Impact of Learning and Diet on Two Distinct Populations of Midbrain Dopamine Neurons

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Dopamine is a crucial component of the neural mechanisms underlying motivation and reward learning, both of which are disrupted in psychiatric disorders. Much of the research in this field has focused on the role of ventral tegmental area (VTA) and its influence on the nucleus accumbens, but there is also evidence that the substantia nigra pars compacta (SNc), a separate dopaminergic region with its own efferent and afferent projections, contributes to guiding motivated behavior. Environmental insults, such as insufficient dietary nutrition, are thought to negatively impact the dopamine system and possibly promote the development of mental illness. These issues have not yet been investigated in the context of dopamine neuronal function during reward-mediated behavior. This dissertation sought to address this gap in our knowledge through two primary objectives. Using electrophysiology in awake-behaving animals, the first aim was to simultaneously record neuronal activity in the VTA and SNc during two reward-related associative learning paradigms to understand the role each region plays in motivated behavior. The second aim was to evaluate the impact of dietary deficiency on dopamine neuronal function and identify any region-specific effects in the VTA and SNc.

In our comparison of VTA and SNc, we employed an instrumental conditioning task, in which the animal executed a nose poke to earn a sugar pellet reward, and a Pavlovian conditioning task, which pairs a previously meaningless cue with a sugar pellet reward. We found that VTA and SNc may play similar roles in both forms of reward learning, possibly driven by common excitatory inputs between the two regions. We then measured correlations in

activity of simultaneously recorded neurons within each region and found that how these correlations fluctuate in response to associative learning differed between regions. In our dietary manipulation experiment, we observed that event-evoked neuronal activity was reduced in animals lacking essential fatty acids, and this reduction is more pronounced in the SNc. Our findings suggest that the SNc may be more vulnerable to environmental insult and its role in reward learning and dysfunction in psychiatric disorders warrant further investigation.

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ACKNOWLEDGEMENTS

I knew when I began graduate school that I would not complete a dissertation on my own. I require a community for support, feedback, encouragement, and discussion to invigorate my soul and inform my work. This community begins with my parents, Stephen Wegener & Lisa Hovermale, and my sister, Lauren Wegener. My parent warned me extensively about the struggles inherent in pursuing a doctoral degree, and supported me unconditionally as I wrestled with those struggles. They never said, “I told you so”, instead offering “What can we do?” and “Just do your best, forgive yourself, and move forward”. They ensured I knew that they were proud of me, and gave me the space to make my own choices and explore their outcome. They also gave me the great gift of an education, without the financial burden that many students carry today. It required sacrifice on their part, and I am incredibly grateful. My sister Lauren gave me perspective on my tiny slice of the world in academia, reminding me that all the work we do here is valuable and impressive. She bolstered my esteem and sent me to every conference, milestone, and social event well dressed, giving me the support I needed to put my best foot forward. I would also like to thank my uncle Chris Wegener and aunt Mary Perkins who generously provided me with a place to stay as I wrote this document in Portland, and their kindness and understanding made completing this endeavor that much easier. I feel immensely lucky to be a part of this family, and could not have achieved this without them.

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As a parting note to other graduate students who are writing their dissertation, as I believe that is the most likely audience for this passage, please know that what you are doing is

incredibly difficult and the struggle you're experiencing is inherent in the journey. You are not alone in this. Do your best, learn from it, and move on.

1.0 INTRODUCTION

Dopamine is a neuromodulator that has been implicated in multiple forms of learning, motivation, and cognitive constructs such as attention, working memory, and decision making (Westbrook & Braver, 2016; Berridge & Kringelbach, 2015; Björklund & Dunnett, 2007; Collins & Frank, 2016; Lammel et al., 2014; Schultz, 2007; Schultz et al., 2015; Wise, 2004). Dopamine has also been implicated in an array of brain disorders including neurological and movement disorders such as Parkinson's disease, and psychiatric disorders such as schizophrenia and ADHD. The longstanding theory in the field has been that different dopamine systems contribute to the symptoms of neurological versus psychiatric disorders. Specifically, dopamine neurons in the ventral tegmental area (VTA), which project to limbic regions such as the nucleus accumbens (Figure 1-1), have been implicated in motivation and reward-related behaviors (Parkinson et al., 2002; Smith, 1976; Schneirla, 1959; Wise, 2009; Lammel et al., 2012; Cohen et al., 2012; Eshel et al., 2015, Flagel et al., 2010; Hamid et al., 2016; Hart et al., 2014; Kim et al., 2014; Kim et al., 2012; Roesch et al., 2007), whereas dopamine neurons located in substantia nigra pars compacta (SNc) which project to dorsal striatum have been implicated in movement related behaviors (Dauer & Przedborski, 2003; Hornykiewicz, 1962; Carlsson, 1964; Marshall et al., 1980; Beninger, 1983). Accordingly, clinical literature has implicated the malfunction of VTA neurons in disorders such as schizophrenia, ADHD and drug abuse (Guillin et al., 2007; Grace et al., 2007; Everitt & Robbins, 2005), and symptoms of movement disorders such as

Parkinson's disease to degeneration of SNc dopamine neurons (Hornykiewicz, 1962; Barbeau, 1974; Palmiter, 2008; Drui et al., 2012). Recent literature, however, is beginning to describe a similar role in reward and motivated behavior processing by both SNc and VTA neuron (Reynolds et al., 2001; Wise, 2009; Rossi et al., 2013; Ilango et al., 2014; Horvitz, 2000). If that is the case, many of our pre-existing theories and purposed mechanisms related to the involvement of dopamine neurotransmission in affect versus movement may have to be re-evaluated.

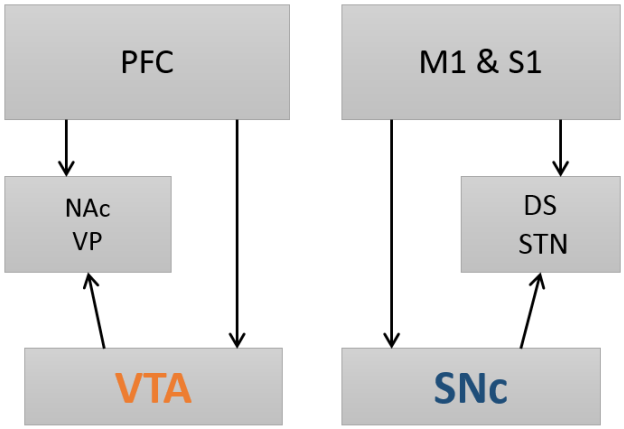


Figure 1-1 A simplified diagram of the circuits examined in this work
Diagram depicting two distinct but related dopamine innervated circuits that were the focus of this dissertation. The question I asked in my thesis was whether two midbrain dopamine neuron groups are regulated differently during associative learning. Abbreviation: ventral segmental area (VTA), substantia nigra pars compacta (SNc), dorsal striatum (DS), subthalamic nucleus (STN), nucleus accumbens (NAc), ventral pallidum (VP), orbitofrontal cortex (PFC), motor cortex (M1), somatosensory cortex (S1).

The overarching purpose of this dissertation was to directly compare the response of neurons in the VTA and SNc to the same salient behavioral events that are relevant to reward-related learning. A second purpose of the work was to determine if a common environmental insult that has been implicated in dopamine-related psychiatric and neurodegenerative disorders affects these neuronal responses. For the first two experiments, we recorded simultaneously from

SNc and VTA neurons during two fundamental forms of learning: Pavlovian and instrumental conditioning. For the second experiment, we performed these simultaneous recordings during conditioning in cohorts of animals chronically deficient in dietary omega-3 fatty acids.

Below I review some of the pertinent literature that inform the aims of this thesis and our approach and experimental design.

1.1 STRUCTURE AND NEUROANATOMY OF THE MIDBRAIN

Research investigating motivation and psychiatric disorders has identified dopamine function as a crucial part of the mechanism behind reward-mediated behavior (Schultz, 1998; Pignatelli and Bonci, 2015; Salamone et al., 2015). Dopamine is released by midbrain neurons which originate from one embryonic cell group and develop into two distinct regions, the ventral tegmental area (VTA) and substantia nigra pars compacta (SNc) (Seiger & Olson 1973; Ungerstedt 1971). Dopamine neurons in both regions exhibit a distinct morphology consisting of large cell bodies which support complex, unmyelinated axonal arbors (Lindvall et al., 1984; Loughlin and Fallon, 1984; Doucet et al., 1986; Björklund and Dunnett, 2007). These axons create projection systems which extend long distances to deliver dopamine to a widespread array of target regions (Lindvall et al., 1977; Goldman-Rakic, 1996; Glimcher 2010). Dopamine can influence these target regions through classic synaptic transmission, or through somatodendritic volume transmission (Cheramy, Lievial, & Glowinski, 1981), and dopamine neurons can regulate each other through electric gap junctions in additon to somatodendritic release (Fuxe et al., 2010; Rice, Patel, & Cragg, 2011; Grace & Bunney, 1983; Baker & Llinas, 1971). This morphology facilitates unique capability of dopamine to influence adjacent neurons and

synchronize to broadcast the same message to functionally distinct areas of the brain simultaneously (Agnati et al., 1992; Zoli et al., 1999; Fuxe et al., 2010).

Anatomical studies of the midbrain have consistently found that dopamine neurons are organized in a functional medial lateral gradient, which is reflected in a reciprocal connection pattern between the striatum and midbrain (Haber et al., 2000, Voorn et al., 2004). This feedback loop heavily influences the electrical activity patterns of striatal and dopaminergic neurons. Tracing experiments established that VTA ascending efferents project heavily to the nucleus accumbens (NAc) and terminates in the portion of the NAc which mirrors the location of the projection neuron's cell body within the midbrain (Beckstead, Domesick, & Nauta 1979; Ungerstead 1971). This mesolimbic circuit, which also includes the thalamus and amygdala, is the central component of addiction and reward research (Berridge & Kringelbach, 2015; Cardinal & Everitt, 2004; Morales & Margolis, 2017). The SNc also sends projections that terminate in the medial half of the striatum or dorsal striatum. This projection pattern contributes to the parallel but overlapping spiral pattern described for cortical-striatal-midbrain to cortical pathways (Haber et al., 2000). Medial portions of SNc are more likely to send projections to ventromedial striatum, and signal the value of rewards and cues (Matsomoto & Hikosaka, 2009). Dorsolateral SNc neurons project to dorsal striatum and are modulated by salient events and information in the environment. Studies delineating these projections from VTA and SNc set a precedent for treating the two regions as distinct systems without significant anatomical overlap. Recently, however, the canonical dichotomy of these two projection systems has been called into question, as optogenetic and viral tracing studies reveal that projections from these areas are more integrated and reiterative than previously suggested (Beier et al., 2015; Lerner et al., 2015). Other studies observed that the VTA and SNc form somewhat of a continuum and do not exhibit

distinct borders between the medial SNc and lateral VTA (Fallon 1988; Beckstead, Domasick, & Nauta 1979; Glimcher 2010), noting that cells within this continuum have similar morphology and projection patterns. Different patterns of anatomical input are thought to contribute to the heterogeneity seen in dopamine neuronal activity (Watabe-Uchida et al., 2012; Matsumoto & Hikosaka 2009; Henny et al., 2014), but also support the possibility that these two regions send parallel information streams to the striatum to influence behavior. The complete separation of the midbrain into two separate circuits which do not influence each is still a matter of debate, a debate which we will examine in the context of our simultaneously recorded data.

1.2 DISTINCT TYPES OF LEARNING PARADIGMS TO ASSESS MOTIVATED BEHAVIORS

We use conditioning paradigms to understand how the brain encodes stimuli and their associated outcomes. In general, when we consider the neuronal underpinnings of motivated behavior, we can approach it from Pavlovian cue-oriented or instrumental action-oriented perspective. Pavlovian conditioning pairs a previously meaningless cue (conditioned stimulus, CS) with an outcome (unconditioned stimulus, US) that provokes an unconditioned response (UR), so that the CS comes to predict the US (Pavlov, 1927; Rescorla & Solomon, 1967; Wasserman & Miller, 1997; Fanselow & Wassum, 2016). It is not sufficient for a CS to be contiguous, it must provide information about the forthcoming effect (Rescorla, 1968). While Pavlovian learning may seem like a thoughtless connection between any two stimuli which co-occur, it is better thought of as the animal finding information in a stimulus that provides understanding and context to an unexpected event occurring in its environment. Rescorla, in his

1987 address is the Eastern Psychological association, provides a potent analogy. “If one thinks of Pavlovian conditioning as developing between a CS and a US under just those circumstances that would lead a scientist to conclude that the CS causes the US, one has a surprisingly successful heuristic for remembering the facts of what it takes to produce Pavlovian associative learning” (Rescorla, 1988). Often the main thrust of Pavlovian conditioning is that, after the association is formed, the CS evoked the same response as the US. For our purposes, this entails a light or sound CS paired with the delivery of a sugar pellet US, which causes the animal to approach the food trough (UR). A Pavlovian association is considered to be established when the CS provokes a conditioned response (CR), which is often the same behavior as the UR, but now coincides with the CS as well as the US. We can measure the associative value of our CS by the occurrences of CR (food trough entries) during the CS presentation. Conditioned responses are the reflection of a cognitive representation, and are therefore more flexible or adaptive than the UR which is often a reflex or unconscious innate behavior (Bolles & Moot, 1972; Rescorla, 1973; Fanselow & Wassum, 2016). Furthering our understanding of this process informs us about how cues in our environment might influence our decisions and neural representations of our experiences. While clearly a valuable tool for understanding association formation, Pavlovian conditioning only addresses a subset of learning processes, which cannot explain all motivated behavior. To make optimal decisions, the brain must discriminate between outcomes that require an action from those predicted by an environmental cue.

This ability to make stimulus-outcome associations lies at the core of our decision-making capabilities as previously formed associations will guide our future behavior (Rescorla & Solomon, 1967; Dickinson and Dawson, 1987; Hall, 2002). Instrumental conditioning requires an animal to perform an action or actions to receive a desired outcome or avoid an aversive

outcome (Thorndike, 1898). A common example might be operating a light switch to illuminate a room. We do not interact with every light switch we encounter in this way, as a Pavlovian association might predict. Instead, we only execute this action to achieve a specific desired outcome, and if that outcome is not desired or unnecessary (such as the light already being on), we do not perform the action. In a laboratory setting, the experimenter can determine what action is required, how many actions are required, and the spatial relationship between the action and the reward. This action is a predetermined CR, in our experiment – a nose poke, often initiated in response to a CS, which signals the window of opportunity for the ascribed action. Instrumental conditioning can inform us about the relationship between effort and outcome, allowing us to quantify the animals' internal value of that outcome. Understanding the mechanism underlying instrumental conditioning explains when and why we choose to execute actions and how we form expectations of specific outcomes following the action.

Employing both instrumental and Pavlovian conditioning approaches in our exploration of dopamine function will provide insight into the mechanism behind multiple aspects of motivated behavior. As mentioned above, Pavlovian conditioning is often thought of as a UR that is transferred from the US to the CS. While this may be accurate in some instances of Pavlovian conditioning (Bolles & Moot, 1972; Rescorla, 1988; Fanselow & Wassum, 2016), it does not apply to instrumental conditioning at all. The action or CR in instrumental learning (a nose poke) does not resemble consumption of a food pellet. Additionally, there is well-established research showing that a CS which signals the availability of an action does not carry the same stimulus-outcome relationship as that of a Pavlovian cue (Rescorla and Solomon, 1967; Colwill and Rescorla, 1990; Hall, 2002). By examining the acquisition and maintenance of both conditioning

paradigms while recording from the midbrain, we can differentiate what role dopamine neural activity plays in associative learning.

1.3 DOPAMINE AND MOTIVATED BEHAVIOR

Pharmacology and lesion experiments provide the data which initially implicated dopamine in multiple forms of motivated behavior including Pavlovian and instrumental conditioning. This line of research originated with observations that lesions of dopamine fibers causes impairment in feeding and drinking behavior (Ungerstedt, 1971; Smith et al., 1972). These effects were limited to nigrostriatal projections, while the mesolimbic fibers were found to be critical for approach behavior (Parkinson et al., 2002; Smith, 1976; Schneirla, 1959), such as the conditioned responses we observe in Pavlovian conditioning. Studies with dopamine antagonists or neuroleptics supported these findings by demonstrating that proper dopamine function was required for the acquisition of an action-outcome (Wise & Raptis, 1986; de Borchgave et al., 2002; Hall et al., 2001; Reynolds et al., 2001) or cue-outcome (Spyraki et al., 1982; Spyraki et al., 1987; Stewart et al., 1984) association, as well as the motivational drive to continue a previously conditioned response (Beninger et al.,1983, Tombaugh 1981; Tombaugh et al, 1980, Wise & Schwartz, 1981; Franklin & McCoy, 1976). Essentially, dopamine is required for the reward to have a reinforcing value. That role comes into play when establishing the connection between a reward and a neutral stimulus in Pavlovian conditioning, as well as motivating an animal to work for a reward in instrumental conditioning. If the reward is not endowed with reinforcing value by dopamine when the neutral stimulus is first presented, the neutral stimuli will have no value, and therefore does not provide useful information (Wise,

2004). After an instrumental conditioning paradigm is learned, dopamine is required for the reward to maintain a value worthy of the work it is being asked to perform (Wise, 2009). In comparing drug-free animals to those under the influence of dopamine antagonists, studies found that instrumental performance only declined in the presence of the drug, and this effect was more potent across multiple days of drug administration (Wise et al., 1978; Smith, 1995; Roberts et al., 1989). These pharmacological and anatomical results confirmed that dopamine function is needed to ascribe a motivational value to food and to drive acquisition or behavioral expression of learned associations (Wise & Rompré, 1989).

Many of the pharmacological and lesions studies described above focused on the role of the nucleus accumbens in drug-seeking behaviors. Lesions in NAc removed the reinforcing properties of cocaine and amphetamine (Roberts et al., 1982; Lyness et al., 1979; Kelley et al., 1997; Smith-Roe & Kelley, 2000), which is often interpreted as evidence that an intact NAc is necessary for reward function. These experiments were performed by manipulating activity in the mesolimbic pathway, but evidence from other NAc lesion studies show that rewarding associations can be acquired and adjusted according to value of the reinforcer in the absence of NAc function (de Borchgrave et al., 2002; Balleine & Killcross, 1994). This suggests a role for other circuits in associative learning and motivation, especially in paradigms which employ naturalistic reinforcers such as food or water (Smith et al., 1972). Specifically, the nigrostriatal pathway is implicated in encoding the incentive value of a food or liquid reward (Wise, 2004; Fouriezos & Wise, 1976; Fourizos et al., 1978; Wise & Raptis, 1986, Lee et al., 2010), not just the physical ability to consume these rewards. Despite this early evidence of reward-related function in SNc, research into SNc neural activity is often limited to motor function in rodents. This arises from the observations of movement dysfunction in Parkinsonian patients, which

results from mass degeneration of SNc dopamine neurons (Dauer & Przedborski, 2003; Hornykiewicz, 1962; Carlsson, 1964), and from the hypokinesia in animals with bilateral SNc lesions (Marshall et al., 1980; Beninger, 1983). Lesion studies such as these provide valuable information about the role of SNc, but do not provide a comprehensive evaluation of VTA or SNc function.

To obtain more information about the function of dopamine neurons during movement and behavior, researchers employ awake behaving electrophysiology. This technique allows us to record electrical activity in single neurons, often while the animal performs a behavior or acquires a cue-outcome association. Some of the first studies recording from dopamine neurons were done in the SNc of nonhuman primates during Pavlovian conditioning. In these experiments, animals were initially presented with an unexpected juice reward while excitatory phasic activity from a selected dopamine neuron was recorded (Ljungberg et al., 1992). Then a cue was introduced which predicted the delivery of the previously unexpected reward. As the reward becomes expected through this Pavlovian conditioning and the cue-reward association is formed, dopamine neurons respond to the earliest reward-predicting cue instead of the expected reward (Mirenowicz & Schultz, 1994; Schultz, 1998, Waelti et al., 2001). This seminal work in dopamine neurophysiology and its role in motivated behavior led to the idea of reward prediction error (RPE). In RPE experiments, rewards which are larger than expected evoke a proportionally larger response from dopamine neurons when that reward is delivered (Schultz, 1998; Tremblay and Hollerman, 1998; Tobler et al., 2005). Alternatively, if the reward is omitted or smaller than expected, the dopamine neuronal activity reflects this with a brief pause in firing (Schultz et al., 1998; Schultz, 2006; Bayer and Glimcher, 2004; Fiorillo et al., 2003; Matsumoto and Hikosaka, 2009). Considering this activity pattern, RPE is thought to function as a learning signal,

representing relevant changes in one's environment. By incorporating these changes, dopamine activity can influence an animal's ability to make better decisions about where to seek out food or when to begin foraging.

It is important to keep in mind that this work establishing dopamine's role in encoding reward-related events and the learning of associated cues was conducted in the SNc neurons of nonhuman primates. Interestingly, much of the research into dopamine and reward now revolves around the limbic system in rodents by closely examining the activity of dopamine neurons in the ventral tegmental area (VTA) and its influence on the nucleus accumbens (Wise, 2009; Lammel et al., 2012; Cohen et al., 2012; Eshel et al., 2015, Flagel et al., 2010; Hamid et al., 2016; Hart et al., 2014; Kim et al., 2016; Kim et al., 2013; Roesch et al., 2007). It is unclear how VTA and the mesolimbic circuit came to overshadow SNc and the nigrostriatal circuit in reward-related learning, but one of the aims of this dissertation was to directly compare VTA and SNc activity during motivated behavior to determine whether these two midbrain regions play similar roles in appetitive associative learning.

1.4 VULNERABILITY OF MIDBRAIN DOPAMINE NEURONS

Environmental factors are known to play a role in the development and severity of psychiatric disorders (Kenler et al., 2003; Tandon et al., 2008). Dopamine neurons appear to be particularly vulnerable to the negative impact of environmental insult (Saxena & Caroni, 2011; Gonzalez-Hernandez et al., 2010; Bondi et al., 2014; Di Monte, 2003; Jackson-Lewis & Smeyne, 2005; Chung et al., 2005). Specifically, dopamine neurons in SNs are vulnerable to large scale cell death, causing deficits seen in patients with Parkinson's disease (Hornykiewicz, 1962;

Barbeau, 1974; Palmiter, 2008; Drui et al., 2012). The source underlying SNc neurons' increased susceptibility to disruption by pesticides, neurotoxins, and pharmacological lesions is yet unknown. Several theories asserting that explanations can be found in cell-type specific characteristics such as increased exposure to oxidative stress (Wang & Michaelis, 2010; Pearce et al., 1997; Brooks et al., 1999), differential calcium activity (Surmeier, 2007; Surmeier et al, 2010; Chan et al., 2009; Hage & Khaliq, 2015; Chung et al., 2005), and high metabolic demands (Wang & Michaelis, 2010; Shaw & Egget, 2000; Cleveland & Rothstein, 2001; Rodriguez et al., 2001) have been put forth as possibilities. These intrinsic physiological and morphological factors may interact with external influences such as toxic exposure, stress, and nutrition to cause the cell death observed in SNc, but not VTA.

Deficiency in omega-3 polyunsaturated fatty acids (n-3 PUFAs) have been identified as an environmental insult that affects cognitive function, possibly aggravating or enabling the emergence of schizophrenia, major depressive disorder, and attention-deficit/hyperactivity disorder (Banni & Marzo, 2010; Wainwright et al., 1994; Connor et al.,1991; Amminger et al., 2010). N-3 PUFAs, including docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA), can be found in leafy greens, wild fish, grass-fed livestock and their products, including eggs, dairy, and meat. Beginning in the 1970's, the American diet shifted towards subsidized, mass-produced corn and grain products with a long shelf life, resulting in an increasingly high proportion of n-6 PUFA, another essential fatty acid, in our food (Simopoulos, 2003; Yamada et al, 2014; Ikemoto et al., 2001). Neither the necessity of these two fatty acids nor the importance of the balance between them were fully recognized at the time (Abbott et al., 2012; Simopoulos, 2003; Holman et al., 1982), and therefore n-3 PUFA was not included in nutritional recommendations put forth by dietary advocates or governmental organizations decades ago.

Dietary studies have shown that n-3 PUFA accounts for ~9.5% of the current average fat intake in the US (Ervin et al., 2004), though records suggest that humans evolved with a much larger portion of n-3 PUFA in their diet. This historic diet resulted in an n-6 to n-3 ratio closer to 1 while the current ratio in Western diets is approximately 15/1 or 16/1 (Simopoulos, 2003). Considering that this ratio began to shift in the 1960's and 1970's, the current young adult population at risk for the development of psychiatric disorders is the second generation experiencing n-3 PUFA deficiency (Passos et al., 2012). Thus, our laboratory has developed a 'second-generation' animal model, in which animals are given a diet which contains either DHA as a precursor to n-3 PUFA or a diet lacking DHA (Bondi et al., 2014). Our previous investigations using this animal model revealed disruption in dopamine protein and receptor expression in the dorsal striatum, but not the nucleus accumbens. While indicative of the impact of n-3 PUFA deficiency on the nigrostriatal circuit, this observation does not identify how neural activity in dopamine neurons is affected.

Previous cellular and behavioral research has demonstrated a profound impact of n-3 PUFA deficiency on the monoamine neurotransmitter system, similar to the environmental factors described above. Studies in dopamine projection regions found that this manipulation affected vesicular monoamine transporter, dopamine transporter and tyrosine hydroxylase expression, as well as increased D2 receptor number and attenuated amphetamine-induced dopamine release in n-3 PUFA-deficient rats (Kuperstein, Eilam, & Yavin 2008; Chalon, 2006; Bondi et al., 2014). These results indicate altered dopamine transmission, but do not directly investigate how dopamine neurons are impacted by dietary n-3 PUFA deprivation. Based on these observations and evidence for SNc greater susceptibility to cell death, we suggest that dopamine neurons in SNc may be more vulnerable to dietary environmental insult compared to

the VTA. By comparing physiological properties of these midbrain regions, we can further isolate the role of an external factor such as diet in the aggravation of prodromal susceptibilities to psychiatric disorders.

1.5 PURPOSE OF DISSERTATION

This dissertation examines the physiological similarities and differences in two distinct midbrain dopamine neuron populations, VTA and SNc. Using simultaneous electrophysiological recordings, we first address basic physiological characteristics of neurons within each region, including baseline firing rate, pharmacological response, and identifying features of recorded units to classify neurons by putative neurotransmitter content. We then explore how neurons in VTA and SNc encode relevant events during two different conditioning paradigms. Pavlovian conditioning provides information into how neuronal activity in these regions represents cue-outcome relationships, while instrumental conditioning requires the formation of action-outcome associations. In understanding how each kind of associative learning is encoded on its own, it becomes easier to decipher how they interact to inform complex behaviors. Lastly, we determine whether the ability to learn an action-outcome association and the encoding of this association by VTA and SNc dopamine neurons is affected by a multi-generational dietary deficiency. This work will advance our understanding of dopamine's role in associative learning, and how it is impacted by environmental insult.

2.0 BASIC PHYSIOLOGICAL COMPARISON OF VTA AND SNC

2.1 INTRODUCTION

Midbrain dopamine neurons have been implicated in multiple aspects of behavior. Dopamine neurons originating in the SNc, which project primarily to basal ganglia regions, are involved in movement execution, action selection, and habit formation (Damier et al., 1999; Graybiel, 2008; Howard et al., 2017). The focus on these nigral functions originates with the observed motor symptoms in Parkinson's disease, which result from massive degeneration of the SNc dopamine neurons. Dopamine neurons in the VTA, which project to limbic regions such as the nucleus accumbens and amygdala, are involved in motivation and reward processing. Recently, however, the concept of heterogeneity within dopamine's role in reward has emerged as several studies are beginning to identify subtle differences within subpopulations of midbrain dopamine neurons (Matsumoto and Hikosaka, 2009; Cohen et al., 2012; Henny et al., 2012; Lammel et al., 2014; Marinelli and McCutcheon, 2014). Until recently, the classic identifier for dopamine neurons has been their strong phasic response to natural and unexpected reward. When an animal encounters a food or liquid reward, dopamine neurons respond with phasic burst firing patterns. When an animal receives less reward than expected, or has an aversive experience, like a mild foot shock, dopamine neurons inhibit their firing, creating a phasic inhibition. While this finding has been reproduced in multiple studies (Bromberg-Martin et al., 2010; Roesch et al.,

2007; Eshel et al., 2015; Margolis et al., 2006), there are also populations of VTA dopamine neurons (30-20%) which do not respond to rewarding stimuli (Marinelli & McCutcheon, 2014; Mirenowicz and Schultz, 1996; Joshua et al., 2008). Additionally, other dopamine neurons respond to aversive or unpleasant stimuli such as a tail pinch or a bolus of quinine water (Horvitz, 2000; Ungless et al., 2004; Matsumoto and Hikosaka, 2009; Bromberg-Martin et al., 2010b). Neurons which respond to aversive events are thought to receive GABAergic input from the lateral habenula or substantia nigra pars reticulata (SNcr) (Henny et al., 2012; Eshel et al., 2015; Menegas et al., 2015), and are present in both midbrain regions. Regardless of the recent observations of heterogeneity of VTA dopamine neurons, there remains the assumption that the "affective" processing of behavioral events may be unique to VTA as compared to SNc dopamine neurons.

Dopamine neurons in VTA and SNc have similar electrophysiological profiles although VTA cells have been reported to have a higher baseline firing rate (Chiodo et al 1984; Grenhoff et al 1988; Marinelli & White 2000; Martig & Mizomori, 2011). Identified dopaminergic neurons in anesthetized animals are known produce bi/triphasic long-duration waveforms at low basal firing rates, and fast phasic response to environmental stimuli (Miller, Sanghera, & German 1981; Grace & Bunney 1984). Phasic responses manifest as bursting activity, defined as 2 or more action potentials of descending peak amplitude produced with interspike intervals (ISI) of 80 ms. This mode of activity is thought to be involved in encoding reward-related learning (see above), and is modulated by interactions between glutamatergic and cholinergic afferents (Kitai et al 1999; Gronier & Rasmussen,1998). Dopamine neurons also exhibit slow and steady spiking activity, influenced by input from limbic and cortical afferents, which is referred to as baseline firing or 'tonic activity' (Chesselet, 1984; Grace, 1991; Howland et al., 2002). This firing pattern

creates low levels of extrasynaptic dopamine, modulating the responsivity of adjacent neurons in both the midbrains and target regions (Floresco et al., 2003).

Dopamine neurons also show a consistent silencing response to administrations of the dopamine agonist, apomorphine, which is reversed by application of haloperidol, a dopamine antagonist (Grace & Bunney 1984; Steinfels, Heym, & Jacobs 1981; Hyland et al., 2002). Many factors can affect these measurements, including electrode type/impedance, level of anesthetic, filtering settings during recording and animal restraints (Anstrom & Woodward, 2005; Hyland et al., 2002; Fa et al., 2003). Studies which established stereotypical dopamine features often compared VTA and SNc neurons, but none of the experiments monitored VTA and SNc activity simultaneously, allowing for variability in the factors listed above. In this dissertation, we strove to eliminate this concern, providing consistency in all recording and environmental parameters to provide confidence that the similarities and differences are accounted for by genuine functional and physiological characteristics.

In addition to dopamine neurons, the midbrain contains GABAergic and glutamatergic cell populations (Kalivas 1993; Nairs-Roberts et al., 2008). Dopamine neurons have traditionally been identified using waveform length, firing rate, and stereotyped response to the environment. These aspects were chosen after being observed consistently in histologically and pharmacologically identified neurons (Grace & Bunney, 1984; Ungless & Grace 2012). They were found to be consistent in anesthetized and awake animals (Hyland et al., 2002), but in both cases a subgroup of dopamine neurons which project to the prefrontal cortex (mesocortical) were found to exhibit faster basal firing rates (~9 Hz) and do not express D2 autoreceptors, making them unresponsive to apomorphine (Chiodo et al., 1984). Many of the studies which established these criteria employed the use of a single electrode, lowered until a neuron which fits the

previously described profile is encountered. While this approach ensures you are recording from a dopamine-containing neuron, it slows our progress in uncovering heterogeneity within this important, multipurpose cell population. Random sampling of neurons in both regions simultaneously eliminates differences due to electrode characteristics, recording parameters, behavioral environment, and internal animal state (i.e. hunger, energy, comfort, etc). Additionally, *in vitro* examination of morphological and electrophysiological properties of neurons found that these features did not reliably predict the neuron's cytochemical identity, especially in the VTA, which contains a large percentage of non-dopamine neurons (Margolis et al., 2006). Optogenetics has eliminated some of this uncertainty because it genetically identifies dopamine neurons and activates or inhibits them in a temporally and spatially specific manner. This approach is by no means perfect though (Lammel et al., 2015), as combining it with electrophysiology creates more complex, difficult, and technically-challenging experiments, which requires time for viral expression, which is not feasible in all animal models. To address this difficulty in this project, we have employed a statistically-based, optogenetically confirmed approach (Cohen et al., 2012; Eshel et al., 2015), augmented by waveform duration and baseline firing rate requirements. We also performed a pharmacological confirmation experiment in a small subset of subjects, the results of which aligned well with the identifications made by our statistical method. Using these rigorous criteria, we aim to definitively compare the basic physiology and pharmacological response of VTA and SNc dopamine neurons.

2.2 METHODS

Surgery and Electrophysiology: Animals were anesthetized with 2-4% inhaled isoflurane and placed in a stereotaxic frame. A midline incision was made, the skull cleaned, and measurements made to mark the location of craniotomies. We placed skull screws and a ground screw to increase the stability of the recording electrodes. After clearing two craniotomies, laboratory-made 8 channel electrode arrays (50μm diameter tungsten wire insulated with polyimide, California Fine Wire Company, Grover Beach, CA) were implanted in VTA (AP -5.3, ML 0.8, DV -7.7) and SNc (AP -5.2, ML 2.2, DV -7.4). Electrodes were slowly lowered to the proper dorsal-ventral coordinate as measured from dura. These electrodes were secured using loctite and dental cement. Toward the end of the surgery, the animal received a subcutaneous injection of ketoprofen (0.3mg/kg) to aid with immediate recovery and the incision site was treated with triple antibiotic ointment. Animals were closely monitored and given access to liquid painkiller for 2-3 days following surgery. During habituation and recordings, animals were connected via a field-effect transistor headstage (Omnetics Connector Corp, Minneapolis, MN) to a lightweight cable and a rotating motorized commutator to allow unrestricted movement during recording (Figure 2-1). Spikes were amplified at 1000x gain, digitized at 40 kHz, and single-unit data was band-pass filtered at 300Hz. Single units were isolated in Offline Sorter using a combination of manual and semi-automatic sorting techniques until each unit is well isolated in state space; minimum acceptable signal to noise ratio approximately 2:1. Neurons are not screened for specific physiological characteristics or response properties prior to recording.

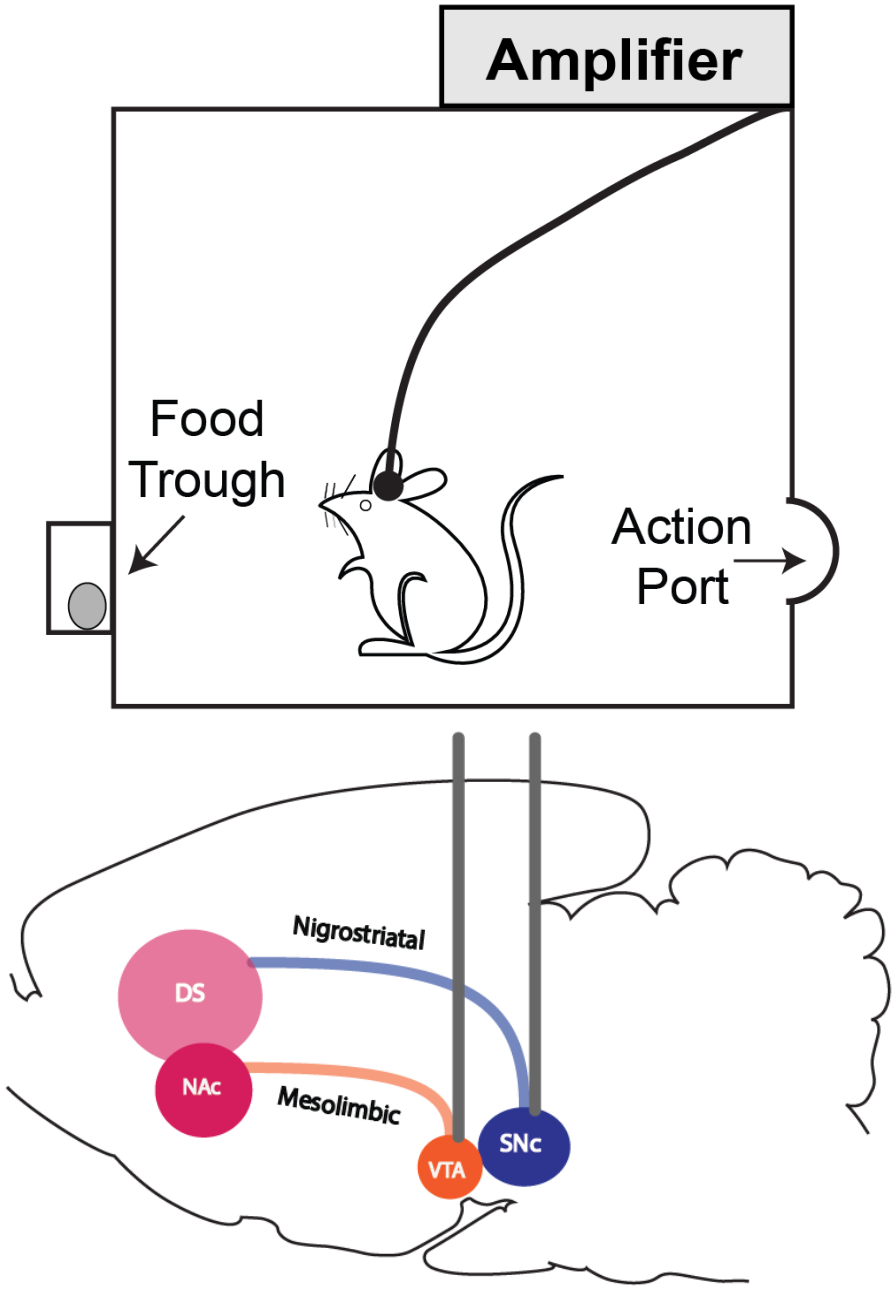


Figure 2-1 Recording apparatus and bilateral implantation schematic
Top: schematic depicts the setup of the implanted animal, chamber, and recording equipment to demonstrate how the subject was connected to the recording system and data was collected. Below: bilateral implantation of mircoelectrode arrays in VTA SN employed in all experiments described in the following chapters.

Data Analysis: NeuroExplorer (NEX Technologies, Madison, AL) was used for preliminary analysis such as crosscorrelograms and perievent rasters. Crosscorrelograms around reward delivery were used to identify neurons captured by multiple electrodes and eliminate them from future analysis. For neuronal activity analyses, firing rates were calculated in 25 ms bins. Baseline rate for individual units was determined using the average firing rate during the middle 3 seconds of the intertrial interval (ITI). Independent samples t-tests assuming unequal variances were used to quantify region-specific differences in single sessions. Isolated single unit data were analyzed with custom written Matlab functions (MathWorks, Nattick, MA). We conservatively classified neurons recorded in consecutive recording sessions as different units, despite any indications that the same units were recorded serially.

Dopamine Classification: We classified units into two types using a hierarchical clustering approach, which has been optically verified by others and described in detail elsewhere (Cohen et al., 2012; Eshel et al., 2015). Briefly, peristimulus time histograms were generated for each unit using 50ms bins spanning the second before and after the event of interest to obtain spike counts for each unit. This activity was then assessed using receiver-operating characteristic (ROC) curves of spike count distributions in 100 ms bins surrounding reward delivery relative to baseline activity (3 seconds from the middle of the 10sec ITI). Principal component (PC) analysis was conducted using the area under the ROC curve. The neurons were mapped in the three-dimensional space comprising the first three PCs, and a Gaussian mixture model was fitted with expectation maximization algorithm (EM) to cluster the neurons into Type 1 and Type 2. Additionally, we conducted a dopamine agonist drug study on a subset of subjects following the final recording session. After a 30-minute baseline recording, we injected animals (n=2) with 0.75mg/kg apomorphine i.p. and recorded for an additional 30

minutes. Responsive units were defined through comparison of interspike interval (ISI) distributions in a nonparametric Kolmogorov-Smirnov test ($p<<0.05$). The direction of modulation after apomorphine (inhibited or excited) was determined by whether the pre- or post-injection distribution had a larger cumulative distribution function.

Histology: After experiments were complete, rats were anesthetized with 5% isoflurane and injected intraperitoneal with 3ml 8% chloral hydrate. The animal was then perfused with 0.9% saline, followed by 10% buffered formalin. Brains were stored in this formalin and transferred to 30% sucrose for at least 24 hours before brains were coronally sliced. Brain were mounted on microscope slides and stained with cresyl violet. Electrode placement in the VTA/SNc were confirmed for all animals who provided electrophysiological data (Figure 2-2).

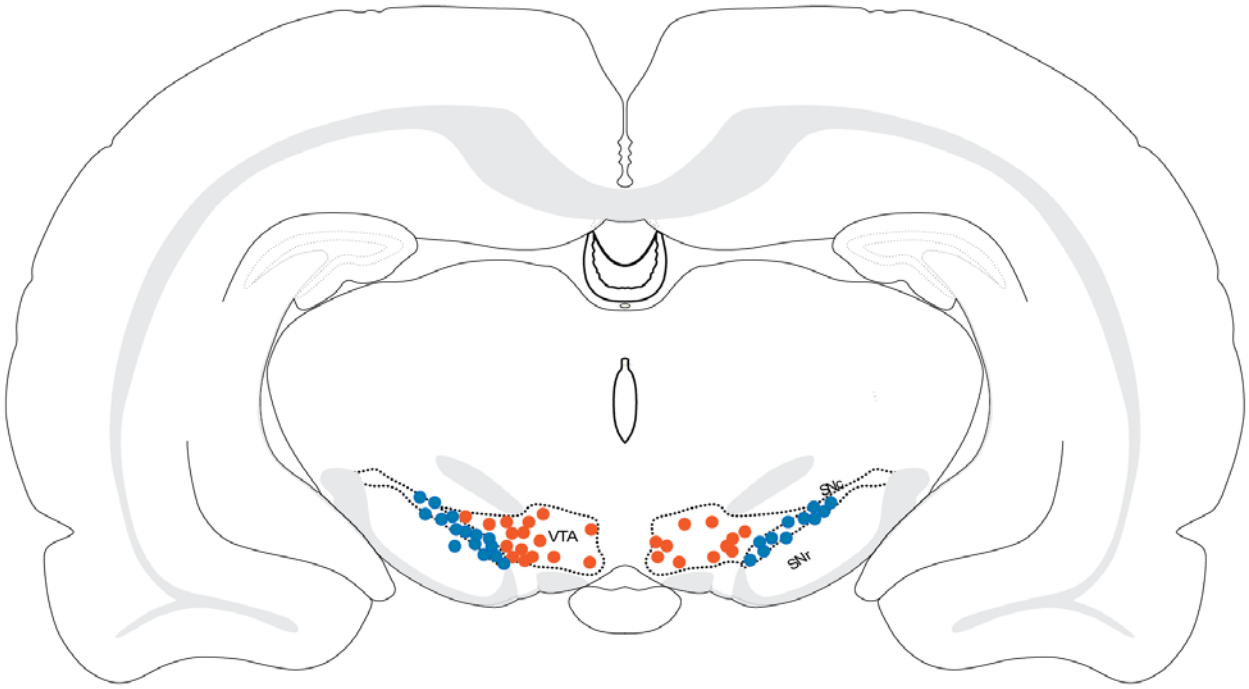


Figure 2-2 Electrode placement confirmation for all experiments
Histological confirmation of electrode placements for all experiments. Placements of VTA recordings are shown in orange, SNc electrodes in blue. Electrode confirmations for experiments employing the FR1 instrumental conditioning paradigm are on the left, and placements from the Pavlovian recording experiment are shown on the right.

2.3 RESULTS

We measured baseline firing rate during the middle 3 seconds of the inter-trial interval of both tasks to investigate whether neurons in the SNc have reduced tonic activity in comparison to VTA as previously reported. Recorded baseline firing rates range from 0.2Hz – 77Hz. Within an individual session, SNc and VTA do not consistently exhibit significant differences in baseline firing. For these comparisons, neurons with a baseline firing rate of >30 Hz were excluded from analysis as such extreme outliers interfered with accurate statistical results. If consistent differences were found within session, it would affect any between-region raw firing rates comparisons and the interpretation of these data. Across all recordings, we encountered fast-firing neurons (putative FSIs) more frequently in VTA (n=32) than in SNc (n=9), as expected from the cell type distribution of each region (Swanson, 1982; Carr & Sesack, 2000b; Yamaguchi et al., 2007; Sesack & Grace, 2010; Tritsch et al., 2014). When we compare baseline firing rates of all neurons recorded (Figure 2-3), we found that SNc has a significant lower baseline (4.72 Hz) than VTA (5.72Hz; independent t-test, $p = 0.00026$). When we compare only Type 1 putative dopamine neurons, the significant difference remains but average firing rates are more similar (SNc = 4.0484Hz, VTA = 4.4528; independent t-test, $p = 0.0011$). This indicates that the overall result is not skewed by the greater number of FSIs found in the VTA as compared to SNc. This result replicates what others have found in the initial physiological characterization of these regions in anesthetized and paralyzed animals (Chiodo et al 1984; Grenhoff et al 1988; Marinelli & White 2000).

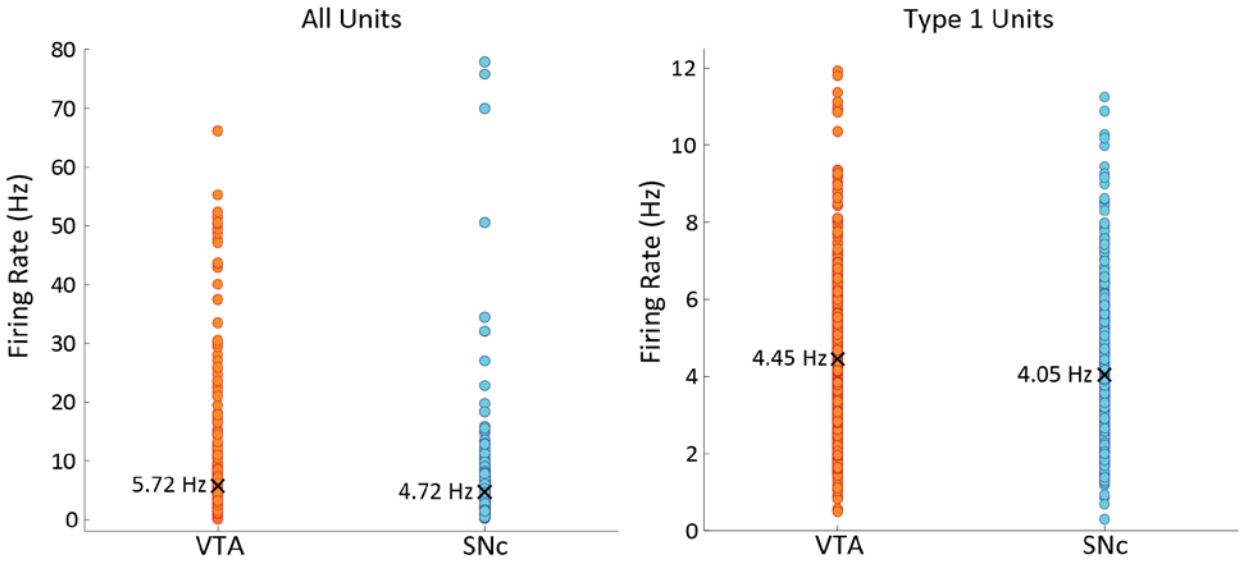


Figure 2-3 Baseline firing rate

Baseline firing rate for both regions using data from the baseline period of both tasks (see Chapter 2). Each data point represents the average baseline firing rate for one recorded unit and the black X marks the population mean for the specified region. Comparison of baseline firing rate (average firing rate during the middle 3 seconds of the ITI) within sessions of both tasks did not reveal any consistent differences between regions. Collectively, comparison of all units recorded did reveal a significantly higher baseline activity in VTA ($p=0.00026$; VTA n=961, SNc n=968). When we limit the comparison to Type 1 putative dopamine neurons, the difference remains ($p=0.0011$; VTA n= 486, SNc n= 498), indicating that the significance is not driven by the higher occurrence of fast-spiking interneurons in the VTA.

After establishing differences during dopamine's tonic firing rate between VTA and SNc, we compared phasic response profiles to behavioral events in VTA and SNc. To eliminate confounding variables, we examined units recorded simultaneously within the same animal (n=20) and their responses to a conditioned cue (CS+) and sugar pellet reward. These units produce a heterogeneous array of responses in both regions (Figure 2-4). Both regions contain units which display the classic sharp phasic response of ~200ms delay to response and 50-100ms duration of response, thought to be typical of dopamine neurons (Schultz, 1997). Other units respond with phasic inhibition during the appetitive stimuli. The SNc contained few neurons which displayed this response, but many SNc units show an inhibition of firing immediately following a phasic activation. A sustained response (150-300 ms duration) profile was present in

both regions during both cue and reward. Both regions also contained neurons which did not respond to any stimuli for either task. This non-responsive population often included fast-spiking neurons, though a small subset of these did show a mild inhibitory response with a slow return to baseline. We will describe these responses in a behavioral context in Chapter 2 but we want to emphasize the heterogeneity present among randomly sampled neurons within each region, which is made possible by not requiring a specific quality or response before beginning recording.

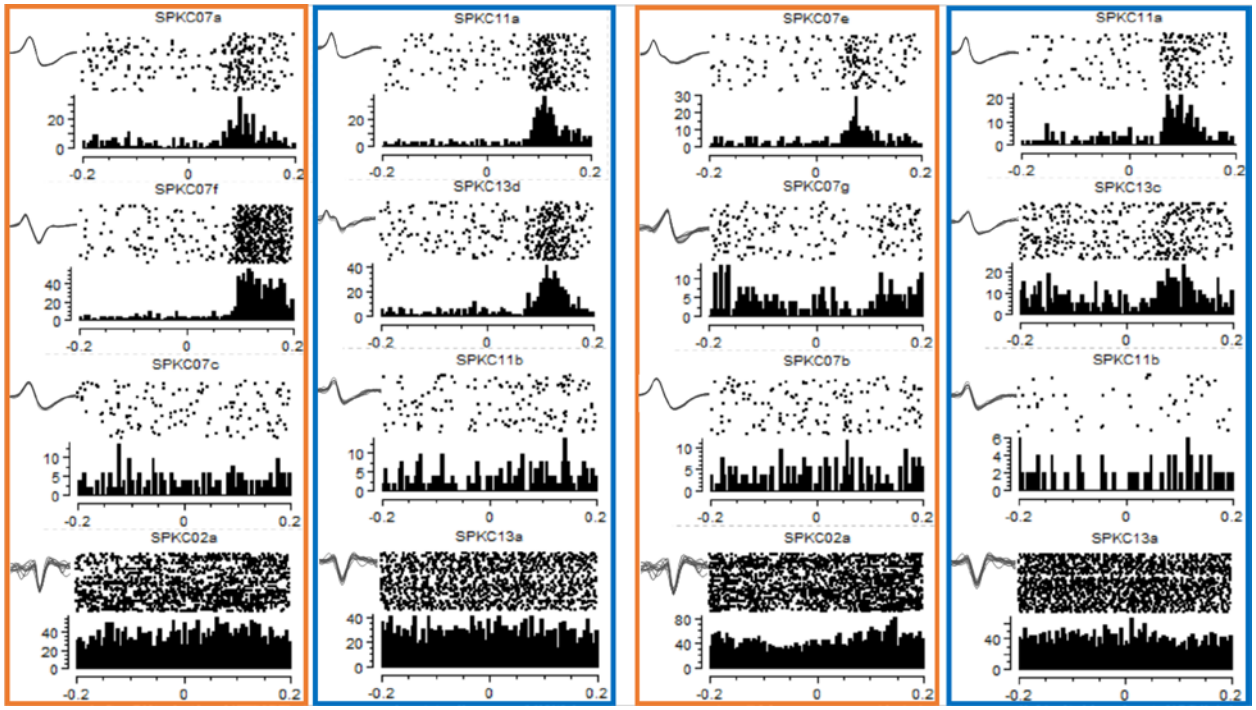


Figure 2-4 Heterogeneity in firing patterns of VTA and SNc neurons
Each plot includes a raster and histogram, which displays activity from simultaneously recorded neurons within the same animal during Pavlovian cue (two columns on the left), and reward delivery (two columns on the right). VTA units are outlined by the orange box, and SNc neurons are outlined in the blue boxes. These raster plots demonstrate that units in both VTA and SNc respond to the same stimuli with a heterogeneous array of activity patterns, including sharp phasic activation or inhibition, sustained phasic activation, or no response. Additionally, both regions contain fast-spiking interneurons as pictured in the bottom row of plots. Each plot is also accompanied by the average waveform for that unit in the upper left corner.

In keeping with the standard of the field, we used a unit's response to reward delivery as a parameter in classifying it as type 1 putative dopamine neurons or type 2 putative non-dopamine (Schultz 1998, Eshel et al., 2014; Cohen et al., 2012). Using the period one second before and after the reward delivery, we constructed a ROC curve for each unit and calculated the area under the ROC curve. The principal components of the area under the ROC curve were graphed for each unit in 3-dimensional principal component state space (Figure 2-5). Graphing the data this way allows us to visualize how the data relate to each other and any clusters present in the data. Our data divided into two distinct clusters regardless of region. The units classified into the type 1 cluster resembled dopamine identified by optogenetics in waveform shape, baseline firing rate, and behavioral response profile (Eshel et al., 2014).

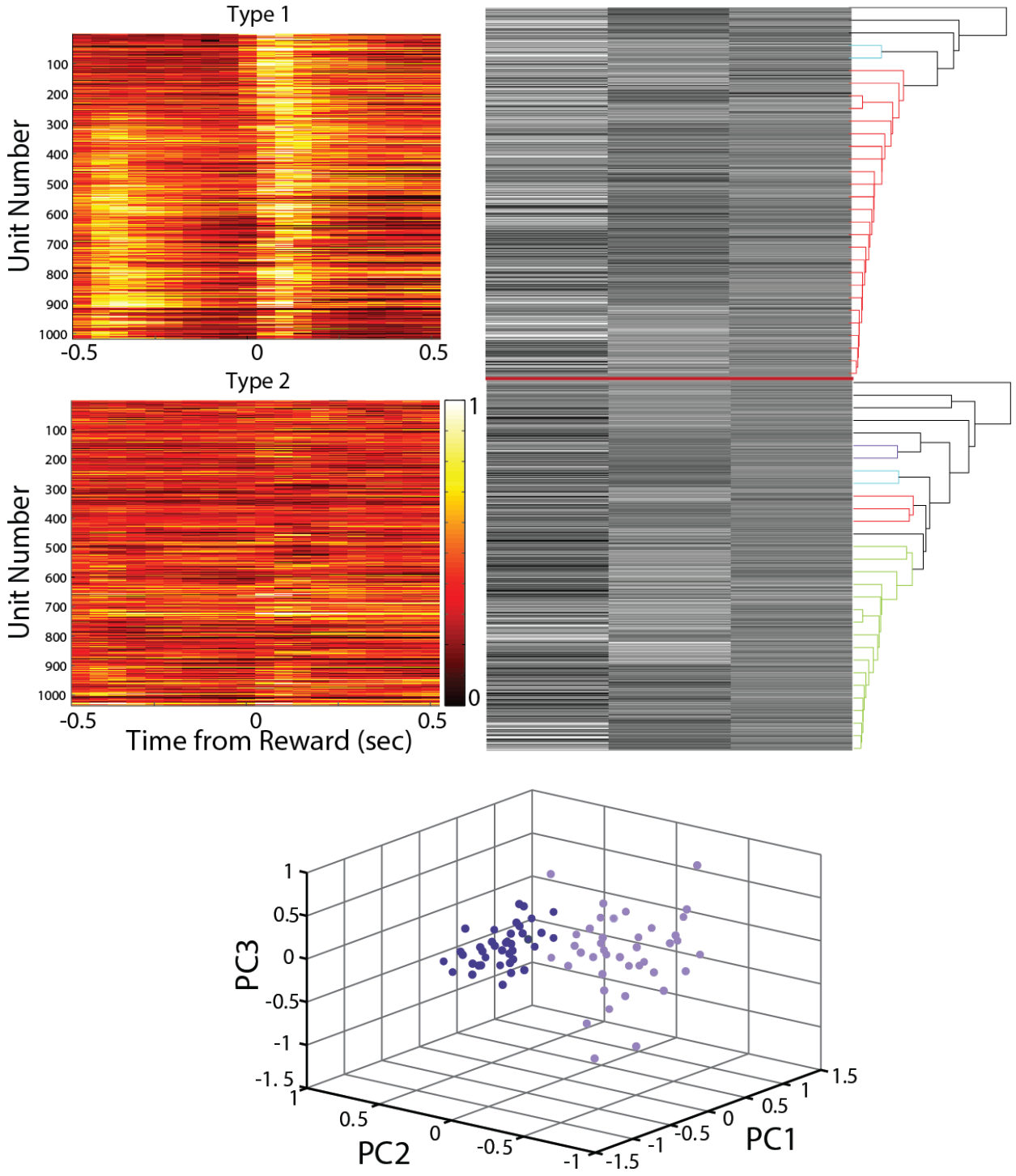


Figure 2-5 Classification of putative dopamine neurons
Two heat plots on the left display auROC for all neurons recorded, type 1 putative dopamine neurons on the top graph, type2 on the bottom. Hotter colors (white/yellow) indicates larger area under the curve. Each row represents a single unit. Principal components (PC) for those same units are depicted to the right, with hierarchical clustering dendrogram on the right of the PC gray scale. Below is an example of clustering between type 1 (dark purple) and type 2 (light purple) units in the state space of the first three principle components.

To confirm we were recording from dopaminergic neurons, we performed a drug study following the final recording session using the D2 agonist apomorphine. After an animal completed the final behavioral recording session, we recorded a 30-minute baseline period to compare with post drug period. Animals spent the majority of the baseline recording time exploring the chamber or at rest. Recorded units responded in possible three ways after the injection (Figure 2-6). Putative FSIs (type 2 units with baseline >30Hz) increase their firing rate, as measured by a decrease in average interspike interval (ISI). Putative dopamine neurons (type 1 units) either decrease their activity to some degree but not entirely, or became completely silent. Out of the 28 units recorded, 19 units responded with reduced spiking activity and 6 responded with increased spiking activity ($p < 1.7 \times 10^{-11}$). Both directions of change in firing rate after apomorphine were observed in both regions. Units began to recover their original pattern of activity 25 minutes post-injection. Units with reduced spiking activity after injection exhibited complex bi/triphasic waveforms similar to those described in other studies.

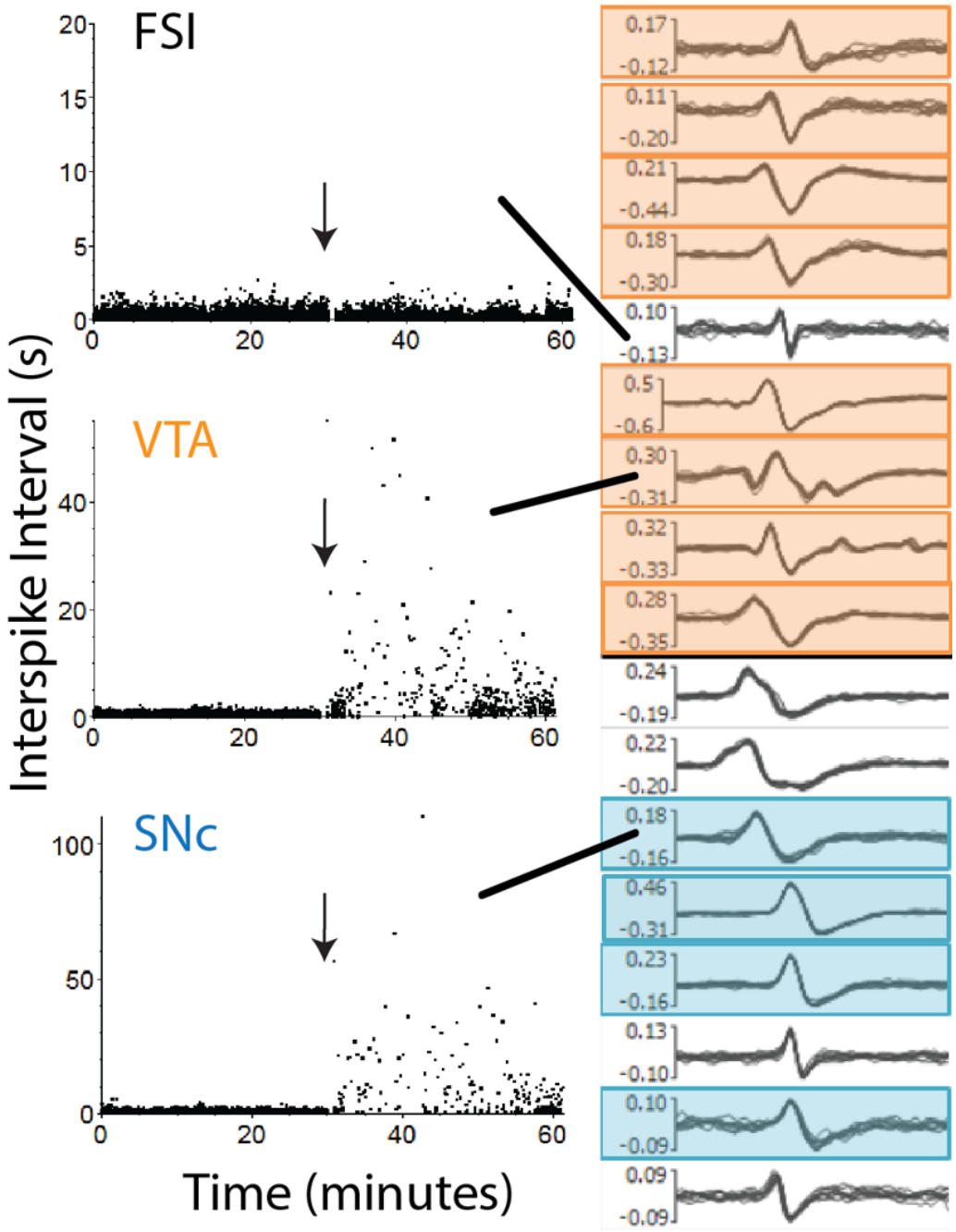


Figure 2-6 Apomorphine identification of dopamine neurons in VTA and SNc
Plots on the left depict interspike intervals over the entire session (30 minutes baseline, 30 mins post injection) for 3 example units, with the injection marked by the arrow. Top: VTA putative FSI which increases spike rate after 0.75mg/kg apomorphine i.p. injection as exhibited by shorter ISI. Middle: VTA putative DA unit significantly increases its interspike interval after injection. Bottom: Example of an apomorphine responsive SN unit. ArrThe waveform traces of VTA neurons that are responsive to apomorphine are outlined in orange and responsive SN unit waveforms are outlined in blue. The corresponding waveform for the example units are connected to their respective ISI graphs by a black line.

2.4 DISCUSSION

We were able to perform simultaneous recordings of multiple units in the VTA and SNc of behaving animals. By directly comparing regions within a single animal, we found that neurons in both VTA and SNc show profound heterogeneity in neural responses to behaviorally relevant events. We further demonstrated that neurons in both regions are sensitive to the dopamine agonist apomorphine and that dopamine classification techniques can be applied to both regions effectively. The only disparity we identified in the physiological characteristics of VTA and SNc dopamine neurons was slight difference in tonic basal firing rates of these neurons.

2.4.1 Baseline firing rate is lower in the SNc

Measuring baseline firing rate is critical for understanding the 'gain' relationship between tonic and phasic activity of dopamine neurons. Instead of a greater phasic response to enhance the signal sent to other regions, it is possible that dopaminergic regions adjust the level of background tonic activity or 'noise' to make the phasic response more prominent (Floresco et al. 2003). We found baseline firing rate to be slightly higher in VTA as compared to SNc. This finding persisted when we limited our comparison to only include Type 1 putative dopamine neurons. This is consistent with other studies in anesthetized and awake rats (Martig & Mizumori 2011; Chiodo et al., 1984; Grenhoff et al., 1988; Zhang et al., 2008, Marinelli & White 2000). This has been shown to be the same across subregions within the VTA and SNc (McCutcheon & Marinelli, 2014). This difference is not necessarily indicative of basis physiological differences in VTA and SN dopamine neurons. Increases in tonic population activity lead to more dopamine

release (Floresco et al., 2003), but this does not necessarily translate into higher levels of extracellular dopamine. Considering this, our finding of significantly lower baseline firing rate in SNc do not necessarily translate into significant divergence in levels of dopamine release. In fact, lower tonic firing rate does not linearly relate to the amount of extracellular dopamine, but instead correlates with the number of units that are tonically active (Floresco et al 2003; Grace 1991; Goto et al., 2007). Homeostatic regulation of spontaneous firing rate is heavily influenced by levels of somatic CA++ (Kim et al., 2012; Pan & Ryan, 2012), which could be the underlying factor that differs between the VTA and SNc (Nemoto et al., 1999; Alfahel-Kakunda & Silverman, 1997). Alternatively, if we consider the influence of adjacent neurons within the midbrain, the larger population of GABAergic fast-spiking neurons in VTA could be responsible (Swanson, 1982; Carr & Sesack, 2000b; Sesack & Grace, 2010; Tritsch et al., 2014), but GABAergic inhibition mainly affects bursting activity (Lobb et al., 2011a, b; Cohen et al., 2012). If an increase in GABAergic tone brought about a disparity in firing rate, we would expect the lower basal firing rate in the VTA. Recruiting previously silent neurons changes the firing rate distribution but does not change the overall average firing rate of the population (Marinelli & McCutcheon 2014), so our result does not suggest a difference between number of spontaneously active neurons as found previously in anesthetized animals (Dai & Tepper 1998, West & Grace 2000). Given that analysis of dopamine neuron activity in awake-behaving animals frequently focuses on changes relative to baseline (Marinelli & McCutcheon 2014, Matsomoto & Hikosaka 2009), not examination of baseline firing rate itself, our simultaneous evaluation of tonic activity provides valuable insight into the functional physiology of midbrain neurons.

2.4.2 VTA and SNc neurons exhibit a similar array of responses after apomorphine administration

We administered apomorphine systemically via intraperitoneal injection, which is known to have similar effects on dopaminergic midbrain neurons as localized infusions (Chiodo, 1988). Both regions contain putative dopamine units that were inhibited by apomorphine. We also observed putative FSIs which decreased their average ISI after i.p. apomorphine. These results are similar to those recorded from nigrostriatal and mesolimbic neurons in anesthetized and awake animals (Freeman & Bunney, 1987; Chiodo, 1988; Clark & Chiodo, 1988). A small subpopulation of midbrain putative dopamine neurons did not respond to apomorphine. While this was surprising, these neurons may be part of a subpopulation that does not express somatic/dendritic autoreceptors (Chiodo et al., 1984), most commonly found on dopamine neurons projecting to prefrontal and cingulate cortices. This population is found in the most medial portion of the VTA, and is generally difficult to access through the sinus vasculature. This justifies not relying solely on apomorphine response to identify dopamine neurons, but instead using as a confirmation of our statistical categorization methodology that has been supported by data from optogenetically 'tagging' dopamine cells. (Cohen et al., 2012; Eshel et al., 2015).

2.4.3 Heterogeneity of firing patterns in both regions

It is well accepted that dopamine neurons have heterogeneous patterns of activity, anatomical projections, and environmental triggers (Roeper, 2013; Brown et al., 2009; Kiyatkin & Rebec, 1998; Watabe-Uchida et al., 2012). It has also been observed that many midbrain

dopamine neurons often exhibit similar overall firing properties (Schultz, 1986; Clark and Chiodo, 1988; Gariano et al., 1989; Robinson et al., 2004). By simultaneously recording in these two regions, we observed a heterogeneous array of neural responses during the same stimulus. This array of possible responses was similar across regions, supporting the theory of overall commonality. In our observations, sustained phasic responses to motivational stimuli were observed more often in the SNc, while sharp phasic activity was more frequent in the VTA, although both patterns were observed in either region. Our future investigations may benefit from a shift toward viewing these regions as a continuum with specialized subpopulations rather than separate, unrelated circuits. Parallel pathways are common in the brain, and allow for mechanisms of compensation and coordination between and across regions (Clark, Hollon, & Phillips, 2012). Basic reward seeking behavior is essential for survival and has a strongly preserved evolutionary base, so it is likely that such an important purpose would be served by multiple systems. Indeed, VTA and SNc neurons are often analyzed together as they do not exhibit significant differences in the measure the study uses (Roesch et al 2007; Martinelli & McCutcheon 2014). This pattern of shared features suggests that both mesolimbic and nigrostriatal circuits should be investigated if we are to fully understand the neural mechanisms behind reward-mediated learning and reward-seeking behavior.

In the subsequent chapter, we will further explore these heterogeneous responses by considering both phasic population responses and neuron-pair coordinated responses during behavioral engagement in associative learning.

3.0 COMPARISON OF VTA & SN NEURAL ACTIVITY IN TWO LEARNING PARADIGMS

3.1 INTRODUCTION

Multiple electrophysiological, optogenetic, and pharmacological studies have investigated and confirmed the role of dopamine neurons in the ventral tegmental area (VTA) and its projections to the ventral striatum (i.e. the mesolimbic system) in affective and emotional processing (Wise, 2004; Pascoli et al., 2015; Salamone et al., 2015; Zhang et al., 2015). For example, animals will consistently work to self-stimulate dopamine in the VTA or its projection terminals with dopaminergic drugs, electric current, or optogenetic excitation (Fibiger et al., 1987; Wise, 1996; Pignatelli & Bonci, 2015). Through these data, many reward-related functions have been limited to this mesolimbic circuit, but there is evidence that other regions might contribute significantly to reward-mediated behavior. In fact, dopamine neurons in another midbrain region, the substantia nigra pars compacta (SNc), are also involved in responses to rewarding events and stimuli (Reynolds et al., 2001; Wise, 2009; Rossi et al., 2013; Ilango et al., 2014; Horvitz, 2000).

Despite functional and anatomical overlaps, the influence of dopamine neurons in the SNc on reward response is less well studied. The vast majority of research involving the SNc concerns its role in motor function, action selection, and the effects of SNc degeneration in

Parkinson's disease (Damier et al., 1999; Beal, 2001; Gurney et al., 2001; Graybiel, 2008). Though the SNc clearly plays a role in movement, its phasic activity is directly related to bodily movement (Horvitz, 2000), so its function cannot be entirely relegated to motor systems. Recently, the canonical dichotomy of these two projection systems has been called into question, as optogenetic and viral tracing studies reveal that projections from these areas are more integrated and reiterative than previously suggested (Beier et al., 2015; Lerner et al., 2015). Behaviorally, self-stimulation studies show that animals will perform instrumental behaviors to receive electrical or optogenetic stimulation to dopamine neurons in the VTA or SNc (Reynolds, Hyland, & Wickens, 2001; Rossi et al., 2013; Ilango et al., 2014). Additionally, in a conditioned place preference paradigm, animals found optogenetic inhibition to either region aversive (Danjo et al., 2014; Ilango et al., 2014). Pharmacologically, infusions of D-1 receptor antagonist into VTA or SNc reduced the amount of effort animals are willing to put forth to receive previously rewarding drug, indicating that the infusions of drug are no longer as valued (Fouriezos et al., 1978; Franklin, 1978; Quinlan et al, 2004; Wise, 2004). Together these experiments suggest that some of the functions traditionally assigned to VTA and mesolimbic circuitry might also generalize to the SNc and nigrostriatal pathway.

The principal aim of the work in this dissertation was to compare the role of VTA and SNc neurons in reward-related learning. The influential work which has implicated the phasic activity of dopamine neurons in motivated behavior has focused on reward prediction error (RPE) signaling. Data supporting this concept show that dopamine neurons respond strongly to unexpected reward during Pavlovian conditioning. During this form of learning, organisms associate an external cue (e.g. tone or light), often referred to as a conditioned stimulus (CS), to an salient outcome (e.g. food). Recording from dopamine neurons has revealed that initially

dopamine neurons respond to this novel food reward with strong phasic activation. But as a rewarding outcome becomes expected during conditioning, the modulation of neuronal activity is transferred to reward-predicting cue. Behaviorally, this change in activity pattern manifests as a decrease in latency to retrieve reward and an increase in approach behavior and attending to the cue (Pessiglione et al., 2006; Flagel et al., 2010; Steinberg et al., 2013). If the reward received is larger than expected, the dopamine neurons will represent that with a proportionally larger response when that reward is delivered (Schultz, 1998; Tremblay and Hollerman, 1998; Tobler et al., 2005). If the reward is omitted or smaller than expected, the dopamine neuronal activity reflects this with a brief pause in firing (Schultz, 1998; Bayer and Glimcher, 2004; Roesch et al., 2007; Matsumoto and Hikosaka, 2009). Considering this activity pattern, RPE is thought to function as a learning signal, representing relevant changes in one's environment. By incorporating the information in these signals into its perception of its surroundings, an animal can make better decisions about where to seek food or when to begin foraging. This hallmark of dopamine neuronal activity usually describes the response to a discrete, reliable reward-predicting CS to which the animal can attend. Importantly, this concept is commonly associated with VTA dopamine neurons and VTA-related models of reward processing (Wise, 2004) whereas it was originally established with recordings from dopamine neurons in the monkey SNc (Schultz, 1998). This divergence arises from anatomical differences between species, as the limbic functions ascribed to the VTA of rats is carried out by the SNc in primates (Düzel et al., 2009). To date, direct comparison of the phasic activity of SNc and VTA neurons during Pavlovian conditioning is lacking, and in fact, SNc neurons are often assigned a role in action execution and habit formation, but not Pavlovian conditioning (Wise, 2009; Everitt & Robbins, 2005). One of the aims of studies described in this chapter was to simultaneously record from

VTA and SNs neurons during Pavlovian conditioning, to determine the pattern of response of dopamine neurons in these two regions during formation of cue outcome associations.

Another fundamental form of associative learning is Instrumental conditioning. Dopamine neurotransmission has been strongly implicated in this form of learning (Ljungberg, Apicella & Schultz, 1992; Everitt & Robbins, 2005; Roesch et al 2007; Rossi et al., 2013; Totah et al, 2013; Hamid et al., 2016). This form of learning is operationally different from Pavlovian conditioning in that the organism must execute an action after it is exposed the environmental cue in order to achieve an outcome. Additionally, Pavlovian conditioning requires a discrete cue while instrumental conditioning does not, though the context of presence of a manipulandum can serve as a cue to evoke an action from the animal. Thus, both forms of learning are "associative" in that they involve a cue or stimulus to predict an outcome but in one (Pavlovian) the outcome appears with no effort from the organism whereas in the other (instrumental) an action must precede the outcome. Electrophysiological recordings during instrumental behavior provide information about how the brain encodes the action-outcome relationship, informing possible mechanisms behind our choices to execute actions and how we know when those actions will result in the desired outcome. In order to make optimal decisions, the brain must discriminate between outcomes that require an action from those predicted by an environmental cue like those used in Pavlovian conditioning. In general, instrumental conditioning, unlike Pavlovian, is thought to be a more flexible form of learning because it allows for animals to adjust their behavior in accordance with the demands of their environment (Dickinson, 2012). Pharmacological studies first established a role for dopamine in instrumental conditioning through the observation that dopamine receptor antagonists attenuated instrumental learning and maintenance in a dose-dependent manner (Wise & Schwartz, 1981). Many of the recent studies

investigating the role of dopamine in instrumental conditioning focus on the dissociation between the initial learning and the habit formation that occurs over training (Ahn & Phillips, 2007; Smith & Graybiel, 2013). Often studies examine and manipulate dopamine in the ventral striatum to affect goal-directed behavior and are interested in the extent to which ventral striatum dopamine encodes effort-related processes (Salamone & Correa, 2012). Dopamine function is inferred by investigating how the ventral and dorsal striatum, which receive afferents from the midbrain, respond during motivational behavior (Parker et al., 2016). While we know that stimulation of the VTA or SNc are sufficient to drive behavior (Rossi et al., 2013; Ilango et al., 2014), we know little about how events during instrumental learning are represented in both regions. Thus, a second aim of the studies described in this chapter was to simultaneously record from VTA and SNs neurons during instrumental conditioning.

The experimental design involved comparing event related phasic responses of VTA and SNc neurons during: 1) an instrumental conditioning task, in which after a cue, animals had to execute a nose poke to earn a sugar pellet reward, and 2) a Pavlovian conditioning task, which paired a previously meaningless cue with a sugar pellet reward. Chronic microelectrode arrays were bilaterally implanted in the VTA and SNc of each animal, enabling us to directly compare activity from both regions across acquisition and maintenance of these two reward-mediated behavioral paradigms. Our single unit recording data reveal striking similarity between VTA and SNc on an overall population level, as well as in the subpopulation of putative dopamine neurons. Differences between these two regions, however, emerged when we computed the changes in functional connectivity between simultaneously recorded neurons.

3.2 METHODS

Instrumental Behavior (FR1): Animals will first be habituated for two days to an operant chamber (Coulbourn Instruments, Allentown, PA) equipped with a food trough and reward magazine opposite a nose-poke port with a cue light and infrared photo-detector unit, and a tone-generating speaker. During experiments, animals will be connected via headstage to a lightweight cable and a rotating commutator to allow unrestricted movement during recording. Spikes are digitized at 40 kHz and high pass filtered at 100 Hz. Similar to fixed ratio one (FR1) tasks used in previous work (Sturman and Moghaddam, 2012; Kim et al, 2016), rats learned to nose poke into the lit port to earn a single sugar pellet reward (45 mg sugar pellet, Bio-Serv, Frenchtown, NJ). Following the nose poke (action), the cue light was extinguished and the reward was delivered after a 1 sec delay (Figure 3-1). Following reward collection, a 10-second inter-trial interval (ITI) occurred before the next trial begins. For each trial, the cue light remained illuminated until the rat responded. Each session lasted 45 min or 100 trials. Proficiency in the task was determined by completion of 100 trials in 30 minutes, latency to perform the nose poke, and latency to retrieve reward.

Pavlovian Behavior: Recording sessions during this behavior were conducted in an operant chamber (Coulbourn Instruments, Allentown, PA) containing a reward delivery trough equipped with an infrared photo-detector unit, a cue light and a tone-generating speaker. In this Pavlovian task (Figure 3-2), either the tone or the light cue (CS+) was presented on the wall opposite of the food trough for ten seconds. After a 500 ms delay following the termination of the CS+, a sugar pellet reward was delivered. The identity of the CS+ (light or tone) was counterbalanced across subjects. The other cue (CS-) yielded no outcome and the trial proceeded immediately into the variable (9-12s) intertrial interval (ITI). After a reward was delivered (45

mg sugar pellet, Bio-Serv, Frenchtown, NJ), the ITI began immediately after reward retrieval by the animal or a 5 second-delay, whichever occurs first. Each conditioning session consisted of 100 trials of each type (rewarded and unrewarded; 200 trials total). This conditioning took place across 10 days, followed by a probe day in which the probability of the learned cues was altered. In this probe session, the previously rewarded cue (CS+) was rewarded on 75% of trials, and the unrewarded cue (CS-) was unexpectedly rewarded on 25% of trials. This allowed us to observe negative and positive prediction error activity in both regions. Learning in this task was assessed by observations of approach behavior, quantified by entrances into the food trough during the CS+ and CS- and latency to retrieve reward (Wan & Peoples, 2008; Kim, Matthews, Moghaddam, 2010).

Electrophysiology/Data Analysis: Data were recorded, sorted, and classified as putative DA or non-DA as described in Chapter 1. Unit firing rates for both behaviors were analyzed in 25 ms bins, smoothed with a Gaussian kernel and aligned with behavioral events. Units with average baseline firing rates >20 Hz were removed from analysis (VTA: n-36, SNc: n = 10) as outliers to avoid spurious statistical differences. Statistical tests were done using activity in a 0.5 sec window around the event of interest (0.25 before and after). Differences between regions within a session and changes in perievent neural activity across sessions was measured with two-way ANOVAs, with Greenhouse-Geisser correction applied when appropriate. When comparing population responses from the same sample of neurons across multiple behavioral events, we applied the Bonferroni correction for multiple comparisons (three events: $p = 0.015$). Responsive neurons were identified by calculating a trial by trial delta distribution for each unit, and using a one-sided t-test to evaluate whether the mean of that distribution was significantly different from zero ($p=0.05$). If it was not significantly difference from zero, the unit was labeled non-

responsive. If the change was significant, the unit was classified based on the direction of the change: inhibited if the direction of change was negative, activated if positive. These criteria were applied to both regions.

Spike Count Correlations: We simultaneously recorded 215, 171, 197, 290, 199, 195 neuron pairs in the VTA (pooled across rats) and 291, 359, 248, 55, 108, 179 pairs in the SNc in instrumental behavior sessions 1- 6, respectively. During the 10 training sessions of the Pavlovian experiment, we simultaneously recorded 525, 533, 569, 495, 492, 460, 156, 259, 177, 141 neuron pairs in the VTA (pooled across rats) and 106, 333, 457, 537, 807, 641, 624, 467, 339, 320 pairs in the SNc. The correlation between each pair of unit's stimulus-evoked spiking activity was analyzed. For these analyses, we did not group unit pairs based on putative neurotransmitter content in order to preserve sufficient sample sizes for reliable analysis. All spike train analysis utilized custom scripts executed in the Matlab environment (MathWorks, Natick, MA). We correlated the trial-by-trial fluctuations in discharge rate between pairs of simultaneously recorded neurons. A Pearson's correlation of spike counts for each pair of units was calculated in the 250 ms following the event of interest. Spike count correlations are sensitive to outliers, so we excluded any trial in which either unit firing rate was >3 SDs away from its mean baseline firing rate (Ruff & Cohen 2014; Kohn & Smith, 2005). Correlations between neuron pairs across trials was calculated as rho and deemed significant at the $p=0.015$ level in the FR1 task and $p=0.0125$ for the Pavlovian Task, correcting for the number of task events we compared within a session.

3.3 RESULTS

3.3.1 Behavioral Data

In the instrumental behavior experiment, rats were trained to perform actions after cue onset for reward according to a fixed-ratio 1 reinforcement schedule (FR1; Figure 3-1). Across 6 conditioning sessions, the number of trials completed increased (session: $F_{5,54} = 8.710$, $p < .0001$), and animals performed nose pokes more quickly following cue presentation (session: $F_{5,54} = 10.036$, $p < .0001$). Latency to retrieve reward after the nose poke also decreased significantly with conditioning (session: $F_{5,54} = 2.707$, $p = 0.03$). These changes across sessions indicate that animals learned the action-outcome relationship through multiple days of conditioning and reached similar levels of peak performance.

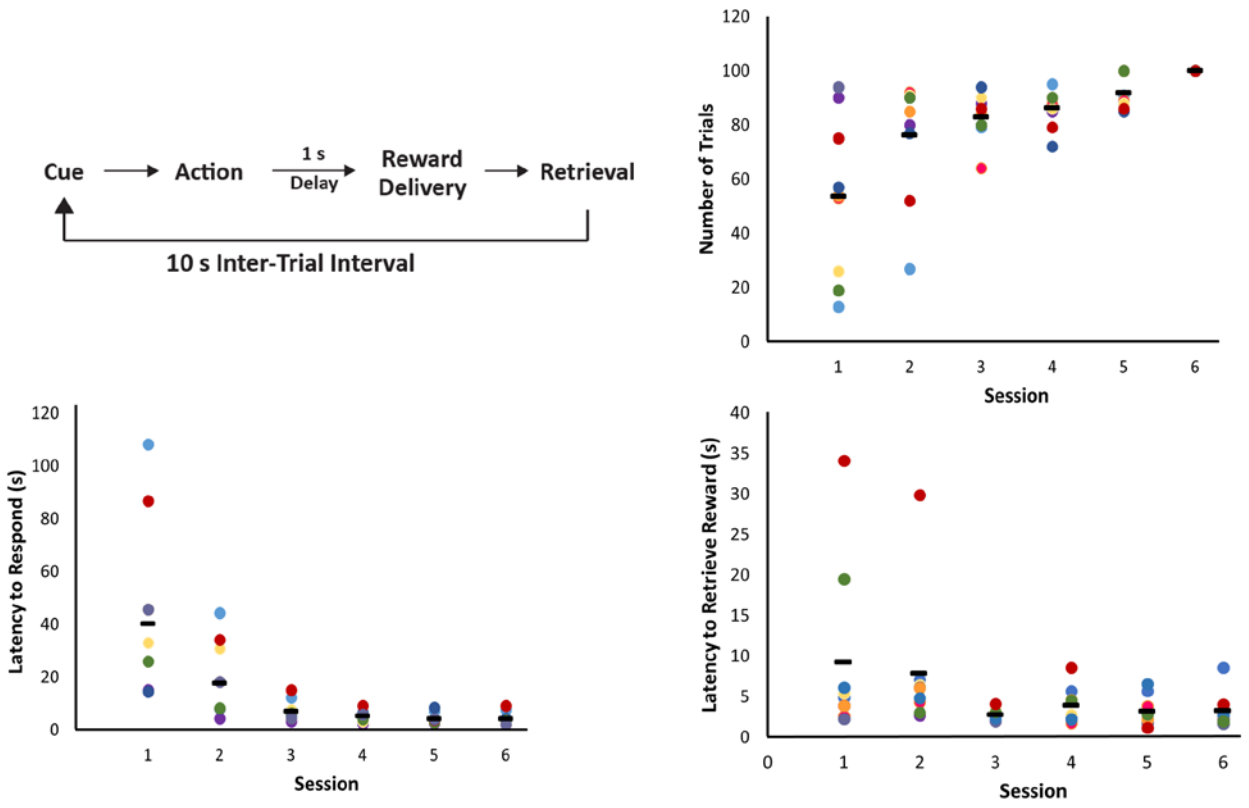


Figure 3-1 Task paradigm and behavioral performance during instrumental recording experiment
Animals were conditioned on a fixed ratio instrumental paradigm in which animals associated a single nose poke into an illuminated port earned one sugar pellet reward. Each trial began with the illumination of the nose poke port, which remained lit until the animal executed the action. Once the animal executed the required nose poke action, the reward was delivered into a food trough on the opposite size of the operant chamber after a one second delay. A 10 second ITI began once the reward was retrieved. Sessions ended when the animal completed 100 trials or 45 minutes elapsed, whichever came first. The animal underwent 6 consecutive sessions of instrumental conditioning. Behavioral plots display each animal as a data point in the column of the indicated session. Animals completed an increasing number of trials across 6 instrumental conditioning sessions. In these same 6 sessions, animals decreased both the latency to perform the nose poke action following the illumination of the port, and latency to retrieve reward.

In the Pavlovian experiment, rats learned a stimulus-outcome pairing through 10 conditioning sessions using either a tone and a light as a reward-predicting cue (CS+) and unreinforced cue (CS-) respectively, counterbalanced across animals (**Fi**gure 3-2). The number of entrances into the food trough was measured during the 10 second CS + and CS- cues. These trough pokes significantly declined across sessions during the CS- (session: $F_{9,83} = 2.764$, $p = 0.007$). Neither trough pokes during the CS+ (Figure 3-2) nor latency to retrieve reward changed significantly across sessions (Figure 3-2). After 10 consecutive sessions, animals underwent a probe session in which the reward contingencies changed so that 75% of CS+ and 25% of CS- presentations were followed by reward delivery, allowing us to assess neural activity during unexpected reward presentation (Figure 3-3). This change in probability also applied to the no reward outcome, meaning 25% of CS+ and 75% of CS- presentations proceeded directly to ITI, enabling us to analyze how the midbrain encodes reward omissions. During this probe session, there were significantly more entrances into the food magazine during the CS+ as compared to CS- (t-test, $p = 0.02$), though there was no significant difference in time to retrieve the reward following either cue (Figure 3-3).

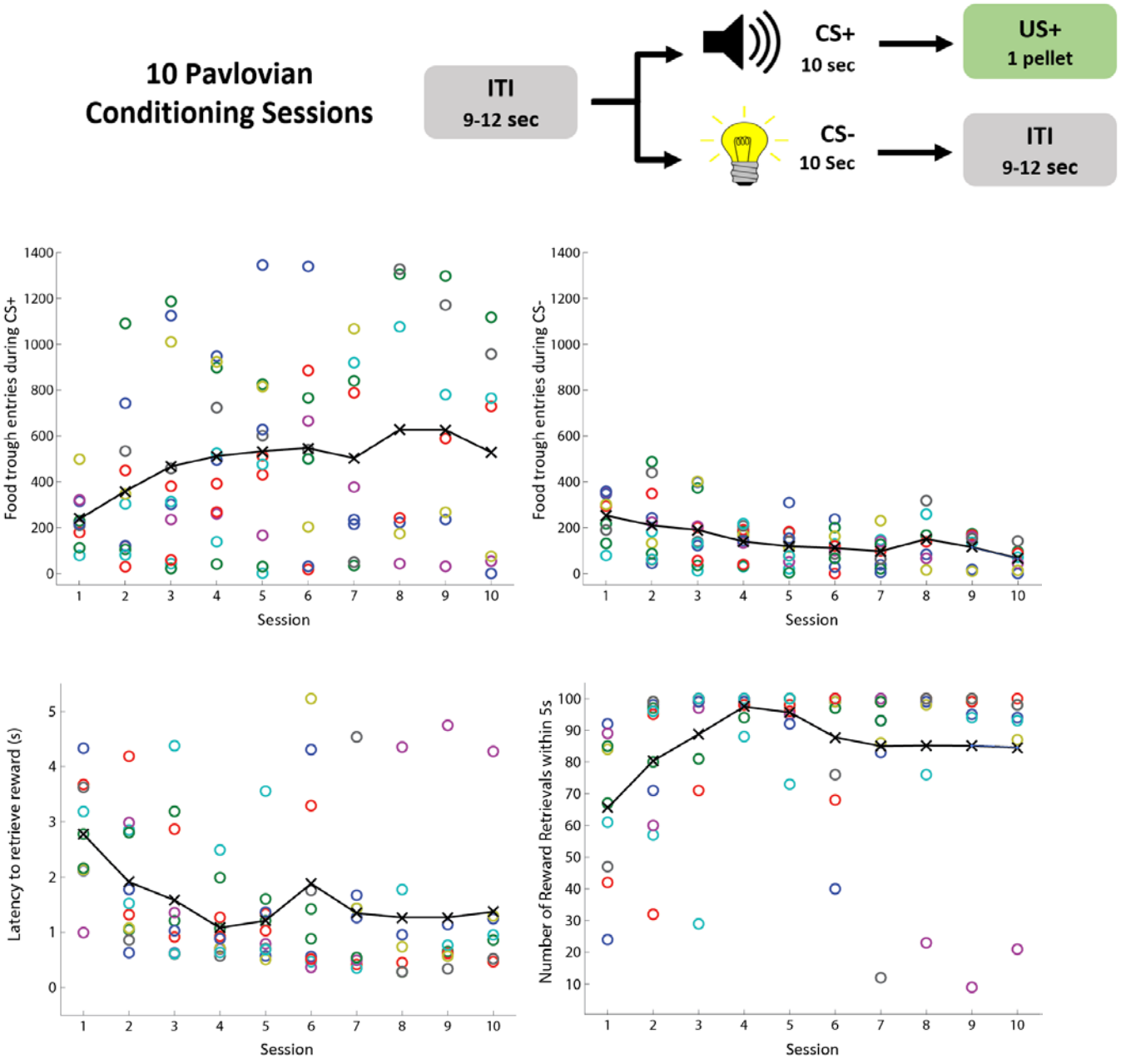


Figure 3-2 Task paradigm and behavioral performance during Pavlovian recording experiment
Animals underwent Pavlovian conditioning during 10 consecutive recording sessions, in which animals associated a light or tone cue with a sugar pellet reward. The cue lasted 10 seconds and was immediately followed by either reward delivery in the case of the CS+ or ITI after CS-. The identity of each cue was counterbalanced across subjects. After reward delivery following the CS+, animals had 5 seconds to retrieve the reward before the trial ended and the ITI began. Sessions ended when the animal completed 100 trials of each type, totaling 200 trials. Entrances into the food trough during the cue were used as a measure of the animal's association between the cue and the outcome. Each data point represents one animal's performance during that session. Average performance within a session is marked by a black X, and these are connected by a black line to display the average trajectory of learning of all subjects across 10 conditioning sessions. Animals increased food trough entries during the CS+ (top left) and decreased during the CS- (top right). Animals also decreased latency of reward retrieval (bottom left), leading to an increased number of reward retrievals within the 5 seconds following delivery (bottom right).

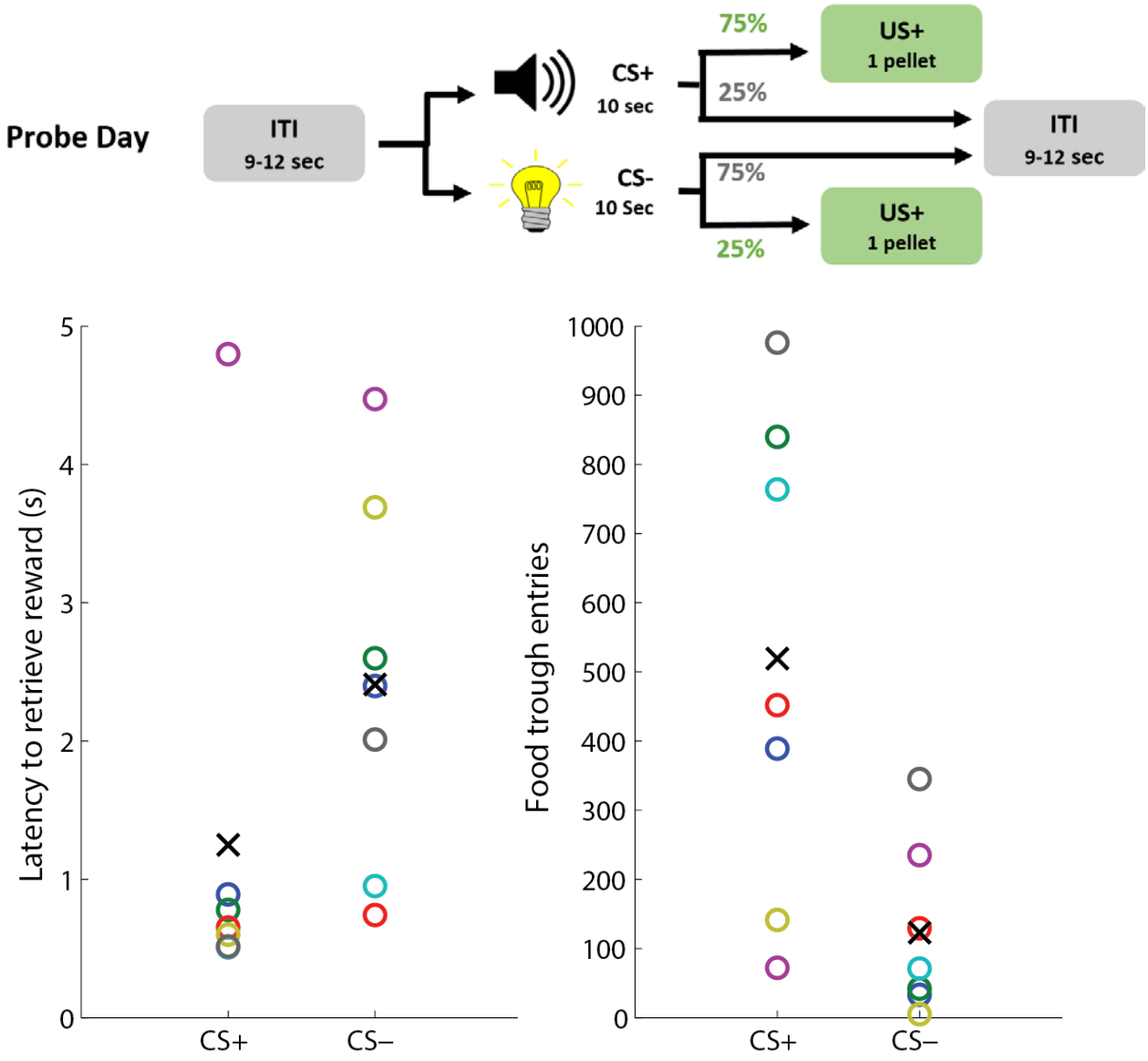


Figure 3-3 Pavlovian conditioning probe day task paradigm and behavioral performance
Pavlovian conditioning was followed by a probe day, the schematic for which is depicted above. In this session, probability of the learned cues was altered so that the previously rewarded cue (CS+) was rewarded on 75% of trials, and the unrewarded cue (CS-) was unexpectedly rewarded on 25% of trials. Learning in this task was assessed using observations of approach behavior, as measured by entrances into the food trough during the CS+ and CS- and latency to retrieve reward (Wan & Peoples, 2008; Kim, Matthews, Moghaddam, 2010). During this session, animals were slower to retrieve reward delivered following the CS-, and enter the food trough less during the CS-.

3.3.2 Population activity

We recorded VTA and SNc neurons in adult animals (n=10) across 6 consecutive instrumental conditioning sessions. We examined the event-evoked activity of all recorded units to understand how the VTA and SNc neuronal population encode the acquisition and maintenance of action-outcome learning. Across sessions, there was no significant difference between cue (Figure 3-4; $F_{5,594} = 1.977$, $p=0.08$) or reward ($F_{5,594} = 1.753$, $p=0.121$) evoked activity in the VTA and SNc. Neither region showed a change in magnitude of response across training (cue: $F_{5,594} = 2.014$, $p=0.071$; reward: $F_{5,594} = 0.595$, $p=0.704$). Within each session, there were no consistent differences in VTA and SNc event-evoked activity. The only exception was session 4, in which significant differences between regions were observed; SNc exhibited significantly stronger phasic response to both cue (reg x time: $F_{19,1653} = 2.641$, $p=0.02$) and reward (reg x time: $F_{19,1653} = 3.18$, $p=0.02$).

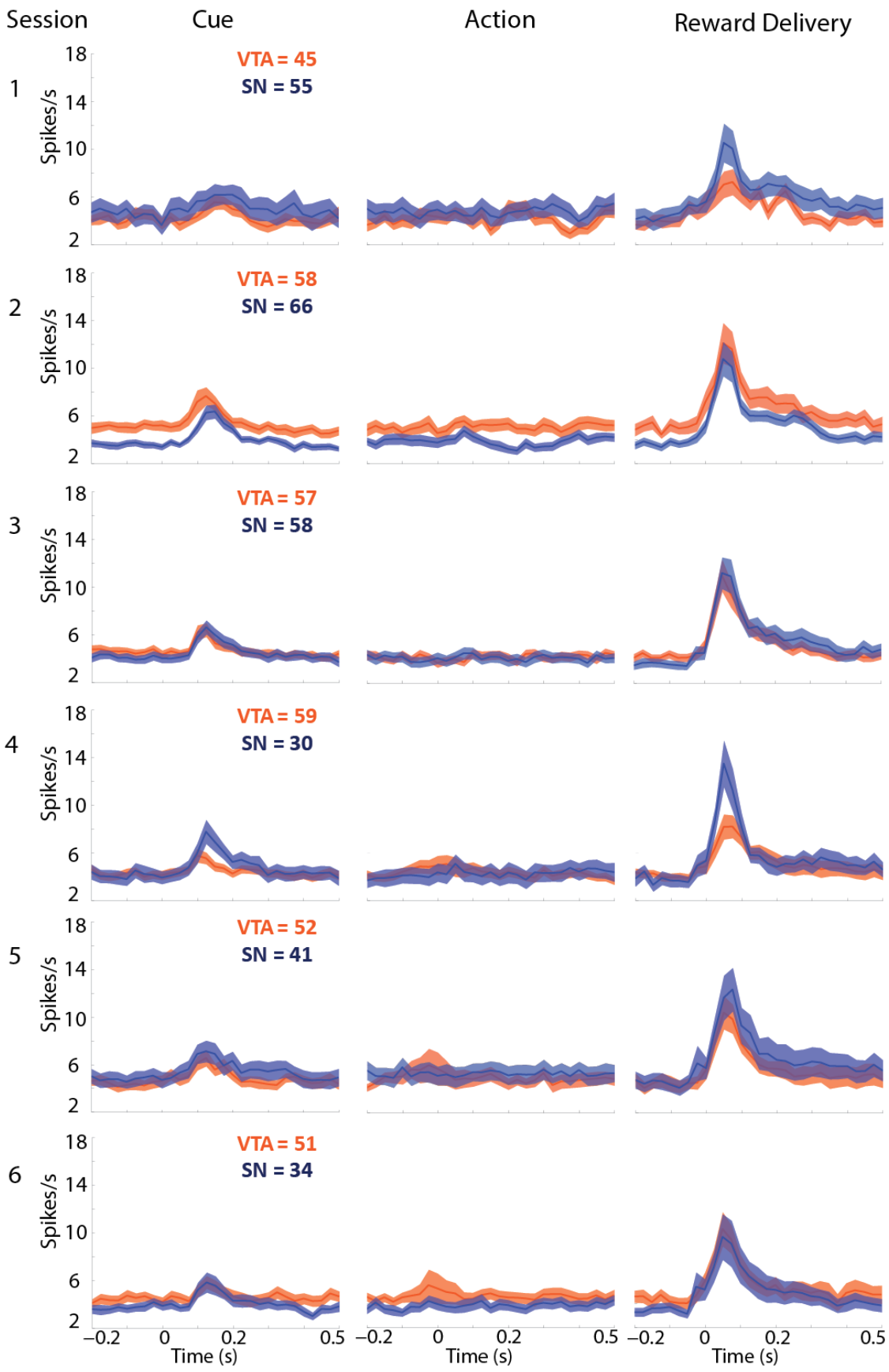

Session
Cue
Action
Reward Delivery
1
VTA = 45
SN = 55
2
VTA = 58
SN = 66
3
VTA = 57
SN = 58
4
VTA = 59
SN = 30
5
VTA = 52
SN = 41
6
VTA = 51
SN = 34
Spikes/s
18
14
10
6
2
−0.2
0
0.2
0.5
Time (s)

Figure 3-4 Session by session raw firing rate population activity in VTA and SNc
Average firing rate in spikes per second with standard error measure in shading for all recorded units during the FR1 experiments. Activity is aligned to the time of the illumination of the nose poke port (left column), execution of the nose poke action (middle column), and reward delivery (right column). The cue and reward evoke phasic activity in both regions, but neither showed any significant activity during the action execution. The number of units recorded in each region during the session is depicted in the upper right corner of the graphs in the left column.

To determine how cue-outcome pairings were encoded, we recorded VTA and SNc neurons bilaterally in adult animals (n=11) across 10 Pavlovian conditioning sessions, followed by one probe day in which outcome contingencies were changed. We analyzed the event-evoked activity of all recorded neurons to assess any differences in VTA and SNc neuronal activity on the population level. Population response in both regions did change in magnitude across training (cue onset: $F_{2, 1401} = 5.052$, p=0.007; cue offset: $F_{2, 1401} = 4.044$, p = 0.02; reward: $F_{2, 1401} = 9.399$, p<0.0005), indicating that midbrain neuronal activity reflects the behavioral changes observed during the Pavlovian task (Figure 3-5). In early learning (sessions 1-3), VTA and SNc neuronal populations were excited by onset and offset CS+, as well as reward delivery. During these task events, there was no observable difference in VTA or SNc population activity (p > 0.3). During intermediate learning (sessions 4-7), VTA and SNc continue to show a strong phasic response, with similar timing and durations through CS+ and reward delivery, though the amplitude of the VTA activity is greater ($F_{19, 6688} = 5.218$, p = 0.002). Across sessions, there was no significant difference between how cue onset ($F_{2, 1401} = 0.0.62$, p=0.94), cue offset ($F_{2, 1401} = 2.537$, p = 0.079) or reward ($F_{2, 1401} = 1.52$, p = 0.219) evoked activity developed during learning in the VTA and SNc.

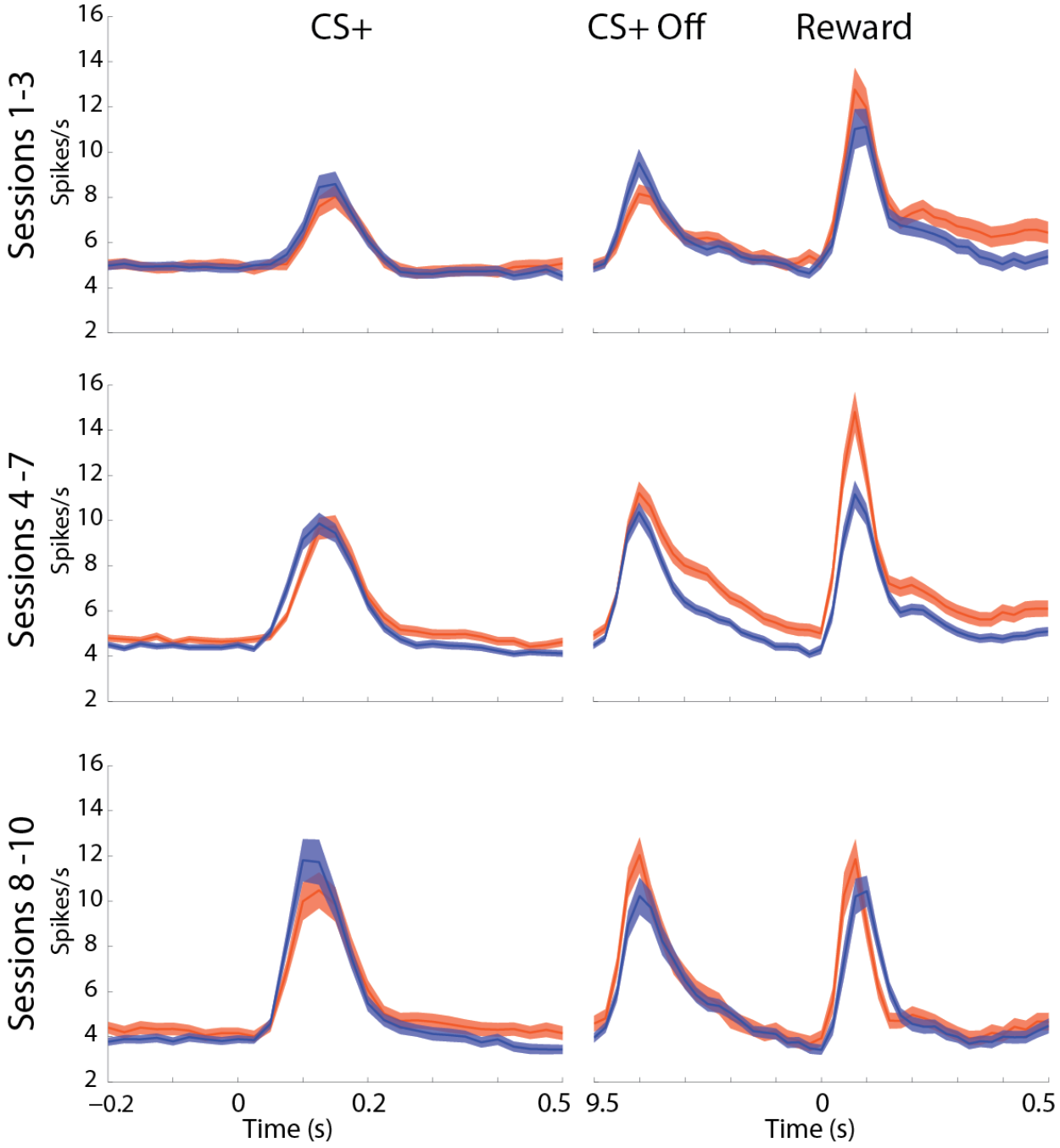


Figure 3-5 Raw firing rate population activity in VTA and SNc during Pavlovian conditioning
Average firing rate in spikes per second with standard error measure in shading for all recorded units during Pavlovian conditioning experiments. Data are grouped into early (sessions 1-3, tow row), middle (sessions 4-7, middle row), and late (session 8-10; bottom row). Activity is aligned to the presentation of the CS+ (left column), CS+ offset, and reward delivery 0.5s after the CS+ (right column). CS+ and reward evoke phasic neural activity in both regions. Population activity in response to the CS+ increased across conditioning sessions, and there was no significant differences between how this developed in each region. The number of units recorded in each region during the session groupings is depicted in the upper right corner of the graphs in the left column.

To understand any differences in how VTA and SNc neurons encode reward prediction error (RPE), we analyzed neural activity during the probe day in which task contingencies changed to produce incidences of unexpected reward and reward omission. We focused our analysis of this session on activity during the CS+, delivery of expected reward, delivery of unexpected reward, and reward omission. Response to these events are archetypical components of dopamine's role in reward, but it is unclear whether this classical response is present across the midbrain. Our data reveal that there is no statistical difference in how neuronal populations in VTA and SNc phasically respond to any of these events (Figure 3-6; $p > 0.13$). The strong phasic activation following an unexpected reward was present in both regions, and neither encode the CS- or the lack of reward after the CS-. When we examine raster plots of individual neurons during reward omission (Figure 3-6), temporary cessation of spiking activity is apparent, but on a population scale the decrease or "pause" in firing is harder to observe.

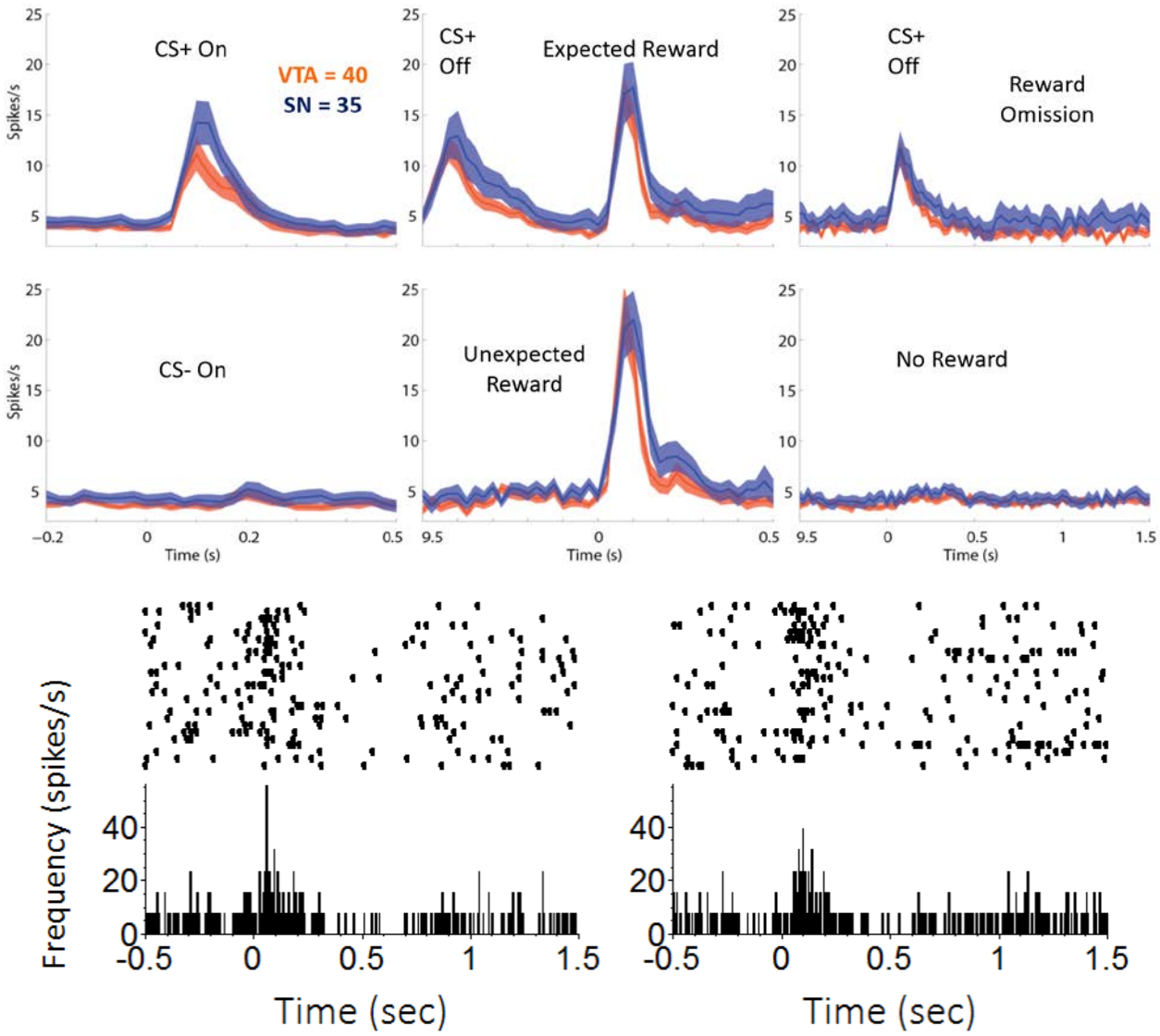


Figure 3-6 Overall population activity in VTA and SNc during Pavlovian conditioning probe day
Average firing rate in spikes per second for all recorded units during the probe day of Pavlovian conditioning. Neural activity from CS+ trials is depicted on the top row, and unit activity from CS- trials is depicted on the bottom row. The number of units recorded in each region depicted in the upper right corner. Neurons from VTA and SNc exhibited strong phasic response to CS+ and reward delivery. Unexpected reward evoked a stronger phasic response from both regions when compared to the amplitude of phasic response during expected reward. Bottom: Raster plots and accompanying histograms for example VTA (left) and SNc (right) neurons during reward omission (upper right population activity plot). These units exhibit the stereotypical pause in firing activity often observed in dopamine neurons when an expected reward is not delivered, conveying a reward prediction error signal.

These overall population analyses consider each region globally, providing information about how dopamine and non-dopamine neurons function as network. Given that neurotransmitter content often indicates specific neuronal physiology and function, we analyzed

putative dopamine units separately to assess whether this important cell population is involved in reward learning across the entire midbrain. Across instrumental conditioning, dopamine-like neurons in VTA and SNc were phasically activated by cue presentation, which signaled action availability, and by reward delivery. Though dopamine neurons in the SNc are known to be involved in motor function, executing a single nose poke during instrumental conditioning did not evoke a phasic response from type 1 SNc neurons during our conditioning paradigm (Figure 3-7). Mean phasic response to cue (session: $F_{5,246} = 3.042$, $p=0.01$) and to reward delivery (session: $F_{5,246} = 2.56$, $p=0.028$) both changed with associative learning as expected from dopamine neurons. There was no significant difference between regions in how dopamine-like neurons responded to either behavioral event across conditioning (cue: reg x session: $F_{5,246} = 1.738$, $p=0.127$; reward: reg x session: $F_{5,246} = 1.197$, $p=0.311$).

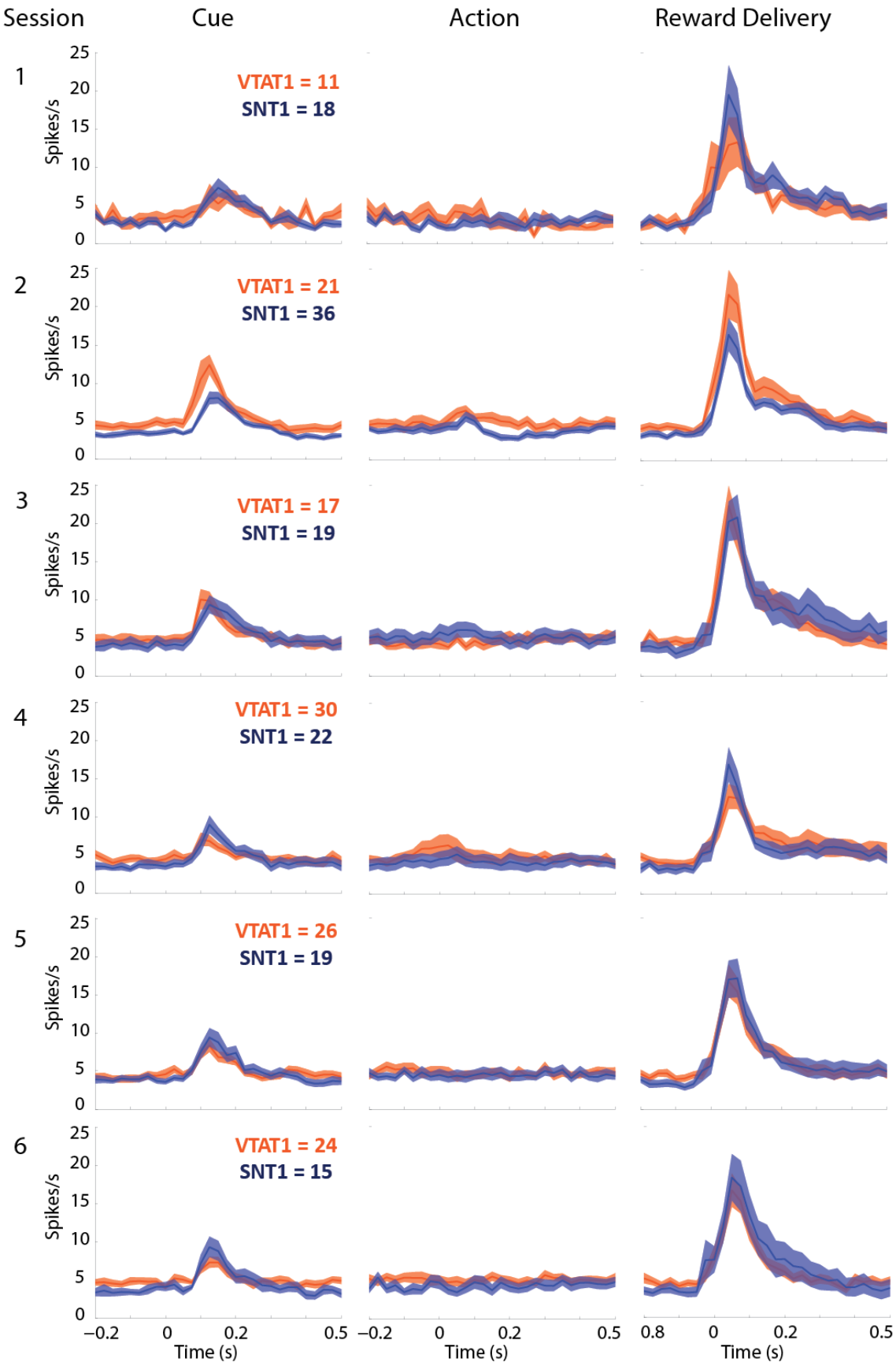

Session
Cue
Action
Reward Delivery
1
VTAT1 = 11
SNT1 = 18
2
VTAT1 = 21
SNT1 = 36
3
VTAT1 = 17
SNT1 = 19
4
VTAT1 = 30
SNT1 = 22
5
VTAT1 = 26
SNT1 = 19
6
VTAT1 = 24
SNT1 = 15
Spikes/s
0
5
10
15
20
25
−0.2
0
0.2
0.5
0.8
Time (s)

Figure 3-7 Population activity of putative dopamine neurons in instrumental conditioning
Average firing rate in spikes per second with standard error measure in shading for putative dopaminergic units during instrumental conditioning. Activity is aligned to the time of the illumination of the nose poke port (left column), execution of the nose poke action (middle column), and reward delivery (right column). Each row of plots represents the activity during the session indicated next to the y-axis. As observed in overall population activity, cue and reward delivery evoke phasic activity in putative dopamine neurons of both regions, while neither showed any significant activity during the action execution. The time course and amplitude of these phasic responses were also similar across regions. The number of units recorded in each region during the session is depicted in the upper right corner of the plots in the left column.

Considering dopamine's well-established role in Pavlovian conditioning (Kelley & Cador, 1988; Schultz 1998; Guarraci & Kapp 1999; Cardinal & Everitt, 2004; Mastumoto & Hikosaka 2009; Darvas et al., 2014), we were interested in how dopamine neuronal activity is contributing to the population response we observed in VTA and SNc. We analyzed putative dopamine units separately, and found dopamine-like neurons in both the VTA and SNc were phasically activated by the onset and offset of the reward-predicting tone or light cue and by reward delivery (Figure 3-8). Mean phasic response to relevant task events (CS+ onset: $F_{2, 720} = 63.223$, $p<0.0005$; CS+ offset: $F_{2, 720} = 150.645$, $p<0.0005$; reward: $F_{2, 720} = 131.112$, $p<0.0005$) both changed with learning as previously observed by others (Ljungberg et al., 1992; Schultz, 1998). Patterns of putative dopamine neural activity were statistically indistinguishable between regions during any task event ($p > 0.095$). There was also no significant difference in how these regions changed their response to CS+ and reward across conditioning ($p > 0.4$). When we analyzed putative dopamine neural activity during the probe day, we again found that there was no measurable difference in how VTA and SNc encode reward prediction error (Figure 3-9).

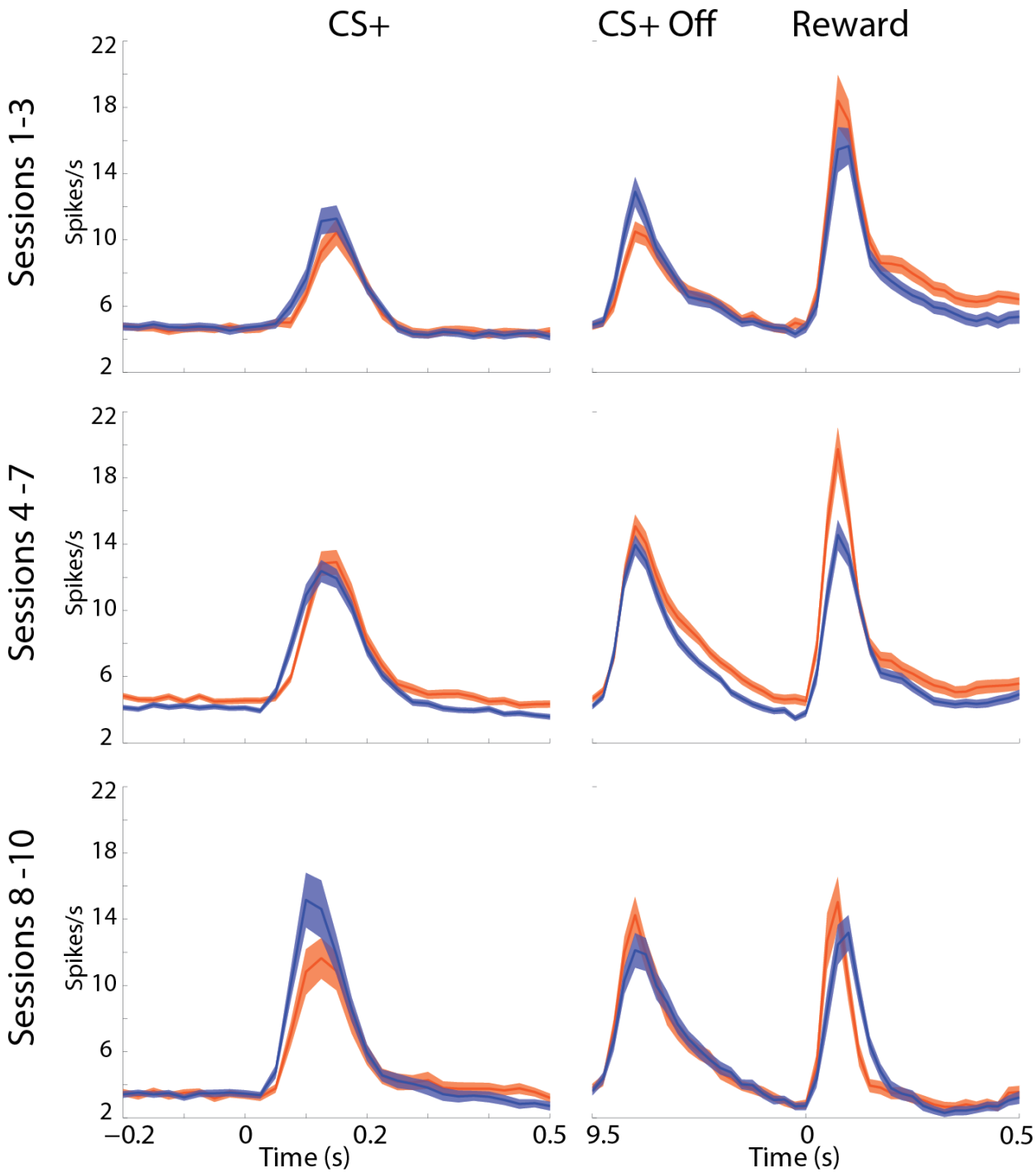


Figure 3-8 Population activity of putative dopamine neurons in Pavlovian conditioning
Average firing rate in spikes per second with standard error measure in shading for putative dopamine neurons recorded during Pavlovian conditioning. Data is grouped into early (sessions 1-3, tow row), middle (sessions 4-7, middle row), and late (session 8-10; bottom row). Activity is aligned to the presentation of the CS+ (left column), CS+ offset, and reward delivery 0.5s after the end of the CS+ (right column). CS+ and reward evoke phasic neural activity in both regions. Population activity in response to the CS+ increased across conditioning sessions, and there is no significant differences between how this developed in each region. The number of units recorded in each region during the session groupings is depicted in the upper right corner of the graphs in the left column.

One component of RPE theory is dopamine's lack response to predicted reward. This finding is based on recordings of prescreened dopamine neurons in animals over-trained in a similar Pavlovian task, and so have formed stronger expectations. While both regions change their response to reward over time, it does not completely diminish. To quantify any difference in magnitude of activation by expected and unexpected reward, we compared these two events within regions. Neither VTA nor SNc exhibited a statistically significant difference in response to anticipated reward following CS+ as compared to the unexpected reward following the CS- when we analyzed the entire population of neurons recorded (Figure 3-6; $p > 0.15$). Considering this feature of activity is thought to be unique to dopamine neurons, we performed the same comparison using only putative dopamine neurons. In this subpopulation, unexpected reward provoked a greater response than expected reward from both VTA and SNc dopamine-like neurons (Figure 3-9; VTA: $F_{1, 48} = 5.626$, $p=0.02$; SNc: $F_{1, 48} = 5.259$, $p=0.03$), a pattern which is consistently observed in dopamine neurons (Schultz, 1997; Cohen et al., 2012).

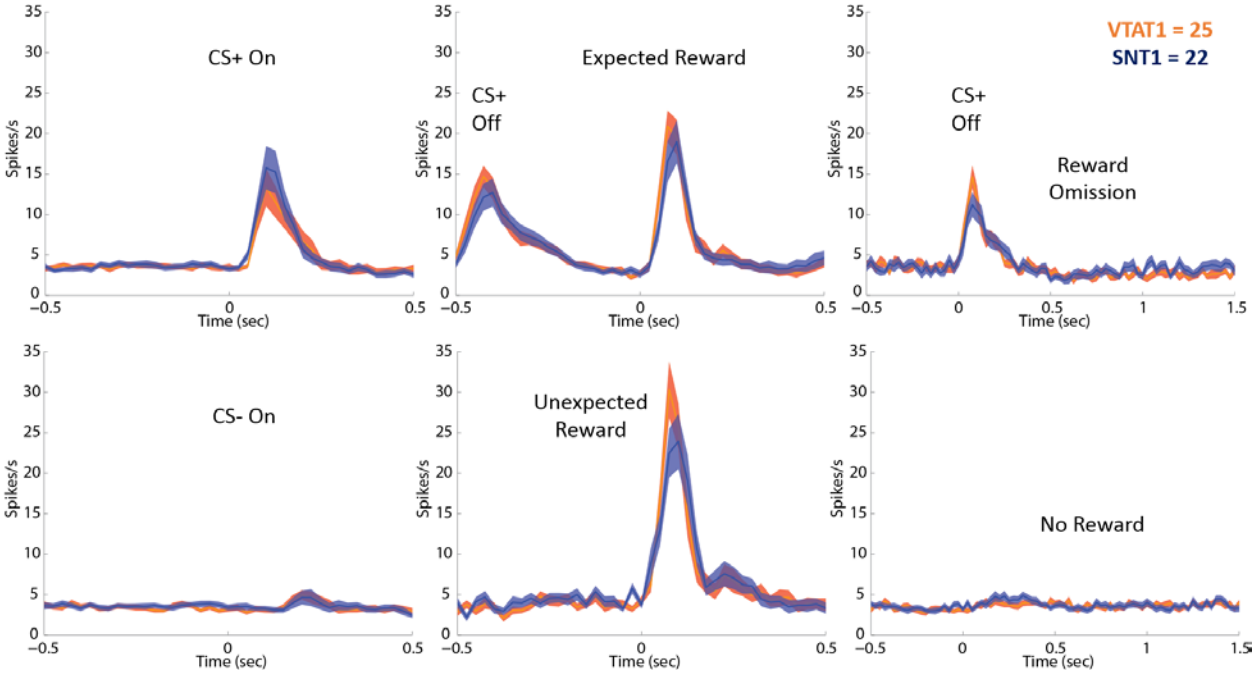


Figure 3-9 Population activity of putative dopamine neurons during the probe day session of Pavlovian conditioning

Average firing rate in spikes per second for putative dopaminergic units during the probe day of Pavlovian conditioning. Neural activity from CS+ trials is depicted on the top row, and unit activity from CS- trials is depicted on the bottom row. The number of units recorded in each region depicted in the upper right corner. As expected, putative dopamine neurons from VTA and SNc exhibited strong phasic response to CS+ and reward delivery. Unexpected reward evoked a stronger phasic response from both regions when compared to the amplitude of phasic response during expected reward. Dopamine neurons are often defined by their strong response to unexpected reward delivery (Eshel et al., 2015; Cohen et al., 2012), so the increase in amplitude as compared to predicted reward replicates what others have found from putative and identified dopamine neurons in rats (Roesch et al., 2007).

3.3.3 Responsive neurons

Average population activity can be useful in understanding how regions function as a whole, but this measure can often obscure subpopulations of neurons with conflicting responses. Consistency across regions in the population responses we observed could be due to populations of a similar size responding at a similar level, or populations of differing size with compensatory differences in strength of response. We isolated and compared these subpopulations between regions by identifying significantly responsive neurons using changes in firing rate during the

event of interest as compared to baseline. To answer whether VTA and SNc produce population responses through similarly sized populations of responsive neurons, we performed chi-square comparisons of the proportion of responsive neurons during each event and each session. There was no consistent pattern of differences in populations of responsive neurons when we compare between regions during instrumental task events (Figure 3-10). When there was a difference within a session, SNc had the larger population of activated neurons (Table 3-1). There was a difference in the proportion of neurons inhibited by reward delivery (χ =4.912, p = 0.027), which is in line with others who have found that VTA neurons are more likely to be inhibited by appetitive stimuli than SNc (Matsumoto & Hikosaka 2009).

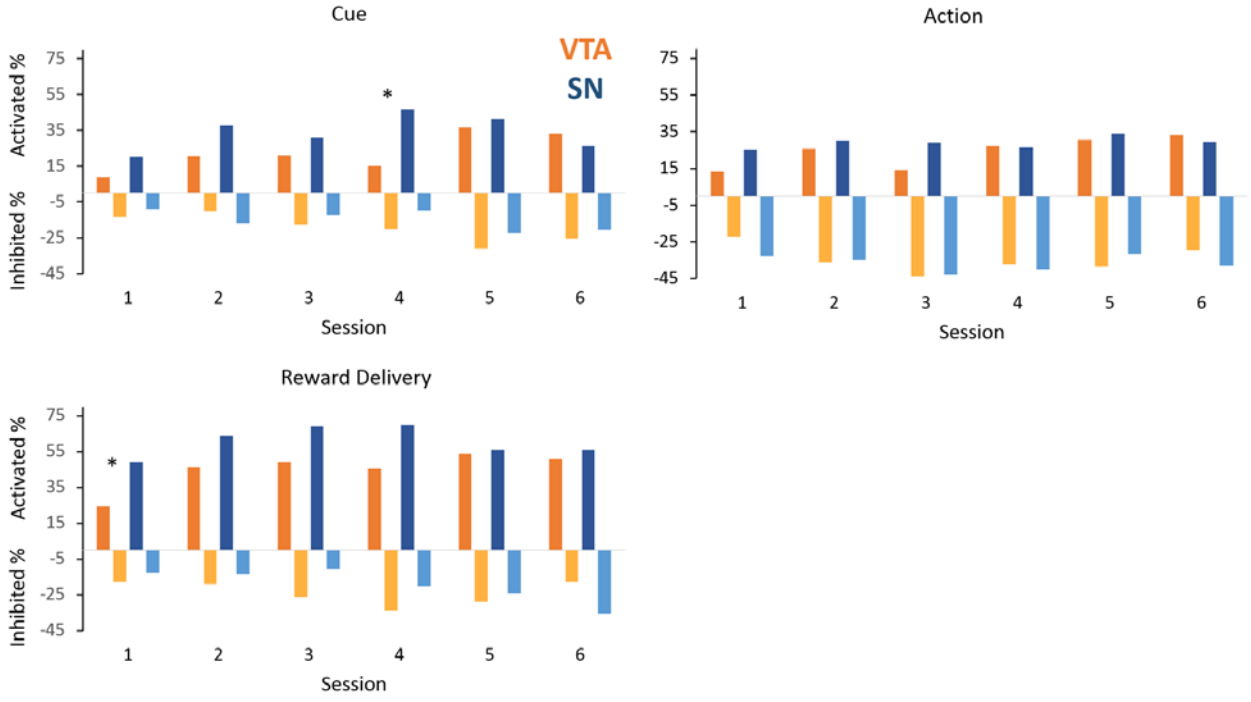


Figure 3-10 Responsive neurons in VTA and SNc during instrumental conditioning
We were interested in whether the similar patterns of population activity we observed across the midbrain were driven by different proportions of responsive neurons with corresponding differences in strength of response to yield the same level of overall population activity. We found no consistent differences in the proportions of activated (darker colors on the positive y-axis) and inhibited (corresponding lighter shades on the negative side of the y-axis) neurons. Any significant differences are marked by asterisks (chi square: p<0.015, correcting for comparisons across multiple events).

	χ^2: Activated units					χ^2: Inhibited units		
Session	**Cue**	**Action**	**Pellet**		**Session**	**Cue**	**Action**	**Pellet**
1	2.396514	2.272727	**6.3813**		**1**	0.45501	1.354818	0.495148
2	4.35693	0.300531	3.650846		**2**	1.042912	0.024884	0.648107
3	1.485534	3.942714	4.683774		**3**	0.684033	0.006689	4.912648
4	**10.23987**	0.002063	4.702256		**4**	1.517036	0.061952	1.857689
5	0.234367	0.119646	0.04692		**5**	0.906907	0.456831	0.231576
6	0.452521	0.144742	0.196759		**6**	0.272436	0.718985	3.415179
	p-values: Activated units					**p-values: Inhibited units**		
Session	**Cue**	**Action**	**Pellet**		**Session**	**Cue**	**Action**	**Pellet**
1	0.121606	0.131668	**0.011533**		**1**	0.499965	0.244438	0.48164
2	0.036859	0.58355	0.056041		**2**	0.307145	0.874657	0.42079
3	0.222911	0.047075	0.030449		**3**	0.408201	0.934814	0.026661
4	**0.001374**	0.963773	0.030123		**4**	0.218069	0.803436	0.172892
5	0.628304	0.729419	0.828512		**5**	0.340937	0.499109	0.630358
6	0.50114	0.703611	0.657349		**6**	0.601702	0.396477	0.064599

Table 3-1 χ^2 results for proportions of responsive neurons in VTA vs SNc during instrumental conditioning
Statistically significant results of chi-square comparisons of proportions of event-responsive neurons during instrumental conditioning are marked in bold ($p<0.015$, correcting for comparisons across multiple events).

Our analysis of response neurons yielded similar results in our Pavlovian conditioning experiment, as there was no consistent difference in responsive neuronal populations (Figure 3-11). Comparable populations encoded all relevant behavioral events during early learning and during maintenance of behavior in the late sessions (Table 3-2). In the middle sessions, the proportion of inhibited neurons was significantly larger in the SNc as the CS+ ends and the reward is delivered (CS= off: $\chi = 9.976$, $p = 0.0016$; reward: $\chi = 17.77$, $p < 0.00005$). The offset of the CS+ during the middle learning sessions also recruited a larger proportion of excitatory responses in VTA than SNc ($\chi = 9.972$, $p = 0.0016$). These results indicate that the VTA and SNc may have slightly altered time courses in their encoding of learned, but still salient, cue-outcome relationships.

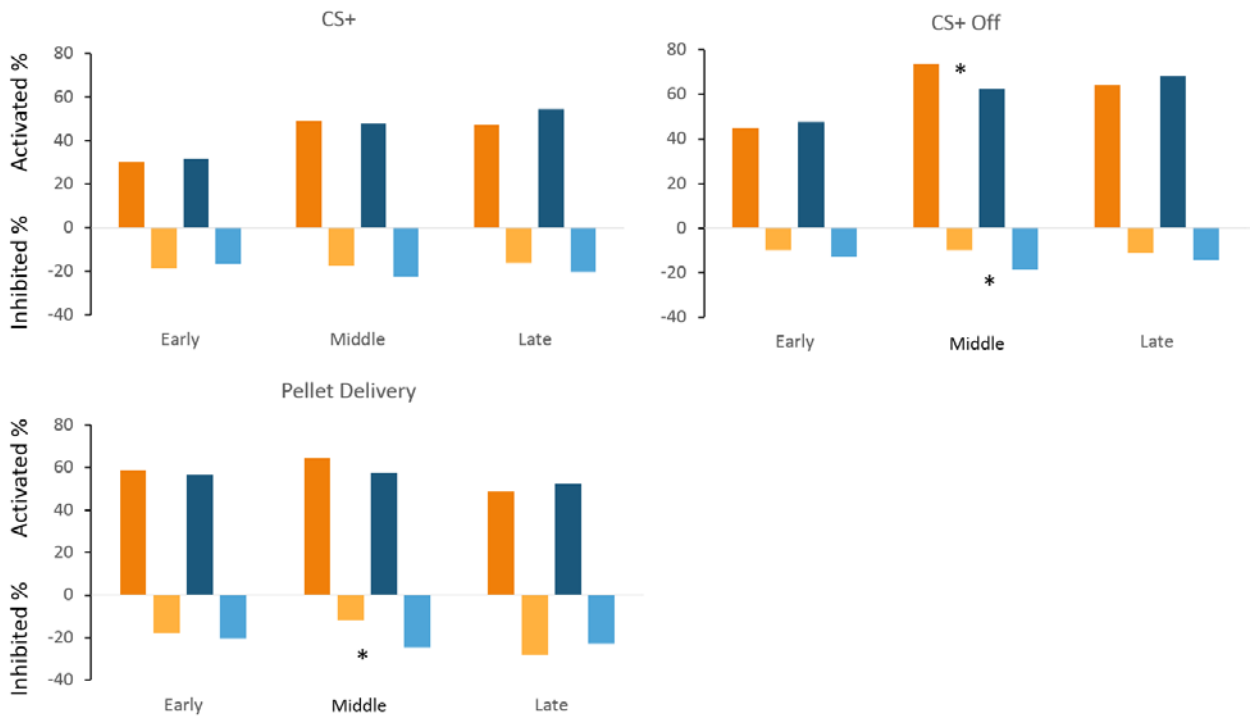


Figure 3-11 Event responsive neurons in VTA and SNc during Pavlovian conditioning
We were interested in whether the similar patterns of population activity observed across the midbrain during the CS+ and reward delivery of Pavlovian conditioning were driven by different proportions of responsive neurons with corresponding differences in strength of response to yield the same level of overall population activity. We found no consistent differences in the proportions of activated (darker colors on the positive y-axis) and inhibited (corresponding lighter shades on the negative side of the y-axis) neurons in VTA (orange) as compared to SNc (blue). Any significant differences are marked by asterisks (chi square: $p<0.015$, correcting for comparisons across multiple events).

	χ^2: Activated Units					χ^2: Inhibited Units		
	CS+	**CS+ off**	**Pellet**			**CS+**	**CS+ off**	**Pellet**
Early	0.11	0.31	0.24		**Early**	0.34	1.02	0.37
Mid	0.039232	**9.972236**	3.11E+00		**Mid**	2.653345	**9.975531**	**1.78E+01**
Late	1.71	0.59	0.43		**Late**	0.94	0.60	1.24
	p-value: Activated units					**p-values: Inhibited units**		
	CS+	**CS+ off**	**Pellet**			**CS+**	**CS+ off**	**Pellet**
Early	0.74	0.58	0.62		**Early**	0.56	0.31	0.54
Mid	0.84299	**0.001589**	7.77E-02		**Mid**	0.103332	**0.001586**	**2.50E-05**
Late	0.19	0.44	0.51		**Late**	0.33	0.44	0.27

Table 3-2 χ^2 results for proportions of responsive neurons in VTA vs SNc during Pavlovian conditioning
Statistically significant results of chi-square comparisons of proportions of event-responsive neurons in each region during Pavlovian conditioning are marked in bold ($p<0.015$, correcting for comparisons across multiple events**).**

3.3.4 Spike correlation data

Neurons which alter their firing patterns in similar ways across trials are thought to be functionally linked or connected. By looking at how these correlation levels change over time we can determine whether VTA and SNc neurons modulate synchronization between neurons in similar ways during associative learning. Spike correlation measures also provide information about how coordinated inputs may regulate neurons within these regions as learning occurs, which cannot be ascertained at the level of population activity. In the first session of conditioning, when associative links have not yet been formed, VTA and SNc showed similar levels of correlation during the cue and action. At cue presentation and while executing the poke during subsequent sessions of the instrumental task, VTA neurons decreased their spike correlation, meaning a reduction is synchronization between neurons within VTA in response to instrumental conditioning (Figure 3-12).

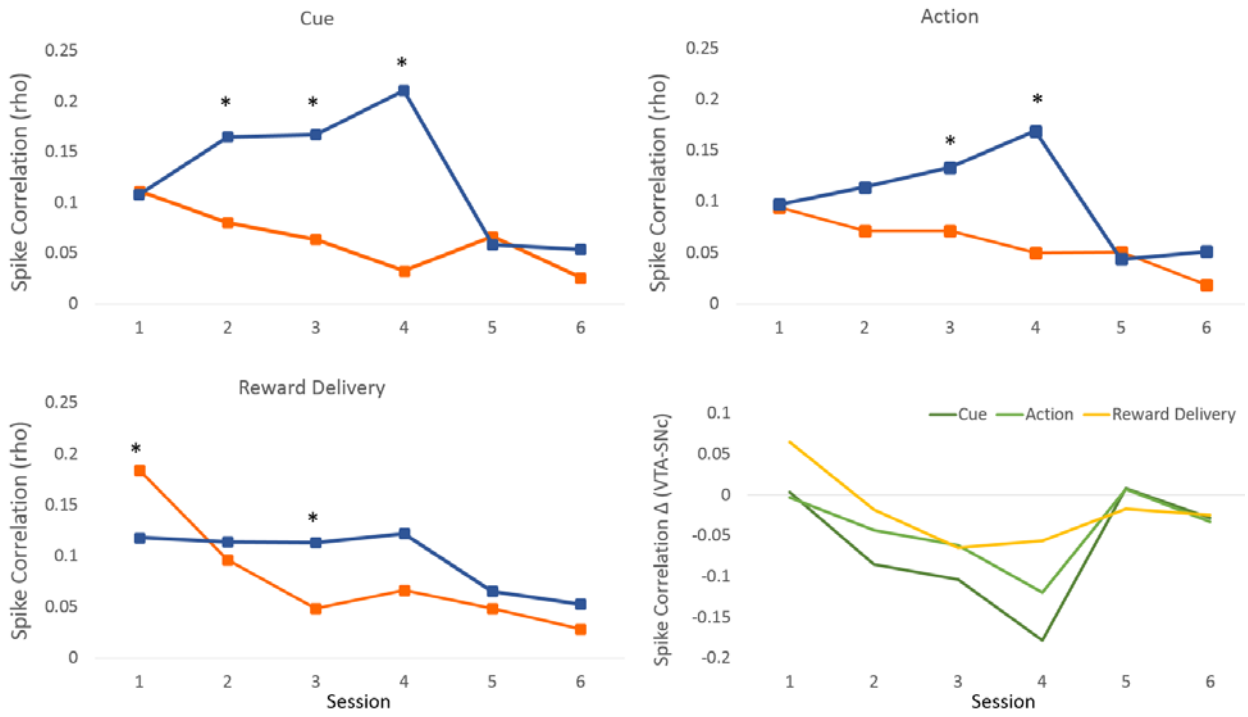


Figure 3-12 Spike correlation in VTA and SNc during instrumental conditioning
Plots depict the average spike correlation, as quantified by Pearson's rho, of simultaneously recorded VTA (orange) and SNc (blue) neurons across 6 sessions of instrumental conditioning. Asterisks mark sessions in which the average correlation significantly differed between regions (t-test, $p < 0.015$). The two regions show opposing patterns of change in correlation structure in response to action-outcome associative learning. Plotting differences between regions (VTA – SNc) in spike correlation (bottom right) show consistently less synchronization in VTA as compared to SNc.

During these same behavioral events, the SNc become more correlated through session 4, but then correlation between simultaneously recorded neurons reduces dramatically during session 5. These differences in trajectory of correlation across learning lead to significantly stronger correlations in SNc spike count correlations during cue presentation in session 2,3,4, as well as during action execution in session 3 and 4 (Table 3-3). This increased synchrony during cue and action in the SNc in the middle sessions of learning could be indicative of the nigral involvement in movement or action choice. This synchrony is diminished in the later behavioral sessions once the task is mastered, and correlational differences between regions also dissipates. Measurements of spike correlation at the time of reward delivery reveal that VTA shows a

reduction in synchronization across regions, as the strong correlations are observed in the early session of conditioning. This pattern is similar to the correlation structure observed in cue and action execution. SNc coordination does appear to be not as strongly modulated by reward delivery following action execution. The divergence in spike correlation during the discriminatory cue and action execution in instrumental conditioning across the midbrain warrants further investigation, as it could define a role for SNc in reward learning that is not shared by VTA.

	1	2	3	4	5	6
Cue	0.970127	**0.00174**	**2.09E-08**	**2.58E-05**	0.626249	0.096456
Action	0.805428	0.324216	**0.000262**	**0.000454**	0.663388	0.040876
Reward	**0.003677**	0.548858	**0.000459**	0.079563	0.251572	0.080319

Table 3-3 Results of t-tests comparing spike correlation between VTA and SN during instrumental conditioning
The p-value for t-tests conducted to identify differences between VTA and SN spike correlation during three events of interest across 6 sessions of instrumental conditioning. Significant results are marked in bold ($p<0.015$).

When we examine the spike correlations of simultaneously recorded neurons during the Pavlovian task, patterns of change across learning are more difficult to define. As in the instrumental conditioning, the most striking differences are found during cue presentation in the middle learning sessions (3-5; Table 3-4). Unlike instrumental conditioning, VTA exhibits the stronger correlations in these sessions, though the difference between regions is not as profound (Figure 3-13). This could indicate that there are aspects of Pavlovian conditioning which more strongly affect the intra-region dynamics of VTA as compared to SNc. Another difference between the conditioning paradigms is the lack of consistent relationship between functional connectivity in SNc during CS+ presentation as measured by spike correlation and session number. Additionally, SNc has drastically different patterns of correlation during the beginning

of the 10 second CS+ as compared to the ending. This could be reflective of the differences in reward proximity between these two events, and the circuits involved in dynamically encoding them. This complexity suggests further investigation is needed to understand whether, and if so how, SNc neurons synchronize to encode the development of cue-outcome relationships.

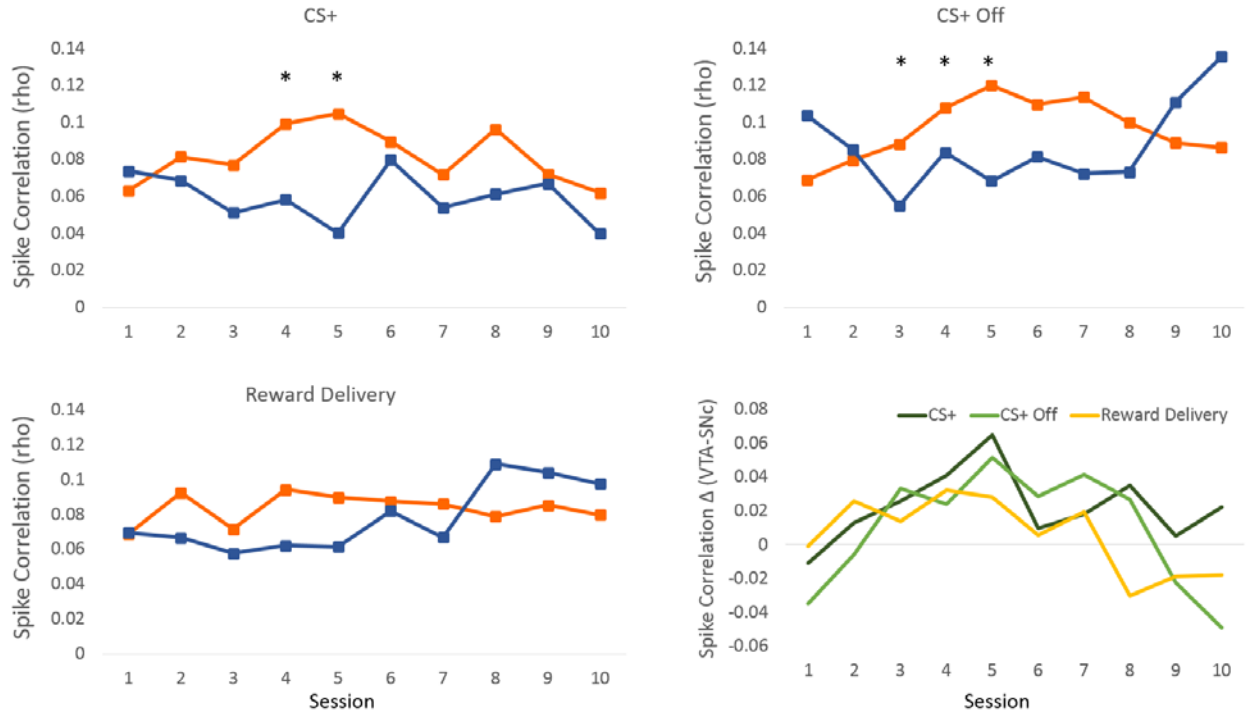


Figure 3-13 Spike correlation between simultaneous recorded neurons during Pavlovian conditioning
Plots depict the average spike correlation, as quantified by Pearson's rho, of simultaneously recorded VTA (orange) and SNc (blue) neurons across 6 sessions of instrumental conditioning. Asterisks mark sessions in which the average correlation significantly differed between regions (t-test, $p < 0.015$). The two regions show opposing patterns of change in correlation structure in response to action-outcome associative learning. Plotting differences between regions (VTA – SNc) in spike correlation (bottom right) show consistently less synchronization in VTA as compared to SNc.

	1	2	3	4	5	6	7	8	9	10
CS+	0.836911	0.0487959	0.178003	**0.0050632**	**0.003013**	0.583443	0.215444	0.437867	0.363682	0.299378
CS+ Off	0.676657	0.1943401	**0.007671**	**4.92E-05**	**3.36E-12**	0.228588	0.200285	0.119483	0.592935	0.127817
Pellet	0.132499	0.1340091	0.165397	0.0220117	0.023654	0.015511	0.803594	0.140736	0.04903	0.363976

Table 3-4 Results of t-tests comparing spike correlation between VTA and SN during Pavlovian conditioning
The p-value for t-tests conducted to identify differences between VTA and SN spike correlation during three events of interest across 10 sessions of Pavlovian conditioning. Significant results are marked in bold ($p<0.015$).

3.4 DISCUSSION

These experiments addressed the question of whether rewards and reward-related stimuli are encoded similarly by VTA and SNc neurons. Using simultaneous electrophysiological recordings from both regions, we observed neuronal activity during two reward-driven behavioral paradigms: Pavlovian conditioning, in which a light/tone cue was paired with a reward, and instrumental conditioning, in which an action earned a reward. Primary measures of interest were phasic responding by VTA and SNc neurons to task stimuli as a function of learning, magnitude of response from putative dopamine neurons in both regions, and the degree of spike correlation in simultaneously recorded pairs. In both tasks, VTA and SNc neurons responded to salient visual and auditory cues in the environment and to delivery of a sugar pellet reward, and neither region exhibited a reliable action-evoked or action-preparatory response during instrumental learning or maintenance. Throughout acquisition of both tasks, both regions exhibited similar patterns of phasic population response as well as putative dopamine neuronal activity. The main observable difference between the two regions was the pattern of change in spike correlations as learning progressed, which likely reflects differences in functional connectivity. These data demonstrate that both the nigrostriatal and mesolimbic circuits contribute similarly to reward-related learning and reward-seeking at the population level, but express different progressions of differentially modulated intra-regional coordination throughout associative learning.

3.4.1 Behavioral findings

For optimal reward-seeking, it is critical that animals understand the relationship between environmental cues, their own behavior, and any available biological rewards. Multiple studies have demonstrated that formation of these associations involves dopamine transmission (Iigaya et al., 2016; Hart et al., 2014; Murray et al., 2007; Steinberg et al., 2013; Schultz, 1998; Glimcher, 2011; Chang et al., 2016). Here, we observed behavior and functional neuronal activity in two distinct forms of associative learning: Pavlovian and Instrumental conditioning. Employing both instrumental and Pavlovian conditioning approaches in our exploration of dopamine function will provide insight into the mechanism behind multiple aspects of motivated behavior. As mentioned above, Pavlovian conditioning is often thought of as a UR that is transferred from the US (sugar pellet) to the CS (light/tone cue). Here we measured Pavlovian conditioning behaviorally by tracking the entrances into the food trough during the CS+ presentation, as this is the approach behavior normally elicited by the US. In instrumental conditioning, one action (a conditioned nose poke) does not necessarily resemble the UR (consumption of a food pellet). Therefore, though these two conditioning paradigms share many common features, such as the formation of associations, reward-seeking, and cue evoked actions, there are fundamental differences between the two paradigms and how we measure learning during each. We found that animals learned cue-outcome and action-outcome associations within the first few sessions and were motivated by the sugar pellet reward in each.

During instrumental learning, we observed an increase in the number of trials completed, and a decrease in latency to execute the action and retrieve reward across sessions, confirming that animals acquired the required action-outcome association. In response to Pavlovian conditioning, animals demonstrated an increase in appetitive behavior throughout training, as

assessed by total entries into the food trough during CS+ compared to CS-, demonstrating that animals learned the contingency between the stimulus and reward. In the probe session of the Pavlovian experiment, we found that the CS+ still yielded more food trough entries and shorter latency to retrieve reward though the CS- was now reinforced with reward in 25% of trials. These results indicate that the change in contingency were not sufficient to significantly shift behavior. By examining the acquisition and maintenance of both conditioning paradigms while recording from the midbrain, we can differentiate what role dopamine neural activity plays in associative learning and reward prediction error (RPE).

3.4.2 VTA and SNc neurons have similar phasic responses to cue and reward

We compared phasic responses in VTA and SNc cells during salient task-relevant events throughout both instrumental and Pavlovian conditioning. These events included cue presentation (Pavlovian CS+ and instrumental cue indicating action availability) and reward delivery during conditioning, with the addition of reward omission and unexpected reward during the Pavlovian probe day. Our instrumental experiment replicated previous VTA findings (Sturman et al., 2012; Kim et al., 2016; Roesch et al., 2007), and demonstrated that the phasic activity consistently observed in the mesolimbic circuit during cue and reward delivery is also present in SNc. In the Pavlovian experiment, neurons in both regions could discriminate between neutral and reinforcing cues, as shown by the lack of phasic activity during the CS- as compared to the strong phasic activation evoked by the CS+. This result suggests that midbrain neurons selectively represent cues that provide salient information about their environment, and do not respond based solely on sensory information, replicating what many have found in dopamine neurons and other reward-related structures (Aosaki et al., 1994; Kawagoe et al., 1998; Tobler et

al., 2003; Waelti et al., 2001). The cue signaling action availability in instrumental conditioning did not activate VTA and SNc neurons as strongly as the Pavlovian CS+, possibly due to the presence of strong auditory and visual cue co-occurring with reward delivery (Fanselow & Wassum, 2015; Pavlov, 1927; Mackintosh, 1975; Kamin, 1969), which was more salient and always resulted in reward. Additionally, the CS+ in Pavlovian conditioning is a more reliable predictor of and has greater temporal congruity with reward delivery than the instrumental cue, which is a secondary reinforcer further removed from the reward itself. We also compared the phasic responses of putative dopamine neurons during these events to determine whether they contributed to the overall population activity in similar ways across the midbrain. Analysis of this subset of the population yielded comparable results as our examination of overall population activity, and did not reveal any differentiation of neural activity between VTA and SNc in either conditioning paradigm. These similarities in neural activity during two distinct conditioning paradigms strongly suggest that VTA and SNc both contribute to reward-related associative learning, and therefore should the entire midbrain be considered as our investigations into mechanisms underlying the motivational and affective symptoms of psychiatric disorders.

These results were somewhat unexpected because much of the research into dopamine and reward revolves around the limbic system, thought of as the VTA and its influence on the nucleus accumbens (Wise, 2009; Lammel et al., 2012; Cohen et al., 2012; Eshel et al., 2015, Flagel et al., 2010; Hamid et al., 2016; Hart et al., 2014; Kim et al., 2016; Kim et al., 2012; Roesch et al., 2007). SNc projections do not interact with traditional limbic structure as strongly as VTA (Beier et al., 2014; Lerner et al., 2014), and is traditionally associated with motor function. This arises from the observations of movement dysfunction in Parkinsonian patients, which results from mass degeneration of SNc dopamine neurons (Dauer & Przedborski, 2003;

Hornykiewicz, 1962; Carlsson, 1964), and from the hypokinesia in animals with bilateral SNc lesions (Marshall et al., 1980; Beninger, 1983). On closer evaluation of the literature, however, suggests that our observations of similar encoding of reward related learning by these regions may be expected. Anatomy studies have shown functional connectivity between the regions and describe a medial-lateral continuum of neurons without the clear boundary we often imagine. Both regions contain reward-responsive populations (Ilango et al., 2014; Parker et al., 2016), and stimulation of either region is sufficient to motivate behavior (Rossi et al., 2013). In previous rodent electrophysiological investigations of the midbrain, small pools of SNc neurons are often included in analysis as the investigators did not find significant differences between the two (Matsumoto & Takada, 2013; Roesch et al., 2007; Morris et al., 2006). Additionally, research into the dorsal striatum, which receives strong input from SNc, suggest that it is more involved with reward-related learning and behavior than previously realized (Palmiter, 2008; Sturman & Moghaddam, 2012; Matthews et al., 2013; Balliene et al., 2007; Wickens et al., 2007) Together with our data, these studies demonstrate that the nigrostriatal system plays a role in reward-mediated behavior, and should be more thoroughly investigated as a potential contributor to motivational dysfunction often found in psychiatric disorders.

3.4.3 Action execution does not elicit phasic response in SNc or VTA

We did not observe reliable phasic responding during action execution in either SNc or VTA during learning or maintenance of instrumental behavior. This is consistent with previous reports from rodents indicating a lack of response from VTA during the peri-action period (Kim et al., 2016, Martig & Mozimuri, 2011). The lack of response in SNc neurons, however, may seem unexpected given the SNc connection to motor function circuits and its involvement in

movement disorders (Dauer & Przedborski, 2003; Hornykiewicz, 1962; Carlsson, 1964; Marshall et al., 1980; Beninger, 1983; Fan et al., 2012). However, our data coincides with the results of recording experiments conducted in cats, monkeys and rodents. These studies also demonstrated that when a discriminatory cue immediately precedes an action leading to reward, the actual action only weakly affects midbrain population activity (Miller et al., 1981; Schultz, 1986; Mirenowicz & Schultz, 1996; Nakahara et al., 2004; Matsumoto & Hikosaka, 2009; Roesch et al., 2007; Bromberg-Martin & Hikosaka, 2010; Takahashi et al., 2011; Totah et al., 2013). A study which required a rat to maintain a lever-pressing response for increasing durations found a subpopulation of SNc dopamine neurons which responded to the initiation or end of an action, but only once a decision-making component had been introduced into the task (Fan et al., 2012). When animals were first trained to perform an action to receive a reward, nigral activity rarely encoded the action execution. Similarly, a study recording single SNc neurons in monkeys found that these neurons were dynamically modulated by the increasing probability of action requirement as time passed, representing the predictions of anticipated changes, but not the action execution itself (Pasquereau & Turner, 2015). In fact, despite SNc involvement in Parkinson's disease motor dysfunction, there is little evidence linking phasic SNc activity with simple action execution (DeLong et al., 1983; Freeman & Bunney 1987; Steinfels et al., 1981; Romo & Schultz, 1990; Pan et al., 2005; Hamid et al 2016). The strongest data suggesting that SNc dopaminergic projections play a role in motor control show phasic activity during internally driven movement, outside of any behavioral paradigm (Barter et al., 2015; Howe & Dombeck 2016; Dodson et al., 2016).) Anecdotally, we did observe large bursts of activity, particularly in SNc neurons, if an animal reared while freely exploring the operant chamber. Our experiments, however, were not designed to continuously track motor behavior,

and therefore we cannot align recorded neural activity to this unprompted behavior. Also, our action-outcome relationship does not involve any measure of uncertainty which has previously been shown to engage SNc neurons (Fan et al., 2012; Pasquereau & Turner, 2015). Our operant data do not exclude a role for SNc in motor function, but add to mounting evidence that the SNc does not drive predictable goal-oriented action execution via phasic population activity.

3.4.4 Caveats to our studies

Data collected during reward-delivery following operant behavior did not diminish across learning as we would have predicted. Dopamine neurons usually lose their response as a reward becomes expected, encoding instead the cue that predicts the reward (Schultz, 1998). In our experiment, neurons in both regions maintained a reward response across all sessions. There are several potential factors which could be responsible for this result. The recorded neural activity could reflect the population response to the sound of the pellet feeder motor or light of the food magazine. These events coincide with reward delivery, and provide a salient visual and auditory cue more proximal to reward than the discriminatory cue signaling availability of action. In our experimental design, these factors cannot be disassociated; this could be accomplished by conducting a session or sessions in which a nose poke action resulted in the sound of the pellet feeder and the light of the food magazine, but no reward. If the phasic neural response remained, the response could then be attributed to these cues instead of the reward in and of itself. It is also possible that our behavioral paradigm does not extend into an “overtraining” phase, during which dopamine neurons usually lose their phasic response to reward (Ljungberg, Apicella, & Schultz 1992; Roesch et al., 2007). These studies are usually performed by reinserting a recording probe every day and lowering until an appropriate neuron is encountered, enabling them to record a

new unit every day across long periods of time. With chronically implanted electrodes, maintaining neurons for more then 8-10 days is technically challenging as neurons adjacent to the electrode wires often die or the electrode site becomes surrounded by glia (Griffith & Humphrey, 2006; Reichert, 2007; McCown et al., 1986). Additionally, our primary aim was to understand how the VTA and SNc acquired the cue-outcome and action-outcome associations, so our experiments focused on acquisition of conditioning, and not the overtraining period often analyzed in other studies. Applying the methods we utilized here to an overtraining paradigm may reveal further differences in VTA and SNc, as the nigrostriatal system is highly implicated in habit formation and execution while the mesolimbic system is not (Graybiel, 2008). These issues should be considered in the design of future investigations into the similarities and differences in the VTA and SNc during reward-related learning.

3.4.5 Spike correlation in distinct neuronal populations and conditioning paradigms

Analyzing changes in amplitude of phasic population response to a specific stimuli only extracts information contained in the rate code of that population. The rate code we examine here, or the information conveyed by the speed and timing of a neuron's spiking activity, is averaged across units, possibly obscuring information contained in the variability between neurons (Zohary et al., 1994; Panzeri et al., 1999). Spike correlation is a measure of the relationship between two simultaneously recorded neurons fluctuating activity across multiple presentations of the same stimulus (Cohen & Kohn 2011; Baeg et al., 2007, Kim et al., 2013; Moghaddam & Wood 2013). This level of analysis captures dynamic relationships between neurons as their activity fluctuates on a trial to trial basis, measuring how network coordination changes in response to learning (Panzeri et al., 1999; Cohen & Kohn, 2011). Increases in spike

correlation can be due to strengthening of synaptic connections between neuron pairs, or more neurons coming online to respond to a stimulus (Baeg et al., 2007). These changes often happen in concert with learning (Ahissar et al., 1992; Schoenbaum, Chiba, & Gallagher 2000), as observed here. We found that SNc encodes instrumental learning through an initial increase in correlation during session 1-4, followed by a decrease towards initial levels of coordination during the last two sessions. This drop cannot be explained by a large decrease in the number of neurons recorded or by a change in the number of fast-spiking neurons recorded, which are known to artificially increase spike correlation measures (Cohen & Kohn, 2011). Instead, this change in synchronization may be reflective of the plateau in behavior observed in session 5 and 6, and the correlation decreases once the association is internalized. Since this pattern is only present in SNc, it may be reflective of the motor component of instrumental conditioning. Additionally, correlation in the SNc is not significantly modulated by reward delivery during instrumental conditioning, an event which does not necessarily coincide with any action from the animal. This interpretation would also account for the differences in coordination structure observed in instrumental and Pavlovian conditioning, as action requirement is the main divergent component between the paradigms. In the VTA, spike correlation decreases across instrumental conditioning during both cue and reward delivery, creating a pattern of change which coincides with behavioral improvement. As the animals completes the required number of trials more quickly, the VTA decreases the level of synchrony among simultaneously recorded neurons. During Pavlovian conditioning, VTA spike correlation during the offset of the cue initially increases and then plateaus at a level slightly higher than the initial correlation measures. This mirrors the pattern of behavior seen during food trough entries and reward retrieval.

Higher spike correlation also means less variability in the information provided by individual neurons (Ghim et al., 2011; Cohen & Kohn, 2011). This means projection targets would receive the same message without having to send converging collaterals from two neurons with different pieces of information. This streamlined system could support rapid learning as less functional connectivity changes are needed before the message becomes heard. These inter-neurons interactions are subtle and do not necessarily affect the signal transmitted by a population, but rather act in a modulatory manner to provide context to the neuron's activity. One possibility is this modulation reflects the influence of executive function, or top-down control (von der Malsburg, Phillip, & Singer, 2010; Moghaddam & Wood, 2014). Another is that this is done locally by changes in the way neurons interact with their immediate neighbors (Constantinidis & Goldman-Rakic, 2002; Kohn & Smith, 2005; Cohen & Kohn, 2011). VTA contain greater heterogeneity in cell types as compared to SNc (Carr & Sesack, 2000 a,b), while SNc has more electrically coupled cells (Grace & Bunney, 1983). These anatomical differences could be a contributing factor to the differences in spike correlation observed here. These possibilities do not preclude each other and are most likely working in concert to contextualize incoming stimuli and environmental changes (von der Malsburg, Phillip, & Singer, 2010). Our data do not indicate which of these factors differs between the VTA and SNc, but it does demonstrate that these subtle interactions are one of the main distinctions between these regions. It is important keep in mind that spike correlation is a measurement of neural plasticity. Dopamine neurons must employ plasticity during associative learning to encode reward prediction error, updating their activity to reflect unexpected changes in the environment (Romo & Schultz, 1990; Schultz & Romo, 1990; Ljungberg et al., 1992; Schultz et al., 1993, Mirenowicz & Schultz, 1996). Thus, dopamine neurons are especially well suited for

examination by this analysis and should be further explored, possibly in relation to the plasticity observed in drug addiction. Understanding how these changes occur and the role of afferent regulation in these changes will be key to deciphering the mechanism behind the brain's ability to coordinate across multiple regions and adapt to new environmental cues and outcomes.

4.0 COMPARISON OF MIDBRAIN NEURAL ACTIVITY IN N-3 PUFA DEFICIENT AND ADEQUATE ANIMALS

4.1 INTRODUCTION

Environmental factors are known to play a role in the development and severity of psychiatric disorders (Kenler et al., 2003; Tandon et al., 2008). Socio-economic status, stress, drug abuse, and epigenetic influence on mental illness have been investigated (Meyer-Lindberg & Tost 2012; Oh & Petronis 2012), but dietary factors have not been thoroughly considered. In particular, some essential fatty acids are critical for proper development and cellular function (Holman 1986; Kidd 2007; Abbott et al., 2012) and cannot be synthesized by humans. Omega-3 polyunsaturated fatty acids (n-3 PUFA), including docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA), can be found in leafy greens, wild fish, grass-fed livestock and their products, including eggs, dairy, and meat. Beginning in the 1970's, the American diet shifted towards subsidized, mass-produced corn and grain products with a long shelf life, resulting in an increasingly high proportion of n-6 PUFA, another essential fatty acid, in our food (Simopoulos, 2003; Yamada et al, 2014; Ikemoto et al., 2001). Neither the necessity of these two fatty acids nor the importance of the balance between them were fully recognized at the time (Abbott et al., 2012; Simopoulos, 2003; Holman et al., 1982), and therefore n-3 PUFA was not included in nutritional recommendations put forth by dietary advocates or governmental

organizations decades ago. Dietary studies have shown that n-3 PUFA accounts for ~9.5% of the current average fat intake in the US (Ervin et al., 2004), though records suggest that humans evolved with a much larger portion of n-3 PUFA in their diet. This historic diet resulted in an n-6 to n-3 ratio closer to 1 while the current ratio in Western diets is approximately 15/1 or 16/1 (Simopoulos, 2003). The ratio between n-3 and n-6 PUFA in the membrane affects the fluidity of the membrane and its metabolic efficiency (Kidd, 2007; Jump, 2002; Else & Hulbert, 2003), G-protein receptor signaling, (Salem et al., 2001), gene expression (Duplus et al., 2000), and the formation of lipid rafts on the membrane (Yamada et al., 2014; Jump, 2002). These changes to the cell membrane fundamentally change how neurons transmit signals, influencing receptor regulation, frequency of synapse formation and/or vesicle fusion events at axon terminals (Darios et al., 2007, Roqueta-Rivera et al., 2011).

Deficiency in omega-3 polyunsaturated fatty acids (n-3 PUFAs) have been identified as an environmental insult that affects cognitive function, possibly aggravating or enabling the emergence of schizophrenia, major depressive disorder, and attention-deficit/ hyperactivity disorder (Banni & Marzo, 2010; Wainwright et al., 1997; Connor et al.,1991; Amminger et al., 2010). Behaviorally, deficiency in n-3 PUFA has been associated with several deficits (Moriguchi et al., 2000; Fedorova & Salem, 2005; Bondi et al., 2014) including increased susceptibility to inflammation (Kidd, 2007; Banni & Marzo, 2009); increased incidence of depressive symptoms (Conklin et al., 2007; Lin et al., 2010), and anxiety or anxiety-like behavior (Larrieu et al., 2012; Liu et al., 2013) in rodents and humans. It is critical to understand the physiological mechanism behind the neural effects of n-3 PUFA deficiency in order to identify the deficits it causes and provide possible mechanisms for the prevention and treatment of psychiatric disorders**.**

Previous cellular and behavioral research has demonstrated a profound impact of n-3 PUFA deficiency on the monoamine neurotransmitter system. To date, research into the neurological impact of n-3 PUFA deficiency focused on the function of dopamine terminals in the striatum, hippocampus and prefrontal cortex. Studies in these regions found decreases in VMAT, DAT, and TH, as well as increased D2 receptor number and attenuated amphetamine-induced dopamine release in n-3 PUFA-deficient rats (Kuperstein, EIlam, & Yavin 2008; Chalon, 2006; Bondi et al., 2014). These results indicate altered dopamine transmission, but do not directly investigate how dopamine neurons are impacted by dietary n-3 PUFA deprivation. The ventral tegmental area (VTA) and substantia nigra pars compacta (SNc) are the main source of dopaminergic projections to the previously studied terminal regions, and are known to be vulnerable to environmental or pharmacological insult (Wang & Michaelis, 2010; Saxena & Caroni, 2011; Gonzalez-Hernandez et al., 2010; Di Monte, 2003; Surmeier, 2007; McCormack et al., 2002). Many of the behavioral changes seen in deficient animals, including hyperactivity, increased distractibility, and self-regulation and inhibition, are associated with malfunction of the dopamine system. These measures provide a useful evidence to support the connection between n-3 PUFA, dopamine, and mental health, but do not elucidate neuronal mechanism behind the changes.

To address this gap in our understanding, our laboratory has established a colony of second-generation n-3 PUFA deficient and adequate animals (Bondi et al., 2014), emulating the current generation of young adults (Passos et al., 2012). Our previous investigations using this animal model revealed disruption in tyrosine hydroxylase (TH) activity in the dorsal striatum, but not the nucleus accumbens (Bondi et al., 2014). This observation suggested that the nigrostriatal as opposed to mesolimbic dopamine cells are more susceptible to this dietary

manipulation. Based on this observation, we hypothesized that n-3 PUFA deficiency has a selective disruptive effect on dopamine neurons in SNc as compared to the VTA. To address this hypothesis, we bilaterally implanted electrodes in the VTA and SNc of second-generation n-3 PUFA deficient (DEF) and adequate (ADQ) animals, allowing us to simultaneously record single unit activity during the FR1 operant behavior described previously (Section 3.3). We analyzed activity during the cue, nose poke action, and reward delivery events, identifying putative dopamine neurons, responsive neurons, and possible differences in functional connectivity across learning. While n-3 PUFA deficient animals exhibited no gross behavioral abnormalities, we found reduced phasic response to cue and reward during early learning. The SNc of deficient animals also contained fewer event-responsive neurons and increased baseline firing rate as compared to adequate animals. When we investigated the effects of n-3 PUFAs on neuronal and network-level function in concert with relevant behavior, SNc neurons exhibited reduced correlation in activity between simultaneously recorded neurons. Additionally, neurons from the VTA and SNc of deficient animals failed to reflect the learned associations from conditioning through changes in their coordination structure. These data indicate that neurons in SNc and this region's involvement in reward learning may be more susceptible to dietary environmental insult than the VTA, and disruptions in functional connectivity could underlie the cognitive impairments commonly seen in n-3 PUFA deficiency.

4.2 METHODS

Subjects: Sprague-Dawley rats were bred in house from two established lines of first-generation n 3-PUFA deficient or adequate dams bred with standard chow fed males. The

second-generation rats (i.e. parents were fed the same diet) used in this study were weaned at postnatal day 21 and continued to receive the adequate or deficient diet (Dyets Company, Bethlehem, PA) of their respective dam. The chow base is composed by weight of carbohydrate (60%), protein (20%), fat (10%), fiber (5%), salts (3.5%), vitamins (1%) and tert-butylhydroquinone (0.002%). Deficient animals received this base diet while adequate animals were fed the same base diet enhanced by ∝-Linolenic acid (∝-LNA), a precursor to docosahexaenoic acid (DHA) found in flaxseed oil. The vitamin, mineral, basal fat, and macronutrient composition of the two diets did not differ (Bondi et al., 2014). This dietary regimen has been previously shown to significantly impact brain fatty acid lipids (Bondi et al, 2014). Rats of both dietary groups were pair-housed in a 12-hour reverse light/dark cycle with the lights turning on at 7pm. Access to food and water was ad libitum.

Surgery, Behavior, Electrophysiology: Surgery and electrophysiological recordings were conducted as described in section 2.3. The behavioral task is described in the instrumental behavior section 3.3.

Data Analysis: Number of trials completed, latency to poke, and latency to retrieve reward served as behavioral indices of learning and performance. Independent samples t-tests were used to quantify diet-related behavioral differences within single sessions. Greenhouse-Geisser corrections were applied in all cases in which unequal variances between groups were detected. Isolated single unit data were analyzed with custom-written MATLAB functions (MathWorks, Natick, Massachusetts). Neural data from animals who completed less than 10 trials in a session were excluded from analysis. Units with a baseline firing rate ≥ 50Hz, as measured in the middle three seconds of the inter-trial interval (ITI), were excluded as outliers (n=6). Unit firing rates during task events were binned (25 msec) and smoothed with a five-point moving rectangular

kernel. A ±0.25-sec window was utilized for the analysis of event-evoked neural responses. Differences in peri-event activity between dietary groups within and across sessions were assessed with two-way ANOVAs.

Spike Count Correlations: We simultaneously recorded 72, 132, 89, 106, 66, 129 ADQ neuron pairs and 208, 73, 335, 305, 395, 315 DEF neuron pairs in the VTA (pooled across animals) in instrumental behavior sessions 1- 6, respectively. In these sessions, we also recorded 242, 264, 232, 40, 37, 26 ADQ pairs and 229, 265, 247, 133, 104, 59 DEF pairs simultaneously in the SN. The correlation between each pair of unit's stimulus-evoked neural activity was analyzed. For these analyses, we did not group unit pairs based on putative neurotransmitter content in order to preserve sufficient sample sizes for reliable analysis. All spike train analysis utilized custom scripts executed in MATLAB (MathWorks, Natick, MA). We correlated the trial-by-trial fluctuations in discharge rate between pairs of simultaneously recorded neurons. A Pearson's correlation of spike counts for each pair of units was calculated in the 250 ms following the event of interest. Spike count correlations are sensitive to outliers, so we excluded any trial in which either unit firing rate was >3 SDs away from its mean baseline firing rate (Ruff & Cohen 2014; Kohn & Smith, 2005). Correlations between neuron pairs across trials was calculated as rho and comparison of rho measures were made with a t-test with no assumption of equal variances and significance set at p=0.015 to correct for multiple comparisons.

4.3 RESULTS

Behavior and learning were evaluated using the latency to respond by executing a nose poke, latency to retrieve the sugar pellet reward, and number of trials completed within a session

(Figure 4-1). Over behavioral training, number of trials completed significantly increased (session: $F_{5,80}$=15.434, p<0.0005) while latency to nose poke (session $F_{5,80}$=9.175, p=0.005) and latency to retrieve reward (session: $F_{5,80}$=6.831, p=0.009) significantly decreased. Analysis revealed no significant difference between dietary groups in latency to nose poke (interaction: $F_{5,80}$=0.534, p=0.514) or number of trials completed (interaction: $F_{5,80}$=1.905, p=0.151). There is a significant difference between dietary groups in latency to retrieve the sugar pellet reward during session 4 (p=0.02) and session 5 (p=0.05) but across all sessions, there was no significant difference in retrieval latency between diets groups (interaction: $F_{5,80}$=0.205, p=0.749). Behaviorally, n-3 PUFA dietary deficiency causes subtle changes which do not have gross impact on simple reward-related learning. These results remove the possibility that behavioral differences are responsible for the discrepancies we observe in midbrain neural activity.

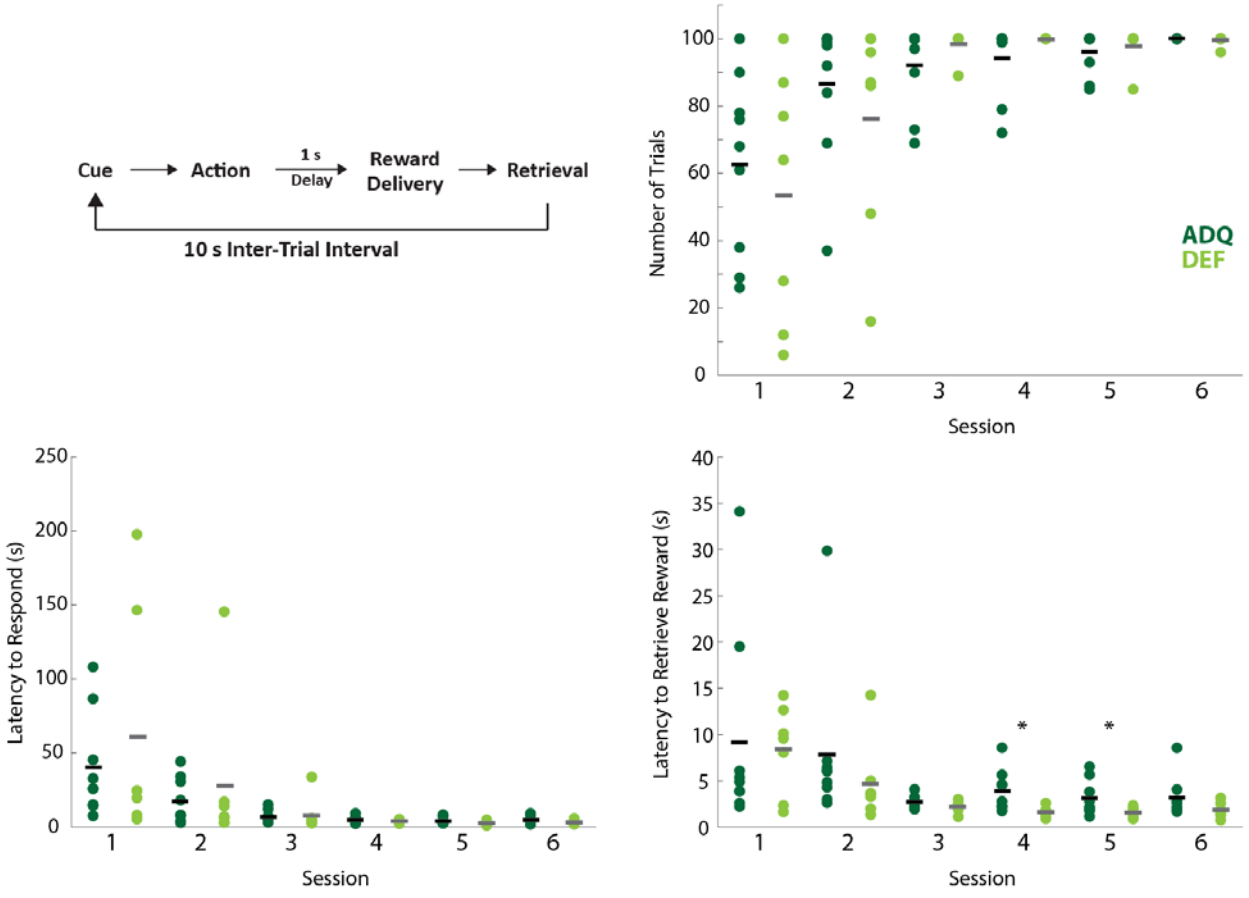


Figure 4-1 Task paradigm and behavioral performance in n-3 PUFA ADQ and DEF animals
Adequate (ADQ; dark green) and deficient (DEF; light green) animals were conditioning on a fixed ratio instrumental paradigm in which animals associated a single nose poke into an illuminated port earned one sugar pellet reward, as described in Section 3.3. Each animal underwent 6 consecutive sessions of instrumental conditioning. Behavioral plots display each animal as a data point in the column of the indicated session. Animals completed an increasing number of trials across 6 instrumental conditioning sessions. In these same 6 sessions, animals decreased both the latency to perform the nose poke action following the illumination of the port, and latency to retrieve reward. There was no significant difference between dietary groups in the way behavior progressed across sessions.

To understand how dietary environment insult affects neural encoding of behavior, we recorded bilaterally from the VTA and SNc neurons of second generation n-3 PUFA adequate (ADQ) and deficient (DEF) adult rats across six operant behavior sessions. When examining the entire population of recorded neurons together, DEF SNc neurons show a significantly diminished response to reward delivery in sessions 1, 2, and 4 (Figure 4-3). They also show a reduced response to the cue in session 2 and 4. Interaction effects between dietary group and event evoked neural activity within a session were not evident in VTA overall population during cue or reward (Figure 4-2).

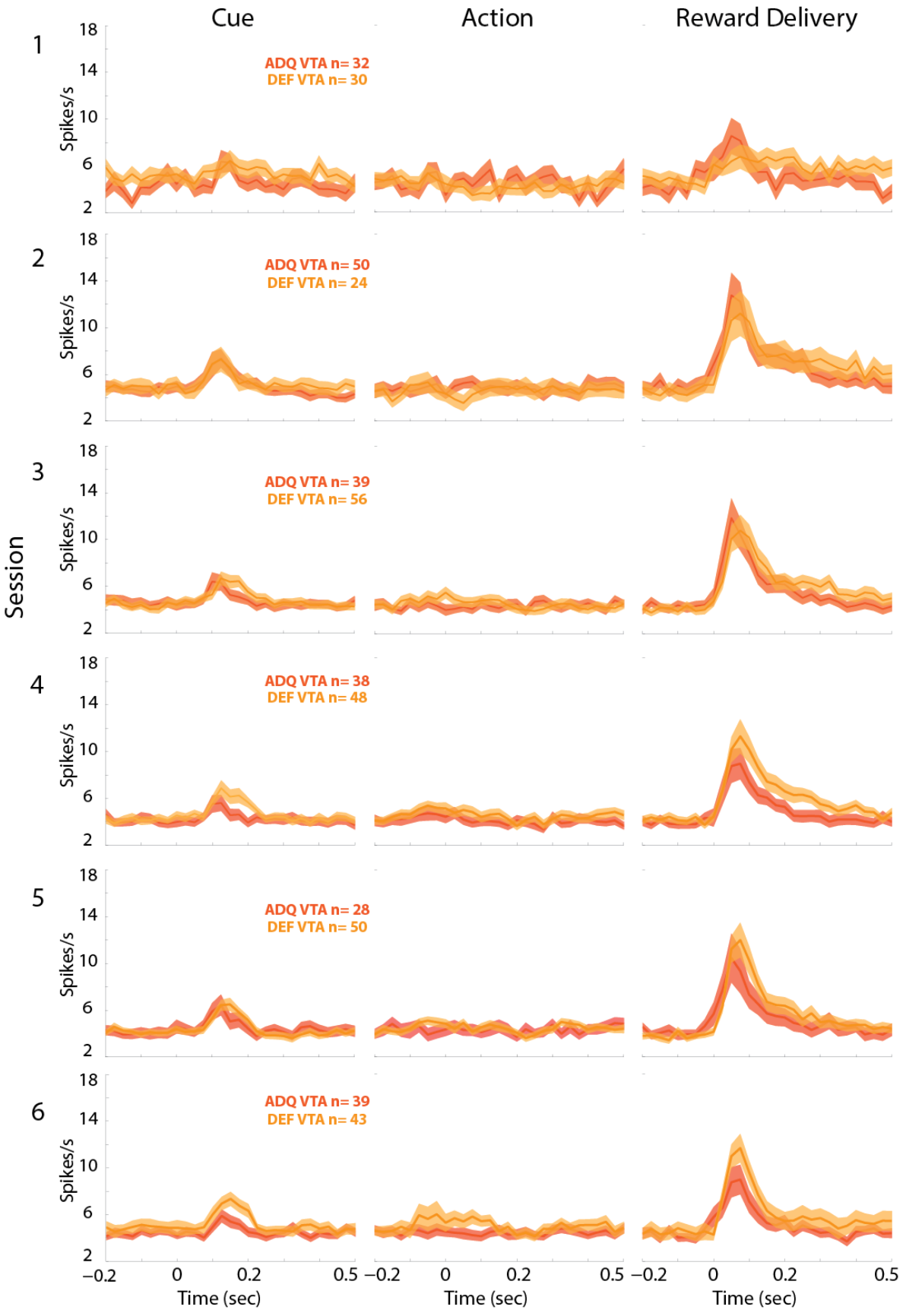

Cue
Action
Reward Delivery
Session
1
2
3
4
5
6
ADQ VTA n= 32
DEF VTA n= 30
ADQ VTA n= 50
DEF VTA n= 24
ADQ VTA n= 39
DEF VTA n= 56
ADQ VTA n= 38
DEF VTA n= 48
ADQ VTA n= 28
DEF VTA n= 50
ADQ VTA n= 39
DEF VTA n= 43
Spikes/s
18
14
10
6
2
−0.2
0
0.2
0.5
Time (sec)

Figure 4-2 Session by session raw firing rate population activity in VTA of ADQ and DEF animals
Average firing rate in spikes per second with standard error measure in shading for all recorded VTA units in ADQ (dark orange) and DEF (light orange) animals during instrumental conditioning. Activity is aligned to the time of the illumination of the nose poke port (left column), execution of the nose poke action (middle column), and reward delivery (right column). The cue and reward evoke phasic activity in both dietary groups, but neither showed any significant activity during the action execution. There was no consistent significant difference between the timing or amplitude of population activation in ADQ and DEF VTA units. Number of units recorded in each region during the session is depicted in the upper right corner of the graphs in the left column.

Across learning, peak neuronal activity in both regions change in response to cue (VTA session: $F_{5,465}=4.153$, $p=0.001$; SNc session: $F_{5,411}=3.169$, $p=0.008$), indicating that both regions play a role in encoding the importance of this cue in guiding behavior. Our analysis of population response across sessions also revealed a significant effect of diet in SNc for both cue presentation (diet: $F_{5,411}=30.846$, $p<0.0005$) and pellet delivery (diet: $F_{5,411}=23.614$, $p<0.0005$). This effect was not present during either event in our comparisons of how VTA activity changes across learning in ADQ and DEF animals, further suggesting that SNc physiology is more vulnerable to dietary deficiency.

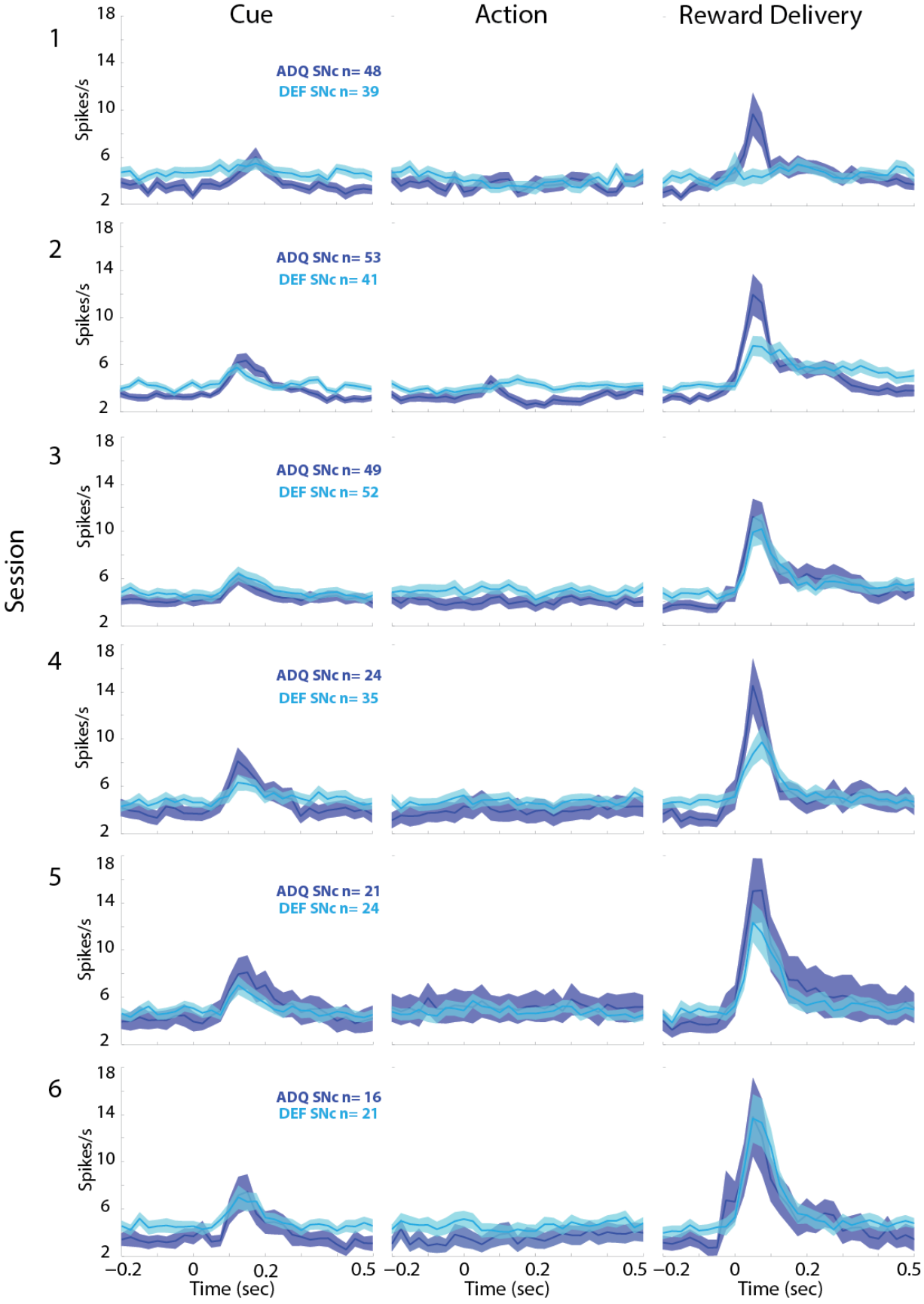

Cue
Action
Reward Delivery
Session
1
2
3
4
5
6
ADQ SNc n= 48
DEF SNc n= 39
ADQ SNc n= 53
DEF SNc n= 41
ADQ SNc n= 49
DEF SNc n= 52
ADQ SNc n= 24
DEF SNc n= 35
ADQ SNc n= 21
DEF SNc n= 24
ADQ SNc n= 16
DEF SNc n= 21
Spikes/s
18
14
10
6
2
−0.2
0
0.2
0.5
Time (sec)

Figure 4-3 Session by session raw firing rate population activity in SNc of ADQ and DEF animals
Average firing rate in spikes per second with standard error measure in shading for all recorded SNc units in ADQ (dark blue) and DEF (light blue) animals during instrumental conditioning. Activity is aligned to the time of the illumination of the nose poke port (left column), execution of the nose poke action (middle column), and reward delivery (right column). The cue and reward evoke phasic activity in both dietary groups, but neither showed any significant activity during the action execution. Our analysis of population response across sessions revealed a significant effect of diet in SNc for both cue presentation (diet: $F_{5,411}$=30.846, p<0.0005) and pellet delivery (diet: $F_{5,411}$=23.614, p<0.0005). Examination of individual sessions revealed an interaction between diet and how neural activity changed in response to reward delivery in session 1 (diet x time: $F_{39,3315}$=3.014 p=0.004), session 2 (diet x time: $F_{39,3588}$=4.202, p=0.002) and session 4 (diet x time: $F_{39,2223}$=2.902 p=0.012). The number of units recorded in each region during the session is depicted in the upper right corner of the graphs in the left column.

We wondered whether the diminished response observed in the DEF animals was due to less neurons responding, or the same proportion of neurons responding at a reduced amplitude. To address this question, we identified neurons which were significantly modulated by task events as compared to baseline activity. We then compared the proportions of activated and inhibited neurons between diets within each region. Once again, we found significant effects of n-3 PUFA dietary deficiency in SNc, but not VTA (Figure 4-4; Table 4-1). These was no observable effect of diet on size of responsive population in the VTA during any session or event. Deficient animals had a significantly smaller proportion of SNc neurons activated by cue presentation (sessions 2 and 4) and reward delivery (session 3) (Figure 4-5; Table 4-2). Differences in these responsive subpopulations were not present in later sessions, once the task had been learned and behavioral performance had plateaued.

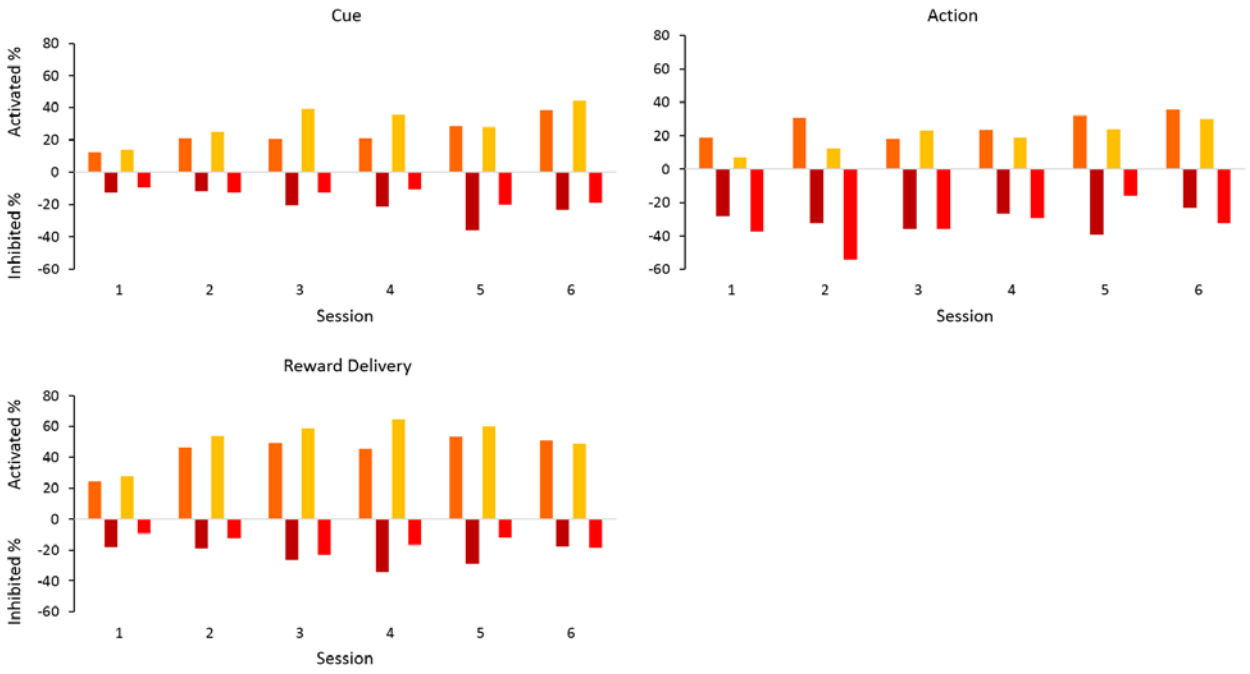


Figure 4-4 Responsive neurons in VTA of ADQ and DEF animals during instrumental conditioning
We found no consistent differences in the proportions of activated (dark orange (ADQ) and light orange (DEF) on the positive y-axis) and inhibited (dark red (ADQ) and light red (DEF) on the negative side of the y-axis) neurons. There were no significant differences found between dietary groups.

	Activated χ2					Inhibited χ^2		
Session	**Cue**	**Action**	**Reward**		**Session**	**Cue**	**Action**	**Reward**
1	0.033542	2.408166	0.098914		**1**	0.196872	0.681323	2.317194
2	0.139888	2.923077	0.000679		**2**	0.014543	3.169491	0.524655
3	3.749765	0.383509	0.242069		**3**	1.11011	0.000336	0.097437
4	2.122298	0.312016	1.255302		**4**	1.869868	0.08568	1.858409
5	0.002894	0.604898	0.303896		**5**	2.324631	5.281973	2.184
6	0.276123	0.297168	0.048897		**6**	0.248909	0.910951	0.005887
	p-values for Activated units					**p-values for Inhibited units**		
Session	**Cue**	**Action**	**Reward**		**Session**	**Cue**	**Action**	**Reward**
1	0.854684	0.120704	0.753136		**1**	0.657258	0.409132	0.127951
2	0.708392	0.087321	0.979211		**2**	0.904013	0.075026	0.468863
3	0.052815	0.535731	0.622716		**3**	0.292058	0.985385	0.754927
4	0.145169	0.576446	0.262542		**4**	0.17149	0.769743	0.172809
5	0.957096	0.436715	0.58145		**5**	0.12734	0.021547	0.139452
6	0.599253	0.585664	0.824994		**6**	0.617844	0.339862	0.93884

Table 4-1 χ^2 results for proportions of responsive neurons in ADQ v DEF VTA
Results of chi-square comparisons of proportions of event-responsive neurons revealed no significant differences between dietary groups ($p<0.015$, correcting for comparisons across multiple events**).**

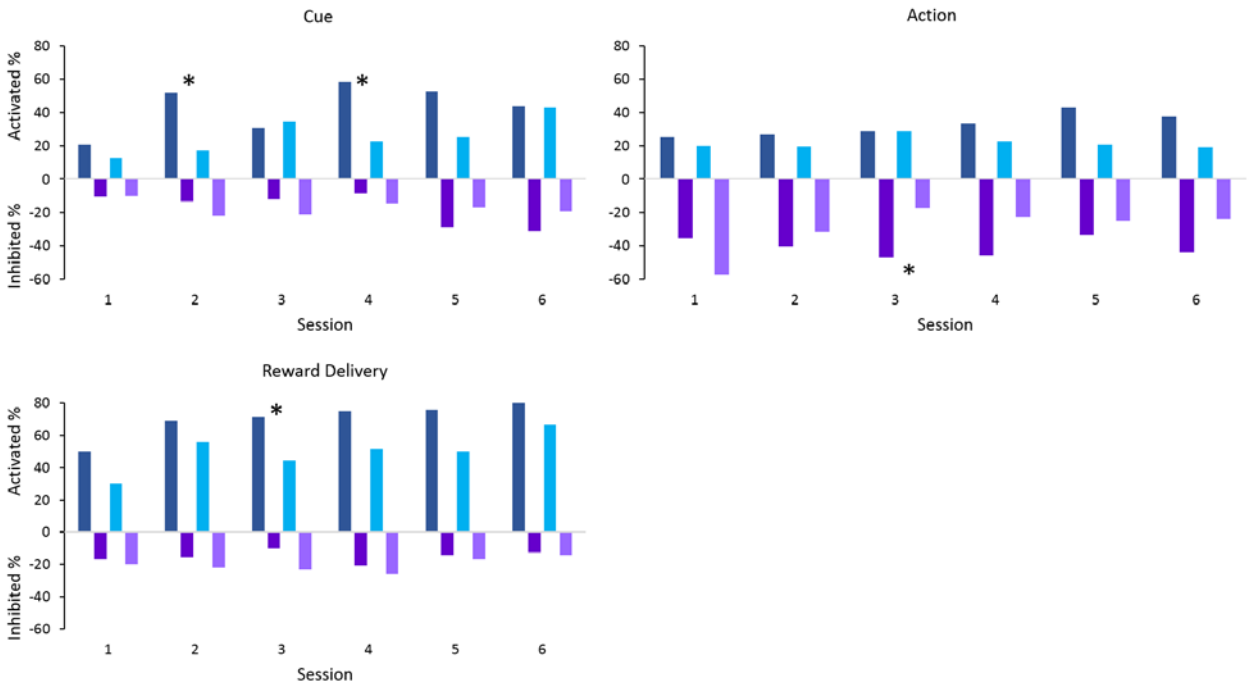


Figure 4-5 Responsive neurons in VTA of ADQ and DEF animals during instrumental conditioning
We found no consistent differences in the proportions of activated (dark orange (ADQ) and light orange (DEF) on the positive y-axis) and inhibited (dark red (ADQ) and light red (DEF) on the negative side of the y-axis) neurons. There were no significant differences found between dietary groups.

	Activated χ2					Inhibited χ^2		
Session	**Cue**	**Action**	**Reward**		**Session**	**Cue**	**Action**	**Reward**
1	1.071537	0.310588	3.610256		**1**	0.004126	4.291528	0.162963
2	**12.00445**	0.697154	1.704829		**2**	1.159958	0.744232	0.661743
3	0.183781	0.00093	**7.632942**		**3**	1.43036	**10.23333**	2.986355
4	**7.662683**	0.790587	3.325621		**4**	0.482405	3.442498	0.187413
5	3.572179	2.534768	3.268307		**5**	0.918367	0.378606	0.048335
6	0.00295	1.567769	0.979244		**6**	0.734564	1.647748	0.024777
	p-values for Activated units					**p-values for Inhibited units**		
Session	**Cue**	**Action**	**Reward**		**Session**	**Cue**	**Action**	**Reward**
1	0.300598	0.577319	0.057424		**1**	0.948786	0.038303	0.686443
2	**0.000531**	0.403742	0.191658		**2**	0.281474	0.388309	0.415945
3	0.668144	0.975669	**0.005731**		**3**	0.231706	**0.001379**	0.083969
4	**0.005637**	0.373923	0.068208		**4**	0.487335	0.06354	0.665078
5	0.058755	0.111363	0.07063		**5**	0.337904	0.53835	0.825986
6	0.956688	0.210531	0.322386		**6**	0.391408	0.199266	0.874925

Table 4-2 χ^2 results for proportions of responsive neurons in ADQ v DEF SNc
Significant results of chi-square comparisons of proportions of event-responsive neurons between dietary groups are marked in bold. ($p<0.015$, correcting for comparisons across multiple events**)**

We are particularly interested in the effect of n-3 PUFA dietary deficiency on the electrophysiological properties and activity of dopamine neurons. Considering the role of dopamine dysfunction in the development and perpetuation of psychiatric disorders, it is important to understand how n-3 PUFA deficiency impacts dopamine specifically. To determine whether this environmental insult specifically affected this important population of neurons, we divided units into putative dopaminergic (Type 1) and putative non-dopaminergic (Type 2). The classification method used has been verified using optogenetics (Cohen et al., 2012; Eshel et al., 2015), and we imposed further requirements in accordance with accepted physiological characteristics. We found that the results of this classification produce divisions of Type 1 and Type 2 neurons in each region which do not reflect the anatomical data (Figure 4-6). This is most likely due to the problems inherent in functional neuronal classification, which are well known in the dopamine electrophysiological field (Ungless & Grace 2012; Margolis et al., 2009). Within this type 1 population, we found no significant difference in the baseline firing rate between dietary groups in VTA. In the SNc, baseline FR of Type 1 ADQ neurons was significantly lower ($p<0.001$) than neurons recorded from DEF SNc.

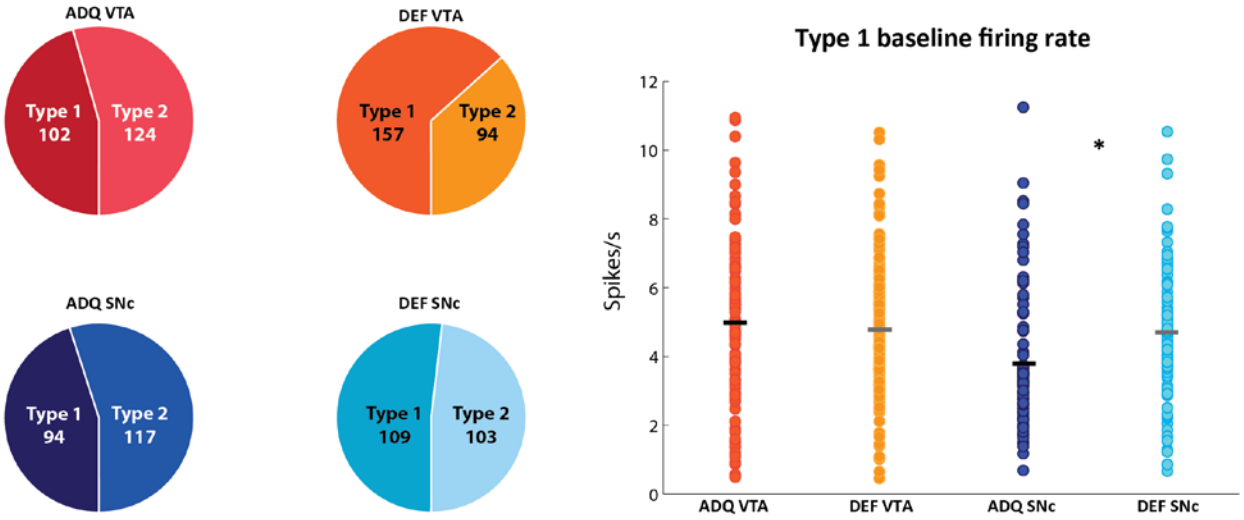


Figure 4-6 Proportions and baseline firing rates for putative dopamine neurons in ADQ and DEF animals
(Left) Proportions of type 1 (putative dopamine neurons) and type 2 (putative non-dopamine neurons) in each region for each dietary group. (Right) Baseline firing rates of Type 1 neurons in the VTA and SNc from ADQ and DEF animals, excluding any putative fast-spiking interneurons with a baseline ≥50 Hz (n=6). Within this type 1 population, we found no significant difference in the baseline firing rate between dietary groups in VTA. In the SNc, baseline FR of Type 1 ADQ neurons was significantly lower ($p<0.001$ marked by asterisk) than neurons recorded from DEF SNc.

Across learning Type 1 neurons in VTA and SNc of all animals display phasic response to cue and reward delivery, but not execution of the nose poke. There are no consistent differences in VTA event-evoked activity between ADQ and DEF type 1 neurons (Figure 4-7), though there is a significantly prolonged response in DEF VTA to pellet within session 3 (diet x ses: $F_{19,912}=3.463$, $p=0.012$). Type 1 units in DEF SNc shows a smaller response to reward delivery, and cue across learning (Figure 4-8; diet cue: $F_{1,191}=7.681$, $p=0.006$; reward: $F_{1,191}=21.644$, $p<0.0005$). The DEF SNc also displays a reduced response to cue during behavioral maintenance in session 5 (diet x time: $F_{19,513}=3.424$, $p=0.013$). What was evident in the overall population response is further emphasized here when looking at only putative dopamine neurons in the SNc. DEF SNc is less responsive to reward-related events, especially during acquisition of the action-outcome relationship. The interaction between diet and reward-

evoked neural activity diminishes in later maintenance sessions as behavior becomes more consistent and purposeful.

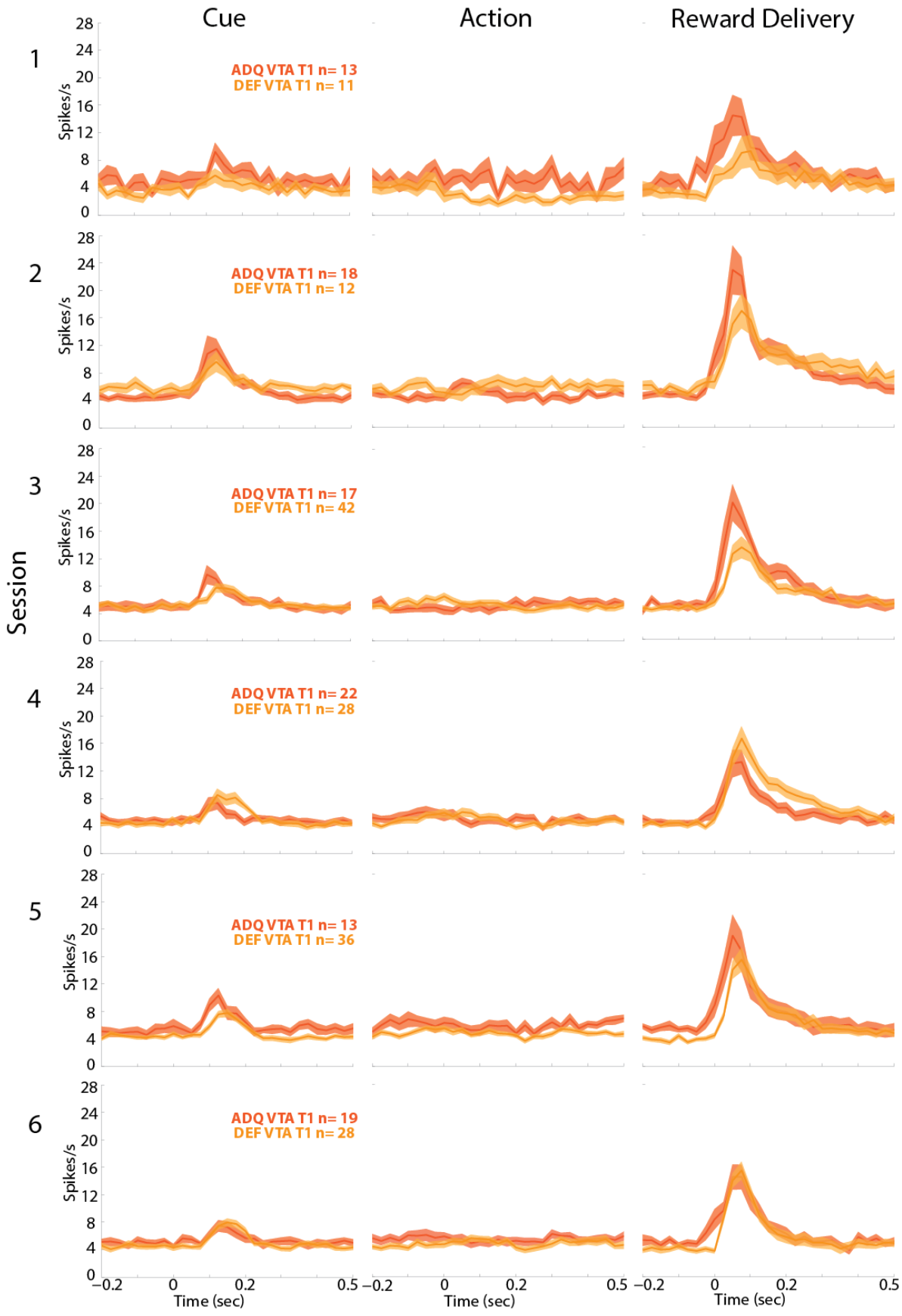
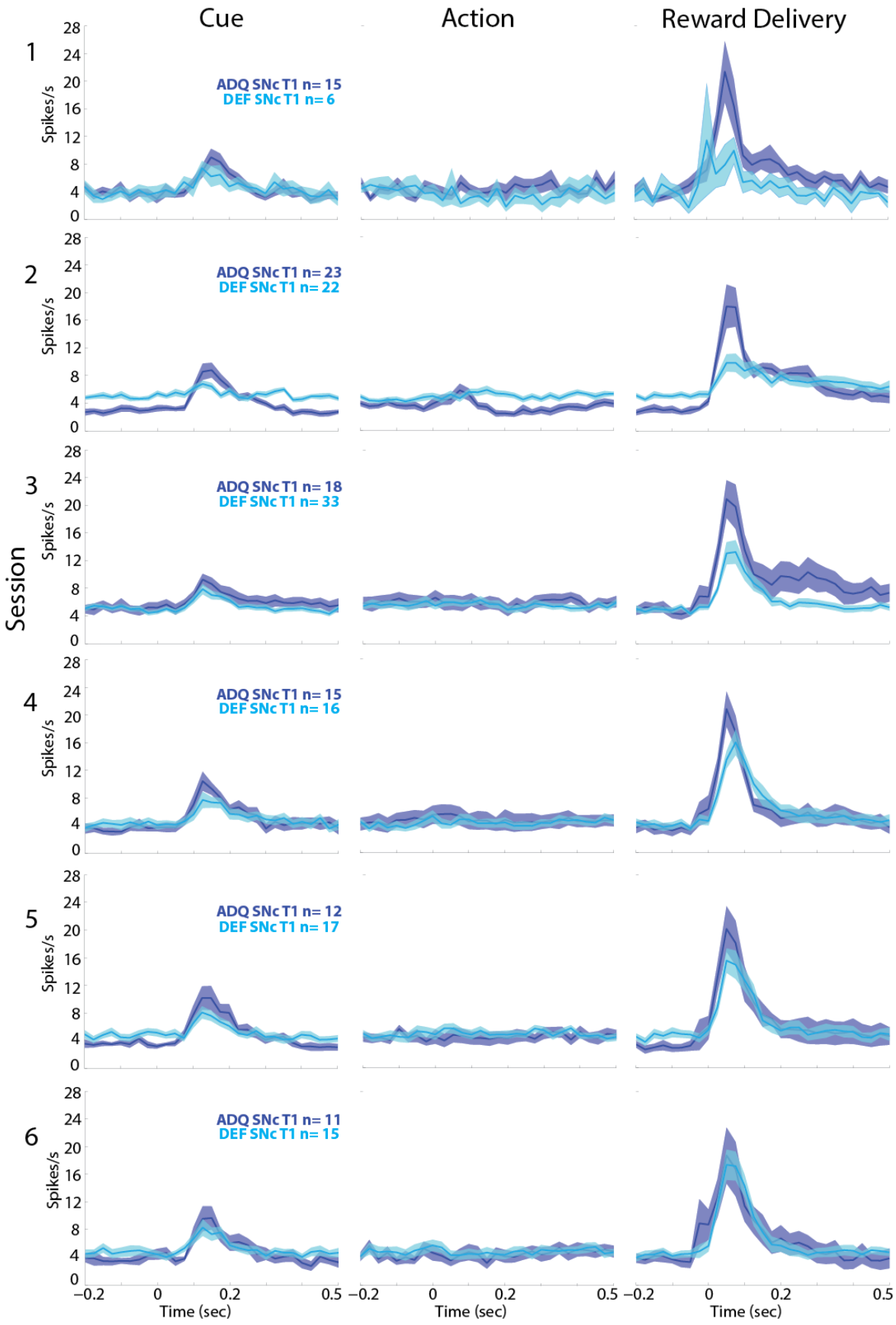

Cue
Action
Reward Delivery
Session
1
2
3
4
5
6
ADQ VTA T1 n= 13
DEF VTA T1 n= 11
ADQ VTA T1 n= 18
DEF VTA T1 n= 12
ADQ VTA T1 n= 17
DEF VTA T1 n= 42
ADQ VTA T1 n= 22
DEF VTA T1 n= 28
ADQ VTA T1 n= 13
DEF VTA T1 n= 36
ADQ VTA T1 n= 19
DEF VTA T1 n= 28
Spikes/s
0
4
8
12
16
20
24
28
−0.2
0
0.2
0.5
Time (sec)

Figure 4-7 Session by session raw firing rate population activity of putative dopamine neurons in VTA of ADQ and DEF animals

Population activity of type 1 VTA neurons in ADQ (dark orange) and DEF animals (light orange) across behavioral sessions. Data are depicted as mean ± SE and aligned to the event of interest, with each event displayed in its own column and each session is in its own row to display how the response to that event changed across conditioning. The number of neurons recorded from each dietary group in each session are depicted in the top right corner of the top row of panels**.**


Cue
Action
Reward Delivery
Session
1
2
3
4
5
6
Spikes/s
ADQ SNc T1 n= 15
DEF SNc T1 n= 6
ADQ SNc T1 n= 23
DEF SNc T1 n= 22
ADQ SNc T1 n= 18
DEF SNc T1 n= 33
ADQ SNc T1 n= 15
DEF SNc T1 n= 16
ADQ SNc T1 n= 12
DEF SNc T1 n= 17
ADQ SNc T1 n= 11
DEF SNc T1 n= 15
0
4
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−0.2
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0.2
0.5
Time (sec)

Figure 4-8 Session by session raw firing rate population activity of putative dopamine neurons in SNc of ADQ and DEF animals

Population activity of type 1 SNc neurons in ADQ (dark blue) and DEF animals (light blue) across behavioral sessions. Data are depicted as mean ± SE and aligned to the event of interest, with each event displayed in its own column and each session is in its own row to display how the response to that event changed across conditioning. The number of neurons recorded from each dietary group in each session are depicted in the top right corner of the top row of panels**.** Type 1 units in DEF SNc shows a smaller response to reward delivery, and cue across learning (diet cue: $F_{1,191}$=7.681, p=0.006; reward: $F_{1,191}$=21.644, p<0.0005). The DEF SNc also displays a reduced response to cue during behavioral maintenance in session 5 (diet x time: $F_{19,513}$=3.424, p=0.013).

In addition to changes in rate encoding, we were interested in how n-3 PUFA deficiency affects functional connectivity. Spike count correlations are used to measure to what extent neurons alter their firing patterns in similar ways across trials. When these measures increase or decrease across learning, it reflects plasticity-induced changes in synaptic strength between neurons or in modulation from cortical afferents. We found that n-3 PUFA dietary deficiency has a profound effect on the strength of these correlations and how they change with improvement in behavioral performance. As with our measures of population activity and responsiveness, these effects were more pronounced in the SNc. While there is an overall decreased level of correlation in the SNc of deficient animals, there are also differences in the trends of change across sessions. The correlations in ADQ SNc during cue presentation tend to increase with learning, creating a significant difference in the level of correlation between ADQ and DEF SNc neurons in sessions 2-6 (Table 4-3). Coordination between neurons in the SNc of DEF animals does not appear to be modulated by afferents as a response to instrumental conditioning, as we observed no consistent relationship between session number and the strength of spike correlation (Figure 4-10). In opposition to our observation in SNc, DEF VTA neurons show an overall higher level of correlation across learning as compared to VTA neurons in ADQ animals (Figure 4-9). This led to significant differences between dietary groups in VTA synchronization, particularly during the cue, but these differences did not have a clear relationship with the progression of conditioning.

Together, these results demonstrate that n-3 PUFA dietary deficiency causes significant alterations in network dynamics during instrumental conditioning, indicating the deprivation of this essential fatty acid affects functional connectivity and interactions between the midbrain and cortical afferents.

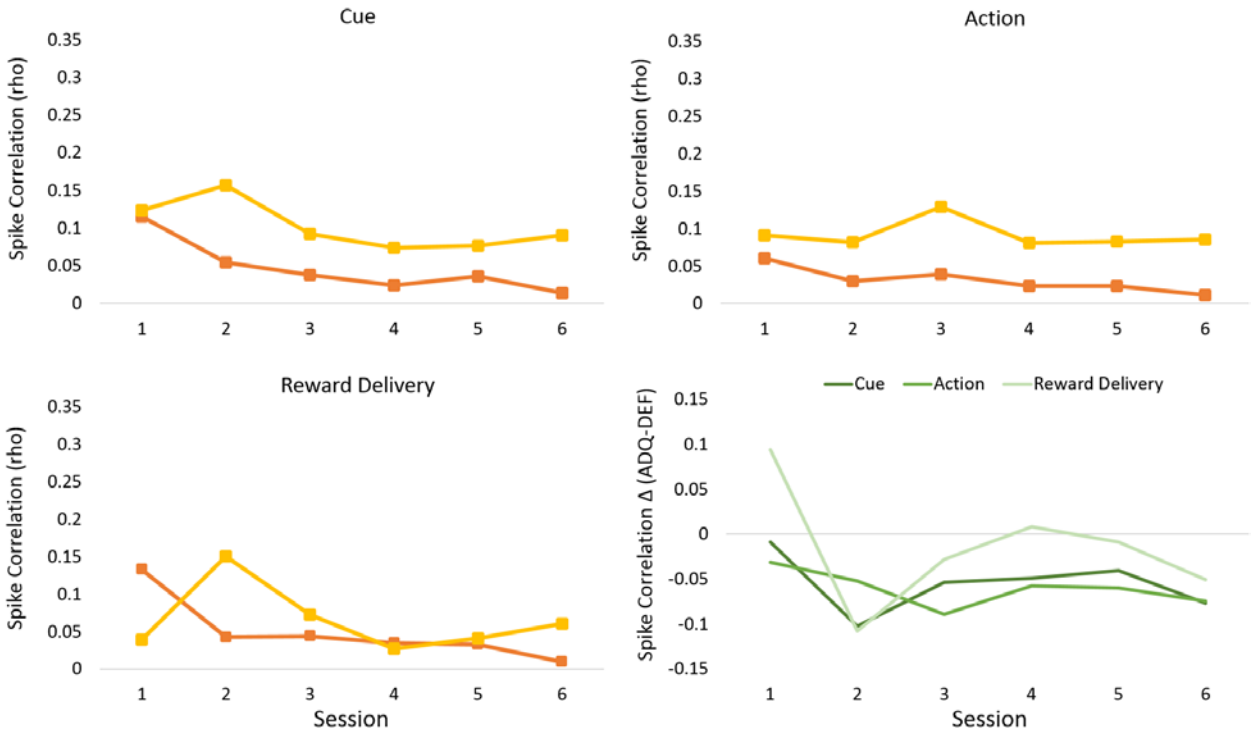

Figure 4-9 Spike correlation in ADQ and DEF VTA during instrumental conditioning
Plots depict the average spike correlation, as quantified by Pearson's rho, of simultaneously recorded ADQ (dark orange) and DEF (light orange) VTA neurons across 6 sessions of instrumental conditioning. Deficient neurons do not significantly modulate their activity with conditioning. Plotting differences between diets (ADQ – DEF) in spike correlation (bottom right) show consistently less synchronization in ADQ VTA as compared to DEF.

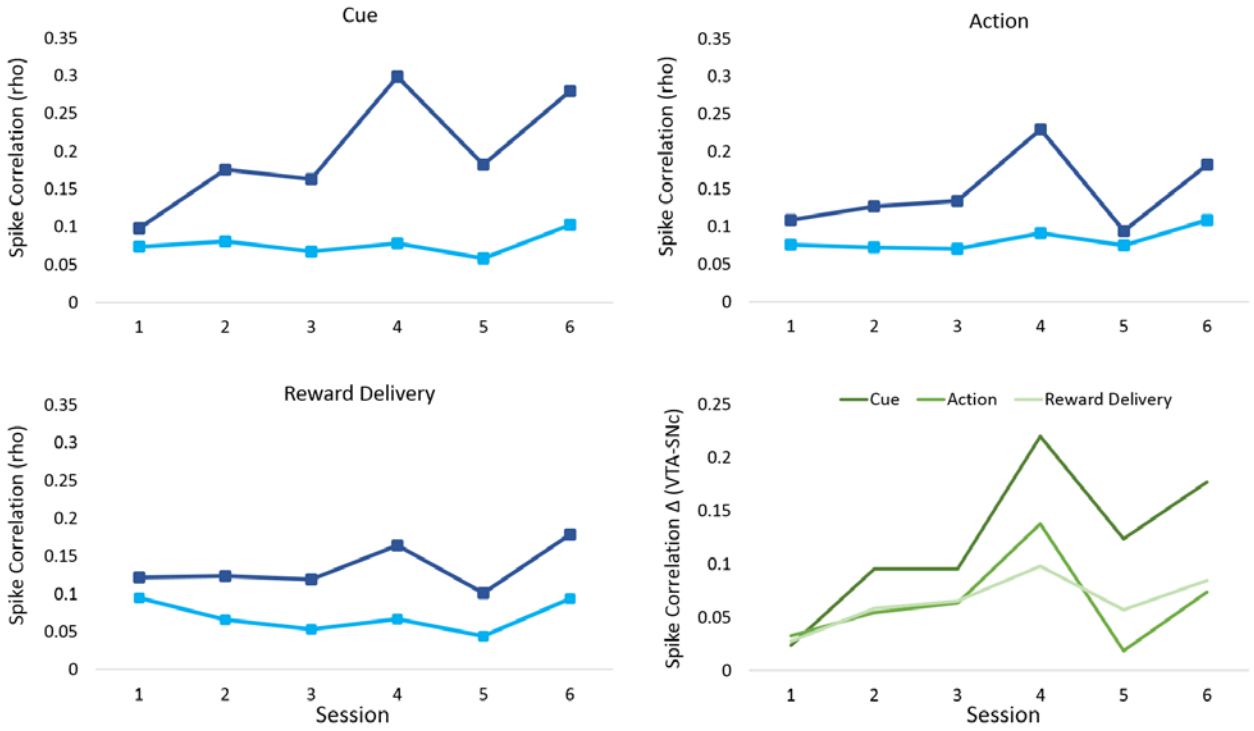

Figure 4-10 Spike correlation in ADQ and DEF SNc during instrumental conditioning
Plots depict the average spike correlation, as quantified by Pearson's rho, of simultaneously recorded ADQ (dark blue) and DEF (light blue) SNc neurons across 6 sessions of instrumental conditioning. As observed in VTA, deficient SNc neurons do not significantly modulate their activity with conditioning. Plotting differences between diets (ADQ – DEF) in spike correlation (bottom right) show consistently more synchronization in ADQ SNc as compared to DEF

	VTA				pvalues	p<0.015
	1	2	3	4	5	6
Cue	0.7439	**0.0007**	**0.0041**	**0.0107**	0.1876	**5.53E-06**
Action	0.5760	0.0523	**1.66E-05**	**0.0033**	**0.0069**	**6.59E-06**
Reward	0.0237	**0.0010**	0.1400	0.8305	0.6862	**0.0003**

	SNc				pvalues	p<0.015
	1	2	3	4	5	6
Cue	0.1921	**2.55E-05**	**1.20E-07**	**2.24E-05**	**0.0039**	**0.0078**
Action	0.1089	0.3298	**8.49E-05**	**0.0019**	0.6222	0.1852
Reward	0.2015	0.1243	**0.0003**	**0.0139**	0.1202	0.1056

Table 4-3 Results of t-tests comparing spike correlation between midbrain ADQ and DEF neurons
The p-value for t-tests conducted to identify differences between dietary groups in functional connectivity across conditioning in VTA and SN neurons. We analyzed spike correlation during three events of interest across 6 sessions of instrumental conditioning. Significant results are marked in bold (p<0.015).

4.4 DISCUSSION

The present study demonstrates that a diet deficient in n-3 PUFAs across generations has a significant impact on the physiology and function of the midbrain dopamine system in rodents. This impact appears to be specific to the nigrostriatal pathway. The subtleness of this effect is consistent with a lack of gross disruption movement or motivated behavior, but may make the system more vulnerable to negative genetic or environmental factors. These data may, therefore, provide insight into the effects of a common environmental insult, which could be a contributing factor to the development or exacerbation of psychiatric disorders.

The disrupted response of SNc dopamine neurons in n-3 PUFA deficient animals was evident through several measures. These neurons had reduced response to salient task events during early sessions of instrumental conditioning. They also show an increase in baseline firing rate, which could affect the efficacy of the signal transmitted by dopamine's phasic response. In addition, functional connectivity, as measured by spike correlation, was diminished between SNc neurons, while VTA neurons in deficient animals were either unaffected or more synchronized.

Our experimental design focused on how this dietary environmental insult affects VTA and SNc neural activity during instrumental conditioning. The choice for this behavioral task was, in part, guided by the fact that this is a task that depends on proper functioning of dopamine neurotransmission. In addition, our previous observations (Section 3) had shown that dopamine neurons in both regions show phasic responses during instrumental conditioning, thus allowing us to potentially detect any subtle changes in encoding of task events by dopamine neurons. A caveat in this task selection may be that our dietary manipulation had little to no impact on behavioral performance during this task. We, however, believe that the choice of a task which does not involved profound behavioral disruptions was a strength as it allowed us to focus on,

how network dynamics and neural responsivity are affected when the behavior is “locked”. Choosing to focus on behavior that is severely disrupted would have provided too many confounding factors for the initial stages of investigations in this animal model. Future studies can elucidate potential neuronal correlates of behavioral disruptions caused by n-3 PUFA deficiency.

To our knowledge, our study is the first to perform awake-behaving electrophysiology in n-3 PUFA deficient animals. We found that fatty acid dietary deficiency across generations causes significant alterations in the physiology of midbrain dopamine neurons, and neurons in the SNc are more severely impacted. This was evident in the higher baseline firing rate we observed in SNc Type 1 neurons. Baseline or ‘tonic’ activity in dopamine neurons is thought to provide gain for the phasic response to salient or rewarding events in the environment (Floresco et al 2013; Hage & Khaliq 2015; Schultz, 2002; Zhang et al., 2009). Higher baseline firing rate could indicate more ‘noise’ in the system, so that a signal would have to be stronger, standing out sufficiently against background activity to provoke a response. This could contribute to the slower development of phasic activity we found in the SNc of DEF animals. Considering that activity in these neurons eventually reached the same levels as their ADQ counterparts, our data suggests that there is a no gross physiological defect causing this delay. It is more likely that the population dynamics, which are known to regulate tonic activity in dopamine neurons (Floresco et al., 2013), have shifted in response to our dietary environmental insult. This possibility is further supported by our spike correlation data, which show that functional connectivity in DEF neurons is not modulated in the same manner as ADQ neurons during instrumental conditioning.

Neurons in SNc of animals on the n-3 PUFA deficient diet show delayed development of phasic response to reward, reduction in number of responsive neurons, and reduced coordination

which does not change to reflect conditioning. The observed differences in spiking activity were often more pronounced in early learning, and diminished as the behavior became focused and consistently executed. Impairments in this stage of conditioning could be indicative of problems in information processing or formation of associations, which may manifest as learning impairments in more challenging behavioral tasks. Reduced overall functional connectivity suggests disruption in the anatomy of neurons in the SNc and how they form synapse with adjacent neurons or alterations in afferent modulation. The dynamic interactions between neurons represented by spike correlations are crucial to cognitive flexibility and coordination between brain regions to appropriately guide behavior. Subtle deficiencies in network interactions could be an underlying cause or contributor to the increased anxiety and distractibility previously observed in these animals (Bondi et al., 2014). Investigations into the impact of n-3 PUFA on SNc anatomy and circuit formation would shed further light on the mechanism behind the observed change in functional connectivity.

Though the amplitude of VTA population response in putative dopamine neurons may be mildly affected, other measures of VTA neuronal function, such as overall population activity and proportion of responsive neurons, did not differ significantly between dietary groups. This finding of differential effects across the midbrain is consistent with other research into the impact of environmental insult on dopamine. VTA neurons are known to survive environmental stress and pharmacological manipulations which selectively obliterate nearby SNC neurons. (Burns et al., 1983; Hung and Lee, 1998; Rodriguez et al., 2001; McCormack et al., 2006). Much of this data comes from research into Parkinson's disease and furthering our understanding of why neurons in the SNc may be more susceptible to the impact of pesticides, oxidative stress, and intrauterine drug exposure (Hirsch, 1992; Saxena & Caroni 2011; McCormack et al., 2002;

Barlow et al 2007). This research in conjunction with our finding supports the pursuit of understanding the role of the nigral system in cognitive and affective symptoms of Parkinson's and other psychiatric disorders where SNc vulnerability may be underestimated.

A wealth of research points to dopamine dysfunction as a key contributor to the debilitating cognitive and emotional effects of mental illness. Additionally, previous work from our lab and others has demonstrated a profound impact of n-3 PUFA deficiency on multiple aspects of the dopamine system, including protein expression, neurotransmission, and behavior (Bondi et al., 2014; Fedorova & Salem, 2005; Kid, 2007; Kuperstein et al., 2008; Zimmer et al., 2002). Our data provide a strong link between n-3 PUFA and dopamine physiology, suggesting that addressing n-3 PUFA deficiency may be a feasible, safe, and cost-effective intervention to support healthy dopamine function. In addition to treating symptoms or "correcting" dysfunction, n-3 PUFA may have potential as a preventive strategy, especially in adolescents at high risk to develop psychiatric disorders such as schizophrenia (Amminger et al., 2010; Amminger et al., 2015; Berger et al., 2007, Fenton et al., 2000; Freeman, 2000; Gama et al., 2012; Kokacya et al., 2015; Mossaheb et al., 2013; Peet et al., 2001; Richardson, 2004; Zugno et al., 2014), mood disorders, (Banni & Marzo, 2010; Chalon, 2006; Conklin et al., 2007; Dyall, 2015; Ferraz et al., 2011; Kidd, 2007; Liu et al., 2013; Su et al., 2015) and ADHD (Dean et al., 2014; DeMar et al., 2006; Fontani et al., 2005; Hamazaki et al., 1996; Karr et al., 2013; Moriguchi et al., 2000; Richardson, 2009, Vancassel et al., 2007; Widenhorn-Müller et al., 2014). Future studies in adolescent models that assess the role of n-3 PUFA supplementation on dopamine physiology are needed to determine whether dietary correction will positively impact the function of the dopamine system.

5.0 GENERAL DISCUSSION

5.1 SUMMARY OF FINDINGS

Our data demonstrate that VTA and SNc are phasically activated by stimuli associated with reward during conditioning, producing similar firing rate output. These similarities do not imply that these midbrain regions are entirely redundant though. In fact, they develop distinct patterns of functional connectivity in response to associative learning, mostly likely through the influence of different coordinated inputs to each region. This ability to dynamically encode salient stimuli by modulating correlation structure is disrupted in n-3 PUFA dietary deficiency, an environmental insult known to disrupt the dopamine system. Measures of dynamic coordination structures during associative learning may provide a functional readout of how n-3 PUFA contributes to dopamine dysfunction in psychiatric disorders.

Using simultaneous recordings from neurons in VTA and SN, we demonstrated that the regions share common electrophysiological and pharmacological characteristics, including baseline firing rate, apomorphine response, and putative dopamine classification. We investigated neuronal activity in both regions during Pavlovian conditioning, in which animals learned to associate a neutral cue with a rewarding outcome, and in instrumental conditioning, in which an animal performed the prescribed action to achieve an outcome. In our observations of neuronal activity during these two behavioral paradigms, we found that VTA and SNc exhibited similar patterns of phasic response to salient task events, such as cue presentation and reward

delivery, on an overall population level and on the level of dopamine neuronal activity. Neither region exhibited an action-evoked or action-preparatory response during instrumental conditioning. In our comparisons of these two distinct dopaminergic regions, the main observable difference between the two regions was the pattern of change in spike correlations as learning progressed, which likely reflects differences in functional connectivity We propose that the divergence in coordination structure between VTA and SN is due to differences in afferent projections, and in how those afferents respond to two distinct forms of conditioning. Altering connections within and between networks of neurons is crucial to the brain's ability to track changes in the environment and encode newly acquired information. This dynamic coordination between midbrain neurons in response to conditioning is disrupted in animals with a dietary n-3 PUFA deficiency. We assert that dysfunction in interactions with other regions of the brain, and the resulting lack of coordination, is responsible for the lack of dynamic encoding to conditioning observed in n-3 PUFA deficient animals.

5.2 COMMON HINDBRAIN INPUT DRIVES PHASIC MIDBRAIN ACTIVITY

Similarities in VTA and SNc phasic activity suggest common bottom-up input driving neurons in both regions. We found that neurons in both midbrain regions respond to relevant cues in the environment as well as the delivery of a sugar pellet reward during two distinct conditioning paradigms. Processing these events would involve short-latency input from the visual and gustatory sensory systems. Anatomical studies have shown that hindbrain nuclei, specifically the superior colliculus (SC) and parabrachial nucleus (PBN) (Figure 5-1), are crucial to these modalities and send strong projections to the midbrain (Watabe-Uchida et al., 2012;

Comoli et al., 2003, Coizet et al., 2007, Coizet et al., 2010; Geisler & Zahm, 2005; Karimnamazi & Travers 1998). Sensory stimuli response latencies in these regions are shorter than those of midbrain dopamine neurons (Overton et al., 2014; Morris et al., 2004; Takikawa et al., 2004), suggesting that the SC and PBN are well-situated to provide short latency sensory input to the VTA and SNc. In fact, simultaneous recordings of hindbrain and dopamine neurons reveal a direct link between the two areas (Overton et al., 2014). These experiments show that disabling the SC and PBN directly affects dopaminergic responses to sensory stimuli (Hajnal & Norgren, 2005; Coizet et al., 2010), including light flashes and sucrose solution consumption. Projections from SC and PBN to midbrain are highly conserved across species (Comoli et al., 2003; McHaffie et al., 2006; May et al, 2009), indicating that these nuclei play a critical role in bottom-up processing of relevant environmental stimuli. Additional studies using optogenetic or pharmacological inactivation of hindbrain terminals in the VTA and SNc would test this model, furthering our understanding of how the SC and PBN might contribute to dopamine's phasic encoding of salient stimuli during conditioning. Elucidating the role of this sensory circuitry in associative learning would further define to commonalities or differences present in VTA and SNc, allowing us to pinpoint sources of dysfunction more easily.

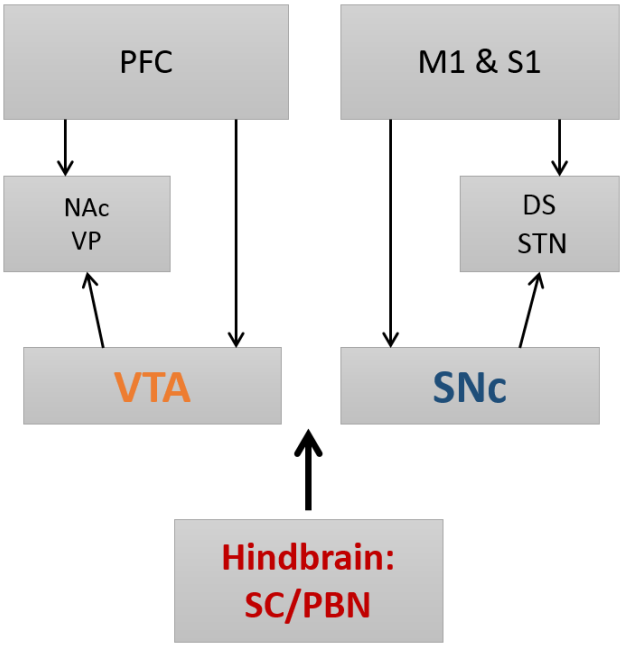

Figure 5-1 A simplified schematic of VTA and SN associated circuits
Diagram depicting two distinct but related dopamine innervated circuits that were the focus of this dissertation. Our working hypothesis was that these two midbrain dopamine neuron groups are regulated differently during associative learning, and that this differential regulation is expressed in terms of changes in neuronal response to salient events. We found that VTA and SNc produce similar phasic responses during salient events of two distinct conditioning paradigms. We propose that this commonality may be driven by common hindbrain inputs, most likely the superior colliculus (SC) and parabrachial nucleus (PBN) Abbreviations: ventral segmental area (VTA), substantia nigra pars compacta (SNc), dorsal striatum (DS), subthalamic nucleus (STN), nucleus accumbens (NAc), ventral pallidum (VP), prefrontal cortex (PFC), motor cortex (M1), somatosensory cortex (S1).

This parallel activation may seem unnecessary, but there is precedent for representing the same information in more than one circuit (Goldman-Rakic, 1988; Alexander et al., 1990; Bernard & Bandler, 1998; Clark, Hollon, & Phillips 2012). VTA and SNc extend efferent projections to different subcortical targets, and it is possible that the information provided by the rate code of spiking activity is required in multiple circuits to adaptively inform behavior. Alternatively, our population activity results could suggest the presence of two parallel circuits conveying learned associations redundantly to preserve important survival information if one circuit malfunctions. It is also important to consider that dopamine is a neuromodulator (Walters & Bergstrom, 1981; Lindvall & Bjorkland, 1979), and dopamine phasic activity is not

necessarily indicative of the excitatory drive we associate with glutamate. Thus, we must be careful in interpreting the purpose of these signals. Additionally, the phasic activity observed in the midbrain may have different impacts based on the microenvironment of the projection terminal (Kawagoe et al., 1992; Wightman et al., 2007; Melchior et al., 2015; Cachope & Cheer, 2014). Unfortunately, we cannot discern projection sites from chronic recordings in the midbrain (Swanson, 1982), though understanding the relationship between phasic activity and the impact on projection targets will be key to full comprehension of our results.

5.3 CONDITIONING AND REGION-SPECIFIC COORDINATION STRUCTURES

Spike correlation is a measure of the relationship between two simultaneously recorded neurons as their activity fluctuates across multiple presentations of the same stimulus (Cohen & Kohn 2011; Baeg et al., 2007, Kim et al., 2012; Moghaddam & Wood 2014). Spike correlation can also be thought of as changes in synaptic weight in response to learning or change in environment. This measure of coordination does not have a direct relationship to population firing activity and can remain stable even when neural activity overall changes (Baeg et al., 2007; Panzeri et al., 1999; Amit, 1997), therefore differences between the regions on this level can coexist with similarities in overall population activity. As two distinct populations of dopamine neurons, VTA and SNc receive different afferent projections from cortical structures, which are often GABAergic (Watabe-Uchida et al., 2012; Loughlin & Fallon, 1984; Carr & Sesack, 2000; Hnasko et al., 2012; Stuber et al., 2012). The effect of these inputs would be more accurately represented by subtle changes in how neurons interact and influence each other than by measuring overall phasic activity (von der Malsburg, Phillips, Singer 2010; Moghaddam &

Wood, 2014). In our model, we represent two possible ways afferents could be modulating spike correlation during conditioning (Figure 5-2). One possible mechanism is structural change, in which already existing connections could be strengthened through Hebbian plasticity. Alternatively, previously inactive inputs or circuits may come online, incorporating new neurons into the coordination structure as behavior changes. It is important to note that our data cannot discern between these two possibilities, but it does demonstrate that the network connections dynamically change with conditioning. Additionally, isolating and manipulating spike correlations to assess its impact on behavior is currently not technically feasible, therefore we cannot draw a causal link between changes in spike correlation and learning. One approach to further define relationship between this measure and the acquisition of an action-outcome or cue-outcome association would be examination of a behavioral measure which fluctuates trial-to-trial, such as food trough entries, in conjunction with the trial-to-trial fluctuations between simultaneously recorded neurons. This would more closely connect the correlation between two neurons to behavior, but would not provide a conclusive result. Dynamic coordination of neurons may be an important component of encoding the newly learned associations, and should be investigated further.

Being able to make cue-outcome or action-outcome connections is crucial to survival in the wild. Animals rely on such associations to guide behavior such as the smashing of a shell to get to the snail inside, or approaching the visual cue of a birdhouse based on its prediction of food. Pavlovian and instrumental conditioning provide unique insight into how cues and associations influence animal behavior (Rescorla and Solomon, 1967; Dickinson and Dawson, 1987; Colwill and Rescorla, 1988; Hall, 2002). Pavlovian conditioning makes sense of a world over which the subject has no control; instrumental conditioning involves the subject asserting

control over the situation to achieve the desired outcome. These aspects of learning and behavior interact and conflict with each other in our daily lives. Our spike correlation data demonstrate that these two conditioning paradigms differentially affect functional connectivity within distinct regions of the midbrain. This could reflect how learned associations in each conditioning paradigm recruit different cortical systems, which in turn modulate different pathways in subcortical neural networks. In understanding how each association is encoded on its own, it becomes easier to decipher how they interact in complex decision-making.

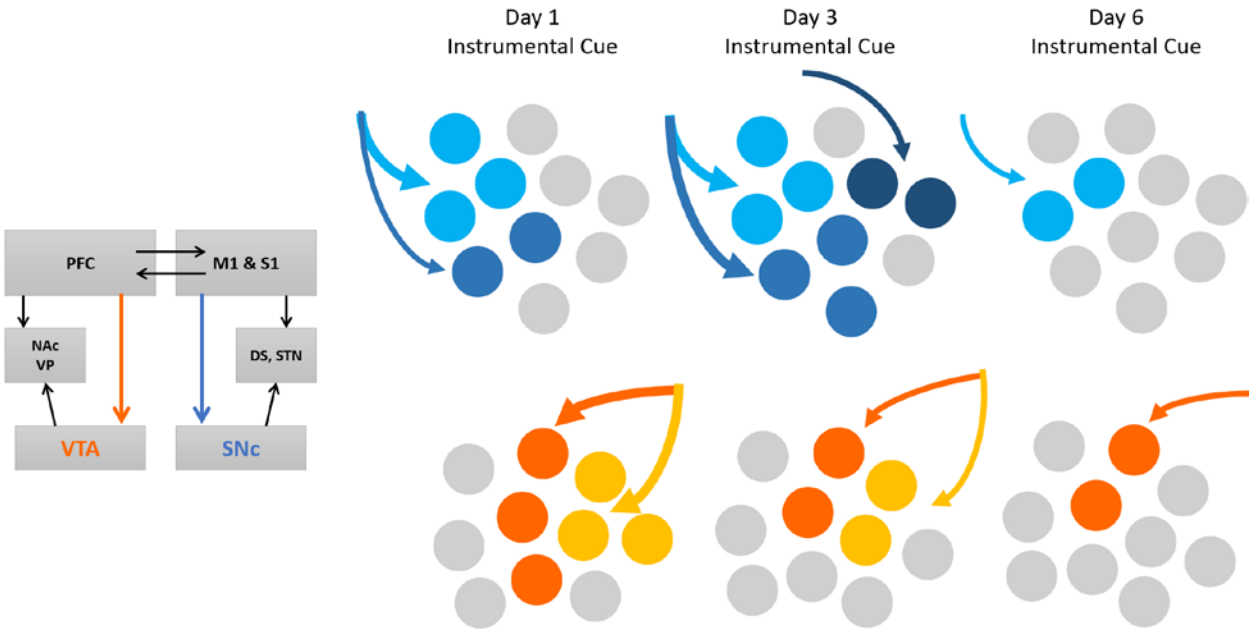

Figure 5-2 A model of coordination structures in VTA and SNc during instrumental learning that support flexible changes in functional connectivity
VTA and SN exhibit similar levels of correlation on the first day of conditioning but then proceed to follow different trajectories of change across learning. We represent two possible mechanisms for how these patterns of change could occur. One possible mechanism is structural change, in which already existing connections could be strengthened through Hebbian plasticity. We represent that here as changing thicknesses of already present arrows. Alternatively, previously inactive inputs or circuits may come online, incorporating new neurons into the coordination structure as behavior changes.

5.4 CHANGES RESULTING FROM DIETARY MANIPULATION AND IMPLICATIONS FOR DISEASE

Deficiency in omega-3 polyunsaturated fatty acids (n-3 PUFAs) have been identified as an environmental insult that affects cognitive function and the dopamine system, possibly aggravating or enabling the emergence of schizophrenia, major depressive disorder, and ADHD (Banni & Marzo, 2010; Wainwright et al., 1997; Connor et al.,1991; Amminger et al., 2010). Our data suggest that this effect may be mediated through the nigrostriatal system, as SNc is more severely affected by n-3 PUFA deficiency as compared to VTA. This is evident in the reduced phasic activity in the overall population and in putative dopamine neurons during early learning. Additionally, we observed a lack of dynamic change in neuron-pair interactions of deficient animals as they learned the action-outcome association. Loss of functional plasticity, in this case, cannot be explained by behavioral differences. This disruption of neural network interactions could be reflective of weakened coordinated input, or an inability to adapt local connections to more effectively convey a signal (Figure 5-3). Despite the static characteristics of neural networks in deficient animals, they did not exhibit behavioral differences. Having parallel systems in place, as described above, may account for the lack of overt behavioral differences we observe in adult animals. By this stage of development, other affective and cognitive networks systems have had time to compensate for SNc dysfunction. Alternatively, networks which incorporate the SNc may try to compensate for disruptions in coordinated activity by increasing dopamine function in target areas. This possibility is supported by our previous observation that deficient animals have heightened levels of tyrosine hydroxylase in the dorsal striatum, which receives dense projections from the SNc (Bondi et al., 2013). If this model is correct, it suggests

that further investigation into the disruption of SNc dopamine function and its link to psychiatric disorders may yield possible treatment and prevention strategies.

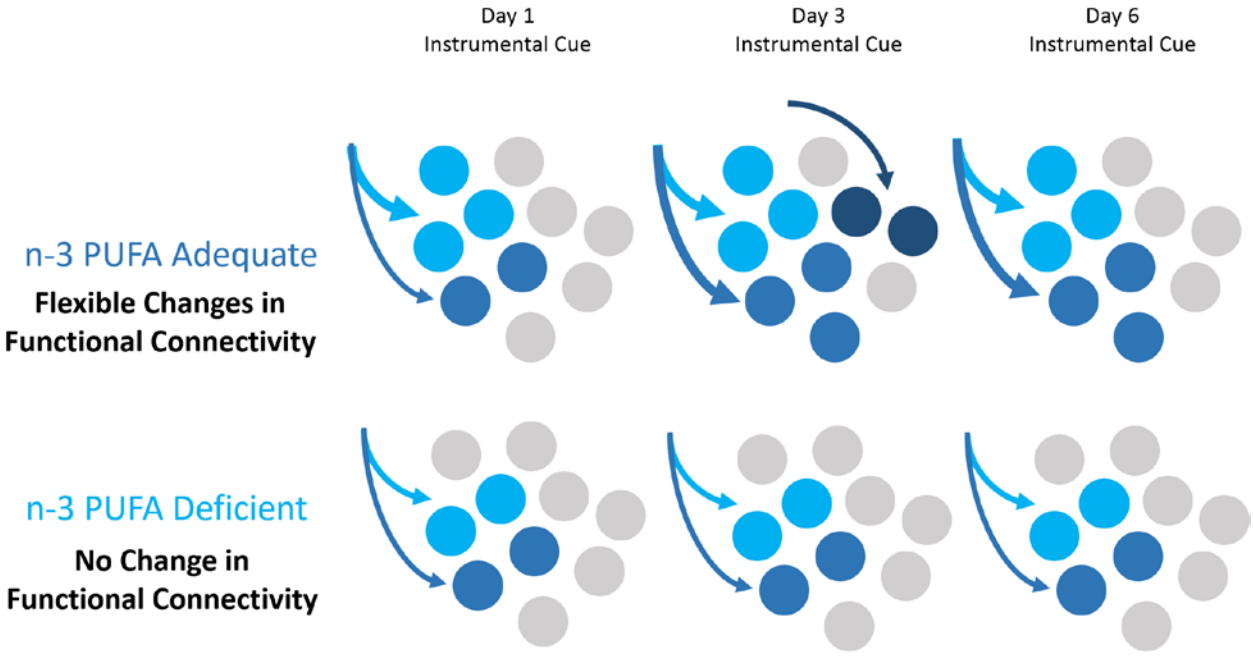

Figure 5-3 A model of coordination structures in n-3 PUFA adequate and deficient SNc during instrumental learning
We propose that SNc neurons in n-3 PUFA deficient do not modulate correlations between neurons during learning due to disruptions in functional connectivity within the region and diminished influence from coordinated input.

5.5 CONCLUSIONS

The purpose of this dissertation was to examine the physiological similarities and differences in two distinct midbrain dopamine neuron populations, VTA and SNc, during two fundamental forms of associative learning. This comparison was done during two conditioning paradigms which engage separate aspects of associative learning, and in response to a common (n-3 PUFA) dietary deficiency. We found that salient stimuli evoked similar robust phasic activity from neurons in the VTA and SNc during conditioning whereas correlations between simultaneously recorded neurons fluctuated differently. These different patterns of functional

connectivity may reflect different patterns of coordinated input, which influences the plasticity that encodes associative learning. We also observed a disrupted pattern of coordinated activity in our dietary deficient model that was more pronounced in the SNc. Additionally, dietary deficiency impaired the ability of simultaneously recorded neurons to encode learned behavior through changes in correlation, suggesting disruptions in functional connectivity across the midbrain. Our findings suggest that the SNc may be more vulnerable to environmental insult and its role in reward learning and dysfunction in psychiatric disorders warrant further investigation.

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