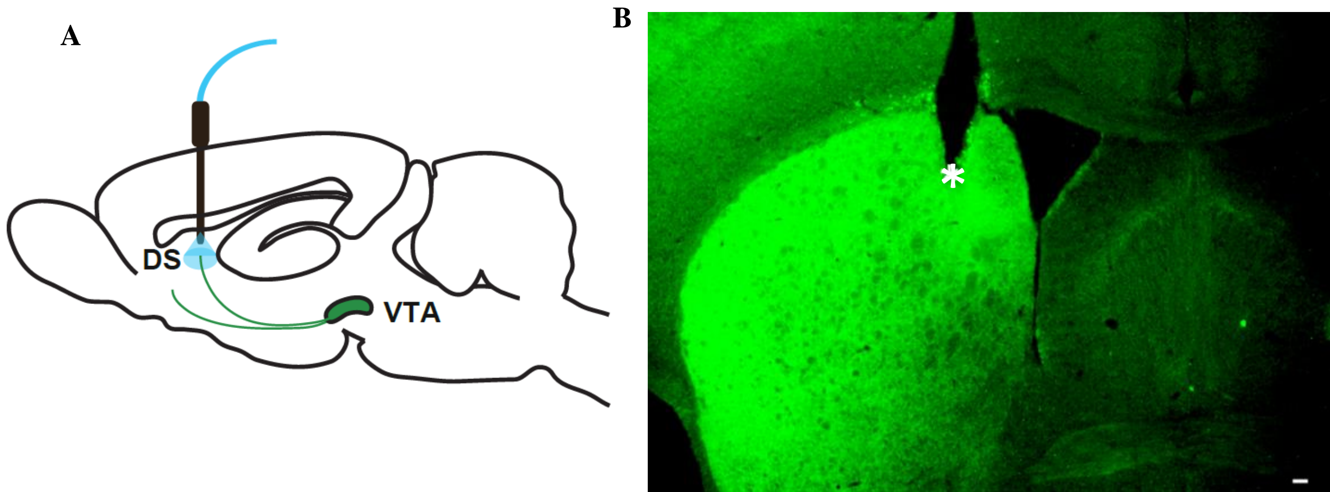


Figure 3.22: Terminal DA stimulation *in vivo* in the DS. (**A**) Schematic diagram for stimulating DA release *in vivo* in the DS. (**B**) Exemplary fiberoptic placement track in behavioral cohort of Dat[IRESCre];Ai32 mutant mice expressing ChR2-EYFP in the DS. Scale bar: 100 µm. Asterisks indicate ventral tip of implant location.

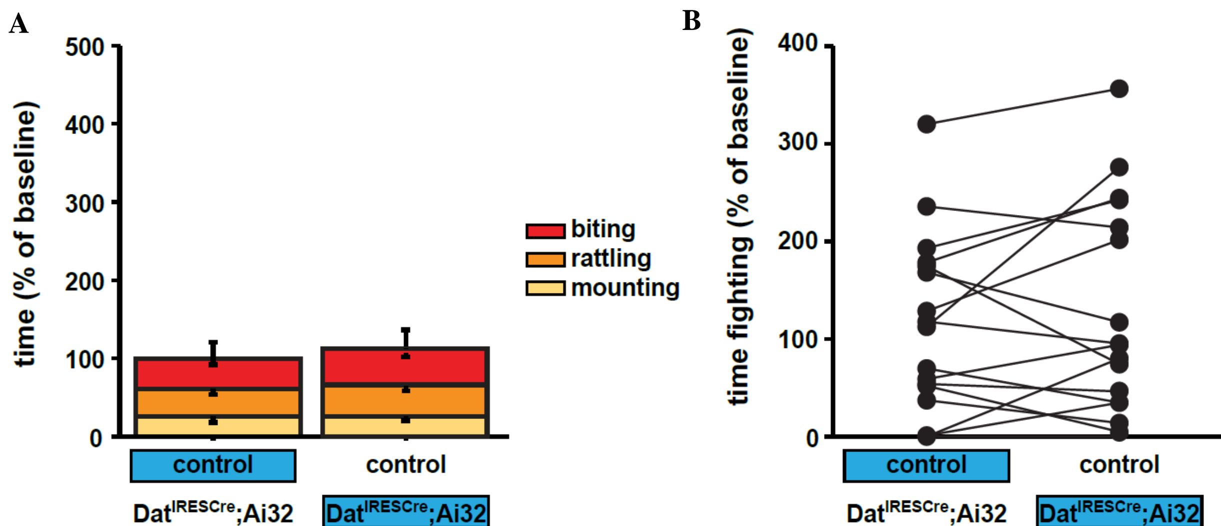


Figure 3.23: ChR2-based optogenetic stimulation of off-target DAergic fibers in the DS has no effect on attack time. (**A**, **B**) No effect of stimulation (blue) was detected during DS stimulation on time spent fighting. mean ± SEM; n = 19 per group.

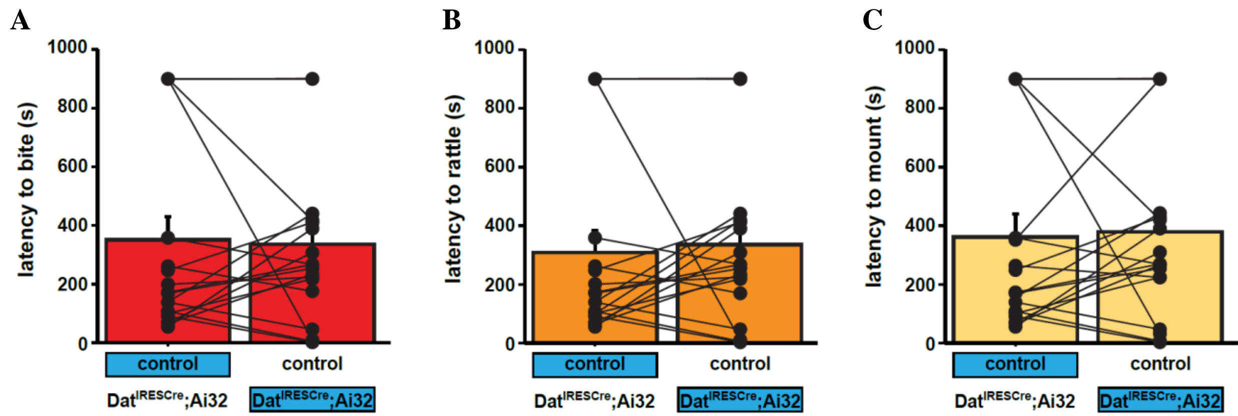


Figure 3.24: ChR2-based optogenetic stimulation of off-target DAergic fibers in the DS has no effect on latency to attack No effect of stimulation (blue) was detected during DS stimulation on latency to (**A**) bite (**B**) rattle and (**C**) mount. mean ± SEM; n = 19 per group.

Considered together, these data reveal dissociation between DAergic input to the NAc and LS, and their respective roles in locomotion/reward and aggressive behavior.

d. Discussion

In summary, my findings reveal how DA promotes aggression via VTA to LS projections, thereby demonstrating a modulatory role of population- and projection-specific DA neurons in a murine model of aggressive behavior. First, we demonstrated that activity of VTA, but not SNc, DA neurons promotes aggression. Second, we immunohistologically screened key aggression nodes to verify VTA-originating DA fibers in the target regions under consideration. We found that the LS and NAc, but the not VMHvl, are key aggression nodes that receive DAergic input from the VTA. Finally, we behaviorally confirmed that DA neuron terminals in the LS, but not the NAc, promote aggression. These data identify a VTA-to-LS DA pathway that directly controls aggression.

Monoamines have long been recognized for their capacity to modulate aggressive behavior. While there is a substantial body of evidence proposing a direct role for the serotonergic system (Takahashi et al., 2010; Nautiyal et al., 2015; Niederkofler et al., 2016), less is known about the explicit role of DAergic circuits in controlling aggression. Previously, I showed that optogenetic activation of VTA DA neurons drives isolation-induced aggression (Yu et al., 2014). As a first step, I built on past data by investigating the population specificity of this function by optogenetically stimulating the two key DA circuit nodes, the VTA and SNc, to examine their role in aggression expression. DA neurons in the SNc and the VTA project extensively to many regions. SNc DA neurons (~90% of the SNc) (Margolis et al., 2006) project largely to the dorsal striatum and VTA DA neurons (~60% of the VTA) (Swanson, 1982; Margolis et al., 2006; Nair-Roberts et al., 2008) project to the ventral striatum (NAc), the prefrontal cortex, the amygdala and the lateral septum, among other regions (Fallon and Moore, 1978; Beckstead et al., 1979; Swanson, 1982; Fields et al., 2007). The SNc, with its dense innervation of the dorsal striatum is heavily implicated

in locomotion control (Graybiel et al., 1994; Groenewegen, 2003). The VTA, however, is associated with the regulation of motivational, cognitive and affective behaviors via its innervation of limbic regions (Alcaro et al., 2007; Lammel et al., 2012; Rive et al., 2013; Morales and Margolis, 2017). In line with this functional division, we hypothesized a predominant role for VTA DA in driving aggression and found that our data supported this claim, with activation of SNc DAergic neurons producing no change in aggression. This dissociation is interesting in light of the fact that D2 receptor antagonists are currently used as first-line treatments in managing aggression, and are thought to achieve their effect via dorsal striatum-mediated sedation (Yudofsky et al., 1987; Brizer, 1988). Hence, one could predict an enabling role for SNc DA in aggressive behavior. But, our data reveal otherwise and warrant that we pay attention to modulation of DA signaling in other regions, perhaps the mesocorticolimbic pathways, which could drive aggression in a manner that is disconnected from locomotion. Finally, our data support the conclusion that VTA and SNc projection neurons target largely non-overlapping neural populations (Steinberg and Janak, 2013) and indicate that the anatomical dissociation may contribute differentially to a range of behaviors, including aggression.

Given the extensive blueprint of VTA efferents, it is unsurprising that its DAergic projections have been implicated in the acquisition and expression of a number of behavioral processes. Indeed, DA modulates behavior by acting on multiple target sites, and so the nature of associated behavioral changes following altered DAergic transmission depends on which terminal field is impacted. As a result, before proceeding with our behavioral investigation of which DAT-positive VTA DAergic projection neurons drive aggression, we first characterized innervation patterns at key target sites that have been associated with aggression, specifically the LS and the VMHvl (Sodetz and Bunnell, 1970; Potegal et al., 1981a; Lin et al., 2011). Moreover, given the

known association between NAc DA release and aggression, we directly explored the VTA-NAc pathway in its regulation of aggression too. Our analysis of endogenous florescence in $Dat^{IRESCre}$;Ai32 mutant mice expressing ChR2-EYFP, revealed distinct DAergic innervation of the LS and the NAc, but not the VMHvl (Refer to Figure 3.12). This falls in line with studies concluding that DA in the hypothalamus is largely located in the arcuate nucleus (a population referred to as the A12 DA nuclei) and is lacking in the VMHvl (Palkovits et al., 1974; Okamura et al., 1988; Romanov et al., 2017). Likewise, our data are congruent with studies that report robust VTA DAergic innervation of the NAc (Fallon and Moore, 1978; Gray et al., 1999). Finally, our histological analysis of LS DA innervation revealed a diagonal band of ChR2-EYP labeling, mapping directly onto a field of TH immunoreactivity previously reported in the LS of rats and mice (Fallon and Moore, 1978; Gall and Moore, 1984; Gaspar et al., 1985).

While our data confirmed previously established DAergic patterns of innervation in the NAc and LS, we next investigated whether these DA afferents were in fact originating from the VTA. To do this we injected, *in vivo*, the retrograde Cre-dependent CAV2 virus conditionally expressing ZsGreen in the LS and NAc and found anatomically discrete clusters of cells in the VTA, and not the SNc. The cells projecting to the LS were concentrated in a relatively more anterior and ventral location in the VTA, than those projecting to the NAc. Moreover, the absence of immunohistochemical evidence of collateralization supported our proposed hypothesis of distinct target sites independently controlling distinct functional outputs.

This dissociation hypothesis was further validated in our behavioral tests of aggression, locomotion and appetitive reward. In general, behavioral activation is largely dependent on which distinct neural circuit is involved, such that release of DA at one terminal may facilitate one set of behaviors and DA release at another terminal may trigger a different behavioral set (Amalric and

Koob, 1987; Nicola, 2007; Robbins and Everitt, 2007; Steinberg and Janak, 2013). For example, a decrease of dorsal striatum DA, decreases the quickness of response in the reaction time test, even if rats are well trained (Brown and Robbins, 1991), but DA depletion in the NAc produces no change in this behavior (Brown and Robbins, 1991; Amalric and Koob, 1993). Conversely, other studies demonstrate how NAc DA depletion produces deficits in food- and drug-related reward behaviors (Parkinson et al., 2002), but DA depletion in the dorsal striatum does not impair reward detection (Panigrahi et al., 2015). These studies reveal that the result of manipulating DAergic activity is greatly dependent on which specific DAergic target site is interfered with. Similarly in our work, we found that local release of DA in the LS directly drives aggression, without affecting reward-based preference and locomotion. In contrast, NAc DA stimulation only affected locomotion and motivational preference, without producing any changes in aggressive behaviors. Our data therefore validate the concept of dissociated anatomical and behavioral circuits, even if they exist under the umbrella of the same neurotransmitter system. In doing so, it further highlights the importance of advancing our understanding of the hyper-DAergic model of aggression from systemic DA manipulations to specific circuit manipulations.

Our identification of a population- and projection-specific DAergic input to the LS that exerts powerful influence over aggressive behavior effectively joins two major streams of aggression research -- the hyper-DAergic model of aggression (Antelman and Caggiula, 1977; Suri et al., 2015) and the classic LS-hypothalamic aggression axis involving “septal rage” (Spiegel et al., 1940; Wong et al., 2016) and the “hypothalamic attack area” (Lin et al., 2011). Moreover, our data are congruent with the functional topography of the LS. Specifically, the anterior LS strongly interfaces with the medial hypothalamus which is directly involved in controlling agonistic behaviors, while the posterior LS connects with the lateral hypothalamic nuclei which

are classically implicated in controlling reward-motivated behaviors (Prado-Alcala et al., 1984; Cazala et al., 1988; Risold and Swanson, 1997). Our stimulation coordinates target the anterior part of the LS. Therefore, even though the LS has historically been implicated in motivational states (Olds and Milner, 1954), our data show that the behavioral impact of manipulating septal activity depends largely on which sub region of the LS is being targeted.

Finally, the identification of the VTA-LS DA pathway also introduces the possibility of distinct projection circuits for different subtypes of aggression, and in doing so, lends credence to the notion that aggression is a multifaceted and dynamic behavior. As described in chapter I, models of aggression are categorized in two dominant forms: (1) reactive aggression which occurs more impulsively in response to perceived external threat and (2) proactive aggression that is more premeditated and directly motivated by a drive for appetitive reward (Rosell and Siever, 2015). VTA DAergic activity is prominently involved in reward coding (Schultz et al., 1997). Hence, one might expect a role for VTA-to-LS DA input in proactive aggression, that is reward incentivized (Couppis and Kennedy, 2008; Falkner et al., 2016; Golden et al., 2016, 2017). However, our findings of DAergic LS input promoting aggression, without affecting reward preference in RTPP, propose a role for DA in reactive aggression instead (Flanigan et al., 2017). On the other hand, work from Scott Russo's lab has identified a functional circuit from the basal forebrain (BF) to the lateral habenula (lHb) that controls the motivational component of aggression. Specifically, they found that activation of BF GABAergic terminals in the lHb, decreases lHb firing, promoting proactive aggression (Golden et al., 2016).

Therefore, our data do not defy previous work that shows increased DA in the NAc during aggression (Tidey and Miczek, 1996; van Erp and Miczek, 2000; Van Erp and Miczek, 2007). To the contrary, they offer insight into the role and timing of DA release during an aggressive

encounter. For example, while NAc DA may not initiate aggression, it might result from activation of the BF-to-lHb circuit, thus mediating the rewarding effects of proactive aggression. Thus, mice who learn to perform an operant task to seek access to a subordinate opponent (Fish et al., 2002; May and Kennedy, 2009; Falkner et al., 2016) or those who develop a preference for a place associated with "winning" (Golden et al., 2016, 2017), are perhaps recruiting the NAc-projecting DA circuit, that is activated through repeated learning and positive reinforcement, as opposed to the single exposure paradigm displayed in our work. Together, these findings indicate that separate pathways drive distinct behavioral components of aggression.

Further supporting a role for the VTA-LS pathway in impulsive aggression are our data where male mice also attack females when stimulated at the level of either cell body or terminals. Typically male mice initiate sexual, not aggressive, behavior with females (Scott, J. Fredricson, 1951; Mugford and Nowell, 1970) and therefore one could argue that the reward value of attacking a female is typically low. In fact, David Anderson's group has described male mice attacking females as displaced aggression towards an inappropriate target (Anderson, 2012). This description is very reminiscent of an impulsive form of aggression seen in humans with intermittent explosive disorder, who display inappropriate and violent outbursts towards targets that don't typically elicit aggression (Rosell and Siever, 2015; Scott et al., 2016). The lack of reward coding together with indiscriminate targeting, further steers our VTA-LS hyper-DAergic aggression circuit away from outcome-oriented and proactive aggression, but more towards abrupt and reactive aggression. Future work will directly assess temporal interplay between spatially segregated DAergic pathways and their post-synaptic targets in regulating complex and dynamic behaviors, including aggression.

Concluding remarks

Considered together, in demonstrating a direct VTA-to-LS pathway that modulates aggression, our data are the first to identify a precise DA aggression circuit. These data advance the hyper-DAergic model of aggression from correlational to causal evidence. However, many questions remain unanswered. For example, what post-synaptic effect is DA producing in LS cells? While we may be activating DAergic neurons, is DA in fact the sole driver of this behavior or is there co-release of other neurotransmitters? What receptors are involved in modulating the behavior? Combining optogenetics with behavioral and electrophysiological pharmacology is a powerful combinatorial technique to probe the mechanism that drives DA-mediated aggression. Specifically, the application of such manipulations to *in vivo* behavioral experiments can be effective for testing the sufficiency versus necessity of VTA-LS activity in driving aggression. The following chapter of my dissertation will employ these strategies to find answers to the aforementioned questions.

CHAPTER IV: Ventral tegmental area-to-lateral septum dopamine modulates aggression through D2 receptor activation

a. Introduction

The post-synaptic target: the lateral septum

The lateral septum (LS) plays a vital role in modulating several social and affective behaviors (Sheehan and Numan, 2000; Sheehan et al., 2004). Therefore, it is important to evaluate dysregulated LS function as an underlying mechanism in disorders of these behavioral groups. In chapter I, I discussed the broad role of the LS in aggression control. In chapter III, I carved out a specific ventral tegmental area (VTA)-to-LS dopamine (DA) circuit that modulates aggression. Given that we've established that the LS plays a causal role in DA-mediated aggression, work in the current chapter aims to understand the mechanism of VTA DA's post-synaptic effect on LS function and aggression. This investigation first warrants an understanding of LS-associated neuroanatomical connections, DA innervation and receptor expression patterns.

The LS receives input from the VTA. This has been illustrated via classic neuronal tracing studies using retrograde tracers such as horseradish peroxidase or florescent neuronal dyes and stains (Fallon and Moore, 1978; Swanson, 1982; Albanese and Minciacchi, 1983; Fallon, 1988). However, in many of these studies the tracers were not cell population-specific and so the labeled cells were often characterized as DAergic only on grounds of conformity in location, size and appearance to VTA DA neurons (Fallon and Moore, 1978). Therefore, these findings reveal connectivity but don't show cell identity, which is important given the cell-type heterogeneity of the VTA (Margolis et al., 2006; Nair-Roberts et al., 2008). To address this limitation, we used conditional cre-driven CavZsgreen virus in $Dat^{IRESCre}$ mice in chapter III to visualize and confirm

a genetically-defined DA connection between the VTA and LS, while also determining axonal collateralization patterns.

In the LS, electron microscopy has revealed that pericellular basket-like arrangements are a characteristic feature of LS afferents (Verney et al., 1987). A close analysis of TH-positive axons reveals dense pericellular terminal baskets that arrange themselves around LS cell bodies and their primary dendrites. The functional importance of the TH-positive baskets lies in the direct synaptic contact between these axon terminals and septal perikarya (Onténiente et al., 1987). Furthermore, there is a temporal aspect to the appearance of TH-positive LS afferents. At postnatal day (P)0 there is a complete absence of these baskets in the LS. At P3 the first formations are detected. Around P9-10, there is a significant increase in the number of TH-immunoreactive baskets (Verney et al., 1987). The increase in numbers, over time, is interesting and suggestive of a rapidly evolving network that modulates the activity of local LS neurons. Finally, the development of perineuronal baskets follows a medial to lateral trajectory, eventually forming a diagonal band of TH immunoreactivity in the lateral part of the septum (Gall and Moore, 1984; Gaspar et al., 1985). The strong neuroanatomical connection between the VTA and the LS is supportive of a key role for VTA DA in regulating the activity and functions of the LS.

The identity of LS neurons that DAergic afferents synapse with was studied using electron microscopy in the rat LS. It was concluded that DAergic afferents chiefly contact a population of GABAergic cells (Onténiente et al., 1987). Another group confirmed that local LS somatospiny cells receive synaptic inputs from TH immunoreactive fibers, which form pericellular baskets around the GABAergic neurons (Jakab and Leranth, 1990). These results demonstrate a key DA-GABA synaptic interface and also indicate a strong presence of GABAergic cells in the LS. Indeed, the LS predominantly consists of long-projecting medium spiny

GABAergic neurons (Sheehan et al., 2004). Wong and colleagues, crossed vesicular GABA transporter (VGAT)-IRES-Cre mice with a GFP reporter line and concluded that 85.2% of cells in the LS were in fact GABAergic as indicated by GFP fluorescence, many of which project to the aggression-associated medial hypothalamus (Wong et al., 2016). Another group used fluorescence in situ hybridization and tyramide signal amplification to detect GABAergic cell bodies using glutamic acid decarboxylase (GAD: the enzyme that produces GABA) mRNA as an indicator. They found that spanning the LS, about 95-98% of the cells expressed both isoforms of GABA synthesizing enzymes GAD65 and GAD67. Additionally, using the neuronal marker NeuN, they established that more than 90% of LS neurons were GABA-immunoreactive (Zhao et al., 2013). These cellular composition and projection characteristics underscore the general inhibitory role of the LS at its target sites.

Finally, evaluating the impact of DA afferents on LS GABAergic neurons requires an assessment of DA receptor expression at the projection site. DA receptor 2 (D2) mRNA is the principal type found in the septum (Meador-Woodruff et al., 1989; Camps et al., 1990; Weiner et al., 1991). Within the septum, D2 mRNA is largely localized in subdivisions of the lateral septal nucleus (Weiner and Brann, 1989; Weiner et al., 1991). Qualitative analysis of relative expression of DA receptor 1 (D1) and D2, as shown in the Allen Brain Atlas, reveals dominant D2 receptor expression in the LS (Figure 4.1).

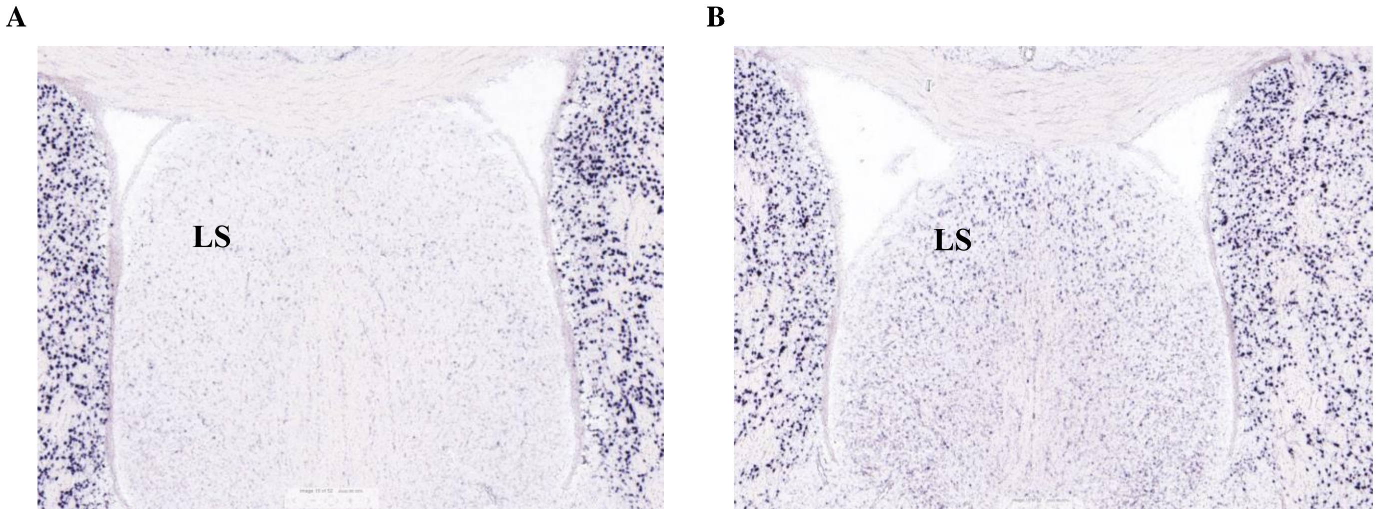


Figure 4.1: DA receptor expression in the LS. Relative expression of (**A**) D1 and (**B**) D2 receptors in the LS. Adapted from the Allen Brain Atlas.

Functionally, an investigation of specific D2 receptor involvement in aggression is very relevant to the current work. Many groups have demonstrated that drugs that manipulate D2 signaling alter aggression in male mice (De Almeida et al., 2005). For example, D2 receptor activation with D2 agonist quinpirole, selectively increased aggressive behavior and decreased social investigation. This outcome was reversed with the use of selective D2 antagonist sulpiride (Puglisi-Allegra and Cabib, 1988). In another study, that genetically classified mice as "low-aggression" and "high-aggression" lines, selective stimulation of D2 receptors with bromocriptine substantially increased aggression in "low-aggression" mice. Conversely, D2 receptor blockade with sulpiride decreased aggression in the "high-aggression" mice. D1 agonist SKF 38393 and the selective D1 antagonist SCH 23390 did not affect levels of aggression significantly (Nikulina and Kapralova, 1992). A study in cats found the same results where D2 agonist, quinpirole, but not the D1 agonist, SKF 38393, facilitated expression of rage. Moreover, these effects were selectively blocked with haloperidol or the specific D2 antagonist, spiperone, but not

with the D1 antagonist, SCH 23390 suggesting once again that rage is mediated primarily by D2 receptors (Sweidan et al., 1990). Evidently, D2 receptors play a key role in DAergic control of aggression. In fact, antipsychotic agents that antagonize the D2 receptor are currently used as first-line treatment in managing aggression in humans (Yudofsky et al., 1987; Brizer, 1988). Therefore, an investigation of D2 receptor signaling, as the probable mechanism of how incoming DA might influence LS activity, and eventually aggression, stands very important.

Inhibition of the lateral septum

The effect of DA VTA afferents on LS neural activity is poorly understood. Due to the dominant presence of the D2 receptor subtype, we predict that the net post-synaptic effect of DA at the LS is inhibition. This is because D2 receptors belong to the family of G protein-coupled receptors that reduce cellular activity by inhibiting the enzyme adenylyl cyclase (AC) via their interactions with inhibitory G proteins (Neve et al., 2004). Congruently, bath application of DA reduces spontaneous LS neuronal activity in an acute slice preparation (Joëls and Urban, 1985). Furthermore, many studies have investigated the impact of antipsychotic medications, which typically antagonize D2 receptors, on the LS and found altered neural activity. For example, acute haloperidol treatment commonly induces c-Fos expression in the LS (Robertson et al., 1994; Guo et al., 1998; Suzuki et al., 1998). The mechanism of antipsychotic-induced stimulation of c-Fos expression in the LS has not been determined. However, compounds that block D2 signaling, could have the net effect of reinstating LS activity after chronic D2-mediated inhibition in hyper-DAergic pathologies.

Reduced LS activity has been reported in many models of psychiatric disorders. For example, blunted LS activity strongly correlates with depression-like phenotypes. Conversely, antidepressants stimulate activity in LS neurons, reversing these symptoms (Chen and Herbert,

1995; Mohammad et al., 2000; Contreras et al., 2001). The LS is responsible for inhibiting fear responses and so depressing LS firing induces fear (Thomas, 1988). Electrical stimulation of the LS prevents the formation of stress-induced ulcers in rats (Yadin and Thomas, 1996), leaving one to speculate if LS inhibition would have an opposite effect in perpetuating the severity of stress. Moreover, there is evidence that inhibition of LS neurons via the action of corticotropin releasing factor on the type 2 receptor (densely expressed in the LS) induces anxiety, while antagonism of this receptor is anxiolytic (Bakshi et al., 2002). Mechanistic confirmation of the same was achieved by stereotaxically iontophoresing CRF onto the LS which indeed inhibited spontaneous spike generation (Eberly et al., 1983). Additionally, nicotine infusion in the LS inhibits overall activity and has an anxiogenic effect, as indicated by performance on social interaction tests and the elevated plus maze (Ouagazzal et al., 1999; Cheeta et al., 2000). Reduction in LS activity, via lesions of the LS, has similar outcomes. Lesions lead to a heightened startle reaction to auditory stimuli (Brady and Nauta, 1953), and exacerbate freezing to cues previously paired with foot shock (Sparks and LeDoux, 1995).

Finally, and most relevant to this dissertation, lesions of the LS have been strongly correlated with aggression and violence. Application of local anesthetic to the LS, and surrounding structures, increased aggression in male rats (Albert and Richmond, 1976). Muricide was also reported following local infusions of lidocaine in the LS (Albert and Wong, 1978). Using optogenetics, Wong and colleagues verified the impact of LS dysfunction on aggression. They found that direct suppression of LS GABAergic activity, reduced downstream inhibition of the medial hypothalamus and increased aggression towards both males and females. These studies provide compelling evidence of the importance of optimal LS function in aggression control.

In considering all the elements of the VTA-to-LS DA pathway, experiments in the current chapter investigate if terminal release of DA at the LS produces D2-mediated neuronal inhibition and heightened aggression. To address this, the work in this chapter combines optogenetics with the more traditional methods of electrophysiological and behavioral pharmacology to test the following aims:

1. First, we aim to evaluate the localization of LS D2 receptors relative to local GABAergic cells:

   Specifically, we hypothesize that D2 receptors are located on LS GABAergic cells. Moreover, D2 receptor expression closely interfaces with DA input to the LS.

2. Second, we aim to assess the post-synaptic effect of DA neuron signaling on LS activity:

   Specifically, we hypothesize that optogenetic activation of DA terminals at the LS will hyperpolarize LS cells. This effect is likely mediated by post-synaptic D2 signaling. **

   **Note: The above aim, in entirety, was executed by Dr. Nao Chuhma, a collaborating electrophysiologist from Dr. Stephen Rayport's lab.

3. Third, we aim to assess the necessity of the LS pathway for VTA-triggered aggression:

   Specifically, we hypothesize that the VTA-LS DA pathway, via D2 activation of GABAergic LS neurons is sufficient, but not necessary, to drive VTA-triggered aggression.

b. *Materials and methods*

Animals

Dat^{IRESCre} (Bäckman et al., 2006) mice and ROSA26-floxedSTOP channelrhodopsin-2-enhanced yellow fluorescent protein (ChR2-EYFP) mice (referred to as 'Ai32' mice; RCL-ChR2(H134R)/EYFP) (Madisen et al., 2012) were crossed to produce experimental cohorts consisting of Dat$^{IRESCre;}$Ai32 double transgenic and single mutant controls on a mixed F1 background (C57BL/6J x 129S2/129SvEv/Tac). For all experiments, controls were littermates that were positive for the Ai32 transgene but negative for the Cre transgene. This Ai line allows for expression of the fusion construct, only following exposure to Cre recombinase. Therefore, driven by the DAT promoter, these mice conditionally express ChR2 (fused to EYFP for fluorescent visualization) in DAergic cells only (Boyden 2005). For behavior, given that female mice do not display territorial aggression, only male mice >4 months of age were investigated. For immunohistochemical studies assessing D2 expression in the LS, *Drd2*-GFP mice expressing GFP under the control of the *Drd2* promoter (Drd2-EGFP, GENSAT - S118Gsat/Mmnc) were used. All mice were housed under a 12:12-hour light:dark cycle (lights on at 7am) in a temperature controlled environment and provided with food and water *ad libitum*. Animal testing was conducted under protocols approved by Columbia University and New York State Psychiatric Institute Institutional Animal Care and Use Committees.

Customization of infusion cannulas

The local infusion set up (Plastic One Inc.) was customized to the specific target in the brain i.e the LS (AP: 0.6; ML: ±0.4; DV: -2.8) (Figure 4.2). The guide cannula directs the internal cannula to the region of interest. Given our coordinates, the length of the guide (stainless steel tube) below the plastic pedestal (threaded pedestal molded around stainless steel tubing; pedestal height

= 9mm) was 3mm (C315G/SPC-3mm). This allowed for a 0.7mm gap between the cranium and the plastic pedestal, which enabled stronger stabilization with dental cement. The remaining 2.3mm of the guide projected into the brain, just above the LS, leaving a 0.5mm gap between the region and the ventral tip of the steel guide. This gap ensured that no direct damage was done by the larger steel tube to the region being investigated, leaving room for the projection of the finer and more precise internal cannula. The internal cannula is inserted through the open top of the guide cannula enabling the injection of fluid at a consistent depth. For our experiments it was designed at 3.5mm to account for the length of the guide (3mm) as well as the aforementioned space of 0.5mm i.e. the projection length beyond the guide tip (C315I/SPC-3.5mm). We also ordered dummy cannulas. The dummy screws onto the guide cannula and is customized to be the exact length of the guide (C315DC/SPC-3mm). While this isn't required, it is recommended to help prevent debris from clogging the guide cannula. It also helps to stop blood and other fluids from retreating into the guide cannula after implantation. Finally we ordered the appropriate connector assembly that is used to tightly connect and secure the internal cannula to the guide allowing for infusion in a freely moving animal (C313CT).

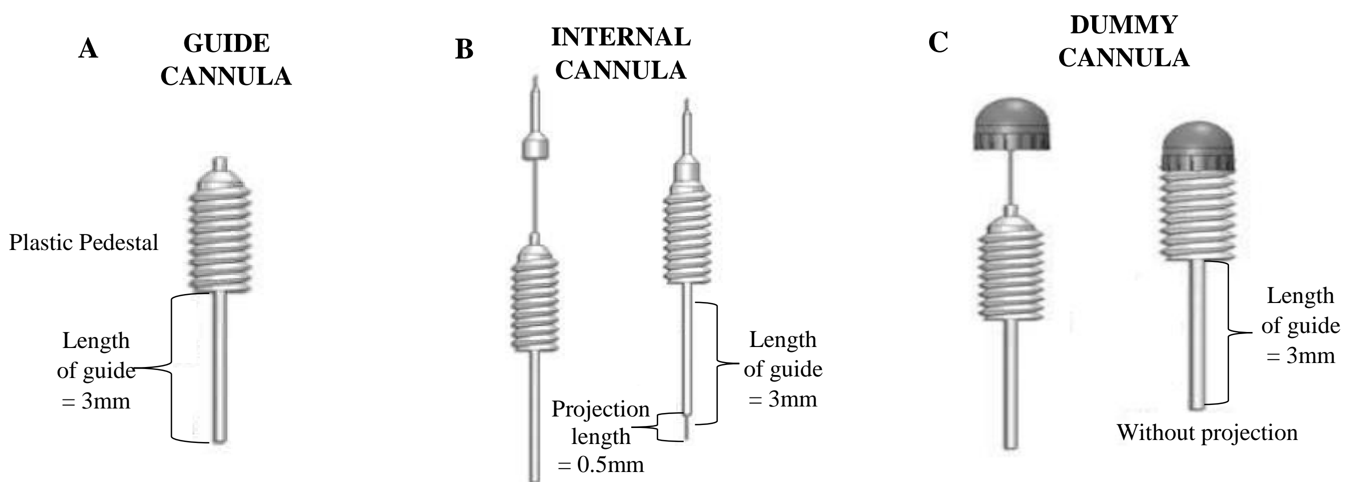


Figure 4.2: Cannulas for local infusions. The infusion set up requires (**A**) a guide cannula (**B**) an internal cannula and (**C**) a dummy cannula that are customized to the target site.

Stereotaxic fiberoptic and cannula implantations

Surgeries were conducted on mice that were at least 2 months in age. Mice were anesthetized with a cocktail of ketamine (10 mg/kg body weight) and xylazine (50 mg/kg) solution via i.p. injections. Animals were then placed in a stereotaxic apparatus and core body temperature was maintained at 37 ˚C with a heating pad. Sterile ophthalmic ointment was applied to the animals' eyes to prevent corneal drying during the procedure. Depth of anesthesia was monitored by toe pinch and corneal reflex before starting, and throughout, the procedure at 10-minute intervals. If necessary, a booster anesthetic, 30mg/kg ketamine alone (30% of initial dose), was administered. The fur on the head was shaved and the area was disinfected with betadine and then alcohol. Once the mouse was fixed, a small incision (approximately 5-10mm) was made in the skin covering the cranium. Stereotaxic coordinates were measured relative to bregma. All behavioral mice were implanted with an optical fiber ferrule over the VTA (AP: -3.5; ML: -0.5; DV: -4.2) and an infusion guide cannula over the LS (AP: 0.6; ML: -0.4; DV: -2.8), in the same hemisphere given that VTA to LS projections are predominantly ipsilateral (Fallon, 1988). Once the cranial coordinates were located, small openings (< 0.5 mm) were drilled at each site. Then, two microscrews were fastened into the skull (Screw 1 AP: 2.5; ML: 2; and Screw 2 AP: -2.5; ML: 2). Next, the optical fiber ferrule was implanted and secured at the VTA using vet bond and dental cement (Dental cement kit; Stoelting). Finally, the standard stereotaxic arm was replaced with a single cannula placement holder (Model 1766-AP, Kopf instruments) and the guide cannula was implanted over the LS. The cannula was inserted D/V: -2.3 from brain surface thus allowing for a 0.7mm gap between the cranium and the base of the plastic pedestal and a 0.5mm gap between the ventral tip of the guide and the LS. As stated before, the usual LS D/V: -2.8 coordinate was

ultimately reached using the internal with an extra projection length of 0.5mm. The cannula was then secured in the skull using vet bond and dental cement (Dental cement kit; Stoelting).

Drug preparation

The behavioral mice were infused with (±)-Sulpiride D2 antagonist (Sigma Aldrich) before the open field test and the isolation-induced aggression test. The sulpiride was dissolved in 0.9% NaCl to achieve a dose of 0.05µg/mouse for every infusion. The final volume of each microinjection, before a behavioral round, was 0.15µl. 0.9% NaCl was used for vehicle (VEH) injections.

In vivo optical stimulation protocol

Before beginning any behavior, mice with fiberoptic implants were briefly immobilized in order to carefully attach the fiberoptic patch cords. These patch cords were in turn connected to a laser (473 nm) adjusted to give an output of 7-10 mw light intensity. The laser output was regulated by a programmable master-8 pulse stimulator through which the pulsing was set to occur at 20-Hz with a 10 ms pulse duration. The stimulator also allowed us to manually control the on and off switch of the laser. Once the patch cord was connected and mice were ready to commence the behavioral assay the laser was turned on.

Behavior

All animals were exposed to the same behavioral paradigms and timeline, starting at 4 months of age. Mice first underwent the open field locomotion assay followed by the isolation induced aggression. So as to allow wash out of any residual effects from the infusions, we allowed a 2 week gap between each behavior. All animals received sulpiride or mock-infusion, VEH microinjections into the LS. Based on previous sulpiride infusion studies, we administered

the drug 15 min before behavioral testing commenced (Couppis and Kennedy, 2008). Each microinjection was 0.15µl in volume and manually infused over 3 min (0.05µl/minute) using a Hamilton syringe (0.5µl; outer diameter: 0.02 inches; inner barrel diameter: 0.104mm Hamilton Co., Reno, NV, USA) and a single syringe infusion pump (Stoelting). The internal cannula was left in place for 2 min after the infusion and the mice were returned to their home cages before testing. All behavioral testing took place during the light cycle between 12:00pm and 7:00 pm. To eliminate odor cues, each apparatus was thoroughly cleaned after each animal.

Open field locomotion test

Naïve cannulated mice were microinjected with either sulpiride or VEH, into the LS, 15 minutes before the open-field test. Locomotor activity, following local infusion, was assessed in plexiglass activity chambers as previously described in detail chapter II. The open field is a standard test for locomotor behavior. It consists of a simple square enclosure that is equipped with infrared detectors to track animal movement in the horizontal and vertical planes. Measure of total distance covered during the assay was used as an index of activity. Infused mice were placed into the center of the open field and activity was recorded over 15 minutes. Testing took place under bright ambient light conditions and total distance travelled was measured.

Isolation-induced aggression test

In order to assess aggressive behavior, we used the isolation-induced aggression paradigm described in chapters II and III, with minor modifications to incorporate the LS infusions. As detailed before, the home cage was divided in half by a perforated partition made of clear plastic. Once again, $Dat^{IRESCre}$;Ai32 mutant mice were paired with controls. Mice were housed for 10 days

before the experiment was performed. On test day, cannulated mice were first injected with either sulpiride or VEH, into the LS, 15 minutes before the test began. For the test, the divider was removed and the treated mice were optogenetically stimulated in the VTA during the 15 minute aggression assay. A cross over design counterbalancing for drug and genotype stimulated was used. The time spent fighting was scored as a sum of the time spent biting, tail rattling and mounting. All fights were video recorded to allow for any additional post-hoc analysis.

Histology

After behavioral tests were completed, mice were euthanized, and genotypes and fiber/cannula placements were confirmed. Animals were deeply anesthetized with ketamine/xylazine and transcardially perfused with phosphate-buffered saline (PBS) followed by 4% paraformaldehyde in PBS. Brains were then removed and post-fixed overnight (4% paraformaldehyde in PBS), and subsequently cryoprotected in 30% sucrose and frozen. Coronal sections (50 μm thick) were cut with a cryostat and fluorescence immunohistochemistry was performed on free-floating sections.

Immunohistochemistry and microscopy

Sections were first rinsed with PBS containing 0.2% TritonX-100 and then blocked with buffer (PBS, 2% bovine serum albumin and 0.2% Triton X-100). This was followed by overnight incubation with primary antibody and eventually conjugated to a fluorophore with the appropriate secondary antibody. The sections were incubated in primary antibody for 24 h at 4 °C, washed with PBS, and then incubated with secondary antibody for 1 h at room temperature. After washing 3x with PBS the sections were incubated with Hoechst 33258, Thermo Fisher Scientific, in a final

concentration of 1μg/ml and transferred into PBS solution with 0.2% TritonX-100. Finally, the sections were mounted with Vectashield mounting medium (Vector Labs, Burlingame, CA, USA).

For GFP, immunohistochemistry was performed using a rabbit primary antibody against GFP diluted in blocking solution 1:1000; Life Technologies, Grand Island, NY, USA. As a secondary antibody we used Cy3 donkey anti-rabbit, diluted in blocking solution 1:350; Jackson Immunoresearch, West Grove, PA, USA. For TH, immunohistochemistry was performed using rabbit primary antibody against TH, diluted in blocking solution 1:1000; Millipore Sigma, Darmstadt, Germany. As a secondary antibody we used Cy3 donkey anti-rabbit, diluted in blocking solution 1:500; Jackson Immunoresearch, West Grove, PA, USA. For GAD-67, immunohistochemistry was performed using mouse primary antibody against GAD-67, diluted in blocking solution 1:1000; Millipore Sigma, Darmstadt, Germany. As secondary antibody we used mCherry donkey anti-mouse, diluted in blocking solution 1:500; Millipore Sigma, Darmstadt, Germany.

For analysis of co-expression of immune-labelled proteins, images were acquired at 40x using confocal microscopy (Leica TCS SP8). All post processing of images was done on ImageJ software (National Institutes of Health).

Slice electrophysiology

These methods were reported by Dr. Nao Chuhma who conducted all slice physiology experiments.

$Dat^{IRESCre}$;Ai32 (P72-93) were anesthetized with a ketamine (90 mg/kg) / xylazine (7 mg/kg) mixture. After confirmation of full anesthetics, mice were decapitated and brains were quickly removed in ice-cold high glucose artificial cerebrospinal fluid (ACSF) (in mM: 75 NaCl, 2.5 KCl, 26 NaHCO3, 1.25 NaH2PO4, 0.7 CaCl2, 2 MgCl2 and 100 glucose, pH 7.4) saturated with mixture

of 95% O_2 and 5 % CO_2 (carbogen). 300 µm coronal sections of the septum (around +0.1mm anterior from bregma) were made by a vibrating microtome (VT1200S, Leica). The sections were incubated in high glucose ACSF at room temperature for at least 1 hour for recovery, then a slice was transferred to a recording chamber (submerged, 500 µl volume) on the stage of upright microscope (BX61WI, Olympus) continuously perfused with standard ACSF (in mM: 125 NaCl, 2.5 KCl, 25 NaHCO3, 1.25 NaH2PO4, 2 CaCl2, 1 MgCl2 and 25 glucose, pH 7.4) saturated with carbogen. Recorded neurons were visualized using enhanced visible light differential interference contrast (DIC) optics with a scientific c-MOS camera (ORCA-Flash4.0LT, Hamamatsu photonics). Recording was performed from relatively large pyramidal/triangular shaped neurons located just dorsal of the fornix covered by EYFP fluorescence of DA neuron terminals in the lateral septum. Only 1-2 slices per animal included the area innervated by DA neurons. Recording patch pipettes were fabricated from standard-wall borosilicate glass capillary with filament (World Precision Instruments). Pipette resistance was 3-7 MΩ. Composition of intracellular solution for firing and the slow EPSC recording was (in mM): 135 K^+-methane sulfonate ($MeSO_4$), 5 KCl, 2 $MgCl_2$, 0.1 CaCl2, 10 HEPES, 1 EGTA, 2 ATP and 0.1 GTP, pH 7.25. Fast current clamp recording was performed with an Axopatch 200B amplifier (Molecular Devices). Liquid junction potential (~10 mV) was adjusted online. Series resistance was 15-27 MΩ and was not compensated. Synaptic responses were evoked by 5 msec duration field illumination with a high-power blue LED (Thorlabs) delivered in a train of five pulses at 20 Hz repeated with 1 min intervals. For pharmacological studies, drugs were delivered by perfusion. Recordings were filtered at 5 kHz using a 4-pole Bessel filter, and digitized at 5 kHz (Digidata 1550A, Molecular Devices). Data acquisition was done using pClamp 10 (Molecular Devices). Data were analyzed with Axograph X (Axograph Science). Average firing frequencies were calculated during 0.5 sec window of pre

stimulation period 0.5-1 sec prior of stimulation onset) or during/post stimulation period (0.1 to 0.6 sec from stimulation onset).Peak amplitudes of PSCs were measured in averaged traces made from 3 consecutive traces.

Data and statistical analyses

Statistical analysis for all behavior in the current study was performed using StatView 5.0 software (SAS Institute, Cary, NC, USA) and Microsoft Excel. For locomotor activity sulpiride vs. VEH infusion data was analyzed using a paired samples Student's t-test. For the fights, a three way analyses of variance (ANOVA) was conducted followed by post-hoc testing as indicated. The criterion for significance for all analyses was *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$. For the physiology, statistical tests were done using SPSS 23 (IBM). Since sample sizes were small, for independent sample t-test, we did not assume equal variance. Sample size estimation was done by G*Power 3.1, with $P = 0.05$, power = 0.9. Effect size was estimated from previous experiments. Results from data analyses are expressed as mean±s.e.m.

c. Results

The main experimental goals of this chapter were to:

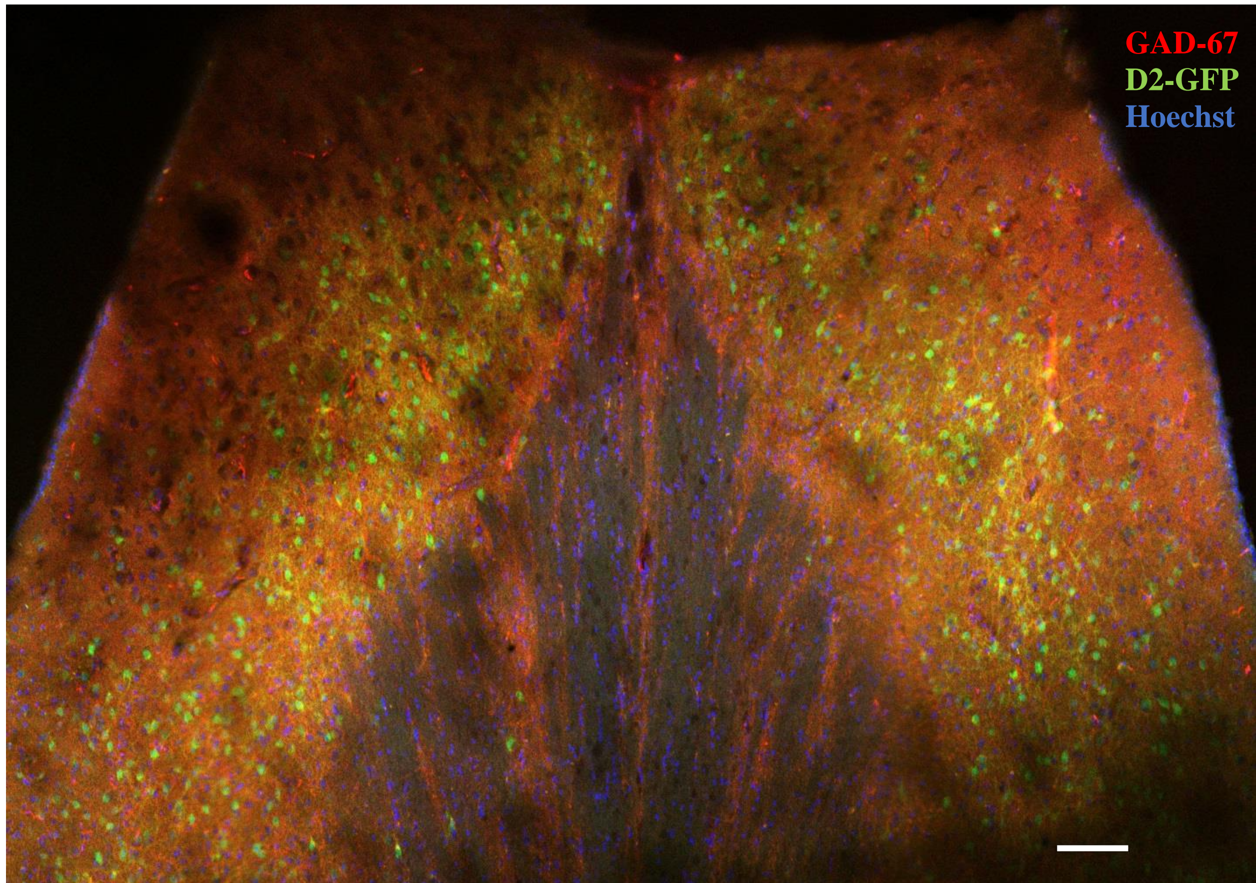
1. Evaluate the expression pattern of LS D2 receptors, relative to local GABAergic cells.
2. Assess the post-synaptic effect of DA neuron signaling on LS activity.
3. Assess, behaviorally, the sufficiency versus necessity of the LS pathway for VTA-triggered aggression.

Using the materials and methods reported in the previous section, we conducted experiments to test the above and found the following:

1. LS D2 receptors colocalize with local GABAergic cells:

Chapter III revealed how direct stimulation of VTA DA terminals at the LS directly increased aggression. In the LS, 85% of the neurons are GABAergic (Wong 2016), and *Drd2* is higher than *Drd1* expression (Weiner et al., 1991). Therefore we predicted that DA would act on LS GABAergic projection neurons via D2 receptors. We confirmed colocalization of D2 receptors in LS GABAergic cells, using immunohistochemistry against prominent GABAergic marker, GAD-67, in mice that express GFP under the control of the *Drd2* promoter (Drd2-EGFP, GENSAT - S118Gsat/Mmnc) (Figure 4.3A). In those same mice, we also detected an expression gradient of D2-GFP that closely matches the input of DAergic fibers into the LS, with peak expression aligned along the diagonal band in the septofimbrial nucleus of the LS (Figure 4.3B)

A



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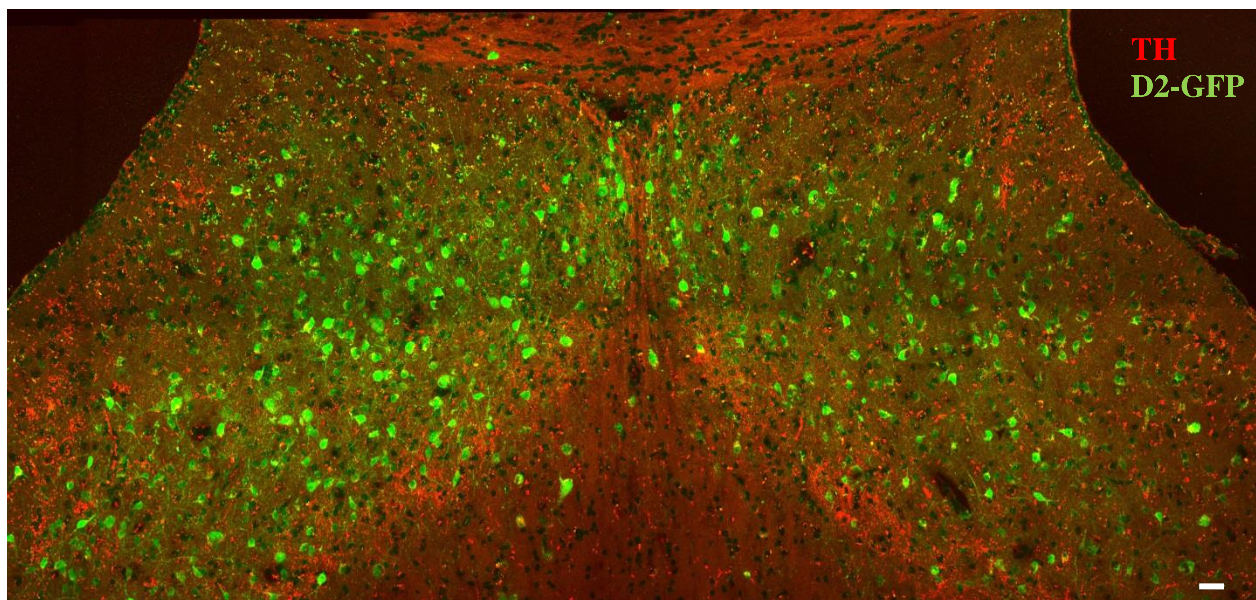


Figure 4.3: LS D2 expression patterns relative to GABAergic cells and incoming DA afferents. (**A**) Colocalization of D2-GFP (green) and GABAergic marker, GAD-67 (red) expressing cells in the LS. (**B**) Diagonal band of D2-GFP expression and TH labeled (red) DAergic input into the LS. Scale bars: 100 μm.

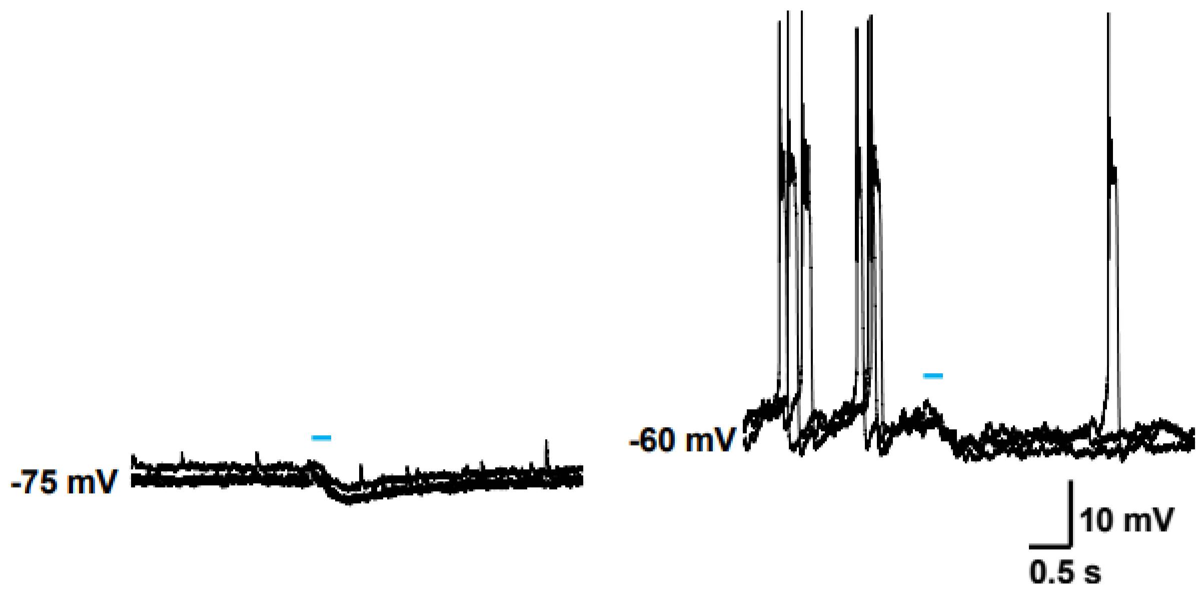
2. DA acts to hyperpolarize LS projection neurons via D2 receptor activation:

In chapter III we found that terminal release of DA at the LS promotes aggression. Furthermore, the previous aim of this chapter provided histological indication of a possible D2-mediated neural correlate. Given these data, we next explored the post-synaptic physiological effect of DA release on LS neurons in acute brain slice preparations. We refined our previous aim's hypothesis to predict that DA would act to hyperpolarize LS GABAergic projection neurons via D2 receptors and thereby reduce downstream GABAergic tone.

The following results of this aim were reported by Dr. Nao Chuhma who conducted all slice physiology experiments.

We recorded from relatively large pyramidal shaped neurons (located dorsal of the fornix) in the LS of $Dat^{IRESCre}$;Ai32 double mutants. Many neurons were not spontaneously active (Figure 4.4A, left), but in most cases current injections depolarized the membrane to cause repeated burst firing (Figure 4.4A, right). Optogenetic train stimulation (5 pulses at 20-Hz, mimicking phasic firing) of EYFP labeled DA neuron terminals caused hyperpolarization in 65% of recorded neurons (n = 40), significantly reducing LS cell firing (Figure 4.4B). Strikingly, theses inhibitory postsynaptic potentials (IPSPs, hyperpolarization) were blocked by 5 min application of D2 receptor antagonist, Sulpiride. The effect was specific to Sulpiride, and not due to cell deterioration, given that we did not detect any differences in the IPSC amplitude during the 5 min recording in control solution (Figure 4.5). Thus, in the LS, DA terminal stimulation causes D2-mediated hyperpolarization, which reduces LS cell firing.

A



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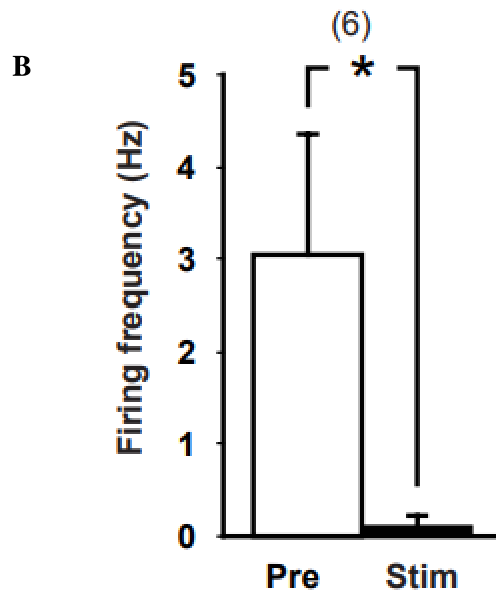


Figure 4.4: DAergic projections from the VTA to the LS hyperpolarize LS neurons. (**A**) Sample traces of IPSCs without current injection (at -75 mV, left) and with current injection to make the neuron fire (at -60 mV, right). 3 traces are superimposed. Blue lines indicate timing of train photo-stimulation (5 pulses at 20 Hz). (**B**) Average firing frequency during 0.5 s window 12 before train stimulation (pre) and during and after train stimulation (post). n of recorded cells = 6. * and *** indicate $p < 0.05$ with paired t-test and $p < 0.001$ with independent sample t-test, respectively.

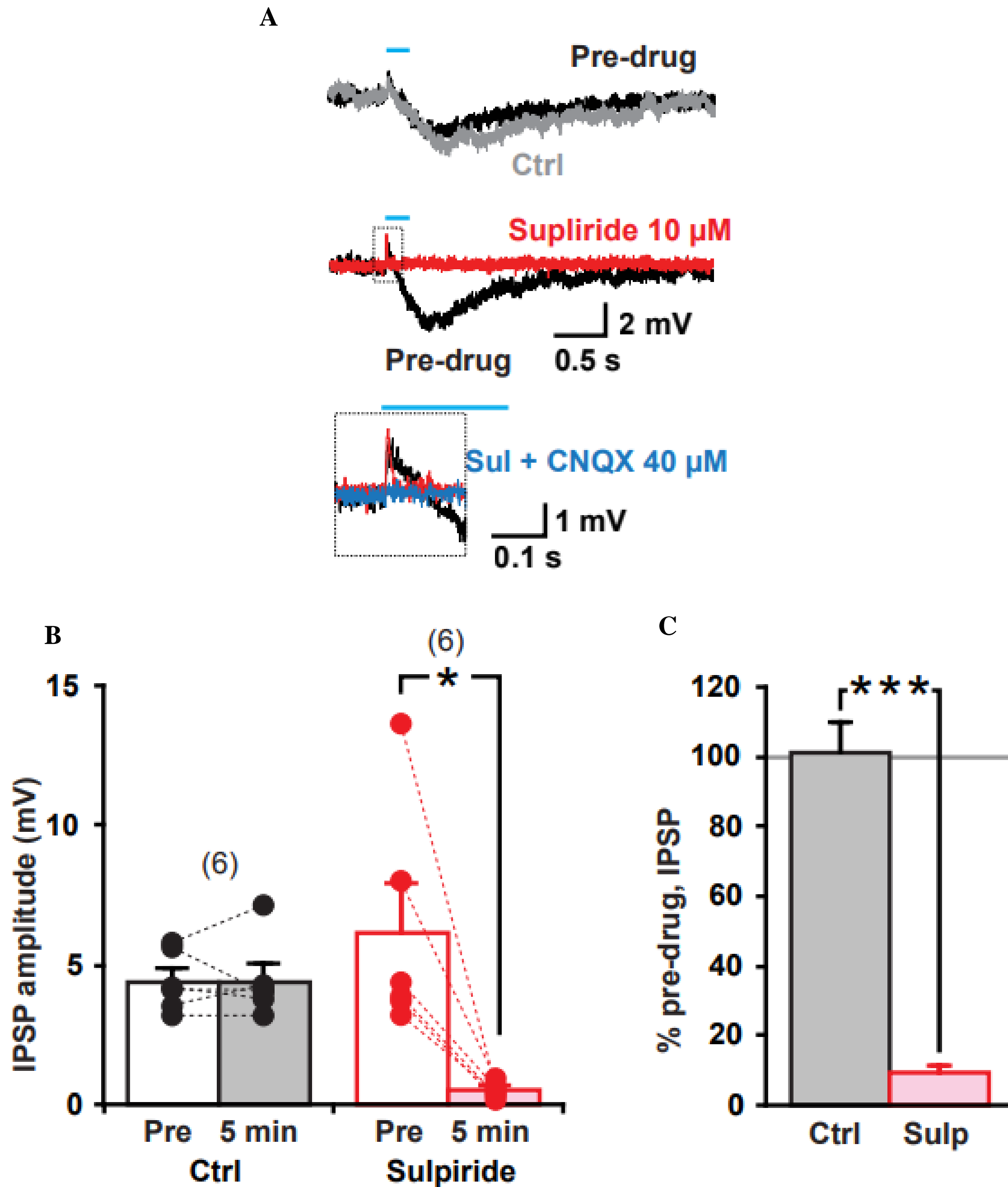


Figure 4.5: DA acts to hyperpolarize LS projection neurons via D2 receptor activation. (**A**) Sample traces of IPSCs without depolarizing current injection before drug application (pre-drug), after 5 min application of control solution without drugs (control), and after 5 min application of sulpiride. The small EPSC in the dashed square of the middle panel is expanded below. The expansion shows a trace after application of sulpiride and CNQX. Each trace is an average from 3 consecutive traces. (**B**) IPSC amplitudes from each cell (circles) and means, for control recording and sulpiride application, during pre-drug period (pre) and 5 min after application (post). Lines connect data from the same recorded cells. (**C**) Percent pre-drug amplitude after application of control solution (gray bar) or sulpiride (pink bar). (**B**, **C**) n of recorded cells = 6. * and *** indicate $p < 0.05$ with paired t-test and $p < 0.001$ with independent sample t-test, respectively.

3. **VTA-LS DA pathway, via D2 activation of GABAergic LS neurons, is necessary to drive VTA-triggered aggression:**

The previous aim confirmed that local DA release at the LS post-synaptically inhibits LS activity via D2 signaling. Inhibition of LS activity has been strongly associated with behavioral pathology, specifically aggression. In this aim, we sought to evaluate the functional role of LS D2 signaling on aggression in awake behaving mice.

For this study one of two outcomes was possible:

a) The VTA-LS DA pathway is *sufficient to* drive aggression: In this outcome, we would conclude that while VTA DAergic projections to the LS promote aggression, other brain regions might also contribute to DAergic facilitation of aggressive behavior.

b) The VTA-LS DA pathway is *necessary* to drive aggression: Alternatively, in this outcome, we would conclude that VTA projections to the LS constitute the dominant pathway involved in DAergic promotion of aggressive behavior.

Before evaluating the impact of LS D2 antagonism on aggression, we assessed if there was any change in general locomotor activity following local infusions of sulpiride in the LS and found no significant differences between the treatment groups ($t_{(19)} = 0.967$, $p = 0.3455$) (Figure 4.6).

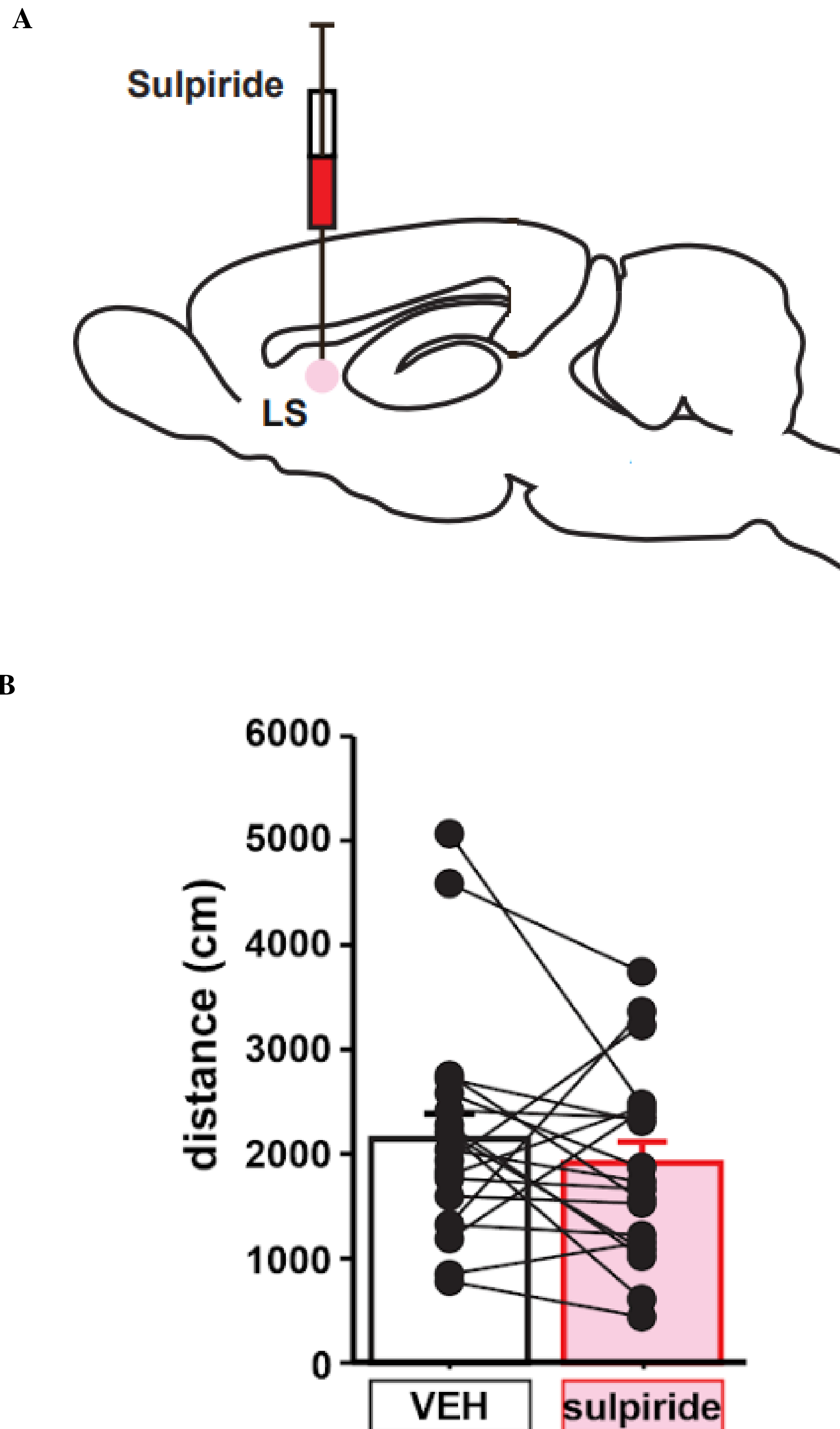
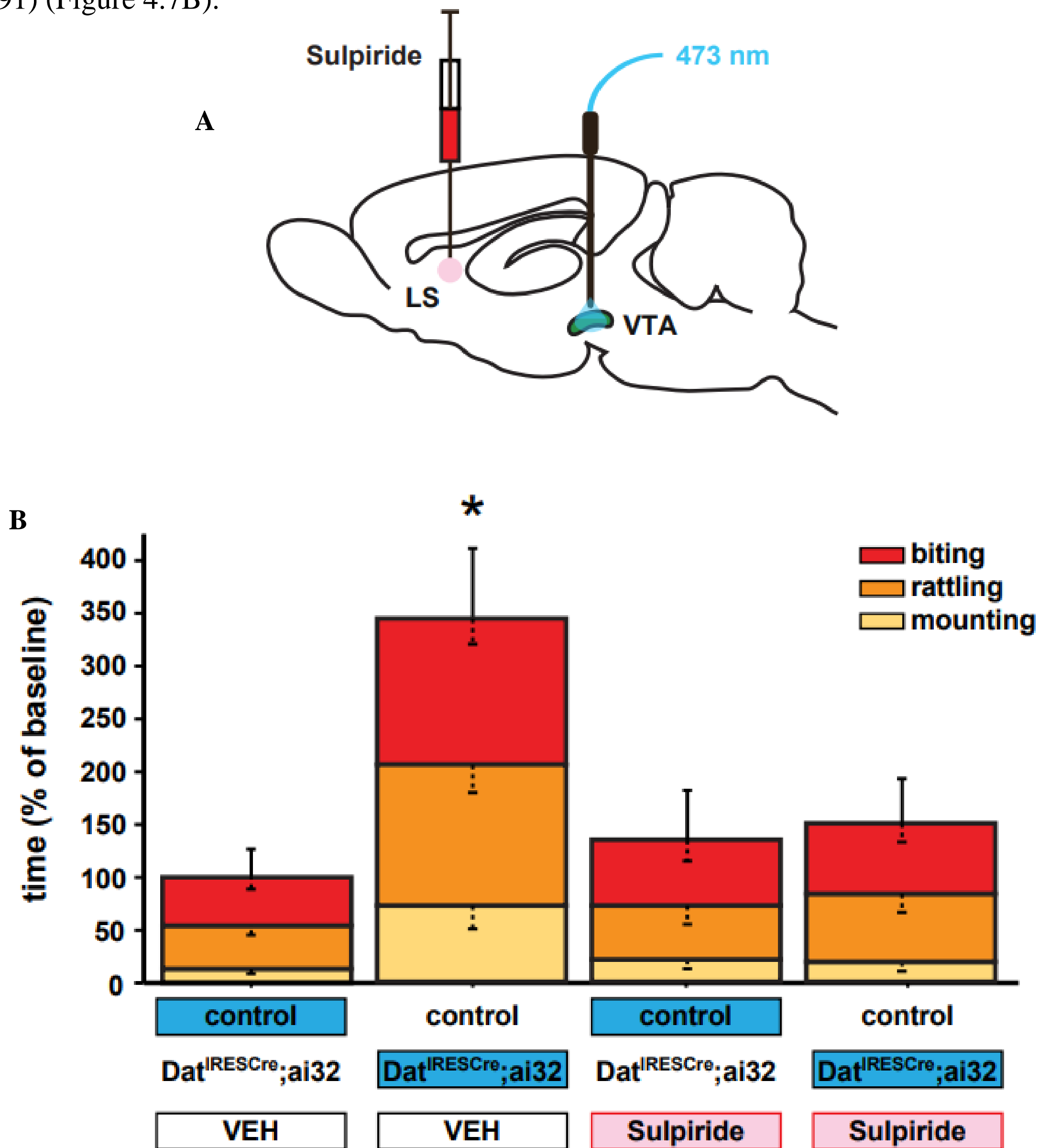


Figure 4.6: The effect of local sulpiride infusion, in the LS, on locomotion. (**A**) Schematic diagram for local infusions of sulpiride in the LS (**B**) No difference in locomotion detected between the groups following local infusion of sulpiride in the LS. VEH, vehicle. *, $p < 0.05$; ***, $p < 0.001$ compared with their respective controls; mean ± SEM; n = 20 per group.

Next, to evaluate the necessity of the LS pathway for VTA-triggered aggression, we infused sulpiride or VEH into the LS, followed by VTA DAergic neuron activation during aggression testing (Figure 4.7A). We found that D2 antagonism in the LS completely blocked VTA-triggered aggression (main effect of treatment: $F_{(1, 10)} = 2.440$; $p = 0.1493$; main effect of genotype: $F_{(1, 10)} = 8.478$; $p = 0.0155$; treatment x genotype interaction: $F_{(1, 10)} = 6.479$; $p = 0.0291$) (Figure 4.7B).



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Time fighting, for individual pairs, broken down by treatment showed a significant difference between genotypes following the VEH mock infusion ($F_{(1, 10)} = 22.419$; $p = 0.008$) (Figure 4.7C, left) but the difference was abolished following sulpiride infusion ($F_{(1, 10)} = 0.053$; $p = 0.8218$) (Figure 4.7C, right). When broken down by stimulation condition there was a significant difference between treatments following mutant stimulation ($F_{(1, 10)} = 4.973$; $p = 0.0498$) (Figure 4.7D, right) as opposed to control stimulation ($F_{(1, 10)} = 0.801$; $p = 0.3918$) (Figure 4.7D, left).

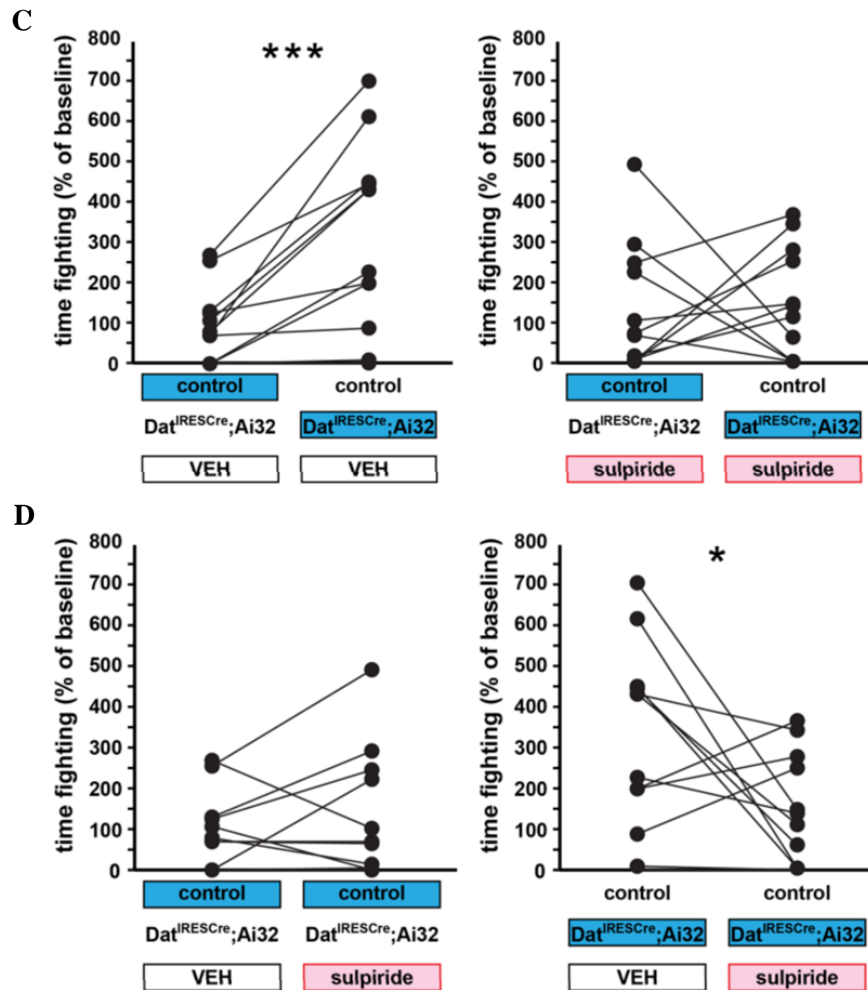
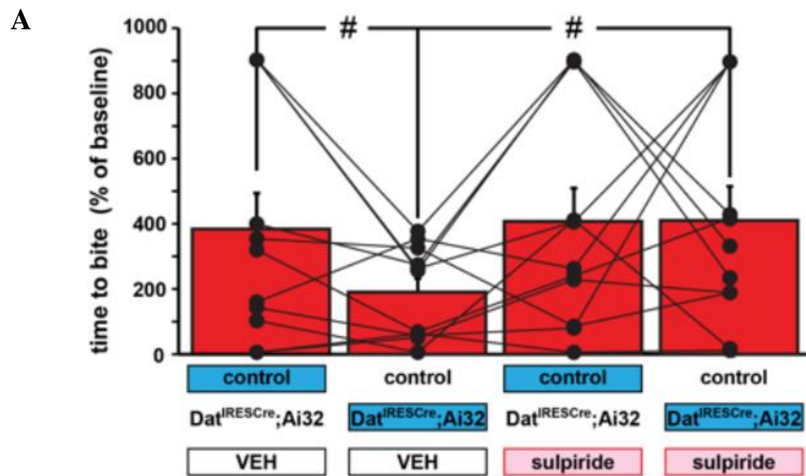


Figure 4.7: Terminal release of DA in the LS is necessary for VTA DAergic activity to increase aggression. (**A**) Schematic diagram for local infusions of sulpiride in the LS followed by optogenetic stimulation (blue) of the VTA to evoke in vivo DA release in the LS. (**B**) Pharmacological rescue of time spent fighting through local D2 receptor antagonism in the LS. Time fighting, for individual pairs, broken down by (**C**) treatment and (**D**) stimulation condition. VEH, vehicle. *, $p < 0.05$; ***, $p < 0.001$ compared with their respective controls; mean ± SEM; n = 11 per group.

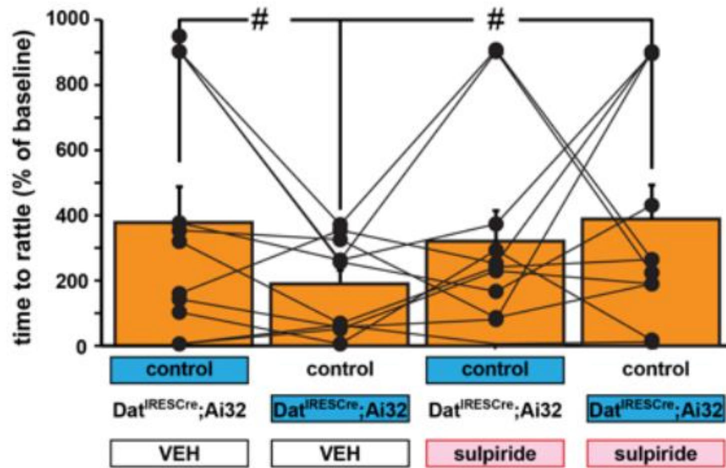
In addition to decreasing the time spent attacking, we found that local D2 antagonism in the LS delayed aggressive bouts by increasing the latency to bite, despite optogenetic stimulation of VTA DAergic cells (Figure 4.8A) (main effect of treatment: $F_{(1, 10)} = 5.669$; $p = 0.0385$; main effect of genotype: $F_{(1, 10)} = 0.888$; $p = 0.3683$; treatment x genotype interaction: $F_{(1, 10)} = 1.755$; $p = 0.2148$). Latency to bite, for individual pairs, broken down by treatment showed a significant difference between genotypes following the VEH mock infusion ($F_{(1, 10)} = 4.806$; $p = 0.0531$) but the difference was abolished following sulpiride infusion ($F_{(1, 10)} = 0.002$; $p = 0.9621$). When broken down by stimulation condition there was a significant difference between treatments following mutant stimulation ($F_{(1, 10)} = 4.911$; $p = 0.0510$) as opposed to control stimulation ($F_{(1, 10)} = 0.065$; $p = 0.8043$).



Additionally, we found that local D2 antagonism in the LS delayed aggressive bouts by increasing the latency to tail rattle, despite optogenetic stimulation of VTA DAergic cells (Figure 4.8B) (main effect of treatment: $F_{(1, 10)} = 1.287$; $p = 0.2831$; main effect of genotype: $F_{(1, 10)} = 0.480$; $p = 0.5042$; treatment x genotype interaction: $F_{(1, 10)} = 2.513$; $p = 0.1440$). Latency to tail rattle, for individual pairs, broken down by treatment showed a significant difference between

genotypes following the VEH mock infusion ($F_{(1, 10)} = 4.747$; $p = 0.0544$) but the difference was abolished following sulpiride infusion ($F_{(1, 10)} = 0.220$; $p = 0.6494$). When broken down by stimulation condition there was a significant difference between treatments following mutant stimulation ($F_{(1, 10)} = 4.017$; $p = 0.0729$) as opposed to control stimulation ($F_{(1, 10)} = 0.313$; $p = 0.5882$).

B



Finally, we found that local D2 antagonism in the LS delayed aggressive bouts by increasing the latency to mount, despite optogenetic stimulation of VTA DAergic cells (Figure 4.8C) (main effect of treatment: $F_{(1, 10)} = 4.868$; $p = 0.0519$; main effect of genotype: $F_{(1, 10)} = 1.456$; $p = 0.2554$; treatment x genotype interaction: $F_{(1, 10)} = 0.462$; $p = 0.5119$). Latency to mount, for individual pairs, broken down by treatment showed a significant difference between genotypes following the VEH mock infusion ($F_{(1, 10)} = 3.972$; $p = 0.0742$) but the difference was abolished following sulpiride infusion ($F_{(1, 10)} = 0.132$; $p = 0.7236$). When broken down by stimulation condition there was a significant difference between treatments following mutant

stimulation ($F_{(1, 10)} = 5.331$; $p = 0.0436$) as opposed to control stimulation ($F_{(1, 10)} = 0.252$; $p = 0.6268$).

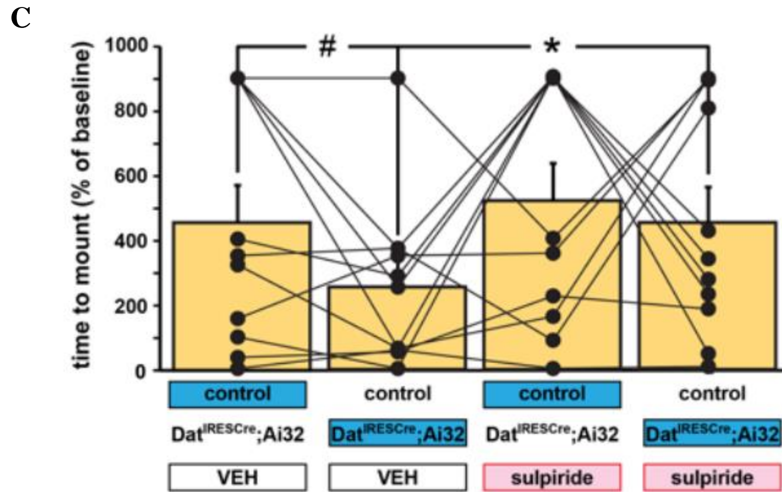


Figure 4.8: Terminal release of DA in the LS is necessary for VTA DAergic activity to decrease the latency to attack. Pharmacological rescue of the latency to (**A**) bite (**B**) rattle and (**C**) mount through local D2 receptor antagonism in the LS. VEH, vehicle. #, $p < 0.1$, *, $p < 0.05$ compared with their respective controls; mean ± SEM; n = 11 per group..

Collectively, our findings demonstrate that the VTA-LS DA pathway, via D2-mediated inhibition of GABAergic LS neurons, is necessary to drive VTA-triggered aggression.

d. Discussion

In summary, the current chapter demonstrates that VTA DA acts on the LS to promote aggression via D2 receptor signaling. First, we demonstrated that LS D2 receptors colocalize with the region's principal cell population – GABAergic neurons. Moreover, D2 expression perfectly aligns with the diagonal band of incoming DA afferents. Second, we electrophysiologically revealed that DA acts on LS cells to hyperpolarize them via D2 activation, a post-synaptic effect that is abolished by D2 antagonism with sulpiride. Finally, we behaviorally confirmed the hypothesis of D2-mediated aggression by completely rescuing the behavior via *in vivo* infusions of sulpiride in the LS. These data strikingly highlight that VTA projections to the LS constitute the dominant pathway involved in DAergic promotion of aggressive behavior.

Chapter III provides evidence of a direct bridge between two prevailing hypotheses in aggression -- the hyper-DAergic model of aggression (Antelman and Caggiula, 1977; Suri et al., 2015) and the classic LS-hypothalamic aggression axis involving "septal rage" (Spiegel et al., 1940; Wong et al., 2016) and the "hypothalamic attack area" (Lin et al., 2011). In this chapter we strengthen the link between the two models by identifying an underlying D2-mediated mechanism of action. This conclusion supports existing work that implicates LS inactivity in violent pathologies. The aggressive rage response following disengagement of the septum (via pharmacological, optogenetic or lesion manipulations) has been described in numerous studies (Brady and Nauta, 1953; Sodetz and Bunnell, 1970; Slotnick and McMullen, 1972; Albert and Chew, 1980; Wong et al., 2016). For example, $GABA_A$ receptor activation in the LS (which also has a hyperpolarizing effect) increases aggression in male hamsters (McDonald et al., 2012). But, inactivation of the receptor decreases maternal aggression in lactating mice (Lee and Gammie, 2009). Optogenetic suppression of LS terminals in the medial hypothalamus increases aggression

(Wong et al., 2016). Lesions of the septum lead to the syndrome referred to as "septal rage" (Brady and Nauta, 1953). Consistent with the LS-associated rage phenomena observed in previous studies, here we showed that D2-mediated LS inactivation induced attack behavior.

In our studies, we characterized circuit connectivity and synapse characteristics, based on defined DA afferents, using optogenetics combined with electrophysiological and behavioral pharmacology. Although DA neurons most commonly release DA, many of them co-release glutamate (Stuber et al., 2010; Tecuapetla et al., 2010), or GABA (Tritsch et al., 2012), which could also contribute to the changes observed. However, our use of D2 receptor specific antagonist, confirms that the behavioral and physiological alterations were specifically caused by actions of DA. Therefore, our controlled synaptic terminal release combined with pharmacology, established a defined, D2-mediated DA circuit, from the VTA to the LS that controls aggression.

The original data in this chapter further implicate a direct role for D2 receptor signaling in aggression. Previous studies frequently used intraperitoneal injections of drugs that affected general D2 receptor signaling. Therefore, anatomical characterization of aggression-related D2 receptors, was thwarted by the absence of behavioral specificity following systemic delivery of DA receptor drugs. One study recognized this limitation and, like us, addressed this by evaluating the impact of local infusion of D2 antagonist sulpiride on aggression (Couppis and Kennedy, 2008). However, in this study they delivered the drug to the NAc in order to directly assess the role of aggression as a positive reinforcer. In manipulating NAc D2 receptor signaling they found that local infusions of sulpiride decreased operant responding for aggression in a behavioral contingency task. However microinjections of sulpiride outside of the NAc had no impact on contingent aggression. In this study, it is evident that mesocorticolimbic D2 receptors in the NAc are involved in the rewarding effects of aggression in mice. This conclusion addresses the

dissociation between the subtypes of aggression, as it pertains to our work. To reiterate, proactive aggression is more premeditated and directly motivated by a drive for appetitive reward. However, reactive aggression occurs more impulsively in response to perceived external threat, with no involvement of a motivational component (Rosell and Siever, 2015). Couppis and Kennedy's work can be directly linked to the proactive subtype, based on their behavioral outcomes. Our data, however, suggest a role for LS D2 signaling in the reactive subtype of aggression, as evidenced by a presence of aggression, but an absence of reward preference, following D2-mediated suppression of LS activity. In both examples, we see direct validation of D2-mediated aggression; however the dissociation in the functional contribution arises when considering the specific region of action. These data once again bolster the idea introduced in chapter III that the type of behavior activated is ultimately dependent on which distinct terminal sites are involved, even if the same neurotransmitter (DA) and receptor (D2) are participating.

Finally, D2 receptor signaling is strongly implicated in governing locomotion (Baik et al., 1995; Kelly et al., 1998; Usiello et al., 2000). However, we have demonstrated a successful rescue of D2-mediated aggression without any confounds related to general motor suppression. This outcome has clinically relevant implications as it offers mechanistic insight into the potential action of antipsychotic agents that attenuate pathological aggression by antagonizing the D2 receptor (Brizer, 1988; Lenox et al., 1992; Chengappa et al., 1999; Dorevitch et al., 1999; Schulz et al., 1999; Rocca et al., 2002). Specifically, in our experiments, local infusions of D2 antagonist, sulpiride into the LS completely rescued the aggression phenotype, without affecting locomotion. These data indicate that clinically established pharmacotherapy, currently used to manage pathological aggression, acts on D2 receptors within the LS to specifically reduce aggression, and not via DA receptor-mediated sedation (Yudofsky et al., 1987; Fava, 1997). This insight provides

a solid foundation for drug development aimed at selectively targeting broadly expressed GPCRs on specific cell types (McCorvy and Roth, 2015). Together our data not only reveal DA circuit mechanism underlying aggressive behavior but also provide guidance for improving diagnosis, prevention and treatment approaches for aggression in human psychiatric disorders.

Concluding remarks

We have established that the VTA-LS DA pathway, via D2-mediated inhibition of GABAergic LS neurons, drives VTA-triggered aggression. In arriving at this conclusion, we are not only bridging two dominant models of aggression, but are also offering mechanistic evidence of the specificity and necessity of this pathway in controlling aggression. However, while we are able to tangibly verify a modulatory role of DA in LS-controlled aggression, our understanding of the downstream impact on the hypothalamus in the "LS-hypothalamic aggression axis" is based on supposition. Here we hypothesize that, like other studies involving septum inactivation and aggression, our manipulation has a net top-down effect of increasing overall medial hypothalamic activity. An interesting speculation worth exploring is that a D2-mediated decrease in LS activity, decreases downstream GABAergic tone and dysregulates the balance between "attack-excited" and "attack-inhibited cells" of the ventral medial hypothalamus, as described by Wong and colleagues (Wong et al., 2016). Future studies examining the sequence of downstream circuit disruptions, following D2-induced LS inactivation, will help test this hypothesis and empirically strengthen the bridge between the two models.

CHAPTER V: General discussion

a. Data summary

The first study, presented in chapter II of this dissertation, explored the long-term consequences of perturbing adolescent dopaminergic (DAergic) signaling, highlighting adolescence as a key sensitive period in DAergic system maturation. Specifically, this chapter provided original data that showed elevated dopamine (DA) levels, via transient dopamine transporter (DAT) blockade from P32-41, impacts adult behavior and DAergic physiology. First, we determined that the DA sensitive window opens at or after P32 and closes at or before P41. Transiently increasing DA signaling during P32-41 increased adult levels of aggression and behavioral sensitivity to amphetamine. Next, we demonstrated that adolescent DAT blockade also potentiated adult response to amphetamine. We concluded this chapter revealing increased DAergic firing rate (*in vitro*), and increased DAergic bursting and population activity (*in vivo*) at baseline, in mice that were treated with a DAT blocker during P32-41. Therefore, this chapter emphasizes how perturbations in periadolescent DAergic signaling enduringly disrupt the function of the DA system, especially as it relates to aggression.

The second study, presented in chapter III of this dissertation, explored the causal role of the DAergic system in driving aggression. Data from the previous chapter lead us to hypothesize a direct DAergic circuit in aggression control, which we tested in a temporally and spatially controlled way, using optogenetics. First, we established that ventral tegmental area (VTA), but not substantia nigra pars compact (SNc), DA significantly increases aggression. Next, we identified that this effect is produced through the lateral septum (LS), but not the nucleus accumbens (NAc), and that the VTA sends distinct and non-overlapping DAergic projections to both regions. Finally, we demonstrated that terminal release of DA at the LS, specifically induces

aggression, leaving other DA-mediated behaviors, like reward preference and locomotion, unaffected. Therefore, in this chapter we established a direct DAergic pathway, from the VTA to LS that actively drives aggression, confirming a causal role for DA in mediating aggression.

The third study, presented in chapter IV of this dissertation, explored the underlying mechanisms of VTA-to-LS DA-mediated aggression. First, we assessed DA receptor expression in the LS and found an abundance of D2 receptors. This led us to hypothesize that LS D2 receptors are key in controlling LS activity following terminal DA release. Next, we conducted a series of experiments that combined optogenetics with electrophysiological and behavioral pharmacology, to assess D2 involvement. We found that terminal DA release, at the LS, activated D2 receptor signaling and hyperpolarized LS neurons, an effect that was abolished by bath application of D2 antagonist sulpiride. Next, we evaluated this model *in vivo*, and found that VTA DA stimulation increased aggression, but local infusion of sulpiride in the LS entirely blocked the behavior, without affecting locomotor activity. Therefore, in this chapter we established that the VTA-LS pathway constitutes the dominant DAergic circuit that controls aggression via D2-mediated inhibition of the LS.

Considered together, while the three studies had their own specific aims, I now explain how the conclusions of each chapter can be synthesized and interpreted together. The central link between the studies is that all of them provide insights on how hyper-activity of the DA system is related to aggressive behavior. Therefore, these findings not only endorse research that proposes a hyper-DAergic model of aggression (Redmond et al., 1973; Antelman and Caggiula, 1977; Suri et al., 2015), but they also advance the model by establishing: i) a developmental component of DAergic aggression control ii) a direct and causal pathway of DA-mediated aggression and iii) a

clinically pertinent, D2-regulated, association with the classic septal-hypothalamic aggression axis (Hess, 1928; Spiegel et al., 1940; Lin et al., 2011; Wong et al., 2016).

b. Mechanistic implications

<u>Pre-synaptic changes</u>

Our studies reveal that transient increases in periadolescent DA signaling impact adult aggression and DA physiology. Moreover, adult DA signaling promotes aggression by inhibiting activity of D2-expressing cells in the LS. Mechanistically, our key conclusions lead us to hypothesize that DA elevations during periadolescence, could confer risk of aggression pathology via increasing (re-) activity of the DAergic VTA-LS pathway (Yu et al., 2014; Suri et al., 2015).

VTA DA activity, and balance between phasic and tonic firing, is determined by the actions of pedunculopontine tegmental nucleus (PPTg), ventral pallidum (VP), and rostromedial tegmental nucleus (RMTg) afferents. These afferents constitute the dominant glutamatergic and GABAergic input regulating VTA DAergic activity (Grace et al., 2007). Therefore, changes in firing patterns could result from altered input balance. Indeed, many psychostimulants acutely act by disrupting the balance of input into the VTA, thereby altering its activity patterns. For example, injections of cocaine in awake animals, causes an increases in firing rate and bursting of DA neurons (Koulchitsky et al., 2012). Single cocaine injections increased the firing rate and bursting activity of VTA DA cells by potentiating the excitatory inputs (Creed et al., 2016). Direct administration of NMDA receptor antagonists into the VTA prevented behavioral sensitization induced by cocaine, strongly implicating a role for altered afferent glutamatergic transmission in the VTA (Kalivas and Alesdatter, 1993). GBR12909 has closely related mechanistic action to psychostimulants like cocaine. Therefore in our experiments, the balance of input to the VTA may

be particularly vulnerable to periadolescent alterations, leading to the protracted *in vivo* burst firing seen in our data. This could lead to stronger activity in outgoing mesocorticolimbic DA circuitry to various terminal sites. Indeed, Sombers and colleagues determined that synaptic overflow of DA in the NAc occurs as a function of burst activity originating from the VTA (Sombers et al., 2009). Likewise, we could investigate DA release in the LS, following our periadolescent manipulation. Therefore, potentiated VTA activity resulting in increased DA release in the LS, is one possible mechanism by which developmental DAT blockade could drive adult aggression.

Our study reports an increase in VTA burst firing. Based on this, we could investigate underlying structural changes as a function of altered firing patterns. One study found that adolescent optogenetic phasic activation of VTA DA neurons promoted mesofrontal DAergic bouton formation in adolescent mice (Mastwal et al., 2014). Specifically, they had two key findings: i) that new bouton formation increased only in response to phasic, but not tonic, stimulation in adolescent mice and the newly formed boutons were present even 24 hours later. Strikingly, phasic activation of VTA DA in adult mice, did not lead to significant differences in meso-frontal bouton formation; ii) that increased DAergic boutons in the mesofrontal circuit was correlated with prolonged frontal local field potential containing high-frequency oscillations (Mastwal et al., 2014), which is indicative of elevated DAergic signaling (Lewis and O'Donnell, 2000; Gireesh and Plenz, 2008; Wood et al., 2012). In sum, these changes reflect potentiated activity in the mesofrontal circuit following adolescent phasic firing. The plasticity found by Mastwal and colleagues (2014) in adolescence highlights this period as a sensitive one (Mastwal et al., 2014). During this period, phasic activity-induced strengthening of DAergic input might also be found in other projection regions, increasing synaptic release at the innervated site. This hypothesis potentially applies to the VTA-to-LS projection, following our periadolescent

manipulation. In the LS, it would be interesting to study the structural correlates in terms of i) DAergic bouton formation and ii) plasticity in DAergic pericellular basket formation, a characteristic innervation pattern found in the LS. This could alter the degree of connectivity between the VTA and the LS, providing a structural base for our aggression behavior.

Post-synaptic changes

Elevations in periadolescent DA could induce lasting post-synaptic effects in the VTA-LS DA pathway. Our data from chapter IV highlight a D2-mediated inhibitory post-synaptic effect of DAergic activity on LS neurons. Therefore, chronically heightened activity in this pathway could sustain inhibition of the LS and lead to long term changes in LS GABA neurons. In fact, prolonged administration of cocaine chronically inhibits LS neurons, hyperpolarizing the resting membrane potential (RMP) (Liu, 2005). Another group had a similar conclusion, following bath-application of cocaine, where a stronger hyperpolarizing effect on the RMP of LS neurons was revealed in subjects who were previously treated, *in vivo,* with chronic cocaine when compared to drug naïve controls (Shoji et al., 1997). This suggests an altered baseline function in the LS, which might induce alterations in local GABA release, following chronic cocaine administration (Shoji et al., 1997). Based on the colocalization of D2 and GAD67 reported in chapter IV, we could explore the LS DA-GABA interface further, following periadolescent DAT blockade. In considering cocaine's inhibitory effect on the LS, it is possible that chronic induction of hyperpolarization drives long-term alterations in LS RMP. In chapter IV we found that terminal DA release in the LS hyperpolarizes LS neurons as well. Mechanistically, this leads us to predict that sensitive period DA elevations would potentiate LS DAergic activity, chronically inhibit the LS, and drive similar post-synaptic RMP alterations.

c. Risk factors and aggression

Our findings suggest that genetic and environmental factors, that increase LS DAergic activity, can be risk factors for aggression-related psychopathology. Moreover, they indicate that elevated periadolescent DA could produce heightened VTA-LS activity. Therefore, it is important to consider factors that could result in increased, developmental DA signaling. Genetic models of aggression, where constitutive or transient increases in DA could contribute to improper LS development, serve as strong models for assessing the role of developing DAergic circuits in aggression disorders. Examples including *MAOA*, *DAT*, and *COMT* loss-of-function mouse lines (Cases et al., 1995; Gogos et al., 1998; Rodriguiz et al., 2004; Scott et al., 2008) may show altered LS development. Some possible structural changes to investigate could be increased pre-synaptic DAergic bouton and pericellular basket formation around LS neurons. Post-synaptically, altered LS D2 receptor expression or increased hyperpolarization of the RMP of LS neurons could be assessed. These changes, could serve as possible neural correlates underlying the increased aggression seen in these mouse lines.

Environmentally, an important factor to consider is drug exposure, either therapeutic or recreational. Psychostimulants increase extracellular DA levels at projection terminals (Berridge and Robinson, 1998; Volkow and Li, 2004). GBR clearly has very close mechanistic action compared to psychostimulants. Therefore we hypothesize other psychostimulants would produce similar outcomes as our experiments. Finally, in addition to psychostimulants, drugs that don't directly target the DAergic system, including nicotine (Mameli-Engvall et al., 2006) opiates (Iwatsubo and Clouet, 1977), and cannabinoids (French et al., 1997), also increase DA neuron burst firing and generate increased phasic DA release (Floresco et al., 2003). Burst firing significantly increases extracellular DA and therefore it stands to reason that these drugs as well

could leave a mark on the developing DA system and aggression, if exposure occurs during the sensitive period.

In summary, genetic and environmental factors that affect developmental DA signaling could exert long-lasting influences on the maturation of the DA system, thus impacting the formation of neurocircuits that mediate aggressive behavior. Refer to Figure 5.1 for an integrated conceptual model of the developmental origins of DA-mediated aggression.

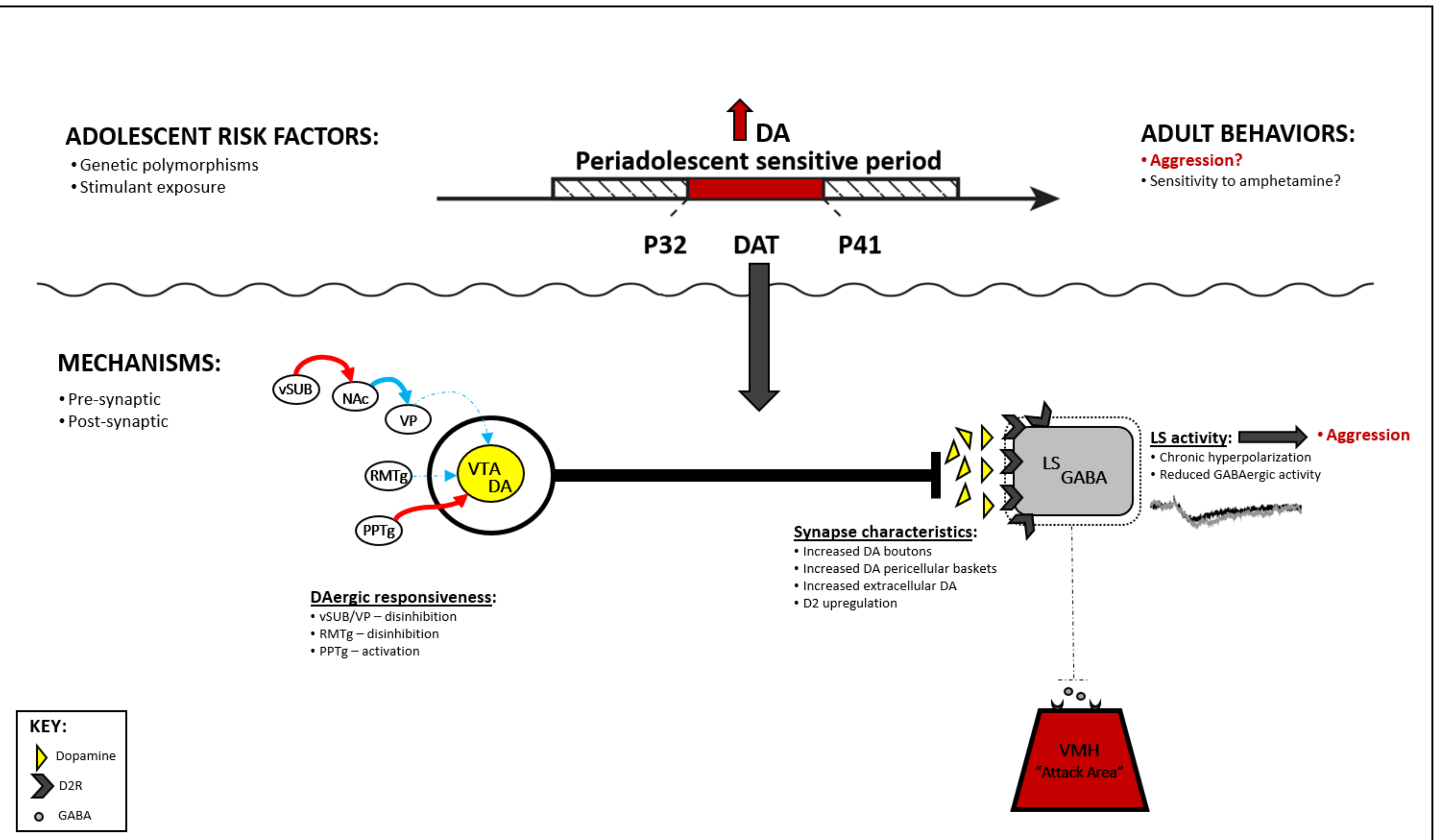


Figure 5.1: Model of developmental origins of DA-mediated aggression. Conceptual framework integrating adolescent risk factors, mechanisms and behavioral outcomes.

d. Future directions

In this final section, I will describe future experiments that may help advance our understanding of the findings presented in this dissertation. In the previous sections of this chapter I hypothesized an association between transient increases in periadolescent DA signaling and increased (re-) activity of the DAergic VTA-LS pathway. The following studies might help test the presence of an association.

In vivo activity recording in anesthetized mice:

In the first set of experiments, we could study the responsivity of VTA DAergic neurons to excitatory and inhibitory input in slice and *in vivo*. We would focus on PPTg, VP, and RMTg afferents because they constitute critical glutamatergic and GABAergic input regulating VTA DAergic activity (Grace et al., 2007). Based on increased DAergic bursting and population activity at baseline, in addition to past work highlighting sensitization to glutamatergic afferents following cocaine exposure (Kalivas and Alesdatter, 1993), we hypothesize that DAergic neurons would be more sensitive to excitatory input after periadolescent DAT blockade.

Inspired by studies that found that direct antagonism of VTA NMDA receptors blocked sensitization induced by cocaine, we could execute similar pharmacology and observe the effect on DA burst firing, in GBR treated animals. If the *in vivo* burst firing observed in our mice is diminished, this would help us strongly implicate a role for altered VTA sensitivity to glutamatergic input, following periadolescent DAT blockade. On the post-synaptic side, we could conduct physiological recordings, both in slice and *in vivo,* in the LS of mice previously treated with GBR or vehicle during adolescence. If GBR treatment does in fact strengthen LS DAergic activity, we would predict chronic suppression of the LS, either through metabolic depression

(Dow-Edwards et al., 1990) or through a persistently hyperpolarized RMP (Shoji et al., 1997; Liu, 2005).

<u>*In vivo* activity recording in behaving mice:</u>

Electrophysiological recordings in anesthetized preparations reveal alterations in VTA-LS DA function and firing patterns, following P32-41 DAT blockade. This data would be further supported by assessing dynamic activity changes in the awake animal, during behavior. One methodology to assess real-time changes, in population activity, in freely moving animals is fiber photometry. This technique would allow us to determine activity dependent alterations in calcium transients in the behaving mouse. Transgenic $Dat^{IRESCre}$ mice could be injected in the VTA with a Cre-dependent adeno-associated virus (AAV1) carrying the GCaMP6 gene, after periadolescent treatment with GBR or vehicle. Using VTA calcium (Ca^{2+}) signals as an index of activity, we could track the role of genetically and anatomically specified pathways, during behavior (Gunaydin et al., 2014). More specifically, we could identify and tightly correlate task-specific (biting, tail rattling and mounting) and pathway-specific signatures in VTA DAergic activity during our test of aggression.

Furthermore, fiber photometry is sensitive enough to measure deflections in Ca^{2+} signals from axonal fibers too, allowing for projection-specific recordings (Saunders et al., 2015). These data would offer us insight into time locked and real-time changes in Ca^{2+} transients only in genetically-specified VTA inputs to the LS, during measures of aggressive behavior. Once again, $Dat^{IRESCre}$ transgenics injected with the AAV1-GCaMP6 virus would be implanted with an optical fiber, but this time in the LS. Given the importance of the VTA-LS projection in DA-mediated aggressive behavior we would predict changes in LS DAergic activity, during a test of aggression in GBR treated mice.

Data in chapter III reveals that largely non-overlapping VTA DA cell populations project to the NAc and the LS, functionally contributing to different behaviors too. Given the heterogeneity in VTA DA cells, it could be beneficial to refine our understanding of local activity to those DA cells which are specifically controlling aggressive behavior. To achieve this a retrogradely transported (specially engineered for monosynaptic transport) and recombinase-dependent GCaMP6 viral vector could be injected into the LS. LS-projecting cell bodies would be labeled and Ca^{2+} transients, only in labeled VTA DA neurons, could be recorded in response to aggression and adolescent treatment (Gore et al., 2013; Rothermel et al., 2013; Saunders et al., 2015).

Based on the outcomes in the fiber photometry studies, we could conclude if periadolescent DAT blockade does in fact amplify task- and pathway-specific activity signatures in mice before, during and after the test of aggression. More precisely, if there is a peak in the activity of only an anatomically confined group of VTA DA cells during aggression, we could label the specific pathway by injecting a retrograde virus into the LS, that also expresses a light-inhibited opsin (Packer et al., 2013), such that those cell bodies alone can be silenced to rescue the aggression seen in GBR treated mice. We predict that results from this experiment would refine our data from a preliminary study where we found that general inhibition of VTA DAergic neurons decreases aggression (Figure 5.2).

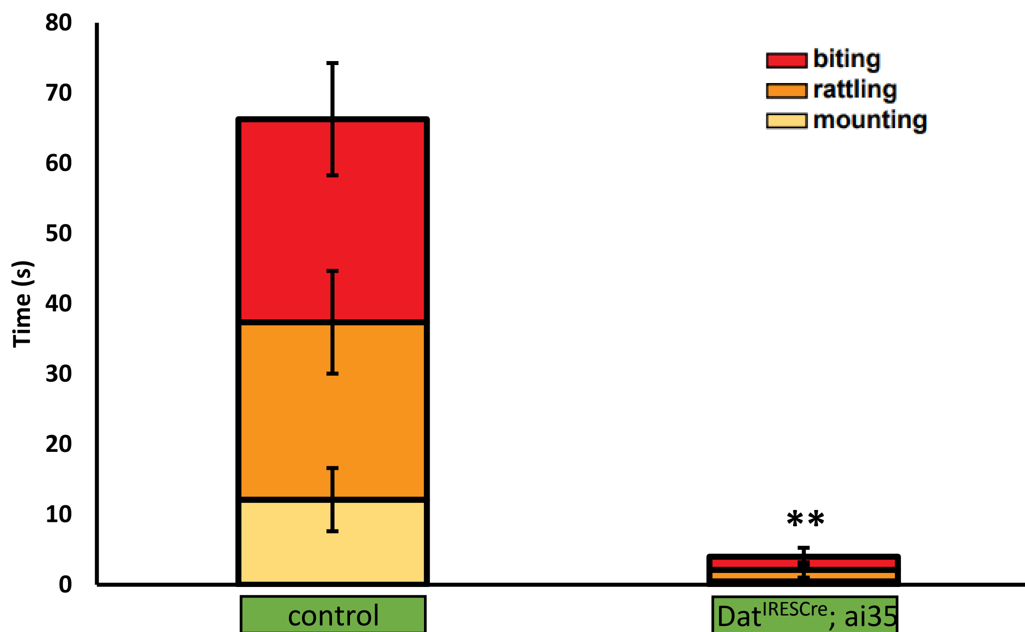


Figure 5.2: Arch-based optogenetic inhibition of DAergic VTA neurons decreases aggression. Reduced aggressive behavior, measured as decreased time fighting, was observed in pairs when $Dat^{IRESCre}$;ai35 mutant mice were inhibited. To achieve this we expressed the light-gated proton pump Archaerhodopsin (Arch), which inhibits neural activity upon photostimulation, in DAergic neurons by crossing $Dat^{IRESCre}$ mice to the RC::LSL-Arch line, which consists of conditional floxed Arch-eYFP targeted to the ROSA26 locus and is abbreviated as "Ai35"(Madisen et al., 2012).*, $p < 0.05$; **, $p < 0.01$ compared with their respective controls; mean ± SEM; n = 10 per group.

In vivo activity imaging in behaving mice:

Neuronal activity in behaving subjects could also be recorded using miniaturized single-photon epifluorescence microscopes (Flusberg et al., 2008; Ziv and Ghosh, 2015). We could conduct high resolution *in vivo* calcium imaging and track the activity of individual neurons in the VTA or the LS using this microscope, which interfaces with chronically implanted lenses (Saunders et al., 2015). Furthermore, we could identify preferential activity in subpopulations of VTA DA neurons during a single exposure aggression encounter like ours versus those who learn to perform an operant task to seek access to a subordinate opponent (Couppis and Kennedy, 2008; Falkner et al., 2016) or those who develop a preference for a place associated with "winning" (Golden et al., 2016, 2017). This study may aid in anatomically parsing out dissociated DA cell

groups involved in premediated versus impulsive aggression. Therefore, microendoscopic *in vivo* calcium imaging would enable us to visualize the heterogeneity or homogeneity of VTA DA cell responding during different types of aggression tasks.

Finally, for both microendoscopy and fiber photometry, it is important to consider that it might be technically challenging to record activity with high signal-to-noise ratio during an aggression assay. If this is the case, it could be valuable to even just analyze baseline differences in genetically defined VTA-LS projections in animals treated with GBR or vehicle in adolescence. Specifically, high resolution imaging could be used to visualize differences in VTA activity at the cell body level but also for imaging difference in new bouton formation at the LS, using DA-projection specific labeling techniques described by Mastwal and colleagues (Mastwal et al., 2014). They found that increased adolescent VTA phasic activity increased bouton formation, elevating DA signaling, in meso-frontal axons. Our follow-up experiment would hypothesize that periadolescent DAT blockade would result in immediate phasic VTA activation, which would promote rapid bouton formation at the LS and increase LS DAergic activity. We would compare bouton numbers in the meso-septal DA pathway of GBR versus vehicle treated mice at baseline, changes that could be quantified and correlated with aggressive behavior. This could serve as one neural correlate, at a target site, underlying the aggression seen in these mice.

Concluding remarks

In conclusion, if the suggested changes in the VTA-LS DA pathway, following periadolescent DAT blockade, are present and causal to the aggressive phenotype, then antagonizing LS D2 receptors in GBR treated mice would be expected to block aggression. Indeed, antipsychotics that block D2 receptors are currently the first-line treatment for aggressive disorders, but they are often discontinued due to their sedative effects (Yudofsky et al., 1987; Miczek et al.,

2002). Strikingly, we found that our D2 antagonist blocked aggression, without affecting locomotion. These data suggest that LS-targeted D2 antagonism would reduce aggression without causing sedation in humans. Of course, local drug infusions are currently difficult to execute in humans. However, with progress in the development of functionally selective allosteric modulators, non-invasive LS D2 targeting might become feasible. These advancements may be able to correct the aggressive phenotype of humans that experienced adolescent alteration in DA signaling, without generating undesirable side effects.

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