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Contributions of the Avian VTA to Behavioral Switching

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Contributions of the Avian VTA to Behavioral Switching

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Contributions of the Avian VTA to Behavioral Switching

by Ritu Kapur

Abstract

Birdsong is a motivated behavior used for courtship and territorial defense. Juvenile male Bengalese finches learn song from their fathers, and over time produce an increasingly accurate copy of their tutor's song. Since it is thought that the ventral tegmental area (VTA) is important in learning and crucial for the production of motivated behavior, we sought to examine the role of the VTA during singing. To probe the function of the VTA we recorded multi-unit activity in adult male Bengalese finches. We observed that neural activity in VTA consistently increased prior to the initiation and termination of song bouts, suggesting that increased activity in this region might mediate behavioral switching. To further test this idea, we coupled VTA recordings with a behavioral manipulation known to cause abrupt terminations of song. We delivered disruptive auditory stimuli during specific notes of ongoing song, which caused song terminations on a subset of trials (Sakata, 2006). Neural activity in the VTA transiently increased at a short latency (10-20ms) in response to the stimulus both during and outside of song. These data indicate that the VTA in singing birds has rapid access to information about salient perturbations of sensory experience, and extend findings indicating that the VTA responds to salient stimuli (Horvitz, 1997). Moreover, we consistently found that neural responses were significantly greater in magnitude on trials in which feedback elicited song termination versus trials in which the bird continued to sing, and the

probability of song termination increased as the level of VTA neural activity increased. Because song termination was associated with neural responses of higher magnitude, we stimulated in VTA during song to test the idea that activity in this region might be causally related to song termination. Song terminations were produced at significantly lower current intensities in VTA than in surrounding regions. One interpretation of these data is that the VTA monitors the environment for salient stimuli and is able to effect a cessation of ongoing motor behavior when environmental conditions favor behavioral switching. This supports the interesting possibility that VTA participates in action selection, and might function in contexts unrelated to reward.

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Introduction

The ventral tegmental area (VTA) first became a subject of intense study when it was identified as part of a medial forebrain circuit that supported intra-cranial self-stimulation (ICSS) (Olds, 1954). Researchers probing this robust and fascinating behavior soon began to hypothesize that the reinforcing effects of medial forebrain bundle stimulation were dependent on dopamine (Phillips, 1973; Corbett, 1980). Because the VTA and the substantia nigra pars compacta (SNpc) are the primary source of dopaminergic innervation within the brain, they became the focus of efforts to understand the neural substrates of reinforcement.

Background. The VTA sends projections to telencephalon, diencephalon, and brainstem, and receives glutamatergic inputs from cortical and limbic areas that descend through the medial forebrain bundle from the hypothalamus (Swanson, 1982; Carr, 1999, 2000). Studies of neuronal populations in VTA report the presence of dopaminergic projection neurons, GABAergic interneurons and projection neurons, and a smaller population of glutamatergic neurons (Johnson, 1992a; Steffensen 1998; Carr, 2000; Yamaguchi, 2007; Nair-Roberts, 2008). For many years it had been assumed that the GABA neurons in VTA were exclusively interneurons and that their primary role was to modulate the output of the dopaminergic projection neurons (Johnson, 1992a, 1992b). Consequently, most studies have focused on the properties of the dopamine neurons in VTA. However, recent studies have demonstrated that VTA GABA neurons project to many of the same target regions as dopamine neurons, which suggests that VTA's influence on downstream targets is modulated by both of these neuronal populations (Carr, 2000a, 2000b).

Experiments designed to probe the function of VTA and the mesolimbic dopamine system generally try to address two broad questions: what drives the neurons in VTA, and what are the functional consequences of VTA activation? Thus far, studies attempting to understand what drives VTA have predominantly focused on the response properties of dopamine neurons (but see Lee, 2001; Steffensen 2001), and because of evidence from self-stimulation studies and the willingness of animals to self-administer drugs of abuse directly into the VTA (Bozarth, 1981; Kiyatkin, 1997; Steffensen, 2006) hypotheses in this field focus almost exclusively on ideas of reinforcement and reward. Studies addressing the functional consequences of VTA activation are also focused primarily on dopaminergic neurotransmission. For example, a large number of studies test the effect of pharmacological manipulations of the dopaminergic system on various parameters of self-stimulation behavior (Koob, 1978; Corbett, 1980; Bozarth, 1981, 1983). Additionally, studies probe the effects of dopaminergic transmission on the activity of downstream targets – primarily the prefrontal cortex and nucleus accumbens (Yang, 1984; Schultz, 2003; Yang, 2006a, 2006b; Robinson, 2007). The resultant hypotheses have implied that DA is necessary or at least permissive for plasticity in striatal and cortical areas, and have focused heavily on reinforcement-related plasticity as an explanation for observed effects.

The Reward Hypothesis. One of the most influential hypotheses about dopamine function is that the activity of dopamine neurons carries signals relating to reward (Wise, 1978; Ljungberg, 1992; Mirenowicz, 1996). An important facet of reward theories is that they deal with the concept of reward in its hedonic sense – that is, a rewarding stimulus is one that induces a *feeling* of pleasure. Perhaps the most influential body of work in this

vein comes from Wolfram Schultz and his colleagues. Their studies explore the properties of VTA neuronal responses to a variety of rewarding stimuli and reward-predicting cues. They report neural responses to unconditioned rewards such as food and juice (Schultz, 1986; Ljungberg, 1992) and to cues predicting reward and the omission of reward (Ljungberg, 1992; Waelti, 2001, Tobler, 2003; also see Bayer, 2005). In addition, they report that VTA activity scales with the probability of expected reward (Fiorillo, 2003). The results of these experiments have been interpreted as supporting the idea that dopamine neurons are signaling about the difference between reward outcome and reward expectation. These "error signals" are then used in reward-related learning. This represents an evolution of the initial reward hypothesis which postulates that dopamine signaling reflects the actual feeling of "pleasure", but it still relies on the notion of hedonic reward to explain VTA activity.

According to the reward hypothesis of dopamine function, plasticity is the major functional consequence of dopamine activity. ICSS in VTA results in a persistent potentiation of stimulation-seeking behavior (Olds, 1954; Corbett, 1980; Singh, 1994; Willick, 1995; Steffensen, 2001). Based on the assumption that stimulation in VTA is "rewarding", Schultz has interpreted cortical reorganization in a sensory area following VTA stimulation (Bao, 2001) as a demonstration of reward-based plasticity (Schultz, 2002). Moreover, it has been postulated that these "reward-related" signals inform higher-order cognitive processes such as decision-making (Bayer, 2005).

Problems with the Reward Hypothesis. While the reward hypothesis of dopamine function initially found wide support, even some of the most influential researchers to propose the idea have re-evaluated their hypotheses in light of recent experimental

evidence (Berridge, 1998). First, it has been clearly demonstrated that non-rewarding stimuli activate VTA dopaminergic neurons. VTA neurons respond to salient visual and auditory stimuli (Horvitz, 1997, 2000) and to aversive stimuli such as hypotonic saline, tail pinch, and foot-shock (Guaracci, 1999; Mirenowicz, 1996; Ungless 2004).

Additionally, dopamine depleted animals seem to be able to experience hedonic pleasure. Early studies reported that lesions of the dopaminergic system resulted in a deficit in ICSS behavior, and were interpreted as evidence that rats were no longer able to feel "pleasure" (Koob, 1978). However, other researchers argued that the deficits in ICSS were due to motor or sensorimotor impairments that interfered with the ability to perform an instrumental task, rather than to the animals' inability to feel "pleasure" (Fibiger, 1976). In an effort to avoid the use of an instrumental task to evaluate the animals' internal state, Berridge and Robinson have conducted taste reactivity tests and found that dopamine depleted rats still exhibit species-specific behaviors that indicate "liking" a sucrose reward (1998). In addition, Hnasko and colleagues have demonstrated that dopamine deficient mice can acquire a conditioned place preference to morphine (Hnasko, 2005), which requires that the mice learn an association between environmental cues and a positive hedonic state.

Another problem for the reward hypothesis arises from studies of ICSS. The reward-based hypothesis posits that electrical stimulation of the VTA is rewarding (and therefore behaviorally reinforcing) because it activates dopamine projection neurons and results in a massive increase in dopamine at the target site. In a direct test of this hypothesis, Garris et al. used fast-scan cyclic voltammetry to record dopamine levels in the nucleus accumbens during ICSS. They found that ICSS did not result in consistent

release of dopamine in target regions, providing evidence against the view that dopamine is the substrate of brain stimulation "reward" (Garris, 1999).

The results of experiments in dopamine depleted animals also call into question the idea that dopamine encodes an error-signal necessary for learning. While it is acknowledged that there are many different types of learning, at least some of them do not require dopamine. For example, 6-OHDA dopamine-depleted rats were able to learn a conditioned taste aversion (Berridge, 1998). Dopamine deficient mice were able to learn a water-maze task and to acquire a conditioned place preference to morphine (Denenberg, 2004; Hnasko, 2005). However, they were unable to perform an instrumental learning task (Robinson, 2007), indicating that dopamine might be required for some aspects of some types of learning.

Incentive Salience. An alternative to the reward hypothesis of dopamine function is Berridge and Robinson's *incentive salience* hypothesis, which makes a clear distinction between "liking" and "wanting". They define "liking" as the hedonic enjoyment or pleasure associated with a reward stimulus, and "wanting" as the drive to engage in approach and consummation of such a stimulus (Berridge, 1998). The incentive salience hypothesis argues that VTA neural activity in the presence of a reward or reward-predicting cue is a reflection of the fact that the stimulus is salient and elicits "wanting". Thus the sensory responses of neurons in dopaminergic brain areas are a reflection of the fact that the stimuli are both *salient* and have some sort of *incentive* motivational power.

The functional consequences of dopamine activity according to the incentive salience hypothesis are related to the idea that dopamine is signaling about salient cues in the environment and facilitating appetitive behavior. Berridge & Robinson state that

"dopamine function in reward often appears to be linked to anticipatory, preparatory, appetitive, or approach phases of motivated behavior", and that this explains why dopamine depletions often result in an inability of the animal to work for a reward (Berridge, 1998; Yun, 2004; Aberman, 1999; Roberts, 1982; Koob, 1978; Fibiger, 1974) without interfering with its ability to experience the hedonic aspects of the reward (Berridge, 1998, 1999) or to learn about the reward (Berridge, 1998; Denenberg, 2004; Hnasko, 2005). According to this formulation, VTA is required for reward seeking elicited by a predictive cue because dopamine is alerting the system to the presence of a stimulus with incentive salience and engaging neural circuitry necessary for approach and consummation.

Action Selection. The action selection hypothesis of dopamine function picks up where incentive salience leaves off, and focuses less on the sensory properties of the neurons (ie, what types of stimuli drive them and to what extent) and more on the functional consequences once the neurons have been activated (Gurney, 2001). This set of hypothesis states that dopaminergic modulation of the basal ganglia helps solve the action selection problems that face all organisms. In their 1999 review, Redgrave and Gurney state that the problem of action selection occurs when two or more competing systems request incompatible actions. An example of this would be the demands placed on the motor system of a rat by the requirements for feeding versus escape – they are mutually exclusive, and in order to survive the organism must select which set of motor behaviors to engage in at the appropriate time. The authors posit that a brain region functioning as a "selector" should meet three basic criteria. First, the "selector" should have the appropriate inputs. In the case of an organism faced with the task of selecting

when to feed and when to escape, the "selector" should be able to receive information about salient stimuli in the environment that are informative about the presence of food or predators. Second, there should be a "common currency" for all competing inputs so that they can be compared. The instantiation of this in a brain region might be that it is able to respond to salient stimuli of many modalities. Finally, the "selector" should "have appropriately connected outputs so as to enable expression of the winning competitor while disabling that of the loser", which in the case of the brain would require that the region be able to arrest ongoing behavior and initiate other motor behaviors. Redgrave and Gurney posit that the role of the basal ganglia is to help solve the problem of action selection, and that the role of VTA and subtantia nigra dopamine is to regulate this process in striatal areas at the level of the corticostriatal synapses. Experimental data make it clear that VTA has a short-latency response to salient stimuli of many modalities. It is also clear that VTA output modulates behavior, but it is not clear whether this modulation is limited to the initiation of goal-directed behavior, nor do we know which subpopulation(s) of VTA neurons might subserve this action selection function, or on what timescale they do so.

VTA and the Basal Ganglia. Basal ganglia thalamo-cortical loops regulate a wide variety of behaviors in vertebrates. The most salient aspect of their function is that the basal ganglia appear to facilitate "the initiation of motor programs that express movement and suppression of competing or non-synergistic motor programs that would otherwise interfere with the expression of sensory-driven or goal-directed behavior" (DeLong, 2000). The canonical basal ganglia loop (see Figure 1a) consists of projections from the

cortex to the striatum, from striatum to the pallidum/SNpr*, from the pallidum/SNpr to the thalamus, and from the thalamus back to the cortex. It is postulated that distinct basal ganglia thalamo-cortical loops regulate the initiation and termination of motor behavior, the planning and organization needed for executive function, and the expression of socially appropriate emotional behavior. Likewise, diseases of the basal ganglia result in motor deficits (Parkinson's and Huntington's disease), disordered or perseverative thoughts (schizophrenia and obsessive compulsive disorder), and disregulation of affect (drug addiction).

It is thought that the dopaminergic nuclei (VTA and SNpc) regulate basal ganglia function at the level of the striatum. Projections from SNpc to dorsal striatum are thought to regulate initiation and suppression of movement, while projections from VTA to ventral striatum, which includes the nucleus accumbens, are thought to regulate reward and motivation. The location of dopamine receptors on the spines of striatal neurons suggests that dopamine modulates their responsivity to cortical inputs. Again, these theories of basal ganglia regulation focus largely on dopaminergic inputs while neglecting the role of the GABAergic projections. Given that VTA GABA neurons project to many of the same target regions as the VTA dopamine neurons (Swanson, 1982; Margolis 2006a, 2006b; Steffensen, 1998; Carr, 2000), it is likely that both of these populations of VTA neurons are modulating basal ganglia function.

Birdsong as a Model System for the Study of the Basal Ganglia. One model system lends itself particularly well to the study of basal ganglia output. Birdsong is a motivated behavior and is learned during a critical period early in the bird's life (Marler,

* SNpr = substantia nigra, pars reticulata

1964). The two major pathways of the birdsong system comprise a thalamo-cortical basal ganglia loop whose output is, in several species, a song that is fairly stereotyped across renditions. Birdsong provides a useful way to probe the function of the basal ganglia because it is a robust, stereotyped behavior that lends itself well to quantification, and changes in behavior can be resolved on a millisecond timescale.

In a manner analogous to motor and premotor cortices in mammals, nuclei in the motor pathway of the song system, HVC and RA[*], are presumed to be the source of premotor and motor control of song (Nottebohm, 1976; Fee & Hahnloser, 2002; Fee, 2004; Leonardo, 2005). These motor nuclei send projections to Area X, a nucleus in the anterior forebrain pathway (AFP) that has both striatal and pallidal characteristics (Farries 2000, 2002, 2005; Carrillo, 2004; Person, 2008). Area X sends projections to the dorsolateral nucleus of the thalamus (DLM), which sends projections to lMAN[†], which in turn projects back to the motor area RA, thus completing a basal ganglia loop. The nuclei of the motor pathway are necessary for song production throughout the bird's life (Leonard, 1976). By contrast, lesions of the AFP cause major disruptions in song learning (Bottjer, 1984; Sorhabji, 1990; Scharff 1991), though they cause only subtle changes in adult song (Bottjer, 1984; Kao, 2006).

In the avian brain, VTA and SNpc send projections to almost all of the major song system nuclei as well as sending strong projections to striatal areas (Lewis 1981; Soha, 1996; Bottjer, 1993; Appeltants 2000, 2002). This suggests that dopaminergic nuclei might also regulate the output of the thalamo-cortical basal ganglia loop used for song production. For example, it has been demonstrated that dopamine is required for the

[*] RA, the robust nucleus of the arcopallium
[†] lMAN, the lateral magnocellular nucleus of the nidopalludim

induction of LTP in Area X (Ding, 2004). Additional evidence that VTA is modulated during singing comes from immediate early gene studies that show activation in VTA after singing in different social contexts (Lynch, 2008; Hara, 2008). Moreover, electrophysiological recordings made in zebra finch VTA suggest that the activity of VTA neurons in modulated differently during song performed in two different social contexts (Yanagihara, 2006).

Because we know that the VTA regulates basal ganglia function, we thought it would be informative to explore this relationship in the context of birdsong. In Chapter 1 of this dissertation, we characterize VTA neural activity during vocal motor behavior. In Chapter 2, we discuss experiments in which pair VTA recordings with salient disruptions of singing behavior. Finally, in Chapter 3, we report the results of experiments in which we stimulate VTA in order to effect a change in vocal motor behavior. By using song as a read-out of basal ganglia function, we hope to probe the role of VTA in the modulation of motivated behavior.

Chapter 1: Modulation of VTA Firing During Singing/Vocal Behavior

INTRODUCTION

Birdsong is a motivated behavior used for courtship and territorial defense. Juvenile male Bengalese finches learn song from their fathers, and over time produce an increasingly accurate copy of their tutor's song (Marler, 1964). Since it is thought that the ventral tegmental area (VTA) is important in learning and crucial for the production of motivated behavior, we sought to examine the role of the VTA during singing by recording in VTA during ongoing song.

Evidence from anatomical studies, patterns of immediate-early gene expression, and physiology in the VTA of songbirds suggests that VTA neural activity is modulated during singing. The VTA is part of the mesolimbic pathway, and provides dopaminergic innervation to both the motor pathway and the anterior forebrain pathway in songbirds (Lewis, 1981; Bottjer 1992; Appeltants 2000, 2002; see Figure 1b). These pathways are the two major circuits comprising the song system and are crucial for the learning and production of song. The anterior forebrain pathway is part of a basal ganglia loop, and juvenile birds with lesions in this pathway are not able to learn a normal song (Bottjer, 1984; Sohrabji1990; Scharff, 1991). Lesions to the major nuclei of the motor pathway cause severe deficits in song production (Nottebohm, 1976). The fact that VTA sends projections to both of these critical pathways suggests that it might modulate vocal motor output or contribute the process of song learning.

Once he has reached adulthood, the Bengalese finch sings a relatively stereotyped song (Figure 1a). Song is composed of many individual notes, or "syllables", which are separated from other syllables by brief periods of silence called inter-syllable intervals

(ISIs) (Figure 1b). A group of syllables that occur together in the same sequence is referred to as a *motif*, and one song bout is usually comprised of several motifs. A bird will often sing the same motif multiple times in one song bout (Figure 1a).

The bird has a version of this song that is specifically directed at females during courtship (Sakata, 2007). It has been suggested in the mammalian literature that VTA dopamine neurons respond to reinforcing cues, including sexual cues (Ljungberg, 1992; Balfour, 2004). As a consequence, birdsong researchers have been investigating the role of dopamine in female-directed song. One microdialysis study found that dopamine levels in Area X (part of the anterior forebrain pathway) increased during directed song when compared to undirected song (Sasaki, 2006). Immediate early gene studies have reported that egr-1/ZENK expression is increased in a subpopulation of VTA neurons when the bird sings female-directed song for courtship purposes (Hara, 2008). In addition, awake behaving electrophysiology recordings made in VTA indicate that VTA neurons fire differently in the context of directed vs. undirected song (Yanagihara, 2006).

While these studies seem to support the idea that courtship-related, reinforcing cues are activating VTA neurons and that this is contributing to the differences between directed and undirected song, they also suggest a broader role for VTA's influence on song. Researchers have reported increased expression of egr-1/ZENK in VTA neurons associated with undirected singing (Lynch, 2008), suggesting that VTA might be modulating song outside of the context of courtship. Yanagihara and colleagues report that VTA responds to salient cues, increases its activity just before initiation of a song bout, and is weakly modulated with song in both directed and undirected contexts (2006).

Taken as a whole, these findings do suggest that VTA is active during song, but perhaps surprisingly, it seems to be the TH- rather than the TH+ dopaminergic neurons that are most clearly modulated. In both IEG studies, the reported increases in egr-1/ZENK occur mostly in TH-, putative GABAergic neurons (Hara, 2008; Lynch, 2008). Similarly, Yanagihara et al. posit that their recordings are made from GABAergic neurons in VTA (2006). While it might be the case that dopaminergic neurons in the VTA respond to reinforcing stimuli and modulate song, it seems to be at least equally likely that TH- neurons in the VTA are modulating song as well, and that they might do so outside of the context of courtship related song.

In order to better understand the relationship between VTA activity and song, we recorded multi-unit activity in singing adult male Bengalese finches and asked whether there were relationships between VTA activity and song.

METHODS

Subjects: We used adult male Bengalese Finches (*lonchura striata var. domestica*) aged 120d post hatch and older. Birds were raised in individual breeding cages until at least 60d, and were housed with other males thereafter until the time of implantation surgery. Animals were housed on a 14/10 light/dark cycle.

Surgery: Stereotaxic surgery was used to implant chronic microdrives whose electrodes could be advanced manually (Hessler, 1999). Each microdrive was built with 2-3 electrodes (FHC UWESEES4NNE 4MΩ) and a low impedance ground. Electrodes were targeted at the VTA (M/L 0.5mm, R/C –3.5mm, D/V -6.2mm from Y_0, beak angle 50^o, intra-aural distance of 3.5-4.5 mm). Implants were built such that the three

electrodes were in as close to a linear conformation as possible, with all electrodes aligned so that they were at the same rostro-caudal extent, covering 300-500 μm along the medial-lateral axis. The stereotaxic measurements outlined above were in reference to the lateral-most electrode.

Birds were allowed 3-7 days to recover from surgery, and were habituated to a recording lead for at least 7 days before experiments began. During this time, birds were housed in individual cages next to conspecifics so that all birds were in visual and auditory contact.

Neural Recordings: Subjects were connected to digital recording equipment (A-M Systems, Sequim, WA) via a flexible lead and op-amp, and signals were routed through a commutator (Dragonfly, Ridgeley, WV), which allowed the birds free range of motion in their home cages. Neural signals were amplified (1000x), filtered (300Hz-10kHz), digitized and recorded (Observer, A. Leonardo, Caltech & C. Roddey, UCSF). Electrodes were advanced in intervals of approximately 80μm and responses to salient probe stimuli (tap on door of soundbox, switching lights off/on) were noted, as was any activity correlated with movement, such as hopping onto the perch, reaching into the food dish, and orienting-related head movements and saccades.

Song recordings: Song recordings were made in a sound-attenuating chamber (Acoustic Systems) that was able to accommodate the bird in his home cage. Audio signals were recorded with a stationary microphone mounted on the ceiling of the chamber, and were filtered (300 Hz –8kHz), digitized, and recorded (Observer) for post hoc analysis. All songs were "undirected" song.

Histology: After experiments were complete, small marking lesions were made to confirm the position of the electrodes. Birds were perfused trascardially with 0.9% saline followed by a 3.7% formaldehyde solution. Brains were dissected, sunk in 30% sucrose, and then frozen and sectioned on a freezing microtome (40µm sections). Every 3rd section was stained for TH (tyrosine hydroxylase, a precursor enzyme necessary for the synthesis of dopamine) according to the following protocol. Free-floating sections were immersed for 5 min. in 1% H2O2 and washed in PBS. Sections were then blocked for 1 hr in PBS containing 5% normal goat serum and .3% triton. Sections were incubated overnight at room temperature with a monoclonal anti-TH antibody (1:10,000, Immunostar 22941) in PBS containing 2% NGS and .3% triton. Incubation with primary antibody was followed by a wash (3x 10 min each) in PBS containing 1% NGS and .15% triton. Sections were incubated for 1.5hr at room temperature in biotinylated goat anti-mouse IgG antibody (1:3000, Vector Labs) in PBS containing 2% NGS and .3% triton. Sections were then washed (3x 10 min each) in PBS containing 1% NGS and .15% triton in preparation for Avidin-Biotin-Complex (ABC) reaction. The ABC reagents were prepared as follows: 2 drops of Vector labs reagent A per 10mL .3% triton-PBS, mixed well, plus 2 drops of reagent B per 10mL, left to mix during half an hour of wash (25 mL per dish, 1-3 brains per dish). Sections were incubated in ABC mix for 1hr at room temperature and washed in PBS (3x 10 min each). Sections were incubated for 5 min in .25mg/mL diaminobenzadine (DAB, Sigma D5637) in 25mM tris with .03% triton. We then added .01% H2O2, and incubated sections for 3-7 min by eye, until TH stain became apparent. Sections were then washed in PBS in preparation for the hematoxylin stain.

Following the stain for TH, free-floating slices were stained with hematoxylin so that lesions in non-TH areas could be clearly visualized and a within-brain scale could be established. Sections were incubated in hematoxylin (1:10, Fisher SH30-500D) and immediately rinsed in dH2O. Sections were then mounted on gelatin-coated slides and dehydrated with 20%, 50%, 70%, then 95% ethanol baths. Slides were incubated in 95% acidified ethanol (5mL 1N HCl in 95mL EtOH) for 5min. and then rehydrated through the same ethanol bath series in reverse order. Slides were incubated in PBS, and drops of 5N NaOH (1-3 drops per 100mL) were added until tissue began to change color to blue. Slides were incubated in this PBS solution for at least 1hr, then dehydrated and cover-slipped with permount.

DATA ANALYSIS

Smoothed, rectified neural traces: The raw waveforms of neural signals in VTA were recorded and stored on computer. Because the signal to noise ratio in VTA was variable across sites and individual neurons were often times difficult to isolate, we used smoothed, rectified neural traces (SMR) as a measure of multiunit activity in the VTA. For each neural trace, the smoothed rectified neural trace was obtained by taking the absolute value of the voltage trace at each sample point (32000 Hz) and smoothing with a 5ms square window. Results obtained with calculations using smoothed, rectified neural traces were qualitatively the same as results obtained at sites using a threshold and counting spikes, and therefore all of the summary data reported here used smoothed, rectified data (see Shea, 2003; Kao 2005 for examples of this treatment of neural data).

Singing vs. Interleaved Non-Singing Baseline: For each experiment, we selected one motif of song and assessed the neural activity during the production of that motif by calculating the area under the curve of SMR data. Instances in which the chosen motif was the first motif or the last motif of song were excluded from the analysis in order to exclude the possibility that levels of neural activity seen at these times were reflecting increases in neural activity related to the initiation or termination of song. These data were compared to the area under the SMR curve for interleaved, quiet trials during which the bird was not singing.

Modulation of Neural Activity with Respect to Initiation and Termination of Vocalization: For analysis of activity with respect to vocalization, spectrograms were visually inspected and the notes of interest were labeled. For analysis of the initiation of song, the first intro note of each bout was labeled as the start of song. Because the beginning of song is often very quiet, we were careful to include only trials for which we could clearly ascertain when the first note was sung, meaning that these song initiations were preceded by at least 1 second of silence. For analysis of the end of song, the last syllable of each bout was labeled as the end of song. Calls with a duration of 100 ms or greater were labeled as well.

For analysis of neural data during initiation of vocal behavior, z-scores were calculated for each 1ms time bin using the mean and standard deviation of the neural data in the baseline period. The baseline period for initiation of vocalization was 500 ms to 250 ms before initiation of song or a call. For analysis of the termination of song, z-scores were calculated for each 1ms time bin during a brief interval of song 500 ms to 250 ms before the end of song.

For individual examples, the average z-score across time (in 20 ms bins) was calculated across trials within an experiment, and this mean was plotted. For summary data, the average z-score across time was calculated for each experiment, and then the mean of all of these average traces was plotted, with the standard deviation of the mean indicated by the error bars.

VTA response to playback of calls: Stimuli for playback were recorded from conspecific males and females. All stimuli were presented to the bird on interleaved trials, and playbacks of call stimuli were interspersed with bouts of vocalization (songs and calls) from the experimental subject. Neural data were analyzed in the same way as for the modulation of neural activity with respect to initiation of vocalization, with a baseline calculated for the window 500-250 ms before the onset of the stimulus.

RESULTS

Multiunit recordings were made in the VTA of adult male Bengalese finches (Figure 2). When experiments were complete, we made two marking lesions in each bird to determine the location of the penetration and to establish a within-brain scale. Brains were then sectioned, stained for tyrosine hydroxylase (TH, an enzyme necessary for the synthesis of dopamine) and examined under a microscope. Recordings made in areas of TH+ cell body staining were categorized as being "in VTA" . Recordings made from areas that were negative for the TH stain were counted as "outside of VTA".

We made recordings in the VTA at 30 sites in 5 birds (Figure 2, red circles = one site, red lines = one penetration with multiple recording sites). As a control, we also

recorded neural activity associated with song in areas adjacent to but outside of VTA (n=7 sites in 5 birds, blue circles and blue lines).

Isolation of single units in the VTA proved to be quite challenging, though we tested electrodes of several types and impedances (100kΩ to 8MΩ). However, even when the signal to noise ratio was low, we noted several trends in the neural signals that were highly correlated with good electrode placement in VTA. First, the overall level of neural activity (as assessed by listening on an audio monitor) increased greatly as we approached and entered VTA. One notable correlate of good electrode placement was the tendency of sites in VTA to respond to salient stimuli, such as tapping on the side of the soundbox or flipping off/on the lights in the experimental cage. These responses were most often observed in the dorsal regions of the VTA. Another consistent observation was that VTA activity increased just before the bird initiated movement, such as reaching into his food dish, hopping onto or off of his perch, or orienting.

It should be noted that these responses were not seen exclusively while in the VTA. Histological analysis showed that similar responses to salient stimuli were sometimes obtained when recordings were made in regions adjacent toVTA through which TH+ fibers were projecting. TH+ cell bodies were sparsely interspersed with projection fibers in these regions. These sites had good signal to noise ratios, and the shapes of the waveforms recorded here were not notably multiphasic, suggesting that these recordings were made from cell bodies rather than from the axons themselves. Because the actual anatomical designation of these areas was unclear, recordings made from these sites are not included here. However, sites adjacent to the VTA through

which there were *no* TH+ axonal projections were clearly *not* modulated by presentation of salient stimuli, and were included as control, "outside of VTA" sites.

While a majority of our recording sites were low signal to noise, we were able to record waveforms corresponding to single units or clusters of units from 6 sites in 3 birds. We aligned waveforms at threshold crossing in order to determine the action potential width (APW) of these neurons and compared these widths to the reported distribution of APW for dopaminergic and non-dopaminergic neurons in the VTA of finches (Gale, 2006; see Figure 3). Though the electrophysiological classification of neurons in VTA based on action potential width and spontaneous firing rate is somewhat controversial (see Ungless, 2004 and Margolis, 2006b), the distribution of APWs in zebra finch brain is clearly bimodal and is highly correlated with cell type (see Figure 3b). All of the waveforms we recorded had APWs of less than 1ms, which suggests that they are TH-, putatively GABAergic neurons (Figure 3a). The waveforms we recorded are similar in shape and duration to those recorded in Yanagihara et al (2007), and correspond well to those reported in Steffensen (1998), which had a mean action potential duration of 500 μsec.

As a first pass at determining whether VTA was modulated during song, we compared neural activity in the VTA when the bird was singing to activity on interleaved baseline trials during which the bird was quiet. We found a significant decrease in neural activity at 10/17 sites (Kolgomorov-Smirnov test, $p<0.05$, see Figure 5). Neural activity during singing was not significantly different from quiet baseline at the other 7 sites, and 0/17 sites increased their firing rates during song.

Despite the overall suppression of VTA neural activity during singing, while collecting data and listening to neural activity associated with song we observed that activity in the VTA seemed to increase around the time of song onset. We therefore aligned neural traces to the first note of song and compared neural activity in the baseline period to the neural activity in the first 500 ms after song initiation (Figure 4b, 4d). We found a significant increase in VTA neural activity at the time of song onset in 10/10 sites (K-S test, $p < 0.01$, Figure 4d). We also noted an increase in activity *prior* to the onset of song, so we compared the neural activity in the 100 ms before the onset of song to neural activity in the baseline period (Figure 4d). We found a significant increase in VTA neural activity in the 100 ms prior to the onset of singing at 7/10 sites in 3 birds (K-S test, $p<0.01$, Figure 5).

Another consistent observation made during data collection was that VTA neural activity seemed to increase around the end of a song bout. To quantify this, we aligned neural traces to the offset of the last note of each song bout (Figure 4c, 4e). We found that the neural activity in VTA was significantly greater in the 500 ms after termination of song than in the 500 ms preceding the end of song (30/30 sites in 4 birds, K-S test, $p < 0.05$, Figure 4e). Again, we noticed that VTA activity increased *prior* to the termination of song, and consistently found that VTA neural activity increased significantly during the last 100 ms of song (28/30 sites in 4 birds, K-S test, $p<0.01$, Figure 4e). In order to determine when the neural activity began to increase, we assessed the time point at which the z-score was 3 standard deviations above the mean of the z-score during song, and found that on average, the significant increase in neural activity occurred 180 ms before the end of singing.

The modulation of VTA activity prior to onset of vocalization was not limited to song. We also noted consistent modulations in VTA activity when the bird made contact calls (Figure 6a). Calls in the Bengalese finch are brief (less than 250 ms), monosyllabic vocalizations used for social communication. Neural activity was significantly greater during calls than it was in the baseline period before vocalization (28/28 sites in 5 birds, K-S test, $p < 0.01$, Figure 6a). Consistent with the results during singing, we saw a significant increase in neural activity in the 100 ms prior to the initiation of a call when compared to baseline rates (28/28 sites in 5 birds, K-S test, $p<0.01$).

Because calls from other birds are a behaviorally salient stimulus, we played recordings of conspecific calls to the bird. Presentation of calls caused significant increases in VTA activity (3/3 sites in 1 bird, K-S test, $p<0.01$, Figure 6b). However, due to experimenter error, one stimulus (a female call) was preceded by 100 ms of ambient noise from the female's soundbox. When this stimulus -- 100 ms of ambient noise from a female cage plus a female call -- was played back to the experimental subject, the increase in VTA activity was correlated with the onset of the recording and a different level of ambient noise, not with the onset of the actual female call.

DISCUSSION

The experiments outlined here demonstrate that VTA activity is modulated during singing. The results of our study confirm and extend previous findings showing 1) a change in overall activity levels in VTA during singing and 2) modulation of neural activity before song initiation (Yanagihara, 2006). In addition, our data show that VTA activity increases before the initiation of contact calls, which suggests that these

modulations in neural activity are not exclusive to singing behavior. Moreover, we saw increases in neural activity just before termination of song and in response to salient stimuli.

The placement of electrodes reconstructed from our histology suggest that our recordings were made the in the VTA (Figure 2). It has been clearly demonstrated that the VTA responds to salient stimuli of several modalities, including light flashes and loud clicks (Horvtiz, 1997, 2000). This responsiveness to salient stimuli was used as a probe during our recording sessions, and was a useful marker in determining electrode placement. In addition, the neural responses we observed before initiation of movement were similar to those reported in the VTA of awake behaving rats (Lee, 2001).

More specifically, the data from our neural recordings suggest that we were recording from GABAergic neurons in the VTA (Figure 3). Using the electrophysiological characteristics of extracellular recordings to classify a neuron as dopaminergic or GABAergic, though common practice, is not without its problems. Most studies, when making this classification, use the duration of the action potential and the rate of spontaneous firing to make statements about neuron type (Grace, 1983, 1989). The most widely used criteria are that dopamine neurons have long action potentials (average of about 2-2.5 ms) and low spontaneous firing rate (less than 10Hz). However, the validity of these assumptions has not been specifically tested in all model systems. For example, even though these criteria are used to identify putative dopaminergic neurons in primates, to date there have been no studies in which these cell have been filled and labeled for TH after they have been recorded from. In rodents, the conclusions of studies that do record, fill, and label cells are not consistent. One such study claims

that the distribution of action potential widths recorded in VTA can be fit with two Gaussian distributions, with longer action potential widths corresponding to TH+ labeled neurons (Ungless, 2004), while another claims that the action potential widths of TH+ and TH- neurons are indistinguishable (Margolis, 2006b).

Fortunately, there has been a careful study reporting on the electrophysiological properties of VTA neurons in the avian brain (Gale, 2006). The researchers recorded from VTA neurons in slice, noted their electrophysiological properties, and then confirmed the identity of the neurons by backfilling and staining for TH. In the zebra finch, there is clearly a bimodal distribution of action potential widths for neurons in the VTA (see Figure 3b), and these two populations correspond closely with TH+ and TH- neurons. For the sites at which we were able to record thresholdable data and isolate waveforms, we compared the action potential widths to the known distributions of action potential widths in the VTA. All of the waveforms we recorded had action potential durations of less than 1 ms, which suggests that these recordings were made from GABAergic neurons in the VTA (Figure 3a). The action potential durations of the neurons we report are short in comparison to the mean of the GABAergic population reported in Gale (2006,). However, they are comparable to those reported in awake behaving recordings made from birds (Yanagihara, 2006) and rats, in which the mean action potential duration was 500μsec (Steffensen, 1998).

The patterns of VTA activity correlated with singing behavior also suggest that we are recording from GABAerigic nerurons. The one other study in which researchers have recorded in the VTA of awake behaving birds posits that their recordings are made from GABAergic VTA neurons (Yanagihara, 2006). We not only see similar waveforms

to those seen in that study, we see similar patterns of VTA activation before song initiation. In addition, the IEG studies reporting modulation of VTA activity during the singing of both directed and undirected song occurs *not* in the TH+ dopamine neurons, but in the TH-, putative GABAergic neurons (Lynch, 2008; Hara, 2008). Taken together, these findings coupled with the results of our experiments indicate that GABAergic neurons in the VTA are modulated in the context of singing.

The finding that VTA activity differs significantly between singing and interleaved quiet baseline trials (Figure 5) confirms the findings of Yanagihara & Hessler (2006). They reported that overall, approximately 75 % of neurons they recorded from were significantly modulated during singing. There was evidence of a significant inhibition of neural activity during singing in roughly 60% of our experiments, but there was never any evidence of a significant increase in activity. In this sense, our results differ from the results of Yanagihara (2006), who reported an increase in neural activity during singing for one subpopulation of neurons and a decrease in neural activity for another. However, our method of assessing neural activity during singing differed from their in one important respect. We used motifs from the middle of song and excluded the first and last motif in each song bout in order to avoid counting the increases in neural activity associated with song initiation and song termination in our estimates. It is possible that this difference in our method of analysis accounts for the differences in our results.

The most striking modulations of VTA activity in the context of singing occurred around the initiation and termination of song (Figure 4). We clearly saw an increase in activity before the onset of singing (Figure 4a), consistent with the results reported in

Yanagihara 2006. They reported a general increase in neural activity starting approximately 1 second before the bird started singing. By narrowing our focus to the 500 ms preceding the initiation of singing, we were able to further determine that the neural activity in the 100 ms preceding song onset is significantly greater than in the several hundred milliseconds before. This tight temporal correlation suggests that VTA activity might play a role in initiating vocal motor behavior.

Similarly, we found a significant increase in VTA neural activity approximately 180 ms before the end of song (Figure 4b). We also observed that high rates of neural activity persisted for at least 500 ms after the end of song. If we consider this novel finding in conjunction with the increase in activity during song initiation and the anecdotal observations that VTA activity increases before the initiation of other motor behaviors, it implies that VTA activity is closely related to the initiation and termination of motor behavior.

This singing-related modulation appears to be different in VTA than in traditional song system nuclei. For example, in a nucleus such as RA, which is part of the motor pathway, neurons produce high-frequency bursts that are tightly time-locked to the acoustical structure of song (Yu, 1996; Leonardo, 2005). By contrast, in the VTA activity increases just before the bird initiates song and falls off until just before song ends, at which point it increases again, suggesting that VTA activity increases when the bird is switching into of out of song.

Modulation of VTA activity does not seem to be restricted to song, however. We recorded neural activity during calls, which are short duration (~150ms), monosyllabic social vocalizations (see Figure 1a). The vocal production of calls utilizes similar

circuitry as song, though the pathways are not entirely overlapping (Marler, 2004; Vicario, 2002, 2004). We looked at both the production of calls and the response to playback of calls to see if VTA was modulated during these vocalizations. We found an increase in activity before the onset of calls (Figure 6a). In addition, we saw an auditory response to conspecific calls when they were played back to awake behaving birds (Figure 6b).

While it is clear from studies in other systems that VTA responds to stimuli predicting reward, it also responds to aversive stimuli and salient stimuli whose valence is not immediately clear (Horvitz, 1997; Guaracci, 1999;Comoli, 2003; Ungless, 2004). Yanagihara et al reported that VTA had a strong response to the curtain opening that predicted the appearance of a female (2006). In keeping with current theories of VTA function, they suggested that this response signaled expectation of reward (Mirenowicz, 2004; Schultz, 2002). Our observations of VTA neural responses to various naturally occurring and experimenter delivered stimuli suggest a broader view. The probes we used to determine whether we were in VTA during a recording session were light taps on the side of the sound box or a flip of the light switch. These stimuli were obviously salient for the birds, and often elicited orienting responses in addition to causing increases in VTA activity. These observations lead us to conclude that VTA responds to a large class of salient stimuli, regardless of their hedonic value. In conclusion, we have found that VTA activity is modulated during song, that VTA activity increases before the initiation and termination of vocalizations, and that VTA responds to salient stimuli. These findings implicate VTA in the initiation and termination of vocal behavior in response to salient cues in the environment.

Figure 1. *Structure of Bengalese finch song*

a) A spectrogram of a typical Bengalese finch song, with time in seconds on the x axis and frequency in kHz plotted on the ordinate. The power at each frequency is denoted by color, with high power indicated by red and low power indicated by blue. In this example, the song bout is preceded by two contact calls. The solid line above the graph indicates the duration of the song bout. Each dash of the broken line above the graph indicates the duration of one motif. As illustrated, a song bout is made up of multiple renditions of one or more motifs. This particular bird sings one motif, but others sing multiple motifs that are interspersed throughout a song bout. **b)** Zoomed in spectrogram of one motif from the example above. Syllables are the individual notes that make up a song, and are indicated by text and arrows. Similarly, the brief period of silence in between syllables -- inter-syllable intervals (ISIs) -- are indicated by text and arrows.

Figure 2. *Histological confirmation of electrode placement in VTA and adjacent areas.*

a) Schematic of the avian brain (sagittal view). The major nuclei of the motor pathway (HVC, RA, nXIIts, RAm/rVG) and the anterior forebrain pathway (Area X, DLM, lMAN) of the avian song system are shown in this schematic, along with the known projections from VTA to these nuclei. **b)** A representative sagittal section through the avian brain, stained for tyrosine hydroxylase (TH). Inset is a lower magnification view of the whole section, with a rectangle denoting the area of higher magnification. **c)** A drawing of the section shown in b), with areas of dense TH+ cell body staining represented by solid gray areas in the drawing. The lines ascending from the rostral end of the area colored in gray represent the TH+ projection fibers that ascend from the VTA to the striatum. TH+ cell bodies were interspersed with these ascending fibers, though the TH+ cell bodies were not as densely packed as within the VTA proper. Red circles connected with a red line indicate the beginning and end of a penetration counted as being "in VTA" (n=1 penetration in 1 bird). The blue circles represent the locations of recordings that were made and counted as being "outside of VTA" (n=3 in 3 birds). Panels **d-f** follow the same convention as b, except that they are coronal sections. Panel d) is the most caudal and f) the most rostral, and sections shown are each 240 μm apart along the rostro-caudal axis. Panels **g-i** follow the same conventions as in panel c), with circles representing the site of a single experiment, and circles connected by lines representing the extent of a penetration. All experiments counted as being "in VTA" are indicated by red circles and lines, and all that were counted as "outside of VTA" indicated by blue circles and lines. All drawings are composites in which the histological

results from multiple birds have been projected onto one representative section. **g)** One experiment outside of VTA in 1 bird (blue circle), 2 experiments inside of VTA in one bird (red circles) **h)** 5 penetrations inside of VTA in 3 birds (5 red lines) **i)** One experiment and one pentration outside of VTA in 2 birds (blue circle, blue circles joined by line). Note that in panel **i)**, one of the sites in a TH+ area was counted as being "outside of VTA". This is because electrodes were in the mesencepahlic central grey (A11), which is comprised of a group of dopaminergic cells that is distinct from VTA.

Figure 3. *Recordings in VTA were made from putative GABAergic neurons.*

Characterization of waveforms recorded in VTA. **a)** Examples of waveforms recorded extracellularly in VTA at 6 different sites in 3 birds. Waveforms were aligned at threshold crossing (n=273, n=310, n=401, n=39, n=862, n=62, n=48, respectively). The average of all of the waveforms for each example is plotted in red over the individual traces. **b)** Reproduction of a figure from Gale, 2006, reporting a bimodal distribution of action potential widths (APW) for neurons recorded in the VTA of zebra finches (intracellular recordings). According to these data, shorter APW (<2.0 ms) are indicative of recordings made from TH-, putative GABAergic neurons. The APW of the neurons shown in a) clearly fall in the TH- portion of the distribution. Though the observed APW from our recordings is short relative to the distribution of TH- neurons shown in b), they are comparable to the 500 μsec APW reported for GABAergic neurons in Steffensen (1998).

Figure 4. *VTA neural activity increases before the initiation and termination of song.*

a) A spectrogram of an individual song with initiation and termination of song indicated by a dashed red line **b)** An example of the average smoothed rectified (SMR) neural data from one experiment in one bird. Neural traces were aligned to the initiation of song (dashed red line) **c)** An example of the average SMR trace from one experiment in one bird for the end of song. Traces were aligned to the offset of the last note of song (dashed red line) **d)** Summary data for initiation of song (n=3 birds, 10 sites). The top panel is a spectrogram zoomed in to the 1 second surrounding onset of song. The bottom panel represents how the z-score changes across this time period. For each experiment, the z-score was calculated by comparing the neural activity at each millisecond to the average of the neural activity in the first 250 ms of the time period shown (before the bird started singing). Z-scores were binned in 20 ms bins, and these binned z-scores were averaged across all experiments. Error bars are s.e.m. The neural activity in the 500 ms following song onset is significantly greater than in the 500 ms of silence preceding it (10/10 sites, $p < 0.01$). Moreover, there is a significant increase in VTA neural activity *prior* to the onset of the first syllable in 7/10 sites ($p < 0.01$). **e)** Summary data for end of song (n=5 birds, 30 sites). The top panel is a spectrogram zoomed in to the 1 second surrounding the end of song. The bottom panel represents how the z-score changes during this time. Z-scores were calculated by the same method as in d), with the exception that they were normalized to neural activity during singing in the first 250 ms of the window shown. Neural activity in the VTA is significantly greater in the 500 ms after the end of song than it is in the 500 ms preceding song's end (n=30/30, $p < 0.05$). Moreover, we see that

neural activity in VTA increases significantly 180 ms prior to the termination of song ($n=28/30$, $p < 0.01$).

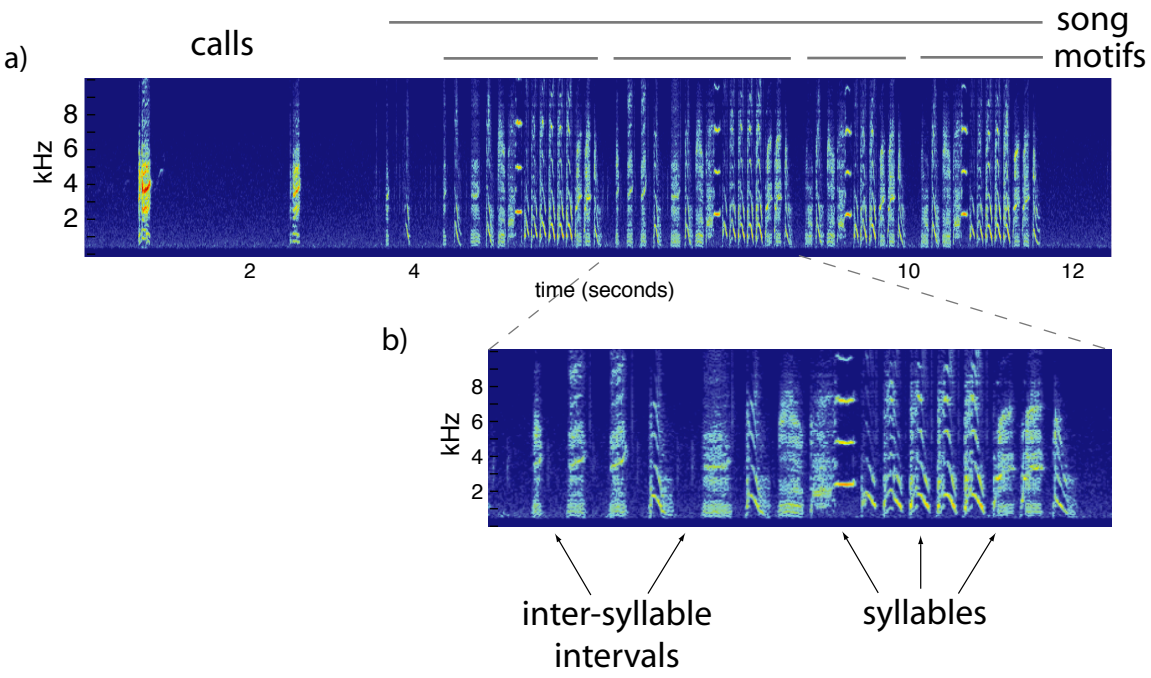
Figure 5. *Neural activity in VTA is inhibited during song.*

In order to compare VTA activity during singing with neural activity outside of the context of song, we measured neural activity in both of these contexts. For each experiment (n=17) we calculated the area under the average smoothed rectified (SMR) trace on quiet baseline trials. Quiet baseline trials were interleaved with song across an experimental session, and were often recorded at times that were several minutes before or after song was sung, thus assuring that any song initiation or song termination-related activity was not included. We compared the area in the quiet baseline condition to the area under the traces recorded during song. We excluded the first and last motifs of song so that our estimate of neural activity was not affected by the changes we see during initiation and termination of song. We found that VTA neural activity was significantly lower during singing than it was on interleaved baseline trials at 10/17 sites ($p < 0.05$). Data points reflecting significant differences between quiet baseline and singing are circled.

Figure 6. *VTA activity increases before the initiation of calls and in response to playback of calls.*

a) Neural activity in VTA increases during calls. The top panel is a spectrogram of the 1 second surrounding the initiation of a species-specific contact call. The bottom panel illustrates how the z-scores of the aligned neural data change across this time. The z-scores were calculated and displayed in the same manner as outlined for Figure 4d, with the first 250 ms of the time period shown serving as the baseline. Neural activity during calling was significantly increased in 28/28 sites ($p < 0.01$). Additionally, the neural activity in the 100 ms preceding the initiation of a call was significantly greater than baseline at 28/28 sites ($p < 0.01$). **b)** VTA neural activity increases in response to playback of a salient stimulus. The top panel is a spectrogram of the 1 second surrounding the playback of a male call to the bird from which we were recording. The bottom panel shows how the z-scores of the neural response change over that time period, and illustrate the auditory response to the stimulus. Z-scores were calculated in the same manner as outlined for Figure 4d, with the first 250 ms of the window shown serving as the baseline. Neural activity increased in response to playback of the stimulus at 3/3 sites ($p < 0.01$).

Figure 1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calls
song
motifs
kHz
8
6
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4
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12
time (seconds)
b)
kHz
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inter-syllable
intervals
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Figure 2

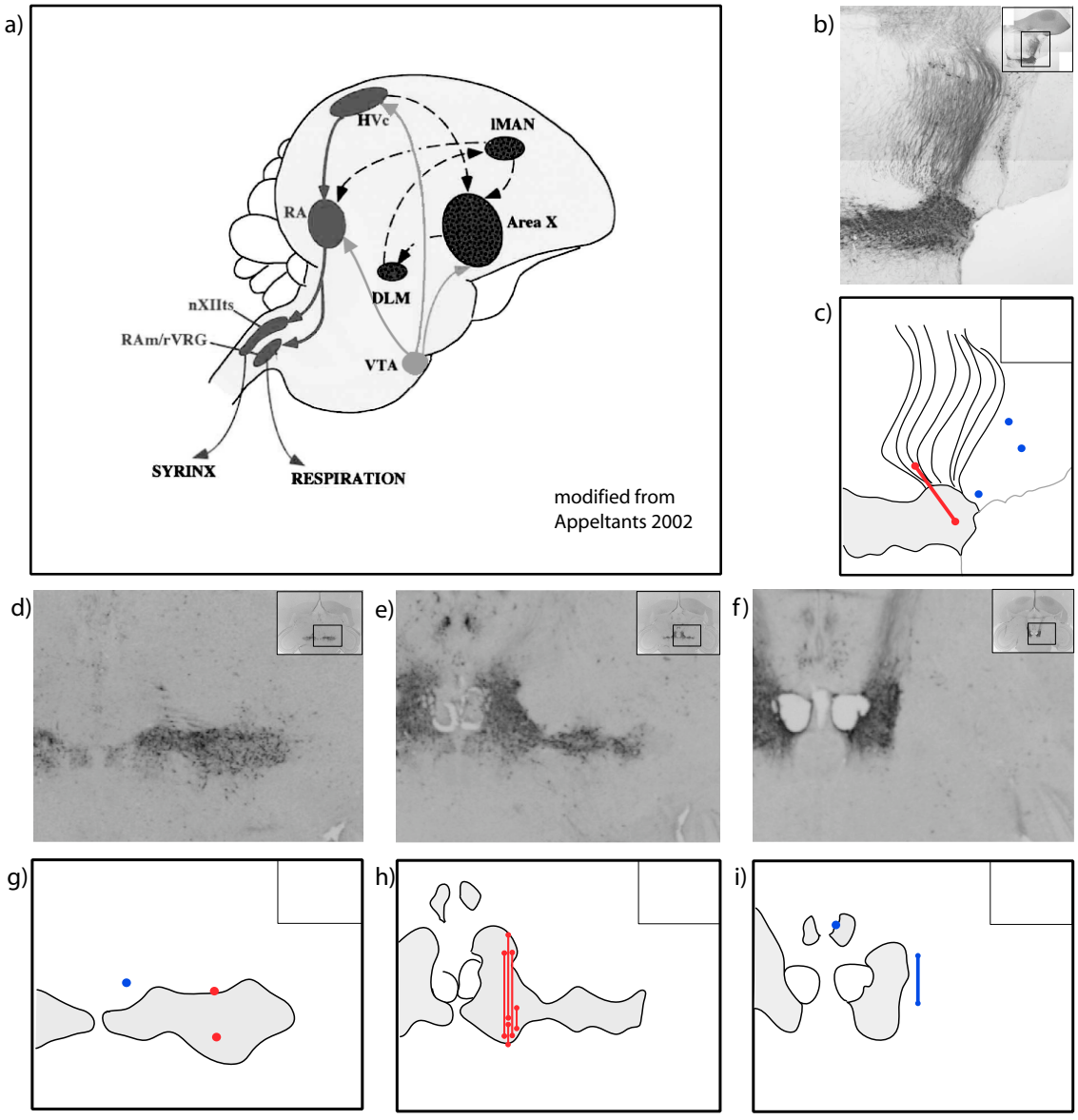
a)
HVc
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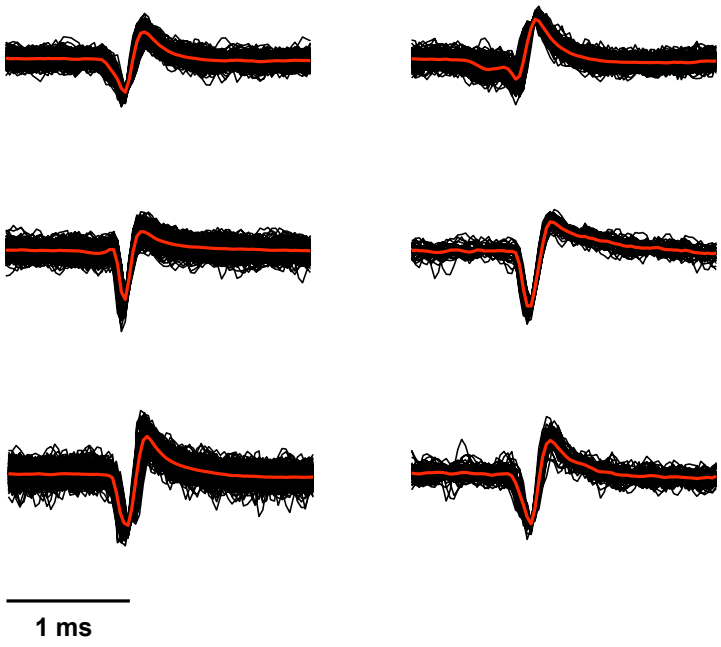
Figure 3

a)



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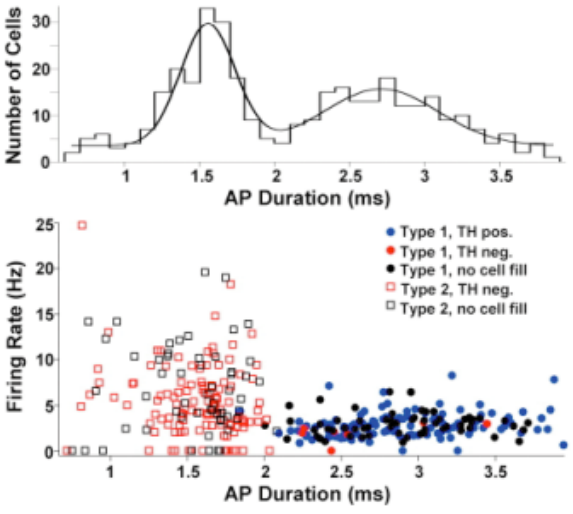

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Figure 4

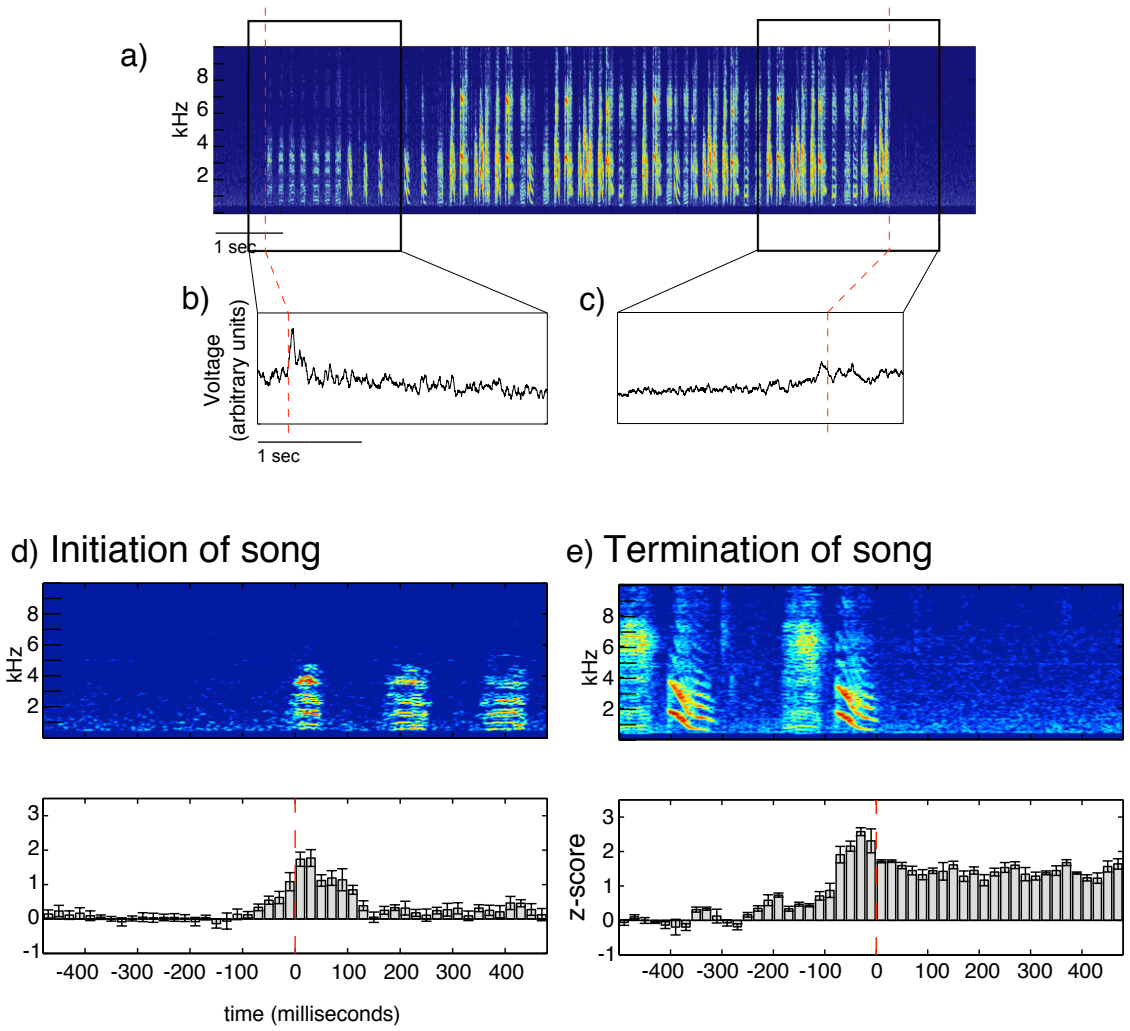
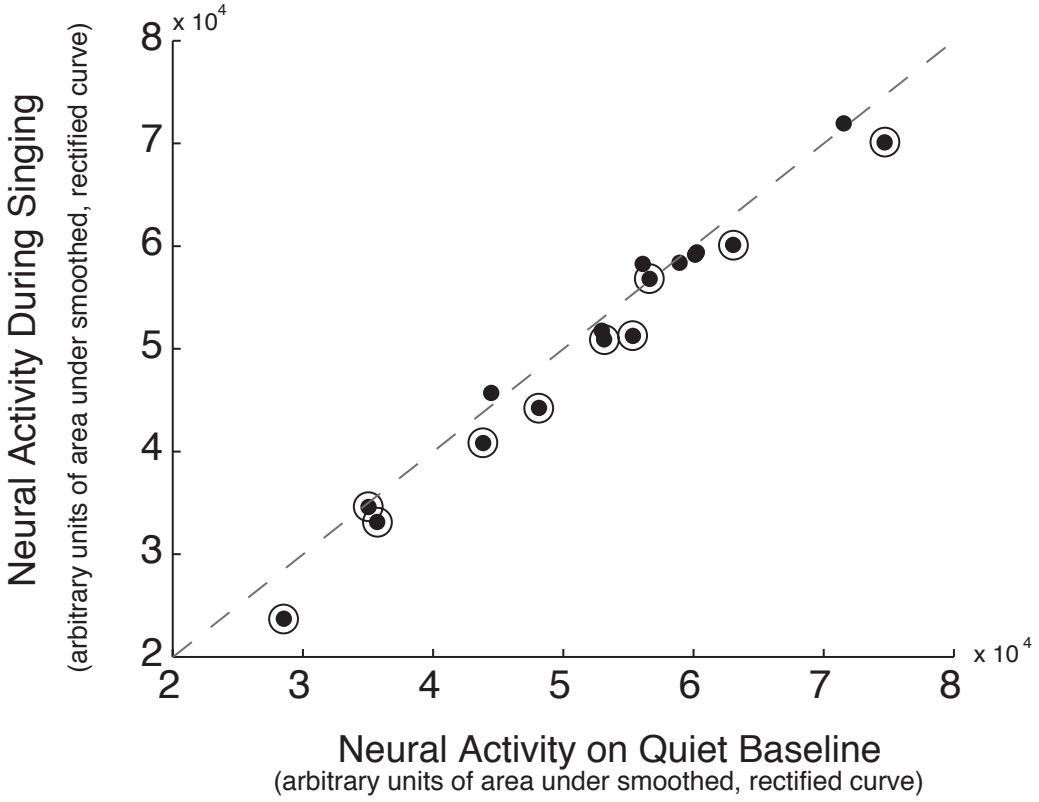

a)
kHz
8
6
4
2
1 sec
b)
Voltage
(arbitrary units)
1 sec
c)
d) Initiation of song
e) Termination of song
kHz
z-score
-400 -300 -200 -100 0 100 200 300 400
time (milliseconds)

Figure 5


Neural Activity During Singing
(arbitrary units of area under smoothed, rectified curve)
x 10^4
8
7
6
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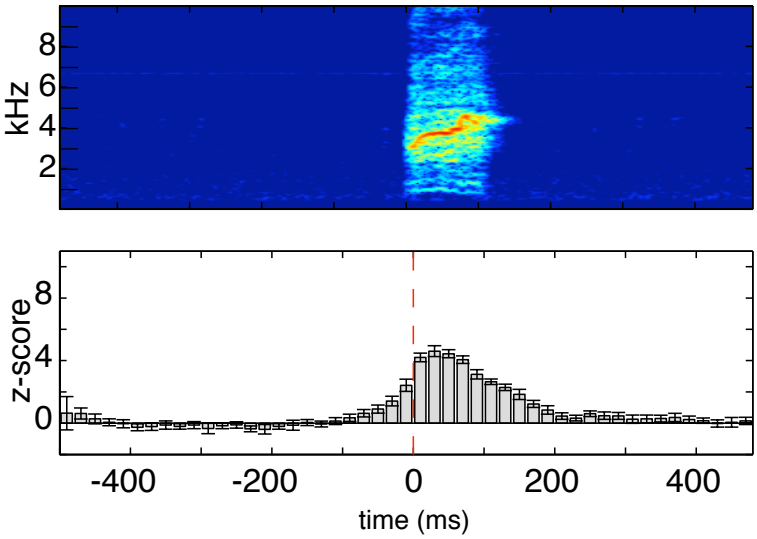
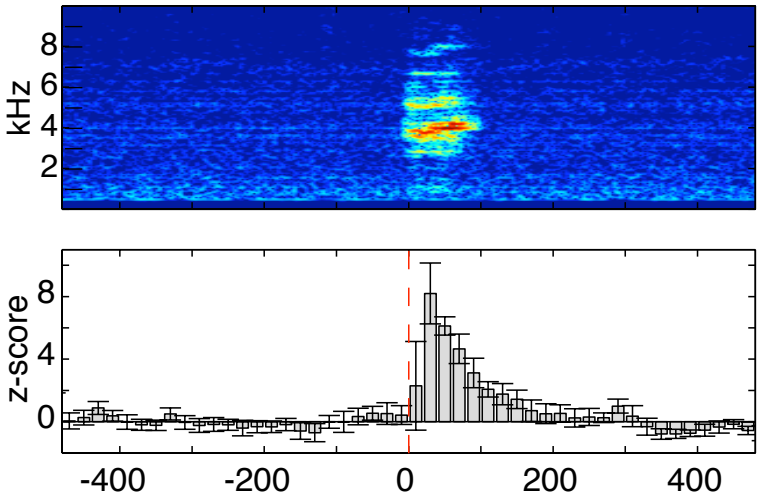
8
x 10^4
Neural Activity on Quiet Baseline
(arbitrary units of area under smoothed, rectified curve)

Figure 6

a) VTA Activity During Calling (Vocalization)



b) VTA Activity During Playback of Calls



Chapter 2: Neural Activity in VTA Increases in Response to Salient Stimuli and Is Correlated with Termination of Singing Behavior

INTRODUCTION

The role of the ventral tegmental area (VTA) in regulating behavior is a subject of intense study. The VTA is the main source of dopaminergic innervation to the dorsal and ventral striatum, and sends diffuse projections to a multitude of limbic structures in the forebrain, including the nucleus accumbens, amygdala, hippocampus, and medial prefrontal cortex. There are many hypotheses regarding VTA function, and chief among these are theories that VTA regulates appetitive behavior. Researchers have posited that VTA signals reward (Olds, 1954; Wise, 1978, 2005), motivation (Aberman, 1999), expectation of reward (Fiorillo 2003; Tobler 2003; Bayer 2005), and incentive salience – "wanting" as opposed to "liking" a reinforcer (Berridge, 1998). Other researchers have supported broader interpretations of VTA function and argued that VTA signals salience regardless of valence (Horvitz, 1997; Comoli, 2003), and that dopamine projections from VTA facilitate action selection in response to salient stimuli (Wickens, 2007; Nicola, 2007).

While it is clear that VTA neurons respond to both conditioned and unconditioned appetitive stimuli (Schultz, 1986), their firing is also modulated in response to mild aversive stimuli (Mirenowicz, 1996) and to stimuli predicting tail pinch (Ungless, 2004) and footshock (Guaracci , 1999). Studies testing VTA responses to non-appetitive

stimuli such as flashes of light and audible clicks have demonstrated that VTA responds to salient stimuli of many different modalities (Horvtiz, 1997; Comoli, 2003).

The findings of several studies elaborate on this responsiveness to salient stimuli and explore the relationship between VTA's sensory response to a cue and subsequent behavior. Interestingly, it seems that not only do VTA neurons respond to cues, their level of activity increases before the initiation of motor behaviors elicited by those cues. A recent study showed that VTA neurons in the male zebra finch responded to cues predicting the presence of a female and maintained high rates of activity during female-directed singing, and that the neurons increased their firing just before the initiation of song (Yanagihara, 2006). When unrestrained rats were trained to nosepoke to a cue for intracranial self-stimulation (Steffensen, 2001) or for heroin (Steffensen, 2006), VTA putative GABA neurons showed an increase in activity 1-2s before the nosepoke and an inhibition during the reward. In a series of primate studies, 45% of VTA neurons increased firing to the reward predictive cue and maintained high rates of firing through *initiation* of a bar press (Nishino, 1987,1991) but not through the entire bar pressing regime and not while the reward was consumed.

The researchers' interpretations of findings from these studies is that VTA responds to reinforcing stimuli and helps initiate motivated behavior, and if there is a motor role for VTA at all, it is due to the goal-directed nature of the behaviors described. However, there is evidence that VTA is involved in motor regulation outside of the context of "reward". While it is true that animals with dopamine depletions show deficiencies in goal directed behavior (Berridge, 1989, 1998; Aberman, 1999; Robinson, 2007), they show grave deficits in locomotor behaviors in general, and are severely

hypoactive (Koob, 1981; Zhou, 1995) while retaining the ability to acquire a conditioned place preference to systemic opiates (Hnasko, 1995). These findings suggest that the role of VTA in responding to cues and regulating motor output might not be strictly limited to goal-directed behavior.

Few studies examine VTA responses outside of the context of reward, but those that do find that VTA neurons are significantly excited or inhibited during a wide range of motor behaviors. Recordings made in awake, unrestrained rats showed phasic increases in firing during locomotion, grooming, head movements, washing of the forelimbs, and shifts in posture during quiet rest (Kiyatkin, 1998). Additionally is has been observed that the discharge of putative GABAergic neurons in VTA of freely moving rats increased with head orienting and forelimb movement (Lee, 2001). The authors of this particular study also note that modulation is less strong during sustained locomotor activity, and more "dramatic" during phasic locomotor activity, suggesting that firing occurs at the onset and offset of behavior rather than throughout.

There is evidence that normal VTA function is necessary not only for *initiation* of motivated behaviors, but for the proper *inhibition* of motor behaviors. For example, small lesions in the VTA of rats resulted in increased levels of locomotion, grooming, and rearing (Koob, 1981; Trojniar, 1999). More recently, it was demonstrated that this hypermotility was caused by selective ablation of VTA GABA neurons (Shank, 2007), which points to tonic regulation of motor behavior by VTA.

The presence of signals in VTA corresponding to both initiation and termination of motor behavior would suggest a role more consistent with behavioral switching than with reinforcement. Although the studies mentioned above hint at a role for VTA in the

termination of motor behavior, there is a paucity of studies probing the direct role of VTA in this regard. In the experiments outlined in Chapter 1, we reported an increase in VTA firing before the initiation *and* termination of singing. These findings lead us to suspect that in addition to responding to generally salient stimuli, VTA is participating in a broad regulation of motor behaviors not strictly limited to reward seeking. To further explore this possibility, we tested the hypothesis that VTA activation is correlated with termination of motor behavior.

We tested this hypothesis by making multiunit recordings in the VTA of adult male Bengalese finches while delivering disruptive feedback to birds during song (Figure 1a). The delivery of a disruptive feedback stimulus during singing has been shown to cause abrupt stoppages of song on a subset of trials (Sakata, 2006), and therefore allows us to look for correlations between VTA neural activity and termination of vocal motor behavior.

METHODS

Subjects: All experiments were performed on adult male Bengalese Finches (*lonchura striata var. domestica*) aged 120d post hatch and older. Birds were raised in individual breeding cages until at least 60d, and were housed with other males thereafter until the time of implantation surgery. Animals were housed on a 14/10 light/dark cycle.

Surgery: Detailed methods for sterotaxic surgery of implants tartgeted at VTA are outlined in Chapter 2 of this dissertation. Briefly, we implanted chronic microdrives whose electrodes could be advanced manually (Hessler, 1999). Each microdrive was built with 2-3 tungsten electrodes (4MΩ) and a low impedance ground. Birds were

allowed 3-7 days to recover from surgery, and were habituated to a recording lead for at least 7 days before experiments began.

Electrophysiology: Detailed methods for neural recordings are outlined in Chapter 2. Briefly, subjects were connected to digital recording equipment via a flexible cable (lead) and op-amp. Signals were routed through a commutator, amplified (1000x), filtered (300Hz-10kHz), digitized and recorded (Observer, A. Leonardo, Caltech; C. Roddey, UCSF). Electrodes were advanced in intervals of approximately 80μm and responses to salient probe stimuli (tap on door of soundbox, switching lights off/on) were noted, as was any activity correlated with movement, such as hopping onto the perch, reaching into the food dish, and orienting-related head movements and saccades. Disruptive feedback experiments were conducted both at sites that were responsive to probe stimuli and sites that were not responsive.

Disruptive Feedback: After lead habituation, each bird's home cage was moved into a recording soundbox. Songs were recorded for 1-3 days, during which period the bird acclimated to the new soundbox. Recorded songs were visually inspected and a target syllable was selected for disruptive feedback perturbation. Syllables that typically occurred at the end of song bouts or at the ends of motifs were excluded from selection. Once a target syllable was selected, we created a template of the syllable using its average power spectrum.

Song was monitored by in-house software (Birdtaf) that compared each note to the template. When a match was detected, a TTL pulse was used to trigger playback of the disruptive feedback stimulus. Stimuli used as the disruptive feedback stimulus included individual syllables from the bird's own song (n=13), syllables from conspecific

song (n=16), and 60 ms bursts of white noise (n=2). Responses to the disruptive stimulus were not significantly different among these categories; therefore data have been pooled across disruptive feedback stimulus type. Only one type of stimulus was used in an experiment. Catch trials, in which the TTL pulse was used to mark the time of the trigger but during which no stimulus was presented, were randomly interleaved with triggered presentation of the disruptive feedback stimulus. As an additional control, we used Birdtaf to send out TTL pulses at regular intervals when the bird was not singing. Half of these baseline trials resulted in the playback of the disruptive stimulus and half in a catch trial. All trials were randomly interleaved. Song, TTL pulses, and neural data were recorded on separate channels (Observer) and analyzed post hoc.

Histology: After experiments were complete, small marking lesions were made to confirm the position of the electrodes. Birds were perfused trascardially with 0.9% saline followed by a 3.7% formaldehyde solution. Brains were dissected, sunk in 30% sucrose, and then frozen and sectioned on a freezing microtome (40μm sections). Every 3rd section was stained for TH (tyrosine hydroxylase, a precursor enzyme necessary for the synthesis of dopamine). Following the stain for TH, free-floating slices were stained with hematoxylin so that lesions in non-TH areas could be clearly visualized and a within-brain scale could be established. Slides were cover-slipped with permount, dried, and examined under a dissecting microscope. Please refer to Chapter 1 of this dissertation for a more detailed description of histology methods.

DATA ANALYSIS:

Smoothed, rectified neural traces: The raw waveforms of neural signals in VTA were recorded and stored on computer. Because the signal to noise ratio in VTA was variable across sites and individual neurons were often times difficult to isolate, we used smoothed, rectified neural traces (SMR) as a measure of multiunit activity in the VTA. For each neural trace, the smoothed rectified neural trace was obtained by taking the absolute value of the voltage trace at each sample point (32000 Hz) and smoothing with a 5ms square window. Results obtained with calculations using smoothed, rectified neural traces (SMR) were qualitatively the same as results obtained at sites using a threshold and counting spikes, and therefore all of the summary data reported here used smoothed, rectified data (see Shea 2003 and Kao, 2005 for examples of other studies using this type of smoothed data). When possible, spikes were analyzed and compared to SMR summary data to determine whether reported effects were consistent across a range of signal-to-noise ratios.

Disruptive Feedback Behavior: To quantify behavioral switching, we visually inspected the spectrograms of song files and looked at behavior after detection of the target syllable and subsequent stimulus presentation. We classified trials into three groups: *catch* trials, during which no stimulus was presented; *sing-through* trials, during which a disruptive feedback stimulus was presented and the bird continued singing; and *stop* trials, during which a disruptive feedback stimulus was presented and the bird stopped singing.

Disruptive Feedback Neural Responses: The neural data for all trials were examined and trials during which there was movement artifact were excluded from the analysis. Data were aligned to the onset of the TTL pulse, smoothed and rectified as

described above. The time window of interest was defined as the first 75 ms after the onset of the stimulus. As a control, we also performed analyses of the neural activity preceding delivery of the disruptive feedback stimulus, for which we used the 300 ms immediately before the presentation of the feedback stimulus.

For each experimental session, we computed trial-by-trial z-scores as well as average z-scores for the whole session. For trial-by-trial z-scores, we calculated the area under the smoothed, rectified curve for each trial in each condition (catch, sing-through, stop). For each trial in the sing-through and stop conditions, we subtracted the average area under the curve for the control condition (catch) and divided by the standard deviation of the catch trials, which resulted in a z-score for each individual trial. For average z-scores, we calculated the average area under the curve for each feedback condition (sing-through, stop), subtracted the average area under the curve for the control condition (catch) and divided by the standard deviation of the catch trials.

Similarly, we calculated the neural response to playback of the disruptive feedback stimulus outside of song by subtracting the average area under the curve during quiet baseline and dividing by the standard deviation during quiet baseline.

PSTHs: For those sites (n=6) whose signal to noise ratios allowed counting of spikes, we computed peri-stimulus time histograms for the same window of interest as the smoothed, rectified neural traces (75 ms after onset of stimulus). PSTHs were calculated using 10 ms bins, and the firing rates were compared across conditions.

Latency to the onset of the neural response: For each experiment, the mean SMR trace in a feedback (sing-through, stop) condition was compared to the mean SMR trace

of the catch trials. The d' value was calculated for each 1ms bin of the trial according to the following equation:

$$\frac{|\mu_{FB} - \mu_{catch}|}{sqrt((var_{FB}+var_{catch})/2)}$$

A shuffle test (1000x, across trial type) was run in order to establish confidence bounds on the d' from the real data. If the d' value from the real data exceeded the 95% confidence interval from the simulation, the neural activity was noted as being significantly different between the feedback trial and the catch trial in that 1ms bin.

Within each experiment, calculations were made for a time window 100 ms before and 450 ms after the onset of the stimulus. The onset to the neural response was defined as the first bin of ≥10 consecutive bins that were above the 95% confidence interval for that experiment. Once this latency was determined, an additional 2.5 ms was added to the average to correct for possible effects of smoothing with the 5 ms square window.

Correlations Between Slowing of Song and Neural Response to the Disruptive Feedback Stimulus: Within an experiment, sing-through trials were analyzed for a possible slowing of song as reported in Sakata (2006). We picked two notes in the stereotyped sequence of song: one note before (within 300 ms) and one note after (within 500 ms) the onset of the disruptive feedback stimulus. The duration of the interval between these two notes was calculated trial-by-trial. The neural response to the disruptive feedback stimulus was computed as the area under the SMR curve from stimulus onset to 75ms thereafter for each trial. We then tested whether there were

significant correlations between the duration of the interval and the neural response to the feedback stimulus.

Behavioral Habituation: We performed two tests to determine whether the bird's behavioral response to the disruptive feedback stimulus habituated over time. First, each experiment was broken up into 1 hr. bins (experiments lasting at least 10 hrs.) or 30 min. bins (experiments lasting at least 3 hrs.). Each experiment was divided into an early phase (first 5 hrs or first 1.5 hrs, respectively) and a late phase (last 5 hrs. or last 1.5 hrs., respectively). We compared the average probability of stopping in early vs. late phases. Second, for experiments lasting at least 10 hrs., we ran a linear regression on the probability of stopping over time and tested for a significant negative slope.

Neural Habituation: Neural activity was calculated as the area under the SMR curve in a 75 ms window after the onset of the feedback stimulus. The neural response within each condition (catch, sing-through, stop) was plotted over trial number. In order to test whether the neural response to the feedback stimulus habituated over time, we performed a linear regression on neural response over trial and tested for slopes significantly different from zero. The neural activity on catch trials served as a control for drift in the electrical signal at the recording site.

RESULTS

Behavioral Response to Disruptive Feedback: Once they reach adulthood, male Bengalese finches sing a song that consists of several notes strung together into relatively stereotyped patterns called motifs. The disruptive feedback paradigm targeted one specific note in a motif for feedback disruption, and every time that this syllable was

detected, the bird either experienced playback of the disruptive stimulus or a catch trial, in which no stimulus was played back (Figure 1).

Approximately 70% of the time, birds continued singing after presentation of the disruptive feedback stimulus. These trials were designated as *sing-through* trials (Figure 1c, middle panel). Though the sensory experience of the bird was different due to playback of the disruptive feedback stimulus, the vocal motor output on catch trials and sing-through trials was comparable. One caveat is that some sing-through trials in some birds were marked by a slight increase in the inter-syllable interval between the syllable receiving the feedback and the subsequent syllable on sing-through trials. A complete description of this slowing of song can be found in Sakata (2006).

Birds stopped singing after the presentation of the feedback stimulus on an average of 30% of trials, and these trials were classified as *stop* trials (Figure 1c, bottom panel). Occasionally, a bird would stop singing after playback of the disruptive feedback stimulus and start again within 500 ms of the playback. In these cases, the bird never continued the interrupted song sequence and instead would start over either with introductory notes or at the beginning of a motif. These trials were counted as stops because the ongoing motor pattern was clearly disrupted. While the vocal motor output on these stop trials was different from the sing-through trials, the bird's sensory experience up until the motor change was comparable, and allowed us to make useful comparisons. A detailed description of the behavioral effects of this disruptive feedback paradigm can be found in Sakata & Brainard (2006).

VTA Neural Response to Disruptive Feedback: We paired the disruptive feedback paradigm with neural recordings in the VTA, and examined the responses to the

disruptive feedback stimulus on catch, sing-through, and stop trials (n=23 sites in 5 birds, Figure 2, Figure 3, Figure 4). VTA neurons responded to the disruptive auditory stimulus with a short latency, phasic activation to the feedback stimulus. The latency to the onset of the neural response was 18ms for sing-through trials and 14ms for stop trials. The latencies to the onset of the neural response were significantly shorter for stop trials than for sing-throughs (paired t-test, $p=0.012$).

The neural activity in a 75-ms window following the delivery of the disruptive feedback stimulus was significantly greater for sing-through trials compared to catch trials at 15/23 sites in VTA ($p < 0.05$, K-S test, mean z-score= 0.4352, see Figure 3a-3c). The neural activity in this same time window was significantly greater for stop trials than for catch trials (21/23 sites, $p>0.05$, K-S test, mean z-score 1.6606, Figure 3a-3c). Moreover, the neural response to the feedback stimulus was significantly greater on stop trials than on sing-through trials at 21/23 sites in VTA ($p < 0.05$, K-S test, Figure 3a-c), suggesting that neural responses of a greater magnitude are correlated to a change in motor behavior.

As a control, we paired the disruptive feedback paradigm with recordings made at sites adjacent to but not in the VTA. We found no significant responses to the feedback stimulus at these sites (n=7 sites in 5 birds) and no correlations with motor behavior (Figure 3d-f). These negative results localize this effect to the VTA.

As mentioned in the Methods section of this chapter, we encountered a broad range of signal-to-noise ratios while recording in the VTA (Figure 4). The results reported above are based on smoothed, rectified data (SMR), and are consistent across sites in VTA. However, for a subset of the data, the signal-to-noise ratio was sufficient

for recording single units or small clusters of multiunit activity (n=6 sites in 3 birds). For this subset of data, we compared the isolated spikes to the SMR data. The results for the spikes were qualitatively similar to the results from the SMR data (Figure 4). All sites showed a significant increase in spike rate in response to the feedback stimulus. Firing rates after delivery of the disruptive feedback stimulus on sing-through trials were significantly greater than on catch trials at 6/6 sites (K-S test, $p<0.05$, ROC=0.7865). Similarly, the firing rates after the disruptive feedback stimulus were significantly greater on stop trials than on catch trials at 5/6 sites ($p<0.05$, ROC=0.8828). Finally, the firing rates during stop trials were significantly greater than those during sing-through trials at 4/6 sites ($p<0.05$, ROC = 0.7487). These data support the idea that our findings are independed of obtained signal-to-noise ratios.

Relationship Between Neural Response and Motor Output: The neural activity in response to the disruptive feedback stimulus is different on sing-through trials and stop trials, even though the sensory input is nominally the same on these trials (See Figure 1c). The same stimulus is being played back in both cases, and the bird is singing the same motif, so auditory and proprioceptive feedback should be the similar across the two trial types. The main difference is that the vocal motor output changes – the bird continues singing in one case but stops in the other. This led us to ask whether there was any relationship between the neural response to the feedback stimulus and subsequent motor behavior. In order to explore this relationship, we binned the z-scores of the trial-by-trial neural responses to the feedback stimulus, regardless of motor outcome. Then for each bin with >10 trials, we calculated the probability that a trial with a z-score in that bin would result in a stop (all experiments, Figure 5; individual experiment, Figure 5 inset).

Our results indicate that the probability of stopping increases as the magnitude of the neural response increases.

As mentioned earlier, delivery of the disruptive feedback stimulus on sing-through trials effects a slowing of song (Sakata, 2006). This slowing is accounted for by an increase in the inter-syllable interval between the syllable receiving the feedback and the subsequent syllable. Because the magnitude of VTA response to the feedback stimulus was highly correlated with termination of song, we sought to ask if there was a relationship between VTA activity and the amount of slowing on a trial-by-trial basis. 3 of 15 sites showed a significant positive correlation between the length of the inter-syllable interval and the neural response to the feedback stimulus, implying that there is no consistent relationship between VTA activity and the slowing of song in this disruptive feedback paradigm.

Baseline Neural Activity and Behavioral Outcome: While analyzing the raw data for each experiment, we noticed a trend in the baseline data, defined here as the 300ms preceding delivery of the disruptive feedback stimulus (Figure 6a). Because feedback and catch trials were delivered on randomly interleaved trials, we expected that the baseline preceding the trigger would be the same for both conditions. As predicted , there is no significant difference between baseline neural activity on trials (*sing-through* and *stop* combined) in which the bird will eventually receive feedback and trials (*catch*) in which he will not (K-S test, $p = 0.1139$; Figure 6b, top panel, Figure 6c). However, when we split the trials based on behavioral outcome, we found that the baseline neural activity on stop trials was significantly greater than on catch trials even before the feedback stimulus was delivered (K-S test, $p<0.001$). Conversely, the baseline neural

activity on sing-through trials was significantly *lower* than on catch trials (K-S test, p = 0.0011). Finally, the baseline neural activity on stop trials was significantly greater than 0.2565, see figure 6b, bottom panel, Figures 6d-f). These data suggest that baseline activity levels in the VTA influence the behavioral response to disruptive feedback (see Figure 7).

Response to the Disruptive Feedback Stimulus Outside of Song: In addition to delivering the disruptive feedback stimulus during song, we delivered it during randomly interleaved periods of silence in order to assess its ability to drive VTA (Figure 8). The neural response to the feedback stimulus was significantly greater when it was delivered outside of song than when it was delivered during singing (K-S test, $p<0.001$; mean z score during singing = 0.395, n=21; mean z-score outside of song = 3.2212, n=23).

Habituation of Behavioral and Neural Responses: Habituation to a stimulus across presentations is a defining characteristic of the startle response. To explore the possibility that the signals we were seeing in VTA were reflective of an acoustic startle response, we tested whether the behavioral and neural responses to the disruptive feedback stimulus habituated over time. First, we analyzed whether the behavioral response to the disruptive feedback stimulus (stopping) occurred less frequently over the course of an experiment. For experiments lasting at least 10 hours, the probability of stopping was not significantly different in the first 5 hrs. compared to the last 5 hrs. of the experiment. We then looked at the first 3 hrs. of all experiments in case the habituation had a shorter time course. The probability of stopping in the first 90 min. of the experiment was not significantly different than the probability of stopping in the 90 min. thereafter. In addition we looked across the time course of each individual experiment,

and found significant negative correlations on only 2/21 experiments, indicating that the behavioral response does not consistently habituate over time.

We also analyzed the neural response to the feedback stimulus across trials and found that only 2/16 sites showed a significant negative correlation. These two sites were not the same two sites at which the behavioral response showed significant negative correlations.

DISCUSSION

The disruptive auditory feedback manipulation utilized in these experiments resulted in changes in the vocal motor behavior of our test subjects, causing birds to abruptly terminate song on approximately 30% of the trials in which the disruptive stimulus was delivered (Figure 1). Because we chose to deliver the disruptive stimulus at a point in song that was not normally associated with the end of vocalization, we are confident that the change in behavior was due to the stimulus. The trials on which the bird received the disruptive stimulus were randomly interleaved with trials that did not receive a disruptive stimulus, and the probability of stopping remained relatively constant throughout the experiment, thus ensuring that the effects on VTA firing reported here are correlated with the behavior of the bird rather than with electrode drift over time. Our results here demonstrate that the disruptive feedback stimulus is a salient stimulus capable of perturbing ongoing singing behavior and confirm the findings of a previous study (Sakata, 2006).

The disruptive feedback stimulus, in addition to causing termination of song behavior, drove short latency (< 20ms) phasic activations of multiunit neural activity

within the VTA. Our results are in agreement with the many studies that have shown that VTA neurons respond to salient stimuli with phasic activation (Ljungberg, 1992; Horvitz, 1997; Comoli, 2003). However, there is one difference. The latency to the onset of the neural response to auditory stimuli in our experiments was shorter than latencies reported in mammals, which are on the order of 80-100ms (Horvitz, 1997). It is possible that this is a species difference – birds are auditory specialists, and Bengalese finches, which weigh an average of 15g have much smaller brains than cats. While latencies of <20ms are short, they are plausible given the timing of the auditory brain stem response for birds (Lucas, 2002; Winer, 2005) and the connectivity of the superior and inferior colliculi with the VTA (Herbert, 1997; Comoli, 2003). It has been demonstrated in mammals that there are direct subcortial pathways from the superior colliculus to the VTA, and that these circuits relay information about salient stimuli to dopamine neurons at latencies shorter than those necessitated by a cortical loop (Comoli, 2003).

It seems likely that the response to the disruptive feedback stimulus seen in VTA reflects salience rather than an appetitive response. Because birds will change their songs in order to avoid a disruptive white noise stimulus (Tumer, 2007), it seems unlikely that birds find the interruption of song positively reinforcing. Furthermore, the short latency to activation suggests that VTA is registering the presence of a salient stimulus before playback of the entire stimulus is complete and an evaluation of its incentive value can be made.

The experiments in this study are the first to report a correlation between neural activity in VTA and the termination of vocal motor behavior. We consistently find that the magnitude of the VTA response is greater on trials resulting in a termination of vocal

motor behavior (stop trials) than it is on 1) trials in which the bird received the disruptive stimulus but continued to sing though (sing-through trials) and on 2) trials in which no stimulus was delivered (catch trials) (see Figures 2 – 4). Since we know that VTA activity increases in response to the disruptive stimulus, it is particularly useful to make a comparison between sing-though and stop trials. The sensory stimulus played back to the bird is the same on both of these trial types, and presumably -- up until the bird stops singing -- his sensory experience is nominally the same. Therefore, it is likely that the greater magnitude of VTA activity we see on stop trials when compared to sing-though trials reflects neural activity associated with the termination of motor behavior.

Earlier in this dissertation, we reported an increase in VTA activity just before termination of unmanipulated song (Chapter 1, Figure 4). The results of the disruptive feedback experiments corroborate this finding, and suggest that increased levels of VTA activity lead to an increased likelihood of stopping. In order to explore the relationship between the magnitude of the response to the disruptive stimulus and a change in motor behavior, we looked at the magnitude of the trial-by-trial response to the feedback stimulus and its relationship to song terminations (Figure 5). There is a clear predictive relationship in that the probability of stopping increases as the neural response to the feedback stimulus increases.

One implication of these results is that VTA activity is correlated with switches into and out of song. Because we saw a correlation between VTA activity levels and vocal motor output, we wondered if signals from VTA might be affecting motor output in a graded fashion. In order to probe this idea, we took advantage of the fact that delivery of the disruptive feedback stimulus on sing-through trials results in an increase in inter-

syllable interval immediately following the stimulus. If VTA is controlling vocal motor output on a syllable-by syllable-basis, one might expect to see a correlation between the level of activity in response to the disruptive stimulus and the length of the motor delay it causes. However, the increase in inter-syllable interval we saw on sing-through trials was not consistently correlated with neural activity in VTA, suggesting that the magnitude of VTA's response to the playback of a salient stimulus may not contribute to this particular modulation of vocal motor production.

While examining the raw data from our experiments, we noticed a striking trend in the baseline levels of VTA activity before delivery of the feedback stimulus (Figure 6b). As expected, the baseline neural activity on trials that got disruptive feedback (sing-through + stop trials) and trials that did not get feedback (catch trials) was the same (Figure 6c). However, when we separated out trials on which the bird stopped once he got feedback, we saw a significant difference in the baseline neural activity of sing-through and stop trials (Figure 6d-f). Feedback trials on which baseline activity was lower than the mean were more likely to be trials on which the bird sang through the feedback stimulus (Figure 6 d-f). Trials on which baseline activity was higher than the mean were more likely to result in stops once the feedback stimulus was delivered, suggesting that the baseline level of activity in this brain region is correlated with probability of termination of motor behavior in response to a salient stimulus. However baseline activity was not the sole determinant of behavioral outcome, as trials with comparable baseline activity levels resulted in both sing-through behavior and song termination (Figure 7).

In an effort to further explore the properties of the sensory response to the disruptive feedback stimulus, we delivered the stimulus to the bird outside of the context of song (Figure 8b). We found that the response to the feedback stimulus outside of song was an order of magnitude greater than it was while the bird was singing (Figure 8d). Vocalization-induced suppressions of sensory responses have been reported in both cortical and subcortical auditory areas, such as auditory cortex, cochlear nucleus, lateral lemniscus, and the inferior colliculus (Elaides, 1993, 2008). This is the first report of signals reflecting an auditory-induced suppression of vocalization in the VTA. However, because auditory responses in the subcortical areas projecting to VTA have been shown to have this property, is unclear whether this phenomenon is a reflection of VTA processing or simply an effect of the peripheral inputs.

Taken in the context of the results outlined above, this finding lends further support to the hypothesis that signals from VTA might be involved in regulating behavioral switching. Singing is an important behavior for a bird. Birds often sing in the contexts of territorial defense or during courtship, and must continue to sing even in the presence of vocalizations from other birds, such as counter-singing by a rival male or contact calls from a potential mate. We have shown both that these types of salient stimuli drive VTA (See Chapter 1, Figure 6) and that the extent to which VTA is activated in response to a stimulus is correlated with whether the bird stops singing (Figure 5). It is possible that this vocalization-induced suppression of the auditory response could help ensure that the bird's vocal motor program is not interrupted prematurely while he is engaged in the important tasks of territorial defense or courtship.

Because the behavioral paradigm we used in these experiments caused abrupt song terminations, we wanted to ask to what extent these signals were reflective of an acoustic startle response. The acoustic startle response consists of a rapid contraction of peripheral muscles coupled with an arrest of ongoing activity (Blaszczyk, 2003). Reported time constants for the onset of the auditory startle response average 6.5 ms (Ison, 1973; Cadenhead, 1999) for the behavioral response and about 10 ms for the neural response (Mortimer, 1973). These time constants overlap with the timescale of the responses we see in VTA. One of the hallmark characteristics of the auditory startle response is that it habituates across presentations of the same stimulus, and experiments in humans as well as in rodents clearly show this effect (Cadenhead, 1999; Blaszczyk, 2003). In order to determine whether the neural signals we saw reflected an acoustic startle response, we looked to see if they habituated across presentations of the disruptive feedback stimulus. We found that the neural responses did not habituate over trials. As an added measure, we tested the behavioral response to see if it habituated across the course of an experiment and found that it did not. Our analyses suggest that the neural signals we observe in VTA are not reflective of those subserving a startle response and instead seem to be signaling the occurrence of a salient event.

Experiments using this same behavioral paradigm while recording from a nucleus in the motor pathway of the song system, HVC, lead us to speculate that the signals we see in VTA could be directly influencing nuclei in the song system (Sakata & Brainard, in preparation). HVC is deeply implicated in the regulation of temporal aspects of song output, and neural activity in HVC is tightly modulated with song (Vu, 1994; Yu, 1996; Hahnloser, 2002). Birds with bilateral lesions of HVC cannot produce song (Nottebohm,

1976), and microstimulation in HVC during singing causes abrupt terminations of singing (Vu, 1994).

The disruptive feedback paradigm causes an inhibition of singing-related neural activity in HVC at a latency of 40-70ms for sing-through trials. On stop trials, the singing-related activity of HVC is completely suppressed after the disruptive stimulus causes the bird to stop singing (Sakata & Brainard, in preparation). The signals we see in VTA occur at a latency of 10-20 ms, which leads us to speculate that the signals we see in VTA could be effecting the inhibition of HVC. A direct connection between VTA and HVC has been established by the use of retrograde tracers injected into HVC (Appeltants, 2002). The anatomical data also support the existence of a GABAergic projection from VTA to HVC, since retrogradely labeled cells in VTA are both TH+ and TH-, though this has not been confirmed with GAD staining. If the signals we see in VTA reflect the activity of GABAergic projection neurons (see Chapter 1 Discussion), it is possible that these neurons are responding to salient stimuli in the environment and broadcasting signals to the song system. It is known that in mammals, the VTA and the substantia nigra pars compacta act to regulate the output of basal-ganglia-thalamocortical loops. The production of birdsong is dependent on just such a loop (Nottebohm, 1976; Bottjer, 1984; Brainard, 2000), and it is perhaps not surprising that it, too, should be regulated by the VTA.

The precise temporal pattern of song and its repeatability across renditions make it a useful readout of basal ganglia activity. In this study, we have found that neural signals in VTA are correlated with termination of motor behavior caused by behavioral interruption. These results corroborate and extend the findings from Chapter 1 of this

dissertation, in which we found increases of VTA neural activity during the initiation and termination of song. Our data also indicate that VTA neurons in singing birds have rapid access to information about salient perturbations of sensory experience, and extend findings indicating that the VTA responds to salient stimuli. Moreover, we demonstrate that there is a strong correlation between level of VTA activity and probability of stopping song. These results imply that VTA responds to salient cues in the environment and regulates motor behavior. However, due to the fact that VTA responds to a broad class of stimuli and is modulated with both the initiation and termination of behavior, we suspect that the signals we see are correlated with action selection rather than with the reinforcing properties of the stimuli used.

Figure 1. *Delivery of a disruptive feedback stimulus during singing results in an abrupt termination of song on a subset of trials.*

a) Experimental set-up. We recorded from the VTA of awake, behaving adult male Bengalese finches. In house software (Birdtaf) monitored the bird's song for occurrences of a pre-selected syllable. When the syllable was detected, Birdtaf sent out a TTL pulse that was used to trigger playback of the disruptive stimulus (either a single out of context syllable or white noise). **b)** Spectrogram of bird's song with examples of the 3 types of trials in the disruptive feedback experiment. On catch trials, the TTL pulse did not trigger playback of a disruptive stimulus. On stop trials, the TTL pulse triggered playback of the disruptive stimulus, which resulted in an abrupt termination of song. On sing-through trials, the TTL pulse triggered playback of the disruptive stimulus but the bird continued to sing through. **c)** A zoomed-in view of the trial types, aligned by onset of the TTL pulse at time = 0. Note that the motor output of the catch trials and the sing-through trials (*top panel, middle panel*) is comparable. This suggests that differences we see between the two trial types reflect a sensory response to the disruptive stimulus. Similarly, the bird's sensory experience on sing-through and stop trials (*middle panel, bottom panel*) is nominally the same until the bird stops singing, which suggests that differences between these two trial types in the window preceding song termination might reflect a motor or pre-motor signal.

Figure 2. *VTA's neural response to the disruptive feedback stimulus is greater on trials in which delivery of the stimulus results in termination of song*.

a) Catch trials, during which the targeted syllable was detected (at time = 0 ms) and no stimulus was played back. *First panel*, a typical spectrogram of the vocal behavior during catch trials. *Second panel*, a representative raw neural trace for catch trials at this site. *Third panel,* a raster plot depicting neural activity across all catch trials in this experiment. Each tick mark represents an action potential, and each row represents one trial. *Fourth panel*, a peri-stimulus time histogram (PSTH) depicting the average firing rate of VTA neurons across catch trials. Firing rates were calculated in 10 ms bins. **b)** Sing-through trials, during which the targeted syllable was detected and the disruptive feedback stimulus was played back (at time = 0 ms). *First panel,* spectrogram of vocal behavior on sing-through trials. On sing-through trials, birds received a disruptive feedback stimulus but did not terminate song. The black bar underneath the spectrogram indicates the onset and duration of the disruptive feedback stimulus. The second, third, and fourth panels represent data from sing-through trials in the same manner as outlined for the catch trials in Figure 2a. **c)** Stop trials, during which the targeted syllable was detected and the disruptive stimulus effected a termination of song. The data for stop trials are shown in all four panels in the same manner as outlined for catch trials and sing-through trials. **d)** Comparison of PSTHs for the three trial types (catch = black, sing-through = blue, stop = red), with the onset of the disruptive feedback stimulus at (time = 0 ms). The neural activity in VTA increases in response to playback of the disruptive feedback stimulus ($p < 0.05$ at this site). Moreover, the magnitude of the response is

greater on trials in which the bird stops than on trials in which he sings through ($p < 0.05$ at this site). Note that the latency to the onset of the neural response in this experiment was at the extreme upper end of the observed distribution.

Figure 3. *Summary data: VTA neural activity increases in response to playback of the disruptive stimulus and is greater on trials in which the stimulus effects a termination of song.*

Inside of VTA: Distribution of z-scores by trial type. The average neural response for each trial type in each experiment was calculated by assessing the area under the average smoothed, rectified (SMR) neural trace. Calculations were made for a 75 ms window after detection of the targeted syllable triggered a TTL pulse. For summary data, z-scores were calculated by comparing the average neural response on disruptive stimulus trials to neural activity in the same time window during catch trials. Within an experiment, the z-score for each trial type represents how many standard deviations away from the mean of the catch trial the data were. Panel **a)** shows the distribution of z-scores for sing-through trials (blue) and stop trials (red) across all experiments in VTA (n=23). **b)** For each experiment, the z-score from the sing-through trials is plotted against the z-score for the stop trials. Data points with values along the abscissa greater than zero represent experiments in which neural activity on sing-through trials was greater than on catch trials (significant at 15/23 sites, $p<0.05$). Data points with values along the ordinate greater than zero represent experiments in which neural activity on stop trials was greater than on catch trials (significant at 21/23 sites, $p < 0.05$). The dashed line represents equal neural activity on sing-through and stop trials. All data points lie above the dashed line, indicating that neural activity on stop trials was always greater than on sing-through trials (significant at 21/23 sites, $p< 0.05$). **c)** The average z-score for sing-through trials on all experiments (blue bar, mean = 0.4352) compared to the average z-score for stop trials on

all experiments (red bar, mean = 1.6606). Error bars are s.e.m. Panels **d – f** correspond to panels a – c for data recorded outside of the VTA (n=7 sites). There were no significant effects of the disruptive feedback stimulus on neural activity at these sites. The average z-scores for sing-through and stop trials was not significantly different than zero.

Figure 4. *Results are consistent across a wide range of signal-to-noise ratios.*

Panels a-d show neural responses for the three trial types in an experiment with a high signal-to-noise relative to our data. Panels e-h show the same data for another experiment with a signal-to-noise ratio more representative of the majority of our data. Figures 4a & 4e, *top panels*, are spectrograms of catch trials in experiments with high and low signal-to-noise, respectively. Figures 4a & 4e, *second panels*, are example raw neural traces for catch trials. Figure 4a, *bottom panel*, is a raster plot of neural firing across trials. Each tick mark signifies an action potential, and each row is one trial. Figures 4b and 4f are comparable data for sing-through trials, and 4c and 4g are comparable data for stop trials. Figure 4d compares the PSTHs of the 3 trial types in the experiment shown in 4a, 4b, and 4c (black = catch, blue = sing-through, red = stop). Panel 4h compares the smoothed rectified neural traces for the 3 trial types in the experiment shown in 4e, 4f, and 4g. A comparison of the effects summarized in panels 4d and 4h reveals that the results are consistent across a range of signal-to-noise ratios. Note: The latency to the onset of the neural response seen in these figures is representative of the data as a whole.

Figure 5. *The probability of stopping on a given trial is positively correlated with the level of VTA neural activity.*

The neural response was assessed for a 75ms window after the onset of the disruptive feedback stimulus. Trial by trial z-scores for neural activity were calculated with respect to the average neural activity on catch trials within the same experiment. The z-scores for all disruptive feedback trials (sing-through trials and stop trials) were binned and plotted against the probability that a trial with a z-score in that bin resulted in a song termination. Only bins with more than 10 trials were analyzed. a) Across all trials in all experiments, the probability of terminating song on a given trial increases as the level of neural activity (z-score) in response to the disruptive stimulus increases (n=4261 trials). b) Within one experiment, (n = 29 trials), the probability of stopping also increases as a function of increasing neural activity in VTA.

Figure 6. *Trials on which* baseline *neural activity is higher are more likely to result in song terminations.*

a) Spectrograms of the 3 trial types. *Top panel*, catch trials; *middle panel*, sing-through trials; *bottom panel*, stop trials. We analyzed the neural activity in the 300 ms *before* the onset of the disruptive stimulus. b) Smoothed, rectified (SMR) neural traces were normalized to the average of the catch trials within each experiment (n = 23 experiments for each trial type). The feedback stimulus was delivered at time = 0. *Top panel*: The average SMR trace for catch trials is plotted in black. The average SMR trace for all trials in which a disruptive feedback stimulus was delivered is plotted in light purple. The width of each trace denotes the standard error. c) The distribution of z-scores for neural activity during the baseline period on catch trials and trials during which a stimulus was eventually played back. Before the onset of the stimulus, the level of neural activity (indicated by z-score, see Methods) in VTA is the same on catch trials (black bars) and trials on which a stimulus will eventually be played back (light purple bars, comprised of sing-through trials plus stop trials). This is as expected, since the disruptive stimulus trials and catch trials are randomly interleaved. d) The distribution of z-scores for neural activity during the baseline period, separated by eventual motor behavior. This figure corresponds to Figure 3a. e) Because z-scores are calculated with respect to catch trials, the average z-score for catch trials is zero. The z-scores on trials that will eventually be stop trials tend to be greater than zero , while the z-scores for the trials that will eventually be sing-through trials tend to be less than zero. f) The average z-scores for baseline firing rates on sing-through trials (blue bar, mean = -0.0857) is significantly

lower than on stop trials (red bar, mean = 0.2565)(n=23, $p<0.001$). These data suggest that baseline levels of neural activity in VTA are correlated with the motor response to the disruptive stimulus once it is played back.

Figure 7. *Baseline neural activity is not the sole determinant of the behavioral response to the disruptive feedback stimulus*.

Within each trial type, trial-by-trial z-scores calculated during the baseline period were binned and plotted against the average z-score of the response to the disruptive stimulus for all trials in that bin. (black line, catch trials, n=2578 trials; blue line, sing-through trials, n= 3179 trials; red line, stop trials, n=1282 trials) The data indicate that the behavioral outcome cannot be entirely predicted by baseline levels of neural activity, since trials with similar baseline z-scores can result in both sing-throughs and stops.

Figure 8. *The response to the disruptive feedback stimulus is suppressed during singing.*

a) The response to the disruptive feedback stimulus during ongoing song. *Top panel.* Spectrogram of a trial in which the disruptive feedback stimulus is delivered during ongoing song. *Middle panel.* A representative raw neural trace from a trial on which the disruptive feedback stimulus is delivered during ongoing song. *Bottom panel.* A raster plot depicting the response to delivery of the disruptive stimulus during ongoing song. b) The response to the disruptive feedback stimulus outside of the context of singing. *Top panel*. Spectrogram of a trial in which the disruptive feedback stimulus was delivered when the bird was not singing. *Middle panel.* A representative raw neural trace from a trial on which the disruptive feedback stimulus was delivered outside of the context of song. *Bottom panel.* A raster plot depicting the neural responses to delivery of the disruptive feedback stimulus outside of the context of song. c) PSTH comparing the response to the disruptive feedback stimulus during ongoing song (blue line) with the response outside of the context of singing (black line). d) Summary data for all experiments in which the disruptive stimulus was delivered during ongoing song (blue bar, n=21, mean z-score = 0.395) compared to experiments in which the disruptive stimulus was delivered outside of the context of song (grey bar, n=23, mean z-score = 3.221). Error bars are s.e.m. The response to the disruptive feedback stimulus is significantly attenuated during singing ($p < 0.001$).

Figure 1

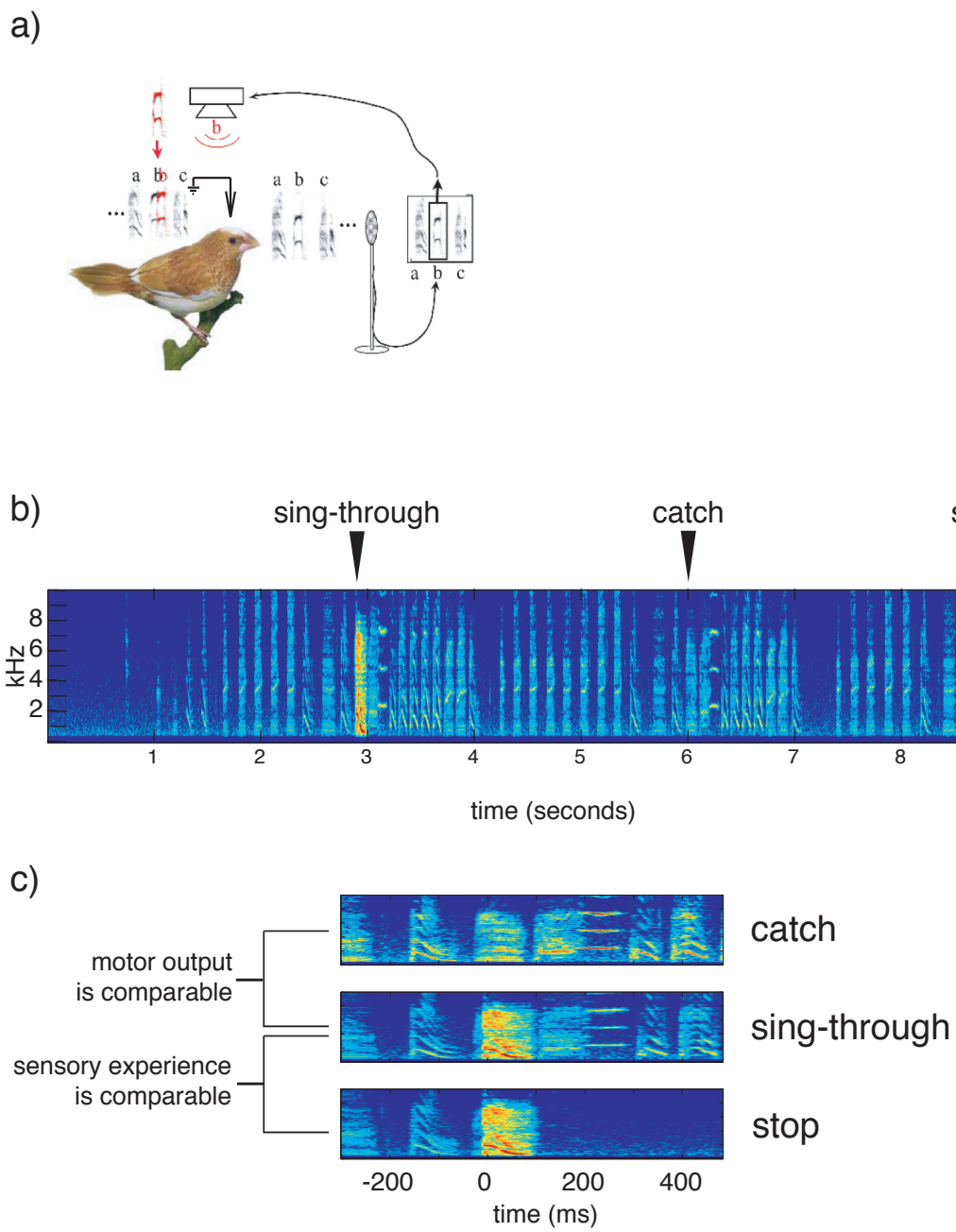

a)
b
a b c
a b c
a b c
b)
sing-through
catch
stop
kHz
8
6
4
2
1
2
3
4
5
6
7
8
9
time (seconds)
c)
motor output
is comparable
sensory experience
is comparable
catch
sing-through
stop
-200
0
200
400
time (ms)

Figure 2

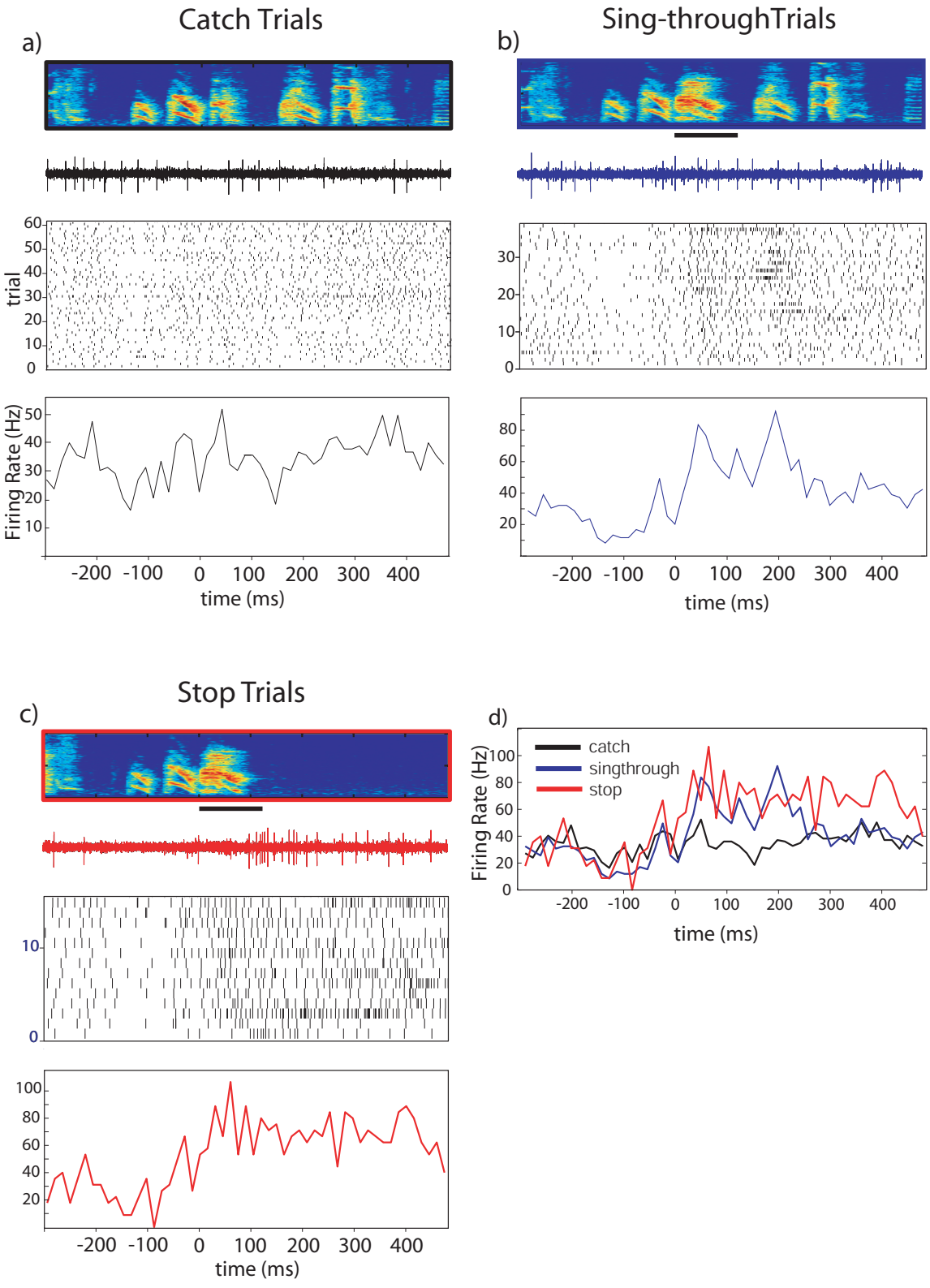
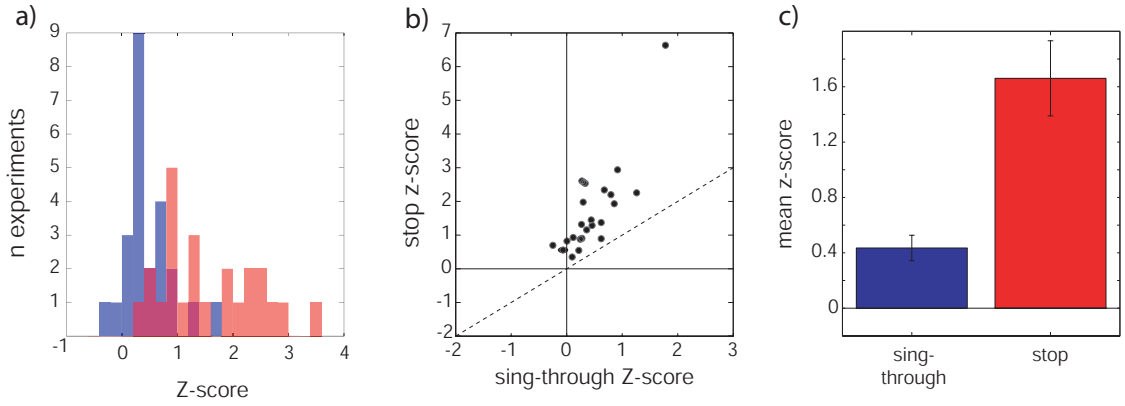

Catch Trials
a)
trial
Firing Rate (Hz)
time (ms)
Sing-throughTrials
b)
time (ms)
Stop Trials
c)
time (ms)
d)
catch
singthrough
stop
Firing Rate (Hz)
time (ms)

Figure 3

Inside of VTA



Outside of VTA

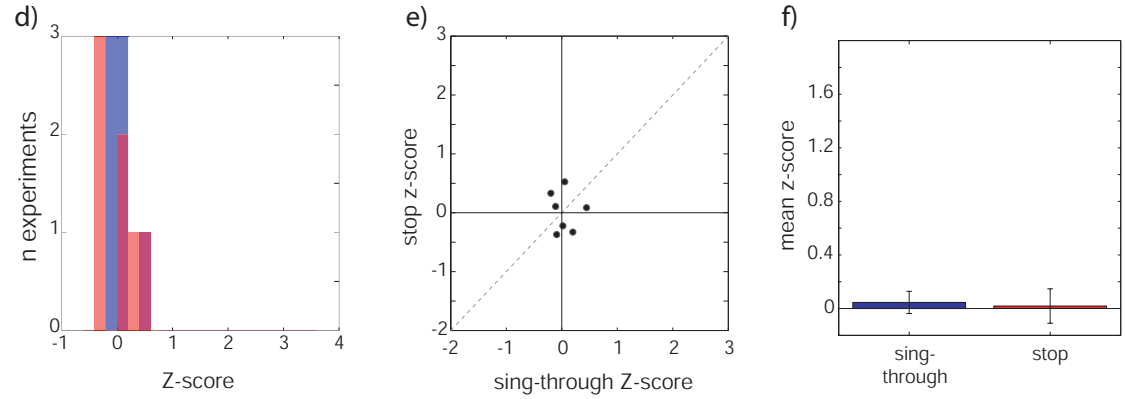


Figure 4

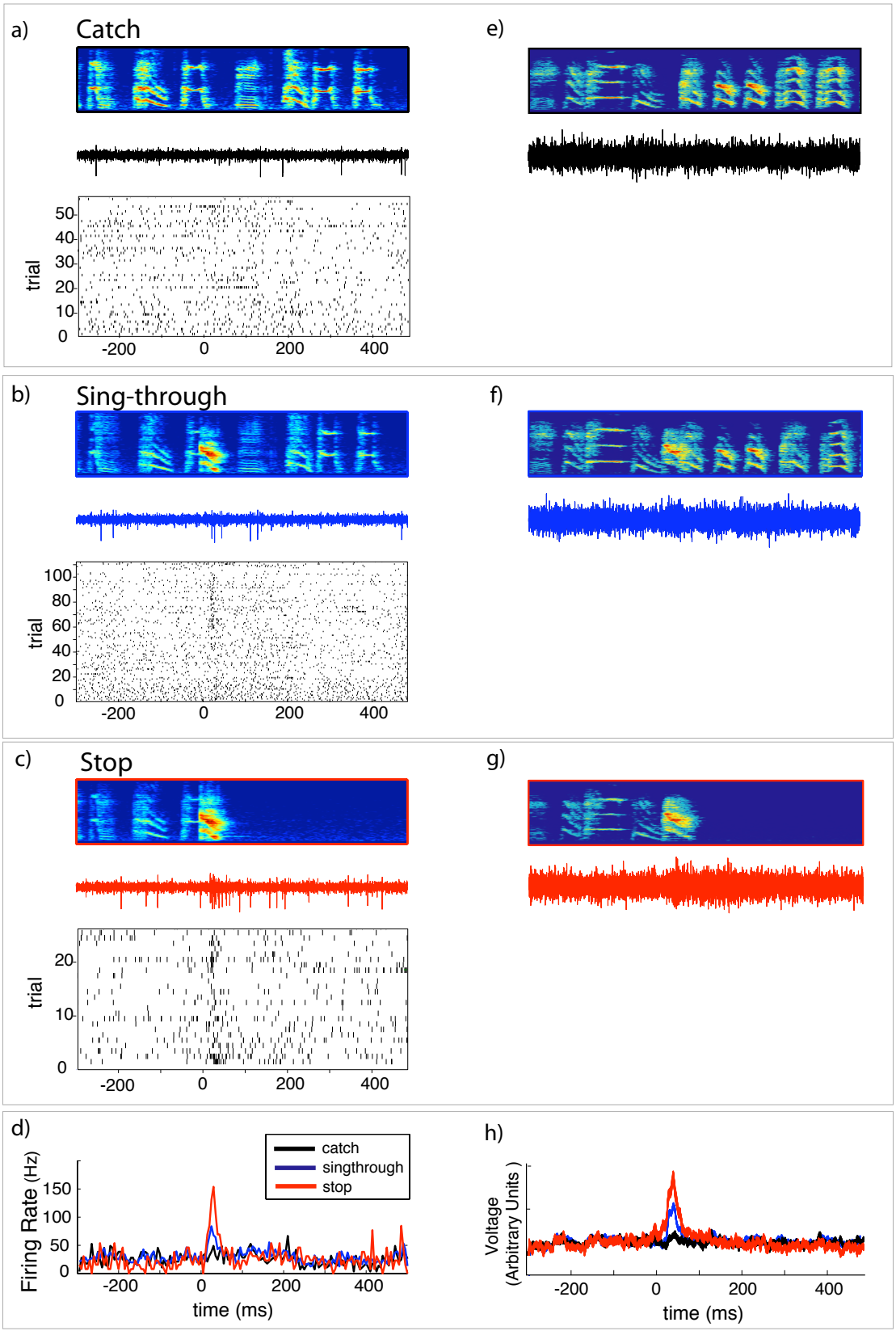

a)
Catch
e)
trial
50
40
30
20
10
0
-200
0
200
400
b)
Sing-through
f)
trial
100
80
60
40
20
0
-200
0
200
400
c)
Stop
g)
trial
20
10
0
-200
0
200
400
d)
Firing Rate (Hz)
150
100
50
0
catch
singthrough
stop
-200
0
200
400
time (ms)
h)
Voltage (Arbitrary Units)
-200
0
200
400
time (ms)

Figure 5

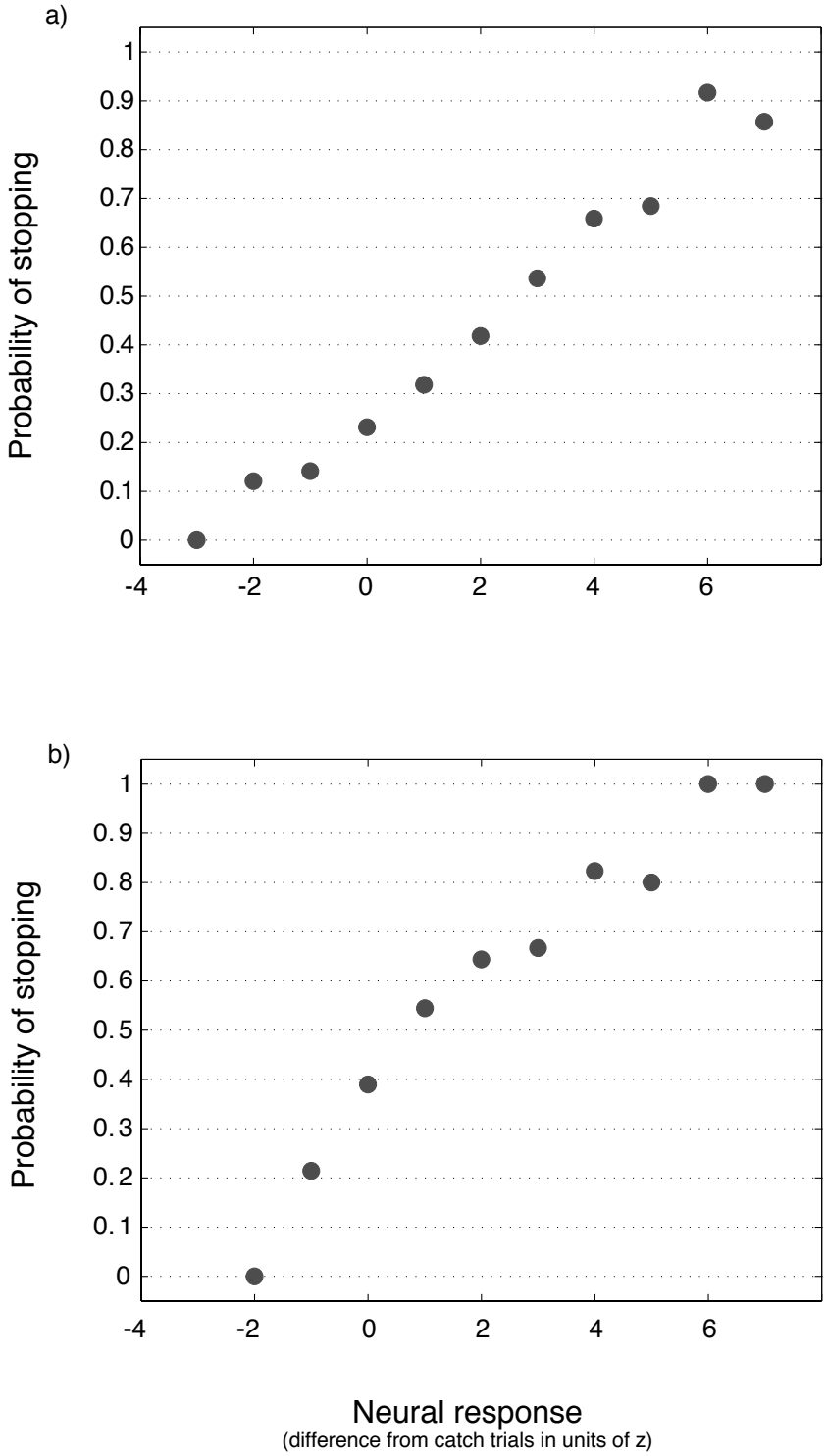

a)
Probability of stopping
1
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
-4
-2
0
2
4
6
b)
Probability of stopping
1
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
-4
-2
0
2
4
6
Neural response
(difference from catch trials in units of z)

Figure 6

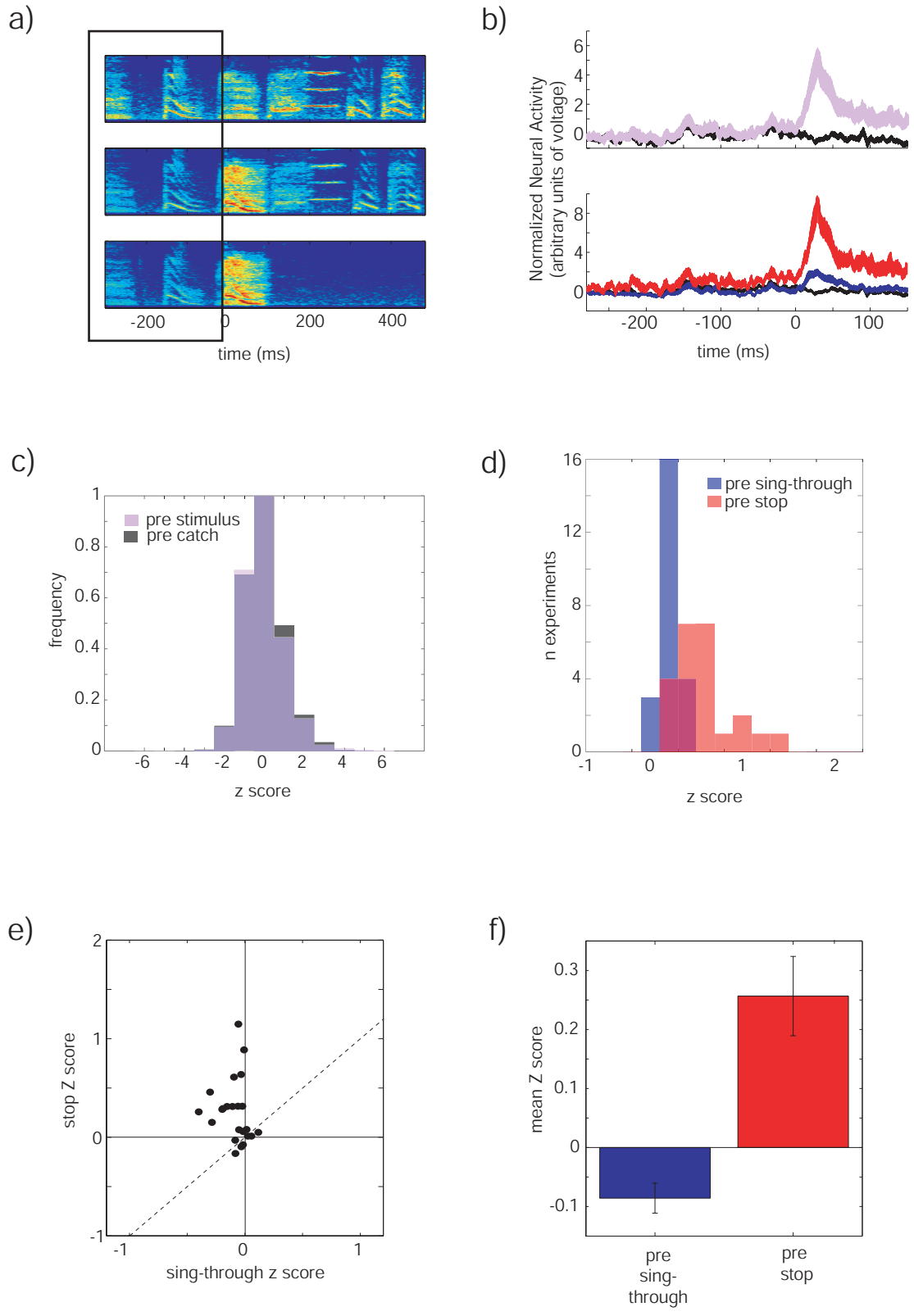

a)
-200
0
200
400
time (ms)
b)
Normalized Neural Activity (arbitrary units of voltage)
6
4
2
0
8
4
0
-200
-100
0
100
time (ms)
c)
pre stimulus
pre catch
frequency
1
0.8
0.6
0.4
0.2
0
-6
-4
-2
0
2
4
6
z score
d)
pre sing-through
pre stop
n experiments
16
12
8
4
0
-1
0
1
2
z score
e)
stop Z score
2
1
0
-1
-1
0
1
sing-through z score
f)
mean Z score
0.3
0.2
0.1
0
-0.1
pre sing-through
pre stop

Figure 7

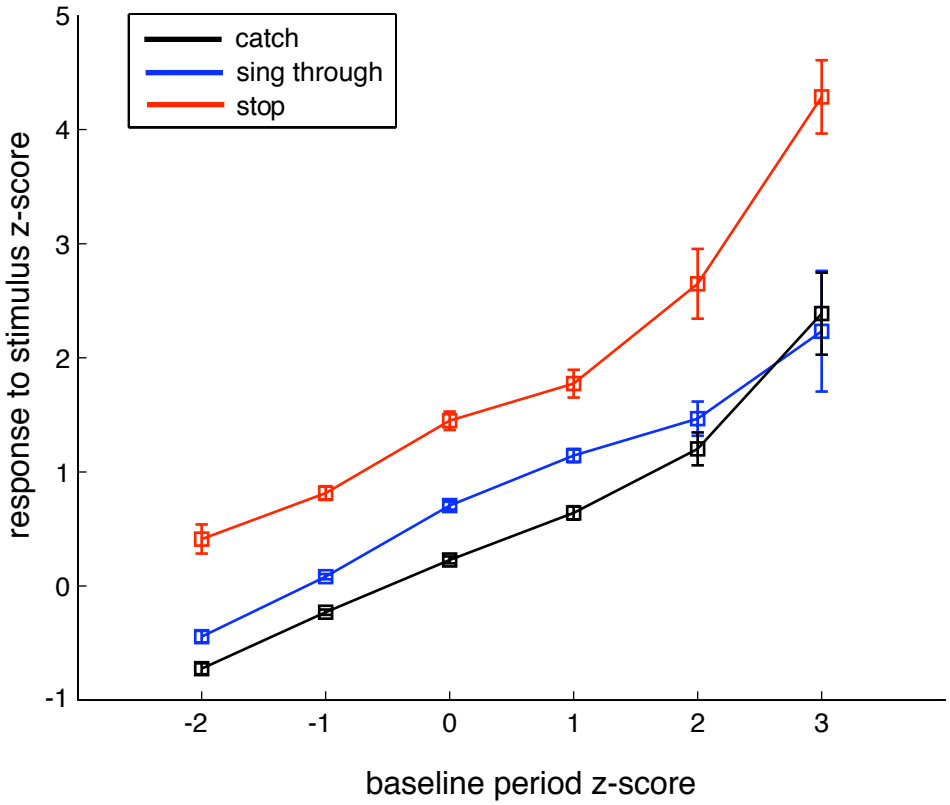
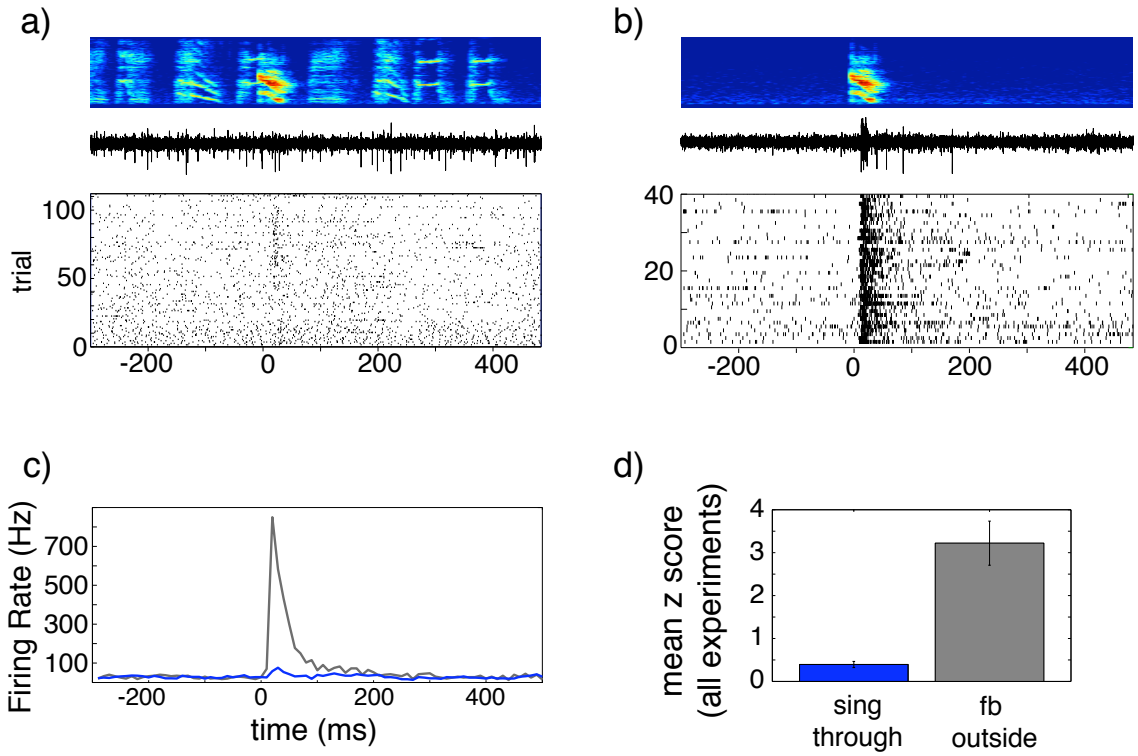

catch
sing through
stop
response to stimulus z-score
baseline period z-score
5
4
3
2
1
0
-1
-2
-1
0
1
2
3

Figure 8


a)
b)
trial
100
50
0
-200
0
200
400
40
20
0
c)
d)
Firing Rate (Hz)
700
500
300
100
time (ms)
mean z score
(all experiments)
4
3
2
1
0
sing
through
fb
outside

Chapter 3: Stimulation in VTA Causes Termination of Song

INTRODUCTION

Stimulation of the ventral tegmental area (VTA) produces reliable operant behavior, and as such intra-cranial self stimulation (ICSS) paradigms have been used extensively to explore the role of VTA and the dopaminergic modulatory system (Olds, 1954, 1969; Phillips, 1973; Fibiger, 1974; Koob, 1978; Corbett, 1980; Garris, 1999; Kilpatrick, 2000; Steffensen, 2001; Wise, 2005). Stimulation in VTA induces a range of motor behaviors in self-stimulation paradigms, and while the increased frequency of these behaviors is attributed to the rewarding properties of brain stimulation, some researchers state that the *hedonic* properties of VTA brain stimulation cannot be ascertained by ICSS (Berridge, 1998). Instead, they argue that ICSS is informative about the *reinforcing* properties of VTA brain stimulation, with a reinforcer being operationally defined as a stimulus for which an animal is willing to make a behavioral response. Given the nature of testing in a laboratory, experiments are designed so that these behavioral responses can be quantified clearly, and as a result, they are often motor behaviors such as lever presses or nose-pokes.

VTA ICSS, then, can be said to potentiate a variety of locomotor operant responses. Animals will lever press (Olds, 1954, 1969; Corbett, 1980; Garris, 1999), nose-poke (Steffensen, 2001), run on treadmills (Burgess, 1991) and lift weights (Garner, 1991) for VTA stimulation. In addition to motor operant responses, one of the most frequently reported correlates of ICSS in the VTA is exploratory behavior, and the presence or absence of stimulation-induced exploratory behavior has been correlated with the extent to which rats learn to lever press for ICSS (Garris, 1999). Similarly, ICSS in

the medial forebrain bundle (MFB) has been shown to activate VTA neurons (Maeda, 1981), and reliably induces exploratory behavior (Mary Christopher, 1968; Miliaressis, 1987).

The effects of VTA stimulation generalize to experimenter-delivered stimulation, which does not require an operant response from the test subject. Experimenter-delivered stimulation induces motor behaviors such as limb movement (Trojniar, 1999), head turns, and sniffing (Cresimanno, 1998). Experimenter-delivered VTA stimulation also causes a potentiation of locomotor response to amphetamine (Ben-Shahar, 1994). Though data on VTA's role in vocal motor behavior are sparse, there is one report of VTA stimulation causing ultrasonic vocalizations in rats (Burgdorf, 2007). These reports and findings from electrophysiological studies (Lee, 2001; Nishino, 1987,1991; Yanagihara, 2006; Kiyatkin, 1998) support the idea that VTA is active during the initiation of motor behaviors, and confirm data presented in Chapter 1 that show an increase in VTA activity before the initiation of singing.

Very little is known about whether VTA activity can inhibit or arrest motor behavior. Lesions of VTA cause hyperactivity (Koob, 1981; Shank, 2007) and potentiation of VTA stimulation-induced motor behavior (Trojnar, 1999), suggesting that VTA has a tonic inhibitory influence on locomotor behavior. Similarly, it has been demonstrated that stimulation in VTA causes an elimination of experimentally induced motor behavior. While

stimulation of the susbtantia nigra pars compacta (SNpC) causes head turns and circling movements in cats, concurrent stimulation in VTA and SNpC eliminates or greatly reduces the circling activity (Piazza, 1989). Along the same lines, VTA stimulation

administered concurrently with the presentation of an acoustic startle stimulus increases the latency of the orienting response (Crescimanno, 1998).

To date, the hypothesis that VTA arrests ongoing motor behavior has not been tested. Results from experiments outlined in Chapters 1 and 2 of this dissertation imply that VTA is involved in the termination of motor behavior. In Chapter 1, we report that VTA neural activity increases just before the end of a song bout. Chapter 2 outlines experiments in which delivery of a salient feedback stimulus causes early termination of singing, and reports that higher levels of VTA neural activity are associated with song terminations as compared to uninterrupted song. Moreover, we find a significant positive correlation with the amount of neural activity in VTA and the probability of stopping. Given these results, we wanted to test the hypothesis that increased levels of neural activity in VTA drive termination of song. In order to test this hypothesis, we stimulated in the VTA of adult male Bengalese finches while they were singing (Figure1a).

METHODS

Subjects: Subjects were adult male Bengalese Finches (*lonchura striata var. domestica*) aged 120d post hatch and older. Birds were raised in individual breeding cages until at least 60d, and were housed with other males thereafter until the time of implantation surgery. Animals were housed on a 14/10 light/dark cycle.

Surgery: Detailed methods for sterotaxic implantation of electrodes targeted at VTA are outlined in Chapter 1 of this dissertation. In summary, we implanted chronic microdrives with electrodes that could be manually advanced (Hessler & Doupe, 1999). Each microdrive was built with 3 tungsten electrodes (4MΩ) and a low impedance

ground. Birds were allowed 3-7 days to recover from surgery, and were habituated to a recording lead for at least 7 days before experiments began. After lead habituation, each bird's home cage was moved into a recording soundbox. Songs were recorded for 1-3 days, during which period the bird acclimated to the new soundbox.

Electrophysiology: After subjects were implanted the chronic microdrives according to the methods outlined in Chapter 1, we listened to and recorded neural signals in the brain in order to determine where along a penetration to stimulate. Birds were connected to digital recording equipment via a flexible lead and op-amp. Signals were routed through a commutator, amplified (1000x), filtered (300Hz-10kHz), digitized and recorded (Observer, A. Leonardo, Caltech; C. Roddey, UCSF). Electrodes were advanced in intervals of approximately 80μm and responses to salient probe stimuli (tap on door of soundbox, switching lights off/on) were noted, as was any activity correlated with movement, such as hopping onto the perch, reaching into the food dish, and orienting-related head movements and saccades.

Stimulation: Once the salience and motor responses along the whole penetration had been mapped, electrodes were retracted to a depth corresponding to a site we estimated was either "in VTA" or "out of VTA". Electrodes were retracted to an "in VTA" site first on half of the birds and an "out of VTA" site first on the other half of the birds in order to control for the possibility that the electrodes were less effective at passing current over time. The birds were then connected to a pulse stimulator (A-M Systems) via a stimulation lead and allowed to sing.

During the experiment, the frequency and duration of the stimulus train were held constant while the experimenter varied the current intensity from trial to trial so that the

various current intensities could be delivered in an interleaved fashion. Electrical stimuli were bimodal, square-wave pulses of 400 Hz. At the start of the experimental session, each bird received 3 trials of stimulation at 20 μA. Thereafter, current intensity was decreased (if necessary) until the bird sang through 10 trials at the same current intensity without stopping, and increased until the bird stopped on 10 consecutive trials or current intensity reached 120μA. Current intensities ranged from 5μA to 120μA, and were interleaved so that a bird experienced current amplitudes from the high and the low end of the range in both the early and the late trials of the experiment. At least 10 stimulation trials were delivered at each current intensity. Once an experiment was complete at one site, putatively "in VTA" or "out of VTA", the bird was allowed to rest for a day before the next experiment was conducted at the second site. For bird #1, stimulation was delivered across two 4MΩ tungsten electrodes. For birds 2 and 3 the stimulation was delivered across one electrode and ground. In bird #3, we stimulated the "out of VTA" site first and the "in VTA" site second.

For two of the birds, stimulation was delivered by the experimenter at random times during song. Data-collection software recorded the birds' vocalizations and the TTL pulse sent out by the stimulator. For bird # 3, stimulation was delivered at the same point in song on each trial by using Birdtaf (see Chapter 2 Methods for more detail). For this bird, songs that were recorded during the 1-3 days before the experiment were visually inspected and a syllable was selected as the target for stimulation. Syllables that typically occurred at the end of song bouts or at the ends of motifs were excluded from selection. Once a target syllable was selected, we created a template for it using its average power spectrum. Birdtaf monitored song online and compared each note to the

template. When a match was detected, a TTL pulse was used to trigger stimulation and was recorded in addition to the bird's song. On the day before the experiment, we recorded one day of baseline data. During basline data collection, song was recorded while Birdtaf detected syllable matches and sent out TTL pulses but the stimulator was not plugged in.

After completion of the initial experiment, we left the electrodes in VTA and recorded the bird's song while he was chronically stimulated for two days. Birdtaf delivered stimulation automatically (400 Hz, 20 ms, 20 μA), and songs and TTL pulses were recorded in Observer.

Histology: After experiments were complete, small marking lesions were made to confirm the position of the electrodes. Birds were perfused trascardially with 0.9% saline followed by a 3.7% formaldehyde solution. Brains were dissected, sunk in 30% sucrose, and then frozen and sectioned on a freezing microtome (40μm sections). Every 3rd section was stained for TH (tyrosine hydroxylase, a precursor enzyme necessary for the synthesis of dopamine). Following the stain for TH, free-floating slices were stained with hematoxylin so that lesions in non-TH areas could be clearly visualized and a within-brain scale could be established. Slides were cover-slipped with permount, dried, and examined under a dissecting microscope. Please refer to Chapter 1 of this dissertation for a more detailed description of histology methods.

DATA ANALYSIS

Percent of songs interrupted by stimulation: During each experimental session, the experimenter noted whether a stimulus train of a given current intensity caused a song

termination on each trial. Song terminations were defined as any cessation of vocal motor behavior occurring within 500 ms of the onset of the stimulation train. Because the sequence of syllables in a given motif is quite stereotyped, trials on which the bird stopped singing and restarted at the beginning of the same or another motif were also counted as terminations. For each current intensity or duration of the stimulus train, the percent of trials on which the bird stopped singing was calculated by taking the number of song interruptions caused by that stimulus and dividing it by the total number of trials of that stimulus that were delivered.

P_{50} current amplitude: For each experiment, we determined the current amplitude at which the probability of stopping was 50%. Once we had empirically determined the probability function (across stimulus intensity), we used a linear interpolation function (MATLAB, MathWorks) to determine the P_{50} current amplitude. Reported P50 values are means for each experimental condition (in VTA and outside of VTA).

Latency to song termination: Bird #3 received stimulation that was triggered by Birdtaf and delivered consistently during the same syllable for all trials. For each trial, the latency to termination of song was quantified by subtracting the time of the onset of the stimulus train from the time of the offset of the last syllable of vocalization and taking the mean across all trials. However, the first time point at which we were sure that vocalization had ceased was at the expected *onset* of the next syllable, and for this reason our estimate of the latency to the termination of singing included the average inter-syllable interval between the note being stimulated and the next note. The average inter-syllable interval was assessed using baseline data recorded on the day before experiments began.

Slowing of song during sub-threshold stimulation: For the experiment in VTA using Birdtaf-triggered stimulation, sub-threshold stimulation trials were defined as trials during which a stimulation train was delivered but the bird did not terminate singing. Inter-syllable intervals between the note targeted for stimulation and the subsequent note were calculated for baseline recording day and for the first day of chronic stimulation.

RESULTS

Stimulation in VTA Causes Song Termination at Low Current Intensities: Stimulation of the VTA caused abrupt termination of singing at low current intensities in 3/3 birds, with an average P_{50} of 13 μA (Figure 1d, 1e).

The Threshold for Eliciting Song Termination is Lower in VTA Than in Surrounding Areas: Though stimulation delivered in VTA caused terminations of song, stimulation delivered at sites outside of VTA also caused terminations of song in 2/3 birds. Lesions made at sites of TH+ cell body staining were classified as being in VTA (Figure 2, red circles, n=3) and lesions outside of these areas were classified as being outside of VTA (Figure 2, blue circles, n=3). Lesions were made at the depths at which the stimulation experiments were performed, and established that we were able to stimulate at least one site in VTA and one site outside of VTA in each bird.

For the two sites outside of VTA at which stimulation elicited song terminations, the probability of stopping increased with current intensity (Figure 3a). At the remaining outside of VTA site, current intensities up to 120μA did not reliably elicit song terminations. A comparison of P_{50} values (inside VTA, mean current intensity = 13μA, n=3; outside of VTA, mean current intensity = 46 μA, n=2) confirmed that stimulation

within VTA caused termination of singing at significantly lower current intensities than stimulation outside of VTA (t-test, $p<0.01$, Figure 3b).

Behavioral Description of Changes To Song: Terminations of song were either total cessations of song for at least 500 ms after delivery of the electrical stimulus (Figure 1d) or an arrest followed by a restart of singing at the beginning of a new motif of song (Figure 1e). Song *motifs* are highly stereotyped sequences of syllables that are repeated several times during the course of one song bout (see Chapter 1, Figure 1a). When birds were stimulated in VTA and restarted song after the initial arrest, they almost always started a new motif instead of continuing on with the next syllable in the sequence. Very rarely (on <5% of trials), stimulation in VTA would cause a truncation of the syllable during which the stimulation was delivered (Figure 1f).

Probability of Song Termination Varies with Parameters of the Stimulus Train: In bird #1 we used a 400 ms long stimulus train. However, because the bird often stopped singing before the end of the stimulus train, it was clear that a shorter stimulus would induce the behavioral effect. We therefore performed an additional experiment in bird #2 at an "in VTA" site in which we held frequency (400 Hz) and current amplitude (30 μA) constant and varied the duration of the stimulus train (10ms – 160 ms). Based the results of this parametric test (Figure 4), we utilized a pulse train with a duration of 20 ms for experiments in birds #2 and #3. We found that for all birds at all sites within VTA, the probability of stopping increased as current intensity increased (n=3 sites, Figure 3a) and as the duration of the stimulus train increased (n=1 site, Figure 4).

Latency to Song Termination: In order to obtain a consistent estimate of the latency to the termination of vocalization, we delivered the stimulus train at the same

point during song on each trial (bird # 3). The cessation of song only becomes apparent when the bird fails to sing the next expected syllable in the sequence, and therefore our estimate of latency was calculated from the onset of the stimulus train to the time of the expected onset of the next syllable. This gives us a conservative estimate of the latency because it automatically includes the duration of the inter-syllable interval in the calculation. Termination of vocalization occurred at an average latency of 73.5 ms (56.1 ms to end of the targeted syllable plus a 17.4 ms inter-syllable interval) from the onset of the stimulation pulse (Figure 3c).

Slowing of Song During VTA Stimulation: In addition to causing song terminations, we found that stimulation in VTA was able to effect a highly localized slowing of song (Figure 5a). After the initial current intensity curve was established, bird #3 was chronically stimulated for a period of 48h at a current intensity of 20μA, which caused stops 100% of the time in the initial tests. Within the first 24h of chronic stimulation, the bird began to sing through the delivery of the stimulus train (Figure 5a, bottom panel). Because trials on which the bird sang through the stimulation were interspersed with trials on which the bird stopped, it can be assumed that behavioral effects were not due to a loss of efficacy of the electrodes in passing current. We compared the length of the inter-syllable interval (ISI) following stimulation with the same ISI in our baseline data and found that stimulation caused a significant increase in the ISI following the target note (t-test, $p<0.01$; control ISI, mean duration=17.4 ms, n=122; stimulated ISI, mean duration=24.05 ms, n=139, Figure 5b).

DISCUSSION

Stimulation in VTA during ongoing song reliably caused abrupt song terminations (Figures 1d, 1e). In order to test whether the effects of stimulation were specific to VTA, we stimulated in brain regions surrounding VTA. We found that stimulation in brain areas outside of VTA resulted in termination of songs in 2/3 birds tested, but that the threshold for eliciting termination of singing behavior was significantly higher in these surrounding regions when compared to stimulation in VTA (Figure 2, Figure 3). It is possible that stimulation in these surrounding regions caused song terminations because of their proximity to VTA, and that the higher threshold to stopping was a reflection of the fact that current had to spread farther to reach VTA. This is supported by the fact that stimulation at the site farthest outside of VTA was not able to reliably induce song stops (see Figure 2b, the most dorsal blue circle). Overall, our results support the hypothesis that stimulation in VTA causes a termination of vocal motor behavior.

In the first two birds tested, we delivered stimulation at random times during ongoing song. This clearly caused the bird to stop singing. However, this method had one drawback. Random delivery of stimulation meant that occasionally stimulation occurred on a syllable that the bird normally sang at the end of a motif or a song bout. Though the stimulation was delivered early enough in a song bout that it was unlikely that the bird would have stopped singing in the absence of a stimulus, we wanted to preclude this possibility altogether. We therefore used software that allowed us to consistently deliver the stimulus train during the syllable of our choosing. The results from this experiment were similar results obtained in the first two birds and confirmed that VTA stimulation delivered during ongoing song causes termination of singing behavior at short latencies.

Delivery of the stimulation train at the same point in song across trials allowed us to probe the motor effects of VTA stimulation more closely. When we compared vocalizations on trials in which the stimulation train did not elicit song termination with those collected during the baseline period, we found that the duration of the inter-syllable interval following stimulation in VTA increased by about 38%. This finding lends support to the hypothesis that VTA is regulating motor output in general, and specifically suggests that VTA has a direct influence on the timing of vocal motor output.

We made a comparison of the thresholds needed to elicit song termination in VTA with those published for other song system nuclei (Figure 6)(Vu, 1994; Ashmore, 2005). A comparison of our results and the results of others indicate that stimulation in VTA causes song terminations at thresholds that are comparable to and in many cases lower than the thresholds found in several song system nuclei. Though one must be cautious about directly comparing results obtained in different settings in different laboratories, in birds #2 and #3 we used the same stimulation parameters reported in these studies (400 Hz, ~20ms stimulus trains).

One possibility is that VTA stimulation is causing disruptions in the activity of HVC. HVC is part of song system motor pathway, and has been implicated in the higher-level motor aspects of song organization such as initiation and sequencing of song (Vu, 1994; Yu, 1996; Hahnloser, 2002). Stimulation of HVC during ongoing song recapitulates the effects of VTA stimulation reported here. Both the latency to the termination of song (70 ms in HVC, 74 ms in VTA) and the restarting of song with a new motif after arrest are comparable (Vu, 1994). Moreover, delivery of a disruptive feedback stimulus during ongoing song elicits firing of putative GABA neurons in VTA (see Chapter 2) and a

concomitant inhibition of neural activity in HVC (Sakata & Brainard, in preparation). Taken together with the finding that VTA sends both TH+ and TH- projections to HVC (Appeltants, 2000), these data suggest that stimulation in VTA causes termination of singing behavior, and may do so by signaling through a nucleus in the motor pathway of the song system.

Stimulation in the VTA, and more specifically ICSS, has often been utilized to study reinforcement related behavior. However, reports from both ICSS and experimenter-delivered stimulation studies make it clear that VTA stimulation has motor consequences. These motor consequences are only partly explained by the hypothesis that VTA is involved in mediating goal-directed behavior. The hypothesis explains the correlation of neural signals in VTA with the initiation of goal directed behavior, but does not account for signals that are correlated with termination of behavior. The experiments outlined here are the first to report that stimulation of the VTA causes a termination of ongoing motor behavior, and as such imply that VTA has a broader role in regulating behavioral output than is currently supposed.

Figure 1. *Stimulation in VTA causes termination of ongoing song.*

a) Experimental set-up. In house software (Birdtaf) monitored the bird's song for occurrences of a pre-selected syllable. When the syllable was detected, Birdtaf sent out a TTL pulse that was used to trigger stimulation in VTA. **b)** Spectrogram of bird's song on a control trial, in which the target syllable was detected but VTA was not stimulated **c)** Spectrogram of a trial during which stimulation was delivered but the bird continued to sing through **d)** Spectrogram of a trial during which stimulation resulted in song termination **e)** example of a trial in which stimulation resulted in a song termination followed by the restarting of a new motif **f)** Spectrogram of a trial in which stimulation led to the truncation of a syllable but not a termination of song. These trials were rare, and occurred less than 5% of the time.

Figure 2. *Histological confirmation of the location of stimulating electrodes in VTA.*

a) A representative coronal section through the avian brain at the level of VTA, stained for tyrosine hydroxylase. Inset is a lower magnification view of the whole section, with a rectangle denoting the area of higher magnification. **b)** A drawing of the section shown in a), with areas of dense TH+ cell body staining indicated by solid gray coloring. Circles represent the sites at which stimulation experiments were performed, with red circles indicating sites that were counted as being "in VTA" (n=3 sites in 3 birds) and blue circles indicating sites that were counted as being "outside of VTA" (n=3 sites in 3 birds). We stimulated at one site in VTA and one site outside of VTA in each bird.

Figure 3. *Stimulation in VTA causes song termination lower current intensities than stimulation outside of VTA.*

a) Percent of song terminations elicited by stimulation as a function of current intensity (μA). For sites in VTA, the percent of stimulation trials resulting in song termination is indicated by solid lines (n=3 sites in 3 birds). The percent of stimulation trials resulting in song termination in the same 3 birds at control sites outside of VTA is indicated by dashed lines. The frequency (400 Hz, n=3 birds) and duration (400 ms in bird #1, 20 ms in birds #2 and #3) of the stimulus train were held constant while the current intensity varied. **b)** The P_{50} current intensity was defined as the current intensity at which the bird would stop 50% of the time when stimulated. The mean P_{50} values for sites inside of VTA are significantly lower (n=3, mean P_{50} = 13μA) than the P_{50} values outside of VTA (n=2, mean P_{50} = 46 μA). Because stimulation outside of VTA in bird # 3 did not reliably elicit song terminations within the range of current intensities tested, this data point was not included in the P_{50} analysis. **c)** A distribution of the latency to song termination for stimulation in bird # 3 (mean latency = 56.1 ms to the offset of vocalization). Because it does not become apparent that a bird has stopped singing until the onset of the next note in the sequence, we added the mean duration of the ISI following the last syllable (17.4 ms) to our calculation in order to arrive at a conservative estimate of the latency to termination of song (73.5 ms).

Figure 4. *The probability of song termination increases with increasing duration of the stimulus pulse.*

At a constant frequency and current intensity (30μA, 400Hz), the percent of trials on which stimulation elicited song terminations increased as the duration of the stimulus train increased (n=1 site in VTA, bird #2). Note that even though our latency estimate was derived from experiments in bird #3, the effect of increasing stimulus duration asymptotes at a similar time course, around 80 ms.

Figure 5. *Stimulation in VTA can effect a slowing of song.*

a) *Top panel*. Spectrogram of a control trial, during which the targeted syllable was detected but no stimulation was delivered. *Bottom panel.* Spectrogram of a stimulation trial, during which stimulation was delivered at time = 0. Time of TTL pulse is indicated by a dashed red line. Initially, stimulation at this site in VTA caused song termination on 100% of trials with these stimulation parameters (400 Hz, 20 μA, 20 ms pulse). However, within 24 hours of chronic stimulation, the bird began to sing through the stimulation. **b)** We compared the durations of the inter-syllable interval (ISI) on control trials with the durations of the same ISI on trials in which the bird received stimulation but did not terminate song. We found that stimulation in VTA significantly increased the duration of the ISI following the stimulation pulse (control trials, n=122, mean duration of ISI = 17.4 ms; stimulation trials, n=139, mean duration of ISI = 20.05 ms, $p<0.01$). The ISI of interest is indicated by an asterisks in the top and bottom panels of a).

Figure 6. *A comparison of the effects of stimulation in VTA with stimulation in song system nuclei*

For the sake of clarity, only one example of stimulation in VTA is included in this graph. These values for the percent of song terminations elicited by stimulation in various song system nuclei are taken from the results reported in Vu (1994) and Ashmore (2005), in which the authors used 400 Hz stimulation pulses that were 16-20 ms in duration. Stimulation in VTA is able to elicit song termination at thresholds that are comparable to or lower than those required in traditional song system nuclei.

Figure 1

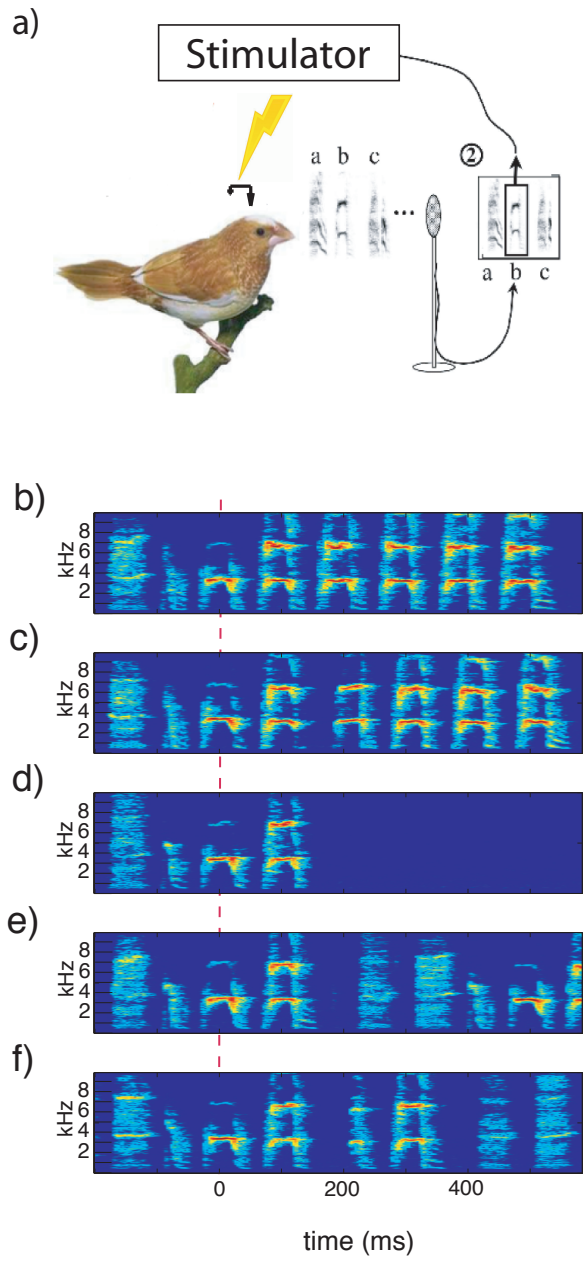


Figure 2

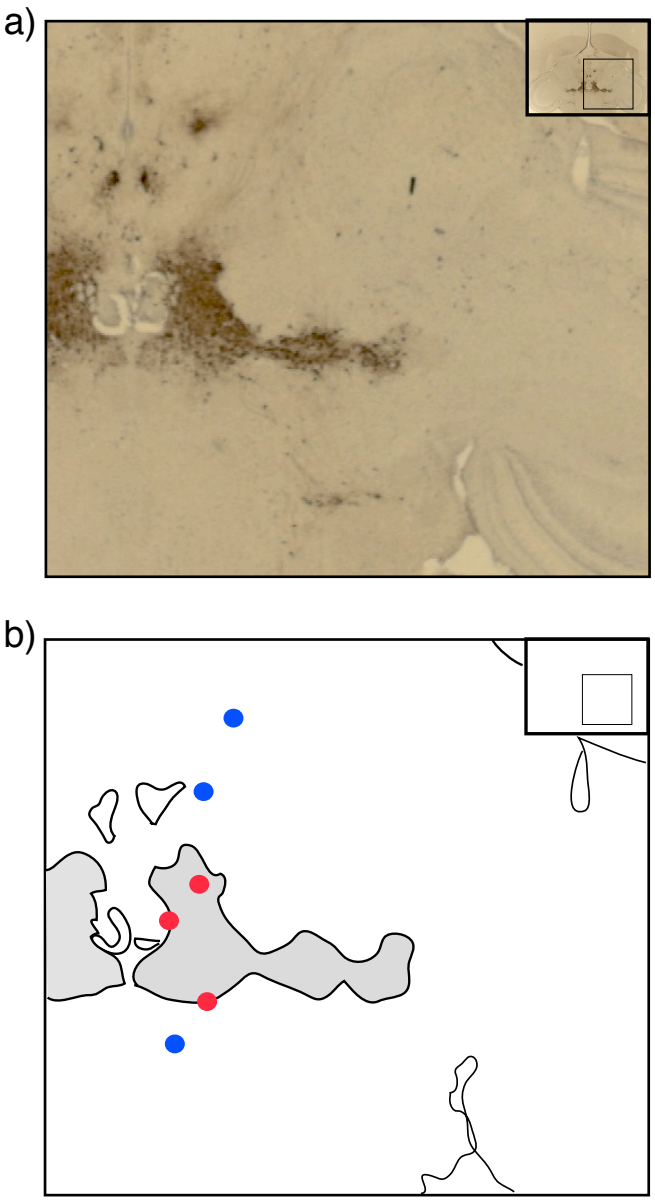


Figure 3

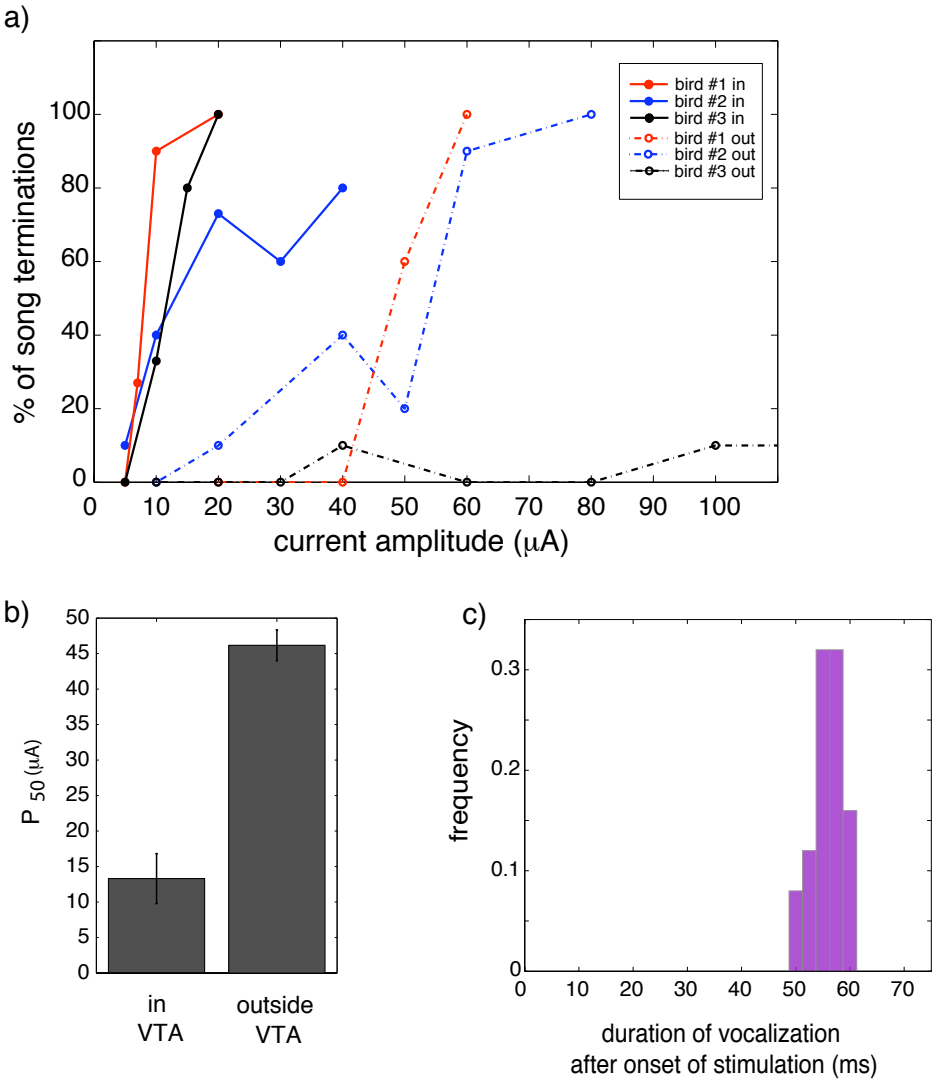

a)
% of song terminations
current amplitude (μA)
bird #1 in
bird #2 in
bird #3 in
bird #1 out
bird #2 out
bird #3 out
b)
P 50 (μA)
in VTA
outside VTA
c)
frequency
duration of vocalization after onset of stimulation (ms)

Figure 4

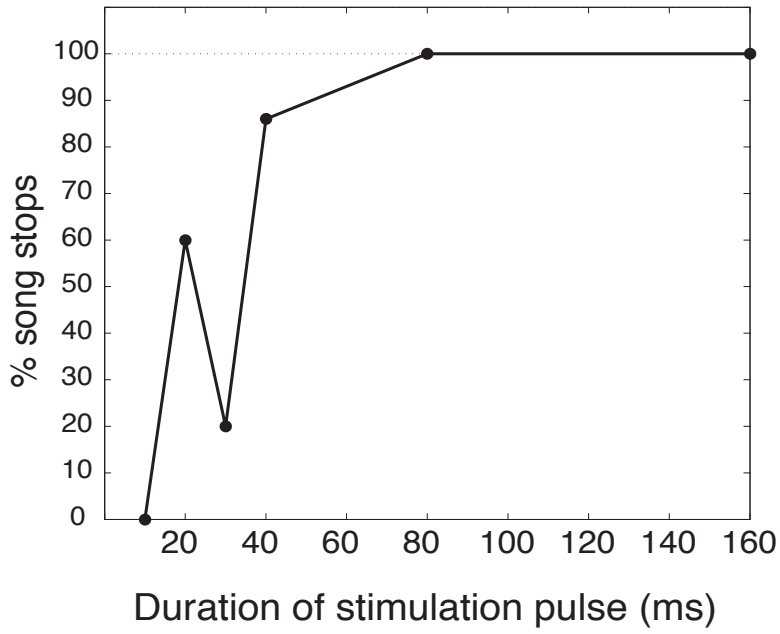
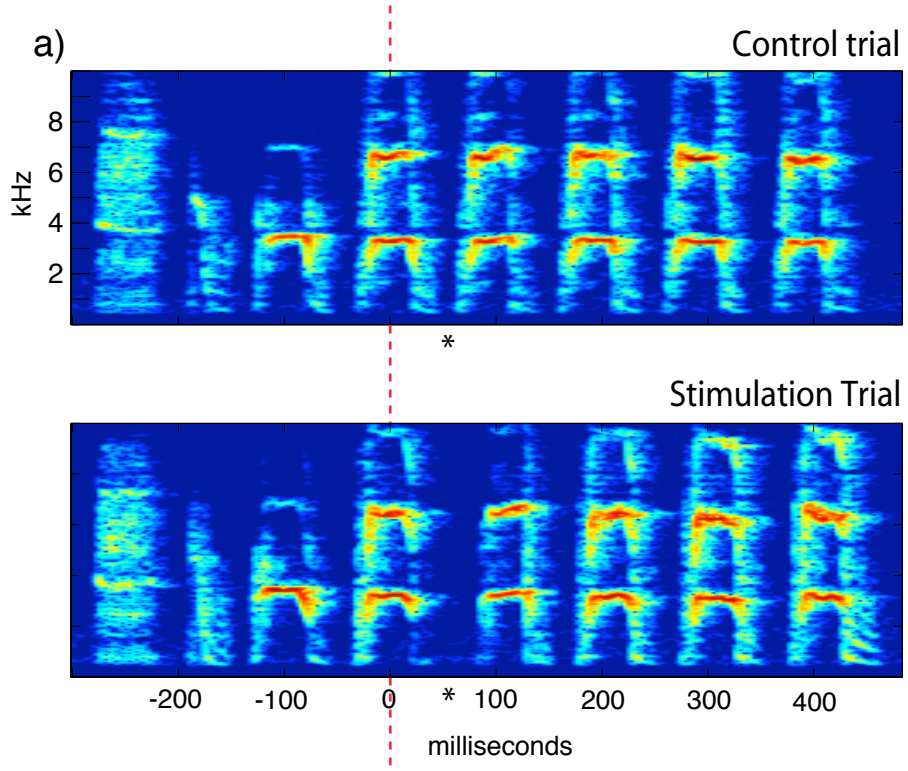

100
90
80
70
60
50
40
30
20
10
0
% song stops
20
40
60
80
100
120
140
160
Duration of stimulation pulse (ms)

Figure 5


a)
Control trial
kHz
8
6
4
2
*
Stimulation Trial
-200
-100
0
*
100
200
300
400
milliseconds

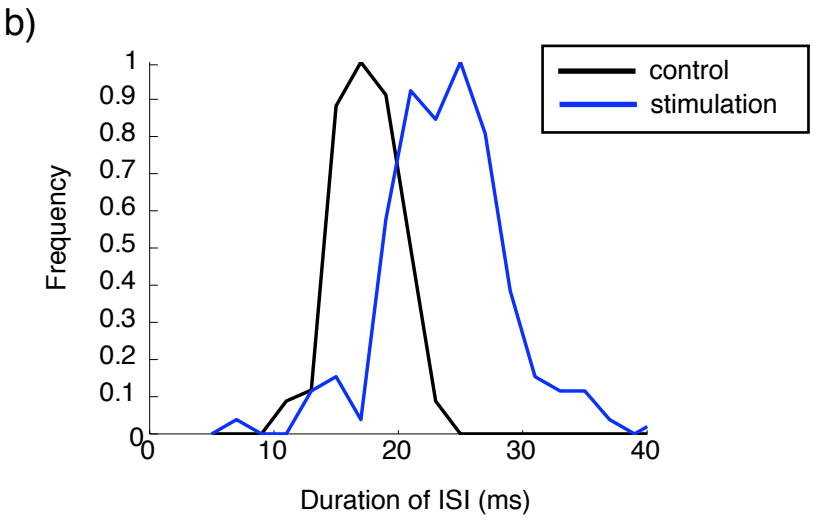
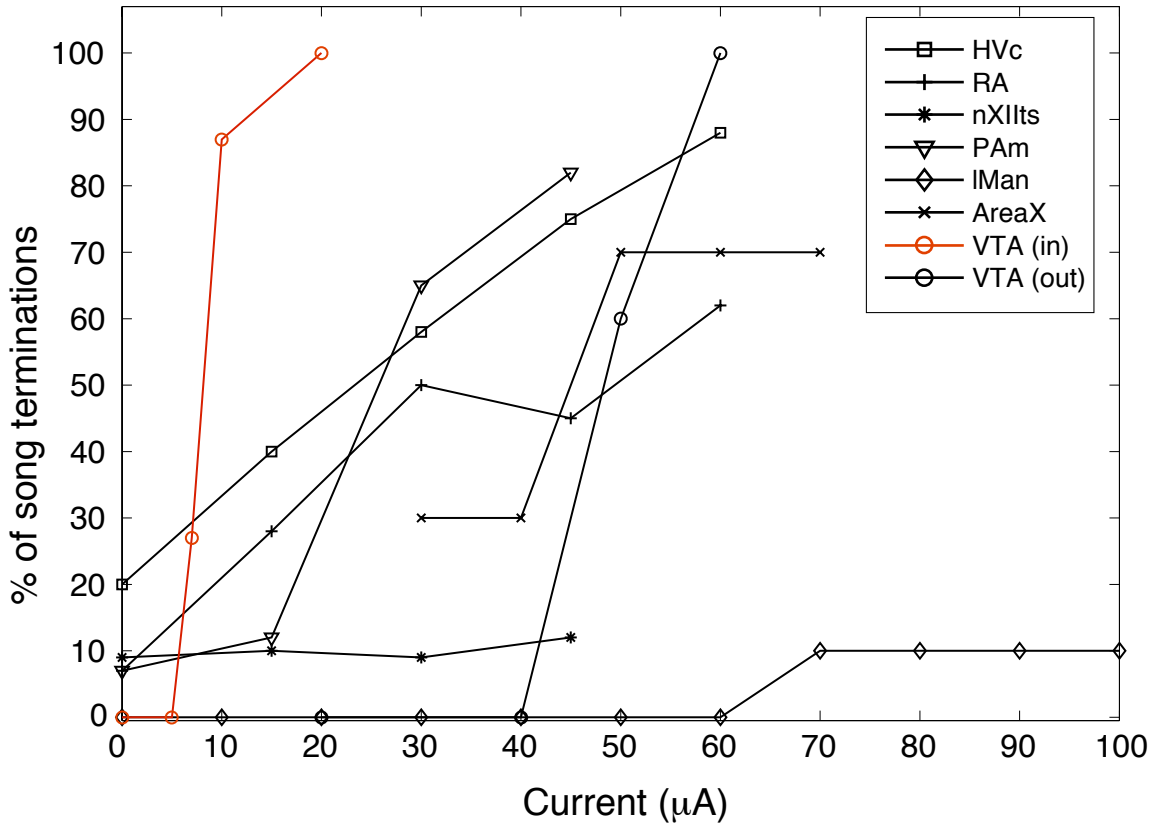

b)
control
stimulation
Frequency
1
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
0
10
20
30
40
Duration of ISI (ms)

Figure 6



Values for song system nuclei are based on results reported in Vu (1994) and Ashmore (2005)

Discussion

The results outlined in this dissertation suggest that the ventral tegmental area (VTA) contributes to behavioral switching in response to salient stimuli. We show that salient stimuli drive responses in VTA (Chapters 1 & 2). We find that these salient stimuli effect behavioral changes and result in the termination of song (Chapter 2). We report that the level of VTA neural activity driven by delivery of a salient stimulus is highly correlated with an increased probability of terminating vocal motor behavior, and moreover that the baseline levels of neural activity in VTA contribute to the likelihood of this switch (Chapter 2). Additionally, we report an increase in VTA neural activity preceding the termination of singing in the absence of any experimenter-delivered salient stimulus (Chapter 1) and suggest that these increases are responsible for song termination. We explicitly test this idea (Chapter 3) and find that stimulation of VTA during singing causes song termination. These results suggest that the role of VTA goes beyond registering the presence of salient or "incentive salience" cues in the environment. They imply that VTA is communicating with motor areas responsible for the initiation and termination of behavior and effecting changes in motor behavior in response to salient stimuli.

VTA neural activity is correlated with initiation and termination of vocal motor behavior. The results of our experiments are the first report that VTA activity increases before the termination of vocal motor behavior in addition to increasing before its initiation. To date, there is only one published study reporting the results of awake, behaving recordings made in songbird VTA (Yanagihara, 2006). One of the preliminary findings of the Yanagihara & Hessler study is that VTA neural activity increases before

the onset of song, and our results confirm and extend this finding (2006). In addition to increasing before the onset of song, we find that VTA neural activity increases before the initiation of calls, which are another socially motivated vocal behavior in birds. Increases in VTA neural activity have been reported before the initiation of a variety of motor behaviors such as nose-pokes for brain stimulation in the medial forebrain bundle (Steffensen, 2001) and drug reward (Steffensen, 2006), and before lever-pressing for food reward (Nishino, 1987, 1991). These findings have been interpreted in such a way as to suggest that VTA responds to reward or incentive salience cues in the environment.

If it is the case that VTA is signaling the presence of a salient or incentive cue in the environment, we might expect to see an increase in neural activity correlated with the initiation of goal-directed or motivated behaviors. According to this formulation, the increase in VTA activity occurs at a time when a motor behavior is being initiated because the cue driving the behavior occurs at that time. Subsequent motor behavior is the result of some downstream process that is informed by the VTA signal registering the presence of the incentive cue, but VTA's role is sensory, and it simply "gates" the flow of information to downstream areas.

However, we find an increase in VTA neural activity correlated not only with the *initiation* of vocal motor behavior, but with its *termination* as well. This suggests a sensorimotor rather than a purely sensory role for VTA. The time course of the signal, which occurs 100 or 180 ms before the start or the end of song, respectively, suggests that it might be premotor to both the initiation and the termination of vocal motor behavior. One caveat to this is that one might suppose the existence of salient cues known only to the bird that cause the bird to stop singing in the same way that they cause

the bird to start singing. In this case, the increase in VTA activity we see before song termination would still reflect a sensory process rather than a sensorimotor one. In an effort to determine whether the signals we saw were sensory or sensorimotor, we utilized the disruptive feedback paradigm described in Chapter 2.

The level of neural activity in VTA is correlated with the probability of terminating song in response to a salient stimulus. We performed a set of experiments in which in which we nominally controlled the sensory input the bird received and looked for differences in VTA neural activity that correlated with the behavior rather than with the stimulus. We reasoned that if VTA activity is simply reflective of a sensory response to a salient stimulus, the same stimulus should evoke the same response, regardless of the motor consequences that follow. Therefore, we brought the behavior of interest – song termination – under stimulus control and probed VTA function in the context of a disruptive feedback paradigm. We found that levels of VTA neural activity reflected not only a response to a salient stimulus, but that they were highly correlated with subsequent behavior.

Though our experiments do not directly test the hypothesis that VTA is activated by salient as opposed to appetitive cues, it is clear that VTA is driven by the stimuli we played to the birds (individual syllables of song and white noise). Neither of these are clearly "appetitive" stimuli, though it is possible that the single syllable, even though it is played out of context, has positive valence for the bird (see Adret 1991, which reports that birds make operant responses for playback of whole songs). When we add to this the direct evidence from other studies that report VTA responses to acoustic clicks, flashes of

light, and light tail touch (Horvitz, 1997; Kiyatkin, 1997), it seems reasonable to assert that VTA responds to non-rewarding stimuli of many modalities.

When we controlled for the sensory stimulus, we saw that higher levels of neural activity in VTA were correlated with higher likelihood of stopping. The results of these experiments suggest that rather than reflecting a purely sensory response, VTA neural activity is a sensorimotor signal, and that some component of this signal reflects premotor activity. Because we see signals during both initiation and termination of vocal motor behavior, the data suggest that the role of VTA is not simply to register the presence of stimuli and initiate goal-directed behavior. VTA might be playing a more general role, in which it responds to salient cues in the environment and initiates or terminates behavior.

Stimulation in VTA results in termination of ongoing song. In order to test the hypothesis that increased activity in the VTA causes termination of vocal motor behavior, we stimulated in the VTA during ongoing song. Stimulation in the VTA caused short latency (74 ms) terminations of song at low current intensities (5-20 μA), demonstrating that increases in neural activity in VTA are able to effect changes in motor behavior. Our data suggest that VTA engages the motor system on a different time scale than has been originally postulated and is able to mediate changes in behavior at times that are on the order of tens of milliseconds rather than "seconds to minutes" (Schultz 2002, 2003, 2007).

Thus far, the bulk of hypotheses regarding dopamine signaling and VTA function have suggested that VTA's role in effecting behavioral change is indirect, and that it has to do with inducing or facilitating plasticity. For example, one type of study is concerned with demonstrating facilitation of LTP in VTA and its downstream targets based on

manipulations of VTA dopamine signaling (Ding 2004; Ungless 2001; Melis 2002; Borglund, 2006). Additional studies focus on cortical remodeling induced by VTA stimulation (Bao, 2001), or the development of locomotor sensitization in response to repeated doses of drugs of abuse (Borgland, 2006). Researchers have also linked VTA signaling to higher-order brain functions requiring plasticity, such as reward based learning (Tremblay, 1998; Mirenowicz, 1994; Waelti, 2001).

The current models of "reward-based" or "reinforcement-based" plasticity have a fundamental premise in common. In the first model, activation of the VTA is thought to increase the probability of reward-seeking behavior. This is not because the VTA is presumed to engage the motor system directly, but because the animal the animal makes the association between the operant response and the feeling of "reward" and *learns* to respond more. Or, in the "reinforcement-based" model, which leaves aside the hedonic aspect of reward, VTA activation leads to an increase in goal-directed behavior because an action (ie, lever-press) becomes associated with some outcome (ie, increased release of dopamine in VTA target regions) and the behavior is potentiated because LTP is induced in downstream areas. VTA signaling is necessary or permissive for the induction of plasticity that increases the probability of the motor response, but is not seen as playing a role in the execution of motor behavior.

By contrast, the results of our experiments indicate that signals from VTA can be used to directly influence motor output within tens of milliseconds. While this result is novel, it is perhaps not surprising. Experimenter-delivered stimulation of the VTA elicits motor behavior (Klejbor, 1999; Martin, 2004), and GABA neurons discharge during movement (Lee, 2001). Lesions of VTA cause hyperactivity that is recapitulated with

selective ablation of GABA neurons (Koob, 1981; Klejbor 1999; Shank 2007), and activation of GABA(B) receptors in the VTA attenuates morphine-induced locomotor activity (Leite-Morris, 2002).

One trend seems to be that VTA activity is correlated with motor behaviors associated with orienting, such as oculomotor saccades (Ljungberg, 2001) and turning of the head (Klejbor, 1999). The injection of glutamate and GABA(A) antagonists into the VTA produces spontaneous turning behavior in a direction ipsilateral to the injection site (Dalia, 1996; Grubb, 2002), and mutant mice with bilateral asymmetries in levels of dopamine show circling behavior and hyperactivity (Lessinch, 2001). One possibility is that VTA might play a role in mediating behavioral arrest and orienting to salient stimuli. Before an animal can engage in goal-directed behavior, it must sometimes terminate ongoing motor programs in order to redirect its efforts. It therefore makes sense that an area registering the presence of a salient cue and initiating behavior should also be able to detect salient cues and arrest ongoing behavior. Beyond signaling the presence of salient or unexpected stimuli, and perhaps in addition to facilitating plasticity in target brain regions, our data show that VTA is able to effect behavioral arrest of the type that would normally precede behavioral switching.

Future Directions. Though the data from this set of experiments suggest that VTA contributes to action selection by mediating behavioral arrest in response to salient stimuli, further experiments are needed to test the validity of this hypothesis. While it is generally accepted that VTA responds to salient stimuli (Horvitz, 2007; but see Ungless, 2004a), it is clear that our understanding would be greatly increased by continued exploration of the types of stimuli that drive VTA neural responses. Though researchers

have made efforts to distinguish between VTA responses to appetitive and aversive stimuli (Mirenowicz, 1996; Ungless 2004), further efforts are needed to understand whether aspects of a stimulus other than valence impact VTA activity. For example, it is not known whether stimuli perceived as "more salient" elicit more firing from VTA neurons. While studies deal with this question in the restricted case of VTA responses to stimuli predicting reward with varying probability (Fiorillo, 2003), it is possible that increasing the amplitude of the playback of a pure tone would also result in increased VTA firing. For example, in songbirds, one might quantify and compare VTA responses to pure tones, which are salient but do not clearly carry valence, and female calls, which are also salient and might serve as appetitive stimuli.

In addition to exploring the properties of the sensory response, further work is needed in understanding the role of VTA in initiation of behavior. One might argue that if VTA is involved in behavioral switching, stimulation in VTA should elicit behavior in addition to arresting it. Though the experiments outlined in this dissertation did not directly test this hypothesis, the results of other experiments speak to it. The results of Yanagihara & Hessler's 2007 study indicate that activations of VTA (caused by a curtain-opening cue) were often followed by an initiation of song. This is a case in which a salient cue drives an increase in VTA firing that is correlated with initiation of singing behavior. A useful next step would be to stimulate in the VTA of quiescent birds and see if this increases the probability of song initiation.

Because VTA sends projections to nuclei in the song system, experiments exploring the relationship between VTA activity and the activity of nuclei in the motor pathway might be useful in further elucidating VTA's role in motor behaviors via its

downstream targets. If sub-threshold stimulation in VTA evokes a suppression of firing in HVC or RA, it is possible that even subtle differences in the motor program can be detected by using song as a read-out. And finally, even though various studies have reported that non-dopaminergic neurons in avian VTA project to the motor pathway, we have yet to establish whether these projection neurons are glutamatergic or GABAergic (Lewis, 1981; Appeltants, 2000, 2002).

Summary. In summary, we find that neural activity in the VTA increases 1) in response to salient stimuli and 2) before the initiation and termination of vocal motor behavior, such as songs and calls. Additionally, we report that higher levels of neural activity in VTA are correlated with a higher probability of terminating song in response to a salient stimulus. Finally, we demonstrate that increasing the level of VTA neural activity by stimulation can cause song termination. Our results suggest that the role of VTA is not limited to signaling about reward-related stimuli in the environment. Rather, we hypothesize that VTA responds to salient stimuli in the environment and effects a cessation of ongoing motor behavior when environmental conditions favor behavioral switching.

References

Aberman, J. E. and J. D. Salamone (1999). "Nucleus accumbens dopamine depletions make rats more sensitive to high ratio requirements but do not impair primary food reinforcement." Neuroscience 92(2): 545-52.

Appeltants, D., P. Absil, et al. (2000). "Identification of the origin of catecholaminergic inputs to HVc in canaries by retrograde tract tracing combined with tyrosine hydroxylase immunocytochemistry." J Chem Neuroanat 18(3): 117-33.

Appeltants, D., G. F. Ball, et al. (2001). "The distribution of tyrosine hydroxylase in the canary brain: demonstration of a specific and sexually dimorphic catecholaminergic innervation of the telencephalic song control nulcei." Cell Tissue Res 304(2): 237-59.

Appeltants, D., G. F. Ball, et al. (2002). "The origin of catecholaminergic inputs to the song control nucleus RA in canaries." Neuroreport 13(5): 649-53.

Ashmore, R. C., J. M. Wild, et al. (2005). "Brainstem and forebrain contributions to the generation of learned motor behaviors for song." J Neurosci 25(37): 8543-54.

Balfour, M. E., L. Yu, et al. (2004). "Sexual behavior and sex-associated environmental cues activate the mesolimbic system in male rats." Neuropsychopharmacology 29(4): 718-30.

Balfour, M. E., L. Yu, et al. (2004). "Sexual behavior and sex-associated environmental cues activate the mesolimbic system in male rats." Neuropsychopharmacology 29(4): 718-30.

Bao, S., V. T. Chan, et al. (2001). "Cortical remodelling induced by activity of ventral tegmental dopamine neurons." Nature 412(6842): 79-83.

Bayer, H. M. and P. W. Glimcher (2005). "Midbrain dopamine neurons encode a quantitative reward prediction error signal." Neuron 47(1): 129-41.

Becerra, L., H. C. Breiter, et al. (2001). "Reward circuitry activation by noxious thermal stimuli." Neuron 32(5): 927-46.

Ben-Shahar, O. and A. Ettenberg (1994). "Repeated stimulation of the ventral tegmental area sensitizes the hyperlocomotor response to amphetamine." Pharmacol Biochem Behav 48(4): 1005-9.

Berridge, K. C. and T. E. Robinson (1998). "What is the role of dopamine in reward: hedonic impact, reward learning, or incentive salience?" Brain Res Brain Res Rev 28(3): 309-69.

Berridge, K. C., I. L. Venier, et al. (1989). "Taste reactivity analysis of 6-hydroxydopamine-induced aphagia: implications for arousal and anhedonia hypotheses of dopamine function." Behav Neurosci 103(1): 36-45.

Blaszczyk, J. W. (2003). "Startle response to short acoustic stimuli in rats." Acta Neurobiol Exp (Wars) 63(1): 25-30.

Blaszczyk, J. W. and K. Tajchert (1997). "Effect of acoustic stimulus characteristics on the startle response in hooded rats." Acta Neurobiol Exp (Wars) 57(4): 315-21.

Borgland, S. L., S. A. Taha, et al. (2006). "Orexin A in the VTA is critical for the induction of synaptic plasticity and behavioral sensitization to cocaine." Neuron 49(4): 589-601.

Bottjer, S. W. (1993). "The distribution of tyrosine hydroxylase immunoreactivity in the brains of male and female zebra finches." J Neurobiol 24(1): 51-69.

Bottjer, S. W., E. A. Miesner, et al. (1984). "Forebrain lesions disrupt development but not maintenance of song in passerine birds." Science 224(4651): 901-3.

Bozarth, M. A. and R. A. Wise (1981). "Intracranial self-administration of morphine into the ventral tegmental area in rats." Life Sci 28(5): 551-5.

Bozarth, M. A. and R. A. Wise (1983). "Neural substrates of opiate reinforcement." Prog Neuropsychopharmacol Biol Psychiatry 7(4-6): 569-75.

Brainard, M. S. and A. J. Doupe (2000). "Interruption of a basal ganglia-forebrain circuit prevents plasticity of learned vocalizations." Nature 404(6779): 762-6.

Burgdorf, J., P. L. Wood, et al. (2007). "Neurobiology of 50-kHz ultrasonic vocalizations in rats: electrode mapping, lesion, and pharmacology studies." Behav Brain Res 182(2): 274-83.

Burgess, M. L., J. M. Davis, et al. (1991). "Intracranial self-stimulation motivates treadmill running in rats." J Appl Physiol 71(4): 1593-7.

Cadenhead, K. S., B. S. Carasso, et al. (1999). "Prepulse inhibition and habituation of the startle response are stable neurobiological measures in a normal male population." Biol Psychiatry 45(3): 360-4.

Carr, D. B. and S. R. Sesack (1999). "Terminals from the rat prefrontal cortex synapse on mesoaccumbens VTA neurons." Ann N Y Acad Sci 877: 676-8.

Carr, D. B. and S. R. Sesack (2000). "GABA-containing neurons in the rat ventral tegmental area project to the prefrontal cortex." Synapse 38(2): 114-23.

Carr, D. B. and S. R. Sesack (2000). "Dopamine terminals synapse on callosal projection neurons in the rat prefrontal cortex." J Comp Neurol 425(2): 275-83.

Carr, D. B. and S. R. Sesack (2000). "Projections from the rat prefrontal cortex to the ventral tegmental area: target specificity in the synaptic associations with mesoaccumbens and mesocortical neurons." J Neurosci 20(10): 3864-73.

Carrillo, G. D. and A. J. Doupe (2004). "Is the songbird Area X striatal, pallidal, or both? An anatomical study." J Comp Neurol 473(3): 415-37.

Coizet, V., E. J. Dommett, et al. (2006). "Nociceptive responses of midbrain dopaminergic neurones are modulated by the superior colliculus in the rat." Neuroscience 139(4): 1479-93.

Comoli, E., V. Coizet, et al. (2003). "A direct projection from superior colliculus to substantia nigra for detecting salient visual events." Nat Neurosci 6(9): 974-80.

Corbett, D. and R. A. Wise (1980). "Intracranial self-stimulation in relation to the ascending dopaminergic systems of the midbrain: a moveable electrode mapping study." Brain Res 185(1): 1-15.

Crescimanno, G., F. Sorbera, et al. (1998). "Inhibitory effect of A10 dopaminergic neurons of the ventral tegmental area on the orienting response evoked by acoustic stimulation in the cat." Brain Res Bull 45(1): 61-5.

Dalia, A., N. J. Uretsky, et al. (1996). "Induction of locomotor activity by the glutamate antagonist DNQX injected into the ventral tegmental area." Brain Res 728(2): 209-14.

Denenberg, V. H., D. S. Kim, et al. (2004). "The role of dopamine in learning, memory, and performance of a water escape task." Behav Brain Res 148(1-2): 73-8.

Ding, L. and D. J. Perkel (2004). "Long-term potentiation in an avian basal ganglia nucleus essential for vocal learning." J Neurosci 24(2): 488-94.

Eliades, S. J. and X. Wang (2003). "Sensory-motor interaction in the primate auditory cortex during self-initiated vocalizations." J Neurophysiol 89(4): 2194-207.

Eliades, S. J. and X. Wang (2008). "Neural substrates of vocalization feedback monitoring in primate auditory cortex." Nature 453(7198): 1102-6.

Farries, M. A., L. Ding, et al. (2005). "Evidence for "direct" and "indirect" pathways through the song system basal ganglia." J Comp Neurol 484(1): 93-104.

Farries, M. A. and D. J. Perkel (2000). "Electrophysiological properties of avian basal ganglia neurons recorded in vitro." J Neurophysiol 84(5): 2502-13.

Fee, M. S., A. A. Kozhevnikov, et al. (2004). "Neural mechanisms of vocal sequence generation in the songbird." Ann N Y Acad Sci 1016: 153-70.

Fibiger, H. C., A. G. Phillips, et al. (1974). "Deficits in instrumental responding after 6-hydroxydopamine lesions of the nigro-neostriatal dopaminergic projection." Pharmacol Biochem Behav 2(1): 87-96.

Fiorillo, C. D., P. N. Tobler, et al. (2003). "Discrete coding of reward probability and uncertainty by dopamine neurons." Science 299(5614): 1898-902.

Gale, S. D. and D. J. Perkel (2006). "Physiological properties of zebra finch ventral tegmental area and substantia nigra pars compacta neurons." J Neurophysiol 96(5): 2295-306.

Gale, S. D., A. L. Person, et al. (2008). "A novel basal ganglia pathway forms a loop linking a vocal learning circuit with its dopaminergic input." J Comp Neurol 508(5): 824-39.

Garner, R. P., L. Terracio, et al. (1991). "Intracranial self-stimulation motivates weight-lifting exercise in rats." J Appl Physiol 71(4): 1627-31.

Garris, P. A., M. Kilpatrick, et al. (1999). "Dissociation of dopamine release in the nucleus accumbens from intracranial self-stimulation." Nature 398(6722): 67-9.

Grace, A. A. and B. S. Bunney (1983). "Intracellular and extracellular electrophysiology of nigral dopaminergic neurons--1. Identification and characterization." Neuroscience 10(2): 301-15.

Grace, A. A. and S. P. Onn (1989). "Morphology and electrophysiological properties of immunocytochemically identified rat dopamine neurons recorded in vitro." J Neurosci 9(10): 3463-81.

Grubb, M. C., J. R. Welch, et al. (2002). "Cocaine self-administration alters the locomotor response to microinjection of bicuculline into the ventral tegmental area of rats." Brain Res 952(1): 44-51.

Guarraci, F. A. and B. S. Kapp (1999). "An electrophysiological characterization of ventral tegmental area dopaminergic neurons during differential pavlovian fear conditioning in the awake rabbit." Behav Brain Res 99(2): 169-79.

Hahnloser, R. H., A. A. Kozhevnikov, et al. (2002). "An ultra-sparse code underlies the generation of neural sequences in a songbird." Nature 419(6902): 65-70.

Hara, E., L. Kubikova, et al. (2007). "Role of the midbrain dopaminergic system in modulation of vocal brain activation by social context." Eur J Neurosci 25(11): 3406-16.

Herbert, H., A. Klepper, et al. (1997). "Afferent and efferent connections of the ventrolateral tegmental area in the rat." Anat Embryol (Berl) 196(3): 235-59.

Hessler, N. A. and A. J. Doupe (1999). "Singing-related neural activity in a dorsal forebrain-basal ganglia circuit of adult zebra finches." J Neurosci 19(23): 10461-81.

Hessler, N. A. and A. J. Doupe (1999). "Social context modulates singing-related neural activity in the songbird forebrain." Nat Neurosci 2(3): 209-11.

Hnasko, T. S., B. N. Sotak, et al. (2005). "Morphine reward in dopamine-deficient mice." Nature 438(7069): 854-7.

Hollerman, J. R., L. Tremblay, et al. (1998). "Influence of reward expectation on behavior-related neuronal activity in primate striatum." J Neurophysiol 80(2): 947-63.

Horvitz, J. C. (2000). "Mesolimbocortical and nigrostriatal dopamine responses to salient non-reward events." Neuroscience 96(4): 651-6.

Horvitz, J. C. (2002). "Dopamine gating of glutamatergic sensorimotor and incentive motivational input signals to the striatum." Behav Brain Res 137(1-2): 65-74.

Horvitz, J. C., T. Stewart, et al. (1997). "Burst activity of ventral tegmental dopamine neurons is elicited by sensory stimuli in the awake cat." Brain Res 759(2): 251-8.

Ison, J. R., D. W. McAdam, et al. (1973). "Latency and amplitude changes in the acoustic startle reflex of the rat produced by variation in auditory prestimulation." Physiol Behav 10(6): 1035-9.

Johnson, S. W. and R. A. North (1992). "Two types of neurone in the rat ventral tegmental area and their synaptic inputs." J Physiol 450: 455-68.

Johnson, S. W. and R. A. North (1992). "Opioids excite dopamine neurons by hyperpolarization of local interneurons." J Neurosci 12(2): 483-8.

Kao, M. H. and M. S. Brainard (2006). "Lesions of an avian basal ganglia circuit prevent context-dependent changes to song variability." J Neurophysiol 96(3): 1441-55.

Kao, M. H., A. J. Doupe, et al. (2005). "Contributions of an avian basal ganglia-forebrain circuit to real-time modulation of song." Nature 433(7026): 638-43.

Kilpatrick, M. R., M. B. Rooney, et al. (2000). "Extracellular dopamine dynamics in rat caudate-putamen during experimenter-delivered and intracranial self-stimulation." Neuroscience 96(4): 697-706.

Kiyatkin, E. A. and G. V. Rebec (1997). "Activity of presumed dopamine neurons in the ventral tegmental area during heroin self-administration." Neuroreport 8(11): 2581-5.

Kiyatkin, E. A. and G. V. Rebec (1998). "Heterogeneity of ventral tegmental area neurons: single-unit recording and iontophoresis in awake, unrestrained rats." Neuroscience 85(4): 1285-309.

Kokkinidis, L. and H. Anisman (1979). "Circling behavior following systemic d-amphetamine administration: potential noradrenergic and dopaminergic involvement." Psychopharmacology (Berl) 64(1): 45-54.

Konishi, M. (1965). "The role of auditory feedback in the control of vocalization in the white-crowned sparrow." Z Tierpsychol 22(7): 770-83.

Koob, G. F., P. J. Fray, et al. (1978). "Self-stimulation at the lateral hypothalamus and locus coeruleus after specific unilateral lesions of the dopamine system." Brain Res 146(1): 123-40.

Koob, G. F., L. Stinus, et al. (1981). "Hyperactivity and hypoactivity produced by lesions to the mesolimbic dopamine system." Behav Brain Res 3(3): 341-59.

Lee, R. S., S. C. Steffensen, et al. (2001). "Discharge profiles of ventral tegmental area GABA neurons during movement, anesthesia, and the sleep-wake cycle." J Neurosci 21(5): 1757-66.

Leite-Morris, K. A., E. Y. Fukudome, et al. (2002). "Opiate-induced motor stimulation is regulated by gamma-aminobutyric acid type B receptors found in the ventral tegmental area in mice." Neurosci Lett 317(3): 119-22.

Leite-Morris, K. A., E. Y. Fukudome, et al. (2002). "Opiate-induced motor stimulation is regulated by gamma-aminobutyric acid type B receptors found in the ventral tegmental area in mice." Neurosci Lett 317(3): 119-22.

Leonardo, A. and M. S. Fee (2005). "Ensemble coding of vocal control in birdsong." J Neurosci 25(3): 652-61.

Lessenich, A., S. Lindemann, et al. (2001). "A novel black-hooded mutant rat (ci3) with spontaneous circling behavior but normal auditory and vestibular functions." Neuroscience 107(4): 615-28.

Lewis, J. W., S. M. Ryan, et al. (1981). "Evidence for a catecholaminergic projection to area X in the zebra finch." J Comp Neurol 196(2): 347-54.

Ljungberg, T., P. Apicella, et al. (1991). "Responses of monkey midbrain dopamine neurons during delayed alternation performance." Brain Res 567(2): 337-41.

Ljungberg, T., P. Apicella, et al. (1992). "Responses of monkey dopamine neurons during learning of behavioral reactions." J Neurophysiol 67(1): 145-63.

Lucas, J. R., T. M. Freeberg, et al. (2002). "A comparative study of avian auditory brainstem responses: correlations with phylogeny and vocal complexity, and seasonal effects." J Comp Physiol A Neuroethol Sens Neural Behav Physiol 188(11-12): 981-92.

Lynch, K. S., B. Diekamp, et al. (2008). "Catecholaminergic cell groups and vocal communication in male songbirds." Physiol Behav 93(4-5): 870-6.

Maeda, H. and G. J. Mogenson (1980). "An electrophysiological study of inputs to neurons of the ventral tegmental area from the nucleus accumbens and medial preoptic-anterior hypothalamic areas." Brain Res 197(2): 365-77.

Maeda, H. and G. J. Mogenson (1981). "A comparison of the effects of electrical stimulation of the lateral and ventromedial hypothalamus on the activity of neurons in the ventral tegmental area and substantia nigra." Brain Res Bull 7(3): 283-91.

Margolis, E. B., H. Lock, et al. (2006). "Kappa opioids selectively control dopaminergic neurons projecting to the prefrontal cortex." Proc Natl Acad Sci U S A 103(8): 2938-42.

Margolis, E. B., H. Lock, et al. (2006). "The ventral tegmental area revisited: is there an electrophysiological marker for dopaminergic neurons?" J Physiol 577(Pt 3): 907-24.

Marler, P. (2004). "Bird calls: their potential for behavioral neurobiology." Ann N Y Acad Sci 1016: 31-44.

Marler, P., P. Mundinger, et al. (1972). "Effects of acoustical stimulation and deprivation on song development in red-winged blackbirds (Agelaius phoeniceus)." Anim Behav 20(3): 586-606.

Marler, P. and M. Tamura (1964). "Culturally Transmitted Patterns of Vocal Behavior in Sparrows." Science 146: 1483-6.

Marler, P. and M. S. Waser (1977). "Role of auditory feedback in canary song development." J Comp Physiol Psychol 91(1): 8-16.

Mary Christopher, S. and C. M. Butter (1968). "Consummatory behaviors and locomotor exploration evoked from self-stimulation sites in rats." J Comp Physiol Psychol 66(2): 335-9.

McCullough, L. D., J. D. Sokolowski, et al. (1993). "A neurochemical and behavioral investigation of the involvement of nucleus accumbens dopamine in instrumental avoidance." Neuroscience 52(4): 919-25.

McHaffie, J. G., H. Jiang, et al. (2006). "A direct projection from superior colliculus to substantia nigra pars compacta in the cat." Neuroscience 138(1): 221-34.

Melis, M., R. Camarini, et al. (2002). "Long-lasting potentiation of GABAergic synapses in dopamine neurons after a single in vivo ethanol exposure." J Neurosci 22(6): 2074-82.

Miliaressis, E. and P. P. Rompre (1987). "Effects of concomitant motor reactions on the measurement of rewarding efficacy of brain stimulation." Behav Neurosci 101(6): 827-31.

Mirenowicz, J. and W. Schultz (1994). "Importance of unpredictability for reward responses in primate dopamine neurons." J Neurophysiol 72(2): 1024-7.

Mirenowicz, J. and W. Schultz (1996). "Preferential activation of midbrain dopamine neurons by appetitive rather than aversive stimuli." Nature 379(6564): 449-51.

Mogenson, G. J., D. L. Jones, et al. (1980). "From motivation to action: functional interface between the limbic system and the motor system." Prog Neurobiol 14(2-3): 69-97.

Mortimer, J. A. (1973). "Temporal sequence of cerebellar Purkinje and nuclear activity in relation to the acoustic startle response." Brain Res 50(2): 457-62.

Munro, L. J. and L. Kokkinidis (1997). "Infusion of quinpirole and muscimol into the ventral tegmental area inhibits fear-potentiated startle: implications for the role of dopamine in fear expression." Brain Res 746(1-2): 231-8.

Nair-Roberts, R. G., S. D. Chatelain-Badie, et al. (2008). "Stereological estimates of dopaminergic, GABAergic and glutamatergic neurons in the ventral tegmental area, substantia nigra and retrorubral field in the rat." Neuroscience 152(4): 1024-31.

Nicola, S. M. (2007). "The nucleus accumbens as part of a basal ganglia action selection circuit." Psychopharmacology (Berl) 191(3): 521-50.

Nishino, H., S. Hattori, et al. (1991). "Basal ganglia neural activity during operant feeding behavior in the monkey: relation to sensory integration and motor execution." Brain Res Bull 27(3-4): 463-8.

Nishino, H., T. Ono, et al. (1987). "Neuronal activity in the ventral tegmental area (VTA) during motivated bar press feeding in the monkey." Brain Res 413(2): 302-13.

Nottebohm, F., T. M. Stokes, et al. (1976). "Central control of song in the canary, Serinus canarius." J Comp Neurol 165(4): 457-86.

Olds, J. and P. Milner (1954). "Positive reinforcement produced by electrical stimulation of septal area and other regions of rat brain." J Comp Physiol Psychol 47(6): 419-27.

Olds, M. E. and J. Olds (1969). "Effects of lesions in medial forebrain bundle on self-stimulation behavior." Am J Physiol 217(5): 1253-64.

Olson, V. G., C. L. Heusner, et al. (2006). "Role of noradrenergic signaling by the nucleus tractus solitarius in mediating opiate reward." Science 311(5763): 1017-20.

Palmiter, R. D. (2008). "Dopamine signaling in the dorsal striatum is essential for motivated behaviors: lessons from dopamine-deficient mice." Ann N Y Acad Sci 1129: 35-46.

Perkel, D. J., M. A. Farries, et al. (2002). "Electrophysiological analysis of a songbird basal ganglia circuit essential for vocal plasticity." Brain Res Bull 57(3-4): 529-32.

Person, A. L., S. D. Gale, et al. (2008). "Organization of the songbird basal ganglia, including area X." J Comp Neurol 508(5): 840-66.

Phillips, A. G. and H. C. Fibiger (1973). "Dopaminergic and noradrenergic substrates of positive reinforcement: differential effects of d- and l-amphetamine." Science 179(73): 575-7.

Piazza, P. V., M. Ferdico, et al. (1989). "The influence of dopaminergic A10 neurons on the motor pattern evoked by substantia nigra (pars compacta) stimulation." Behav Brain Res 31(3): 273-8.

Redgrave, P., T. J. Prescott, et al. (1999). "The basal ganglia: a vertebrate solution to the selection problem?" Neuroscience 89(4): 1009-23.

Redgrave, P., T. J. Prescott, et al. (1999). "Is the short-latency dopamine response too short to signal reward error?" Trends Neurosci 22(4): 146-51.

Roberts, D. C. and G. F. Koob (1982). "Disruption of cocaine self-administration following 6-hydroxydopamine lesions of the ventral tegmental area in rats." Pharmacol Biochem Behav 17(5): 901-4.

Robinson, S., A. J. Rainwater, et al. (2007). "Viral restoration of dopamine signaling to the dorsal striatum restores instrumental conditioning to dopamine-deficient mice." Psychopharmacology (Berl) 191(3): 567-78.

Sakata, J. T. and M. S. Brainard (2006). "Real-time contributions of auditory feedback to avian vocal motor control." J Neurosci 26(38): 9619-28.

Sakata, J. T., C. M. Hampton, et al. (2008). "Social modulation of sequence and syllable variability in adult birdsong." J Neurophysiol 99(4): 1700-11.

Salamone, J. D. (1994). "The involvement of nucleus accumbens dopamine in appetitive and aversive motivation." Behav Brain Res 61(2): 117-33.

Salamone, J. D., M. Correa, et al. (2005). "Beyond the reward hypothesis: alternative functions of nucleus accumbens dopamine." Curr Opin Pharmacol 5(1): 34-41.

Sasaki, A., T. D. Sotnikova, et al. (2006). "Social context-dependent singing-regulated dopamine." J Neurosci 26(35): 9010-4.

Scharff, C. and F. Nottebohm (1991). "A comparative study of the behavioral deficits following lesions of various parts of the zebra finch song system: implications for vocal learning." J Neurosci 11(9): 2896-913.

Schultz, W. (1986). "Responses of midbrain dopamine neurons to behavioral trigger stimuli in the monkey." J Neurophysiol 56(5): 1439-61.

Schultz, W. (2002). "Getting formal with dopamine and reward." Neuron 36(2): 241-63.

Schultz, W. (2007). "Behavioral dopamine signals." Trends Neurosci 30(5): 203-10.

Schultz, W., L. Tremblay, et al. (2003). "Changes in behavior-related neuronal activity in the striatum during learning." Trends Neurosci 26(6): 321-8.

Shank, E. J., P. K. Seitz, et al. (2007). "Selective ablation of GABA neurons in the ventral tegmental area increases spontaneous locomotor activity." Behav Neurosci 121(6): 1224-33.

Shea, S. D. and D. Margoliash (2003). "Basal forebrain cholinergic modulation of auditory activity in the zebra finch song system." Neuron 40(6): 1213-26.

Sohrabji, F., E. J. Nordeen, et al. (1990). "Selective impairment of song learning following lesions of a forebrain nucleus in the juvenile zebra finch." Behav Neural Biol 53(1): 51-63.

Steffensen, S. C., R. S. Lee, et al. (2001). "Responses of ventral tegmental area GABA neurons to brain stimulation reward." Brain Res 906(1-2): 190-7.

Steffensen, S. C., S. H. Stobbs, et al. (2006). "Contingent and non-contingent effects of heroin on mu-opioid receptor-containing ventral tegmental area GABA neurons." Exp Neurol 202(1): 139-51.

Steffensen, S. C., A. L. Svingos, et al. (1998). "Electrophysiological characterization of GABAergic neurons in the ventral tegmental area." J Neurosci 18(19): 8003-15.

Swanson, L. W. (1982). "The projections of the ventral tegmental area and adjacent regions: a combined fluorescent retrograde tracer and immunofluorescence study in the rat." Brain Res Bull 9(1-6): 321-53.

Swerdlow, N. R., D. L. Braff, et al. (1986). "Central dopamine hyperactivity in rats mimics abnormal acoustic startle response in schizophrenics." Biol Psychiatry 21(1): 23-33.

Tobler, P. N., A. Dickinson, et al. (2003). "Coding of predicted reward omission by dopamine neurons in a conditioned inhibition paradigm." J Neurosci 23(32): 10402-10.

Tobler, P. N., C. D. Fiorillo, et al. (2005). "Adaptive coding of reward value by dopamine neurons." Science 307(5715): 1642-5.

Tremblay, L., J. R. Hollerman, et al. (1998). "Modifications of reward expectation-related neuronal activity during learning in primate striatum." J Neurophysiol 80(2): 964-77.

Trojniar, W. and I. Klejbor (1999). "Facilitatory effect of unilateral lesion of the ventral tegmental area on locomotor response to stimulation of the contralateral ventral tegmental area: involvement of GABAergic transmission." Brain Res 842(2): 419-30.

Tumer, E. C. and M. S. Brainard (2007). "Performance variability enables adaptive plasticity of 'crystallized' adult birdsong." Nature 450(7173): 1240-4.

Ungless, M. A. (2004). "Dopamine: the salient issue." Trends Neurosci 27(12): 702-6.

Ungless, M. A., P. J. Magill, et al. (2004). "Uniform inhibition of dopamine neurons in the ventral tegmental area by aversive stimuli." Science 303(5666): 2040-2.

Ungless, M. A., J. L. Whistler, et al. (2001). "Single cocaine exposure in vivo induces long-term potentiation in dopamine neurons." Nature 411(6837): 583-7.

Vicario, D. S. (2004). "Using learned calls to study sensory-motor integration in songbirds." Ann N Y Acad Sci 1016: 246-62.

Vicario, D. S., J. N. Raksin, et al. (2002). "The relationship between perception and production in songbird vocal imitation: what learned calls can teach us." J Comp Physiol A Neuroethol Sens Neural Behav Physiol 188(11-12): 897-908.

Vu, E. T., M. E. Mazurek, et al. (1994). "Identification of a forebrain motor programming network for the learned song of zebra finches." J Neurosci 14(11 Pt 2): 6924-34.

Waelti, P., A. Dickinson, et al. (2001). "Dopamine responses comply with basic assumptions of formal learning theory." Nature 412(6842): 43-8.

Wickens, J. R., J. C. Horvitz, et al. (2007). "Dopaminergic mechanisms in actions and habits." J Neurosci 27(31): 8181-3.

Wise, R. A. (1978). "Catecholamine theories of reward: a critical review." Brain Res 152(2): 215-47.

Wise, R. A. (2005). "Forebrain substrates of reward and motivation." J Comp Neurol 493(1): 115-21.

Yamaguchi, T., W. Sheen, et al. (2007). "Glutamatergic neurons are present in the rat ventral tegmental area." Eur J Neurosci 25(1): 106-18.

Yanagihara, S. and N. A. Hessler (2006). "Modulation of singing-related activity in the songbird ventral tegmental area by social context." Eur J Neurosci 24(12): 3619-27.

Yang, C. R. and G. J. Mogenson (1984). "Electrophysiological responses of neurones in the nucleus accumbens to hippocampal stimulation and the attenuation of the excitatory responses by the mesolimbic dopaminergic system." Brain Res 324(1): 69-84.

Yang, P. B., A. C. Swann, et al. (2006). "Chronic methylphenidate modulates locomotor activity and sensory evoked responses in the VTA and NAc of freely behaving rats." Neuropharmacology 51(3): 546-56.

Yang, P. B., A. C. Swann, et al. (2006). "Sensory-evoked potentials recordings from the ventral tegmental area, nucleus accumbens, prefrontal cortex, and caudate nucleus and locomotor activity are modulated in dose-response characteristics by methylphenidate." Brain Res 1073-1074: 164-74.

Yu, A. C. and D. Margoliash (1996). "Temporal hierarchical control of singing in birds." Science 273(5283): 1871-5.

Yun, I. A., K. T. Wakabayashi, et al. (2004). "The ventral tegmental area is required for the behavioral and nucleus accumbens neuronal firing responses to incentive cues." J Neurosci 24(12): 2923-33.

Zhou, Q. Y. and R. D. Palmiter (1995). "Dopamine-deficient mice are severely hypoactive, adipsic, and aphagic." Cell 83(7): 1197-209.

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