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The Responses of Ventral Tegmental Area Neurons to Appetitive and Aversive Conditioned Cues

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The Responses of Ventral Tegmental Area Neurons to Appetitive and Aversive Conditioned Cues

by

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DISSERTATION

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Abstract

While the ventral tegmental area (VTA) and its dopamine (DA) projections have been implicated strongly in reward processing, and more tentatively in fear conditioning, the response time course of all of the many types of VTA neurons to rewards, aversive events, and cues predictive of such outcomes is still unclear. To this end, extracellular recordings of VTA neural activity were conducted during appetitive and aversive Pavlovian conditioning in awake, behaving rats. Two populations of VTA neurons were excited by a visual reward-predictive cue: a Congruent population excited by reward, as well as an Incongruent population inhibited by reward. The Congruent population displayed cue response characteristics often ascribed to presumed DA neurons, such as learning-related enhancement, extinction-related decrement, correlation with motivation, and a negative reward prediction error signal. The Incongruent population did not display these cue response characteristics, and may encode the sensory salience of the cue. In the Pavlovian fear conditioning procedure, phasic responses to visual and auditory cues predicting footshock delivery were found. The proportion of cue inhibitions, and the strength of cue excitations, encoded behaviorally expressed cue-elicited fear. Conditioned phasic responses to appetitive and aversive cues were very similar, and most cells excited by the fear-conditioned cue were excited during reward consumption. Taken together, our findings suggest that VTA neurons whose responses change with learning are not generally selective for the hedonic valence of events, but may instead reflect the motivational salience of both rewarding and aversive events.

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Chapter 1

Introduction

"We may lay it down that Pleasure is a movement, a movement by which the soul as a whole is consciously brought into its normal state of being; and that Pain is the opposite."
- *Aristotle (384 BCE – 322 BCE)*

"It is within the experience of everyone that when pleasure and pain reach a certain intensity they are indistinguishable."
- *Arnold Bennett (1867-1931)*

We often hear of the thin line between love and hate, between pleasure and pain – mankind has long recognized the similarities between the subjective experiences of positive and negative events. This may be surprising in light of the fact that they are, in the most obvious sense, opposing forces. Sigmund Freud formally proposed this "pleasure principle": namely, that we are continually driven to seek pleasure and to avoid pain. However, this formulation cannot address the many exceptions to this rule. It is apparent that humans engage in many behaviors that result in pain or other aversive consequences, or a mixture of pain and pleasure; aside from the extreme example of sexual masochism, there are the more everyday examples of strenuous exercise, over- or under- indulgence in food or drink, and addictive behaviors such as drug abuse. Nor is this behavior restricted to humans, with our large over-thinking brains and complicated cultural environments; even rodents will learn to lever-press for shocks if they are predictive of food or drug reward (Pearce and Dickinson, 1975; Pelloux et al., 2007).

Some have proposed that the positive or negative valence of emotional events (their "goodness" or "badness") is derived from our cognitive interpretation, using social and environmental cues, of physiologically ambiguous internal stimuli. This two-factor

theory of emotion was based initially on the observation that a subject's physiological arousal, induced by administration of adrenaline, could be subjectively experienced as either positive or negative based on situational cues (the behavior of an experimental confederate) (Schachter and Singer, 1962). In another study, male subjects who met an attractive woman when they were on a high bridge pursued her more often than subjects who met her after they had finished crossing the bridge, perhaps because they attributed their high arousal state to her charms rather than to their fear of falling (Dutton and Aron, 1974). While more recent research on emotions has shown that this model may be overly simplistic, its power to describe some aspects of subjective experience is still striking. Most of us can attest to the fact that many symptoms of intense love or other positive excitement are similar to the experience of fear (stomach turning, palms sweating, knees buckling). However, these effects appear largely peripheral; how are rewarding and aversive events processed differently in the brain? Since we are, in the end, generally able to distinguish events as "good" or "bad", neural processes must exist which mediate the assignment of these labels, and, when necessary, elicit relevant behavioral responses.

Learning about reward and punishment

To survive in natural environments, organisms must learn to approach stimuli which predict positive outcomes (food, water, sex, shelter) and avoid stimuli which predict negative outcomes (pain, hunger, thirst, attack from predators). This requires animals to learn (a) *salience* - which stimuli in an environment are behaviorally relevant and deserve their attention, and (b) *valence* - whether these stimuli predict "good" or "bad" events. It has been shown in a variety of species that stimuli paired with rewards or punishments

begin to take on rewarding or aversive properties of their own. In the first laboratory demonstration of classical (Pavlovian) conditioning using reward, Ivan Pavlov was able to show a dog will salivate when presented with a cue predicting food (Pavlov, 1927). At around the same time, John Watson demonstrated similar results with learned fear (Watson and Rayner, 1920). In this study, an unlucky small child (Little Albert) learned to fear a white rat (and other objects resembling it), after only a few sessions in which he heard a loud (startling) noise while the rat was present. In both cases, the cues (Pavlov's "bell" and Watson's white rat) were initially neutral, but responses naturally elicited by the food reward (salivation) and the loud noise (fear) were transferred to the cues through associative conditioning. In the decades since these initial findings, much research has been directed toward the neural substrates of learning about rewards and punishments, but there continues to be a great deal of debate about whether these different kinds of learning are mediated by distinct or overlapping neural systems.

Dopamine: a neural substrate of reward, salience, or motivation?

The neurotransmitter dopamine (DA) is released by two major populations of neurons originating in the midbrain: the ventral tegmental area (VTA) and the substantia nigra (SN). DA, particularly when released in areas well-established as important for reward learning, such as the nucleus accumbens (NAc) and the prefrontal cortex (PFC), has been proposed by many to be a neural substrate of reward. This hypothesis, probably most clearly stated by Wise and colleagues as the "anhedonia hypothesis" of neuroleptic action (Wise, 1982; Wise et al., 1978), argues that DA antagonists "blunt the hedonic impact of rewards" at lower doses than those at which they cause motor impairment. It is clear that

a variety of reinforcing stimuli, including food (Bassareo and Di Chiara, 1997), sex (Damsma et al., 1992), and drugs of abuse (Yokel and Wise, 1976), induce DA release in the brain. Lever-pressing (Wise et al., 1978; Yokel and Wise, 1976) and conditioned place preference (Spyraki et al., 1982) for these reinforcers are attenuated by DA antagonists, and rats will learn to lever-press for DA agonists (Yokel and Wise, 1976).

A more recent line of work has suggested that, rather than signaling reward, dopamine instead signals reward prediction. Schultz et al. have shown in primates that increased firing of a majority of presumed DA neurons in an appetitive operant conditioning task correlates initially with reward consumption, but that as the animal learns the association of the predictive cue with the reward, the excitatory neural response shifts to the cue (Ljungberg et al., 1992; Mirenowicz and Schultz, 1994). Unpredicted reward continues to elicit firing in these neurons, and, most interestingly, there is an inhibition in firing at the time of the expected reward when the conditioned cue is presented but reward is omitted. With extensive over-training, to the point that the animal responded in what appeared to be a habitual manner, firing to the predictive cue also disappeared (Ljungberg et al., 1992). This led the authors to suggest that DA encodes a "reward prediction error signal", such that DA neurons increase firing when unpredicted reward (or its associated cue) occurs, decrease firing when expected reward is omitted, and show no change in firing when an expected reward occurs. These responses do not appear to be species-specific; another study tested the reward prediction error model in rats trained on an appetitive Pavlovian conditioning procedure, with results in general agreement with those from primate studies (Pan et al., 2005). A recent functional MRI study in humans found that VTA activity was consistent with a role in

positive, but not negative, reward prediction error (D'Ardenne et al., 2008), although it should be noted that BOLD responses should not be understood as a direct measure of neural firing, particularly the specific activity of DA neurons.

An alternative interpretation of the data obtained by Schultz et al. is that DA is required for the "attribution of incentive salience to otherwise neutral events" (Berridge and Robinson, 1998; Berridge, 2007). These authors suggest that reward processes can be separated into "wanting", the motivation to acquire a reward, and "liking", the hedonic impact of the reward. They argue that DA is not required for "liking" because DA depletions of over 99% in rats, while inducing aphagia and disrupting operant responding for reward, do not disrupt normal orofacial responses (their measure of "liking") to sweet or bitter solutions, reactions which are disrupted by some other forms of aphagia. This is interpreted to mean that, while DA-depleted animals no longer "want" food, water, drugs, or other rewards, they still "like" them. This fits well with the observation that DA transporter "knockdown" mutant mice, with 70% elevated levels of synaptic DA, show enhanced "wanting" (performance of operant responding), while showing no greater "liking" reactions to the sucrose reward (Pecina et al., 2003; Cagniard et al., 2006). This hypothesis is also supported by evidence that responses in presumed DA neurons, as well as NAc DA release, in operant appetitive tasks are seen in advance of and during operant responding (lever-pressing), but fall off sharply during receipt of the reinforcer (Kiyatkin and Gratton, 1994; Kiyatkin and Stein, 1995; Richardson and Gratton, 1996; Kiyatkin and Rebec, 2001; Roitman et al., 2004). It has also been noted that DA antagonists in human addicts do not attenuate the hedonic effects of drugs, but rather reduce drug craving (Berridge and Robinson, 1998) and that DA release in the NAc in normal

subjects and Parkinson's patients correlates better with "wanting" than with "liking" drugs (Leyton et al., 2002; 2005; Evans et al., 2006).

While there is substantial evidence in rodents that dopamine release is seen to conditioned appetitive cues for food (Bassareo and Di Chiara, 1997; Richardson and Gratton, 1998; Phillips et al., 2003; Roitman et al., 2004; Day et al., 2007; Cheng et al., 2003) and drug (Di Ciano et al., 1998; Ito et al., 2002) reward, and that such release is required for behavioral responses as well as cue-related firing in the nucleus accumbens (Yun et al., 2004a,b), concerns have been raised about reward-specific hypotheses of DA function. First, it is unclear how the well-established phasic responding of presumed DA neurons to novel salient stimuli (Horvitz, 2000, 2002; Ljungberg et al., 1992) can be explained by these hypotheses. While it is possible that a novel salient stimulus is predictive of reward, it is equally likely that it is predictive of punishment – an experimental animal cannot know in advance that the tone with which it is presented is followed by sucrose rather than a shock. Even more problematic for reward hypotheses is evidence showing DA release to aversive stimuli (Abercrombie et al., 1989; Blackburn et al., 1992; Wilkinson et al., 1998; Young, 2004) and associated cues in Pavlovian conditioning paradigms (Wilkinson et al., 1998; Yoshioka et al., 1996; Young, 2004; Young et al., 1998). Additionally, DA release in the NAc correlates with the number of behavioral avoidance responses emitted during an operant shock avoidance task in which performance is also disrupted by DA depletion (McCullough et al., 1993). It is worth noting that these studies measuring DA release used microdialysis, which has a time resolution of minutes, and therefore could not detect phasic changes in DA transmission. In fact, it is admitted by proponents of the "reward error prediction" hypothesis that there

are tonic increases in dopamine to aversive stimuli (Schultz, 2002), and it is often suggested that these increases could reflect "relief" responses to aversive stimulus offset. However, even aversive stimuli that were present throughout the dialysate collection period induced DA release (Jackson and Moghaddam, 2004; Young, 2004), and one report in rabbit has found excitation in some presumed DA neurons to conditioned aversive stimuli, although it found inhibitions in others (Guarraci and Kapp, 1999). In addition, in primate, a small proportion (11-15%) of presumed DA neurons show phasic excitations to mild aversive stimuli such as air puff to the hand or hypertonic saline in the mouth (Mirenowicz and Schultz, 1996). These results are usually not addressed because these same neurons also show reward- and salience-related activity, but it is difficult to make generalizations about these data due to the obviously mild nature of these aversive events. Other work, in anaesthetized rats, has shown an inhibition of firing to foot pinch in 10/12 immunohistologically-identified DA neurons recorded (Ungless et al., 2004), but due to the anaesthetized preparation, it is unclear what these data suggest about DA responses in awake, behaving animals. Thus, while it is likely that DA is released in aversive situations, there is no consensus about the similarity of this response to DA release in appetitive paradigms.

The implication of dopamine in the neural response to novel and aversive events has caused several authors to propose theories of DA function that are not selective for reward or reward-related learning. Horvitz (2000) argues that DA may encode a general prediction error irrespective of valence, showing increased release to all salient or arousing unexpected events. He also suggests that DA release to such salient stimuli gates glutamatergic input to the striatum, enhancing the efficacy of strong inputs while

attenuating weak input (Horvitz, 2002). The possibility that DA thus improves the "signal-to-noise ratio" in the striatum is consistent with a large body of electrophysiological experimental data (Nicola et al., 2000; 2004; Hjelmstad 2004). Redgrave et al. (1999; 2006; 2007) have argued that rather than signaling error in the prediction of future reward, DA is critical for "the process of switching attentional and behavioural selections to unexpected, behaviorally important stimuli". Others have suggested that the role of nucleus accumbens DA is "to facilitate flexible approach responses [to rewards or safety from punishment] by modulating incentive motivation processes" (Ikemoto and Panksepp, 1999). Similarly, one major critic of the anhedonia hypothesis has stated that NAc DA "is involved in higher order motor and sensorimotor processes that are important for activational aspects of motivation, response allocation, and responsiveness to conditioned stimuli" (Salamone et al., 1997; 2003). Indeed, Berridge and Robinson (1998) have suggested that DA's possible role in aversive situations could be due to its mediation of "motivational salience" rather than "incentive salience" in a reward-selective sense.

The VTA: more than just dopamine

While much attention has been given to the role of DA in learning, and its specificity to rewarding situations, many VTA neurons are non-DAergic (Swanson, 1982; Margolis et al., 2006b), and very few studies have examined their function. Though it was long assumed that non-DA VTA neurons were interneurons, it has since been shown that there are GABAergic VTA neurons which project to the PFC and NAc (Steffensen and Henriksen, 1998; Carr and Sesack, 2000, Margolis et al., 2006a). Glutamatergic VTA

neurons also exist (Kawano et al., 2006; Yamaguchi et al., 2007) and VTA stimulation induces glutamate release in the PFC and NAc (Chuhma et al., 2004; Lavin et al., 2005). VTA presumed GABAergic neurons are inhibited by drugs of abuse and brain stimulation and are sometimes excited prior to responding for such rewards (Steffensen et al., 2001, 2006; Stobbs and Steffensen, 2004; Lassen and Steffensen, 2007). There is also some pharmacological evidence that non-DAergic VTA neurons may play a role in mediating reward processing (Nader and van der Kooy, 1997; Laviolette and van der Kooy, 2001). However, only the activity of presumed DA neurons in the VTA has been examined in any detail in learning tasks. Unfortunately, it has been demonstrated recently that the classical criteria used to identify DA neurons *in vivo* exclude some tyrosine hydroxylase-positive neurons and include some tyrosine hydroxylase-negative neurons (Margolis et al., 2006b; Fields et al., 2007; Lammel et al., 2008; Luo et al., 2008; but see Grace et al., 2007). There is a paucity of research investigating the responses of VTA neurons without attempting to select for a certain neurochemically-defined population, but this is the only way to get a complete picture of this region's activity during learning. We therefore recorded single-unit activity in the rat VTA without *a priori* selection criteria to characterize the responses of all types of VTA neurons to conditioned stimuli predicting sucrose reward or shock in Pavlovian conditioning paradigms.

We found two populations of cue-excited VTA neurons in the appetitive Pavlovian conditioning procedure: a Congruent population excited by reward, as well as an Incongruent population inhibited by reward. The Congruent population displays cue response characteristics often ascribed to presumed DA neurons, such as learning-related

enhancement, extinction-related decrement, correlation with motivation, and a negative reward prediction error signal. The Incongruent population does not display these cue response characteristics, and may encode sensory properties of the cue.

We also found phasic VTA neural responses to cues predicting footshock delivery. The proportion of cue-inhibited cells, and the strength of cue responses in cue-excited cells, reflects the fear expressed behaviorally by subjects to the cues. These phasic responses appear very similar to those seen in VTA neurons to reward-predictive cues, and most cells excited by the fear-conditioned cue were excited during reward consumption, suggesting that these VTA neurons are not generally selective for the hedonic valence of events.

Chapter 2

Neuronal Responses to Appetitive Pavlovian Cues in the Ventral Tegmental Area

Abstract

Excitatory phasic responses to reward-predictive cues develop with Pavlovian conditioning in putative dopamine neurons in the ventral tegmental area (VTA). We recorded VTA neural activity in awake, behaving rats and found that a reward-predictive visual cue phasically excites neurons that are excited by reward ("Congruent" cells). Surprisingly, excitatory cue responses are also seen in a second class of neurons inhibited by reward ("Incongruent" cells) with similar electrophysiological properties. The short-latency component of Congruent cue responses is unrelated to reward-prediction, while overall response strength is enhanced as animals learn that the cue predicts reward, and on trials with conditioned approach. Accordingly, response strength decreases with extinction of behavioral conditioning. Additionally, Congruent cells are inhibited by omitted reward. Incongruent neurons show neither reward-predictive nor motivation-related properties, and may encode attention to, or salience of, the sensory cue. These findings demonstrate that VTA encoding of reward-predictive cues involves two functionally distinct neuronal populations.

Introduction

The ventral tegmental area (VTA) is a source of input to many brain areas involved in reward and motivation (Swanson, 1982), and inactivation of this region decreases reward-directed behavior (Yun et al., 2004a; Di Ciano and Everitt, 2004). Dopamine (DA) is released from VTA afferents in target areas such as the nucleus accumbens (NAc) and prefrontal cortex (PFC) during reward consumption, as well as during the presentation of reward-predictive cues (Phillips et al., 2003; Roitman et al., 2004; Day et al., 2007; Cheng et al., 2003; Di Chiara, 2002; Ito et al., 2002; Richardson and Gratton, 1998; Bassareo and Di Chiara, 1997). DA antagonists administered systemically (Beninger and Phillips, 1980; Wise and Schwartz, 1981), or into the NAc or PFC (Di Ciano et al., 2001; Hernandez et al., 2005; Baldwin et al., 2002), impair acquisition of conditioned responding to reward-predictive cues. Together, these findings indicate that VTA DAergic projections are important for reward learning.

The neural responses of presumed DA neurons in the VTA have been recorded during appetitive conditioning tasks in primates (reviewed in Schultz, 1998; 2002), and to a lesser extent in rats (Pan et al., 2005; Pan and Hyland, 2005; Roesch et al., 2007), and are consistent with a role in reward-related behaviors. In primates, increased firing in a majority of presumed DA neurons in the VTA and substantia nigra pars compacta (SNc) during appetitive operant and classical (Pavlovian) conditioning tasks correlates initially with reward consumption, but as the animal learns the cue-reward association, the excitatory reward response diminishes and an excitatory response to cue onset emerges (Ljungberg et al., 1992; Mirenowicz and Schultz, 1994). However, these neurons still respond to unpredicted reward, and also show a firing decrement during omission of

expected reward. Hence, DA neurons have been conceptualized as providing a reward prediction error signal (Schultz, 2002). A recent study tested the reward prediction error model in rats trained on an appetitive Pavlovian conditioning procedure. Although the reward response was still present after the cue response appeared, these authors report findings in general agreement with those from primate studies (Pan et al., 2005). However, the reward prediction error hypothesis has been questioned as phasic responses in the presumed DAergic population occur at presaccadic latencies (70-100 ms) and are of short duration (100-200 ms), and therefore are considered to occur too quickly for an event to be identified as rewarding (Redgrave et al., 1999). These authors have suggested that phasic DA responses instead may play a role in pre-attentive processing of salient stimuli related to behavioral actions (Redgrave and Gurney, 2006; Redgrave et al., 2007).

A major limitation of the above studies is that the neuronal population examined was restricted to presumed DA cells. The classical criteria used to define a neuron as DAergic *in vivo* generally include long action potential duration (>1.5 or 2.0 ms), low firing rate (<10 Hz), and sometimes inhibition by a DA D2 agonist. It is notable that, while over 50% of VTA neurons contain tyrosine hydroxylase *in vitro* (Margolis et al., 2006b; Swanson, 1982), and are therefore DAergic, only a small minority of VTA neurons recorded *in vivo* fulfill classical criteria for DA neurons. Furthermore, recent studies in VTA slices and anesthetized rats show that the classical criteria exclude some tyrosine hydroxylase-positive neurons and include some tyrosine hydroxylase-negative neurons (Margolis et al., 2006b; Fields et al., 2007; Lammel et al., 2008; Luo et al., 2008; but see Grace et al., 2007). This raises the possibility that the responses of some non-DA neurons have been attributed to DA neurons, and vice versa, in VTA recording studies in

awake behaving animals. There is also a large proportion of non-DAergic VTA neurons (up to 45%; Swanson, 1982; Margolis et al., 2006b) whose role in appetitive Pavlovian conditioning has not been examined. Some of these neurons are likely to be GABAergic VTA neurons which project to the PFC and NAc (Van Bockstaele and Pickel, 1995; Carr and Sesack, 2000, Margolis et al., 2006a). Intriguingly, the presence of glutamatergic VTA neurons has also been histochemically confirmed (Kawano et al., 2006; Yamaguchi et al., 2007), and VTA stimulation induces glutamate release in the PFC and NAc (Chuhma et al., 2004; Lavin et al., 2005).

We therefore recorded single-unit activity in the rat VTA without *a priori* selection criteria to characterize the responses of all types of VTA neurons to a reward-predictive cue during acquisition of appetitive Pavlovian conditioning. Here, we show that learning-correlated changes in the strength of cue responses occur in cue-excited neurons that are excited by reward consumption, which we termed Congruent cells. We propose that the short-latency component of the Congruent cue response reflects the salience of the cue, while the duration and magnitude of the response encodes its reward-predictive properties. Congruent cells also display a negative reward prediction error signal when expected reward is omitted and an enhancement of cue responses when motivation to retrieve reward is greater. We also identified a novel neuronal population with excitatory cue responses, but inhibited by reward consumption. Interestingly, this second cue-excited population, termed Incongruent cells, exhibits electrophysiological properties similar to Congruent cells. However, cue responses in this population lack learning-induced changes and modulation by motivation, and may therefore encode attentional or other sensory processing variables. Taken together, our results provide a

significant resolution to the critical issue of whether VTA firing represents a reward prediction error or pre-attentive processing of salient stimuli.

Materials and Methods

Animals and Surgery

Male Long-Evans rats (Harlan, Indianapolis, IN) weighing ~325 g were implanted with microelectrode arrays directed unilaterally at the VTA (AP -5.4; ML +2.0; DV -8.0) under isoflurane anesthesia. The arrays, consisting of eight 50 μm diameter insulated tungsten electrodes (NeuroBiological Laboratories, Denison, TX), were affixed to the skull with dental cement. After 7-10 days of recovery with ad libitum food and water, rats were water-restricted for 3-5 days prior to the first recording session, and water restriction was maintained until the end of the experiment. For the first 4 Paired rats, water restriction consisted of 1 hour access to ad libitum water immediately following daily recording sessions. The remaining animals were water-restricted by allowing continuous home cage access to 20 ml of water daily (the mean water intake in 1 hr by rats deprived for 23 hrs). Weight was monitored daily, and all procedures were approved by the Gallo Center Institutional Animal Care and Use Committee and were in accordance with National Institutes of Health guidelines.

Behavioral Testing

Behavior and neural activity were measured in a behavioral chamber in which there was a small cue light on the wall opposite a reward delivery port equipped with a photobeam emitter and detector to record port entries. A recording cable attached to a freely rotating

commutator at the top of the chamber was plugged into the rat's headstage, allowing the rat unhindered access to all areas of the chamber. To shape reward port behavior, all rats were first given 1-2 days of magazine training, a 1.5 hr session in which unpredicted 10% liquid sucrose was delivered in the reward port with a variable intertrial interval (ITI; mean ITI=1 min). In subsequent daily 2-hr sessions, Paired animals were presented with a 3 s flashing light CS (0.1 s on; 0.4 s off; 6 flashes) immediately followed by delivery of 0.1 mL of the 10% sucrose US over 3 s. Trials were presented with a variable ITI (mean=1 min), with each trial commencing only after sucrose from the previous trial was consumed for consistency of reward volume across trials. For Unpaired animals, the cue and sucrose were presented for ~ the same number of trials as the Paired group in an explicitly unpaired fashion (CS and US separated by at least 11 s, mean ITI=1 min). Paired rats were trained at least until the session in which the learning criterion was reached (Learned = $p<0.0001$, paired t-test comparison, for a given session, of port entries during the CS compared with port entries during a 3 s pre-CS baseline period). Paired animals required 7-13 sessions (mean 9.65 ± 0.96 SEM; median 9) to reach this criterion. Two Paired animals were excluded because they did not reach this criterion within 14 days. Unpaired animals were trained for 12 sessions, and Day 9 was selected for comparison with the Learned session in Paired animals. After reaching the learning criterion, Paired rats were given 2-3 daily extinction sessions in which the CS was presented but sucrose was not delivered. In 4 Paired animals, full extinction sessions followed a day of within-session extinction, in which sucrose followed CS presentation for the first 30 trials, but was omitted for all subsequent trials. This group also received 2 sessions in which sucrose was omitted for 1/3 of the trials, and then an additional

conditioning session with all trials reinforced, prior to extinction. For both Paired and Unpaired animals, "conditioned responding" to the cue is quantified as the percentage of cue presentations during which a port entry response was made normalized to port entries during a 3-second pre-cue baseline period. Extinction was defined as <10% conditioned responding for both between- and within-session extinction. To compare the level of conditioned responding between sessions, t-tests ($p<0.05$) were used when data were normally distributed; otherwise the non-parametric Mann Whitney Rank Sum test ($p<0.05$) was used. To compare the level of conditioned responding over the course of within-session extinction, a paired t-test ($p<0.05$) was used.

Histology

After completion of the final recording session, rats were deeply anesthetized with isoflurane after small electrolytic lesions (19μA current for 10 s) were made at the end of each electrode from which single units were recorded. Brains were removed and fixed with 10% formalin with 3% potassium ferrocyanide and then submerged in 0.01M phosphate buffer with 25% sucrose and 3% potassium ferrocyanide. 50 μm coronal sections were taken through the extent of the VTA and processed immunohistochemically for tyrosine hydroxylase reactivity as follows: free-floating sections were incubated in 3% hydrogen peroxide (10 min), rinsed, incubated in 50% alcohol (20 min), rinsed, and then incubated in normal donkey serum (10% in PBS, 30 min), before being incubated overnight in the primary antibody (rabbit anti-tyrosine hydroxylase polyclonal antibody, AB152, Chemicon, 1: 700 in PBS with 0.1% Triton-X100). Incubation with secondary antibody (biotinylated donkey anti-rabbit antibody,

Jackson ImmunoResearch, 1:300) for 2 hr was followed by incubation in ExtrAvidin-peroxidase complex (Sigma; 1:2500 in PBS) for 2 hr. Peroxidase was histochemically visualized with diaminobenzidine (DAB, Fisher). Sections were examined under a light microscope to verify that electrode placements (lesions marked with potassium ferrocyanide) were within the borders of the VTA as defined by the rat stereotaxis atlas (Paxinos and Watson, 1998).

Single-unit discrimination and characterization

Neural recordings were amplified, filtered (0.4 kHz low-cut and 5 kHz high-cut), and digitized using commercial acquisition software and hardware (Plexon Instruments, Dallas, TX). Single units were isolated based on consistent waveform shape and principal component analysis (Plexon Instruments), with autocorrelograms and interspike interval histograms used for additional rectification. Action potential waveform lengths were measured from initial inflection to first trough, and baseline firing rate was measured in the 10 s pre-cue baseline period over the session. A One-Way ANOVA on Ranks ($p<0.05$) was used to compare baseline firing rates and waveform lengths in different sessions. To compare baseline firing rates and waveform lengths of different neural populations within the same session, t-tests ($p<0.05$) were used when data were normally distributed; otherwise the non-parametric Mann Whitney Rank Sum test ($p<0.05$) was used.

Analysis of neural responses

Neural data were analyzed from a total of 16 rats (n=8 Paired and Unpaired) with electrode placements in VTA and identifiable single units, that met learning criteria. A phasic cue response was considered significant if one or more 100 ms bins in the 500 ms after cue onset were statistically significant relative to a 500 ms pre-cue baseline ($p<0.01$ using the nonparametric Wilcoxon signed-rank test; MatLab, Mathworks, Natick, MA), and the direction of the response was determined by examining perievent histograms in NeuroExplorer (Plexon Instruments). A small number of phasic responses (<15%) consisted of mixed excitation/inhibitions; in all cases, the magnitude of the excitation exceeded that of the inhibition, so these mixed responses were classified as excitations. To assess the neural response to sucrose, a variable was first created in NeuroExplorer to mark the time at which the animal received the sucrose, which was at the time of sucrose delivery itself only if the animal was already in the reward port, and was otherwise at the time of the first port entry after sucrose delivery. Because some units displayed phasic neural responses to port entry within 1 s of entry, a sucrose response was considered significant if one or more 1 s bins in the 2-5 s after sucrose receipt were significantly different from a baseline period -15 to -10 s before sucrose receipt ($p<0.01$, Wilcoxon signed-rank test). For analysis of sucrose and omitted sucrose in the sucrose omission session, neural activity was examined in the 2-5 s period after sucrose delivery or omission. Pearson Chi-square tests ($p<0.05$) were used to compare the proportion of responsive neurons between sessions and groups.

The magnitude of cue excitation was determined for each neuron by normalizing firing rate (FR) in 100 ms bins trial-by-trial using z-scores as follows:

$$Z=\frac{\text{FR in given bin} - \text{mean FR within an interval from -10 to +0.5 s around cue onset}}{\text{SD of FR within an interval from -10 to +0.5 s around cue onset}}$$

For comparison of neural responses on sucrose delivery and omission trials, z-scores were computed for one 3 s bin after the time of expected sucrose delivery, relative to a baseline period -15 to -10 s before sucrose receipt. The normalized magnitude of the neural response was compared between groups and sessions using t-tests ($p<0.05$). For within-session comparisons of response magnitude of the same cells, a paired t-test ($p<0.05$) was used. For analyses of neural activity with 10 ms resolution (to determine the latency and length of excitatory cue responses), a 10 ms bin within the 500 ms phasic cue response window bin was considered as showing a response only if its firing rate was >2 standard deviations above the mean baseline firing rate in the 10 s prior to cue onset. Cue excitation onset was defined as a response in 2 of 3 consecutive bins, and offset was defined as two consecutive non-response bins. Response length was equal to the time of response offset minus the time of onset. A 2-way repeated-measures ANOVA ($p<0.05$) was used to determine the effect of stage of training on the time course of responses with 10 ms resolution. To compare lengths and latencies of neural responses between groups and sessions, t-tests ($p<0.05$) were used when data were normally distributed; otherwise the non-parametric Mann Whitney Rank Sum test ($p<0.05$) was used.

Results

To examine VTA responses to reward-predictive visual cues, we measured neural activity in multiple daily sessions during Pavlovian appetitive conditioning or an unpaired control procedure (Figure 2-1A). Paired rats learned over several sessions that a flashing light (conditioned stimulus, CS) predicted delivery of liquid sucrose (unconditioned stimulus, US) in the reward port. This learning was reflected in an increase in anticipatory reward port entries during cue presentation (Figure 2-1B; for behavior across training, see Figure 2-2). Unpaired rats received presentations of the same light cue and sucrose reward in an explicitly unpaired manner such that the cue was not reward-predictive; this group did not develop conditioned port entry responding (Figure 2-1B).

We recorded the activity of 227 neurons histologically confirmed to be within the VTA (Figure 2-1C) from 16 rats in multiple behavioral sessions. Baseline firing rates did not differ significantly between behavioral groups or over days (One-Way ANOVA on Ranks $H(6)=6.065$, $p=0.416$).

VTA neural excitations to a salient cue degrade unless the cue is paired with reward

Both phasic excitations (Figures 2-3A, 2-4) and inhibitions (Figure 2-4) were seen to the cue in both Paired and Unpaired groups, typically within the first 500 ms after cue onset. On Day 1, Paired rats showed phasic cue excitations (20/36 cells, 55.6%) and inhibitions (3/36 cells, 8.3%), generally from the first trial onward (Figure 2-4). A similar proportion of cells had cue excitations (21/47 cells, 44.7%; Figure 2-3B) and inhibitions (5/47 cells, 10.6%) on Day 1 in Unpaired rats. Paired rats required 9 days on average to meet the learning criteria ("Learned" session, see Methods), so this session was compared to

Figure 2-1. Experimental Paradigm and Behavioral Results

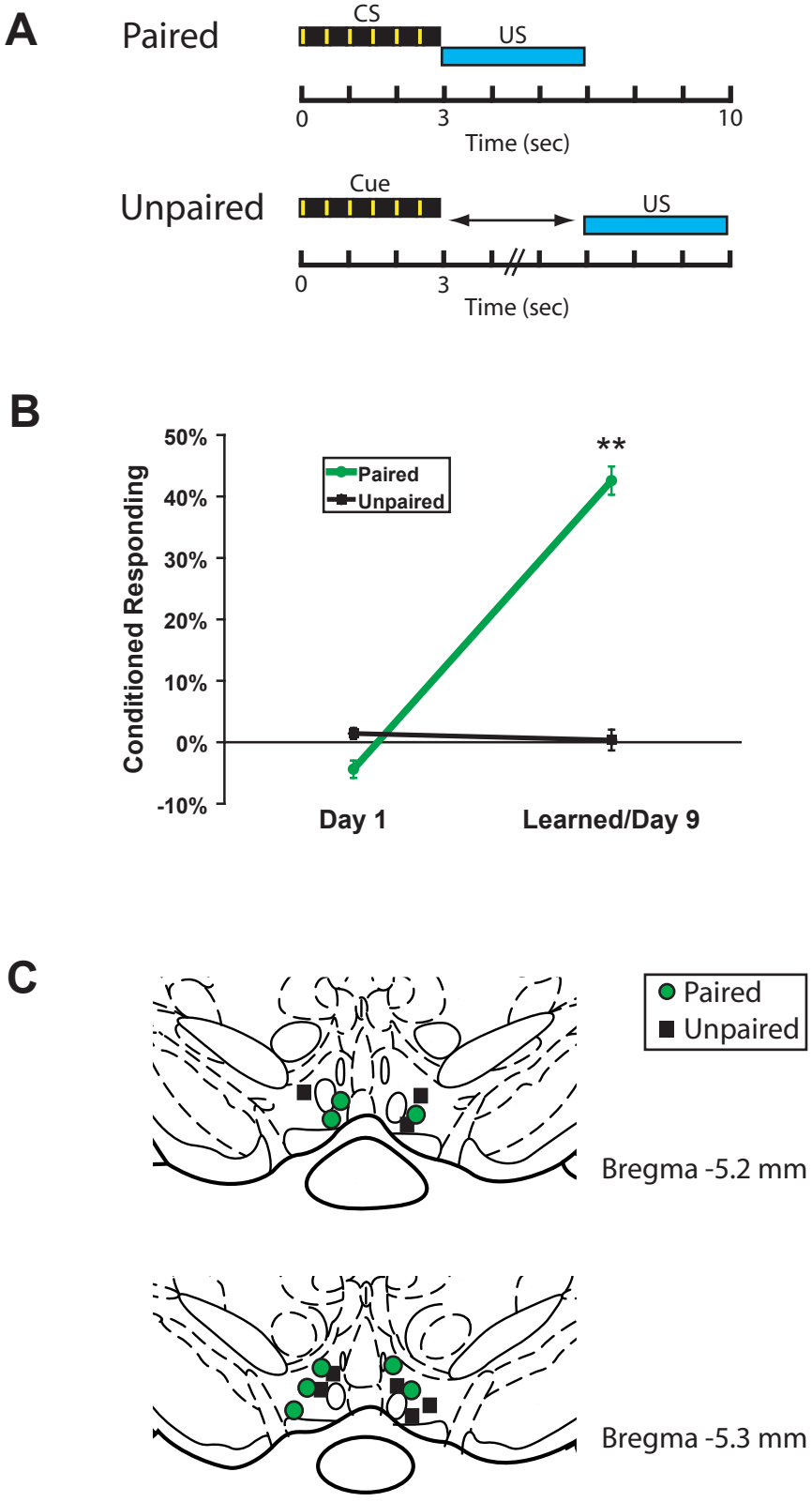


Figure 2-1. Experimental Paradigm and Behavioral Results

(A) For Paired rats, US (10% sucrose) delivery immediately followed each presentation of the CS (flashing light: 0.1 s on, 0.4 s off, 6 flashes in 3 s). For Unpaired rats, US delivery had a variable temporal relationship with the flashing light cue, such that the Cue had no reward-predictive value. (B) Paired animals developed conditioned responding to the CS over days (**$p<0.001$), while Unpaired animals did not. Values reflect mean (±SEM) percent of cues during which a port entry was made, normalized to port entries during a 3 s pre-cue baseline period. (C) Location of electrode tips in Paired (green circles) and Unpaired (black squares) animals.

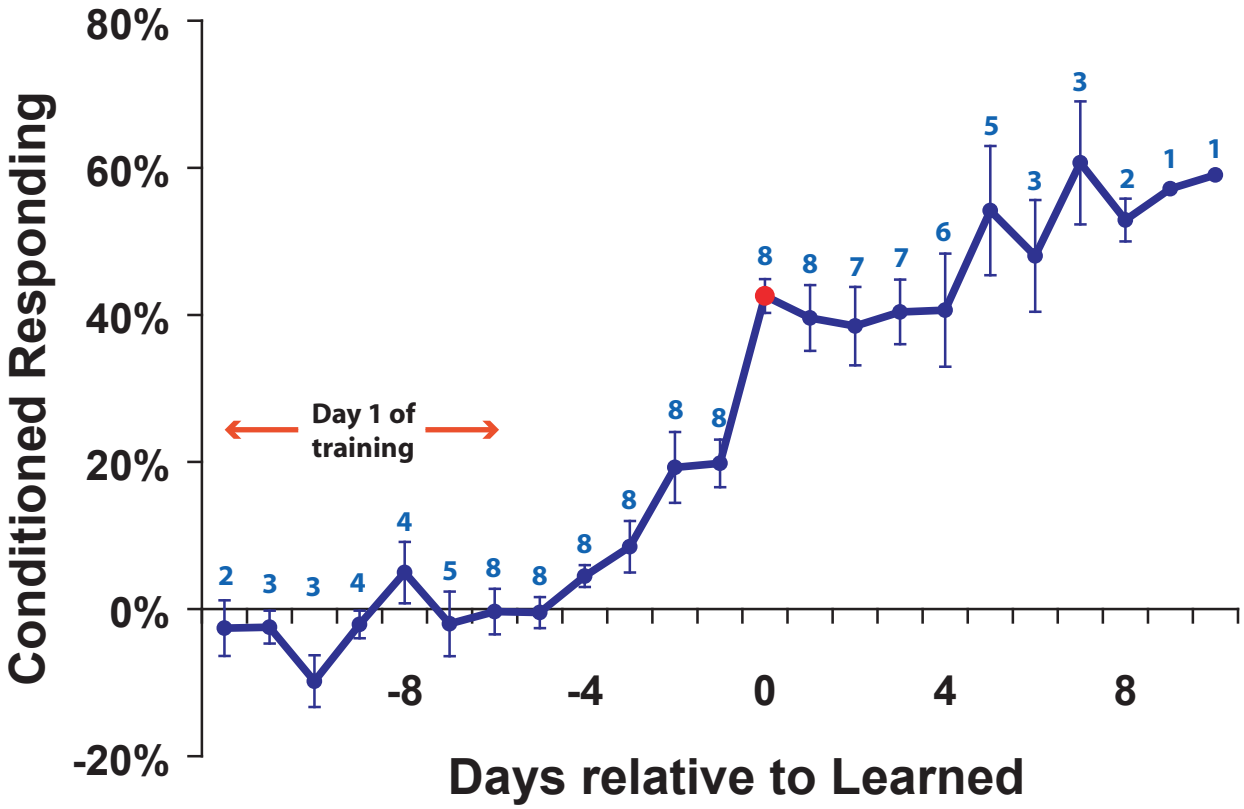


Figure 2-2. Development of conditioned port entry responding to cue in Paired animals

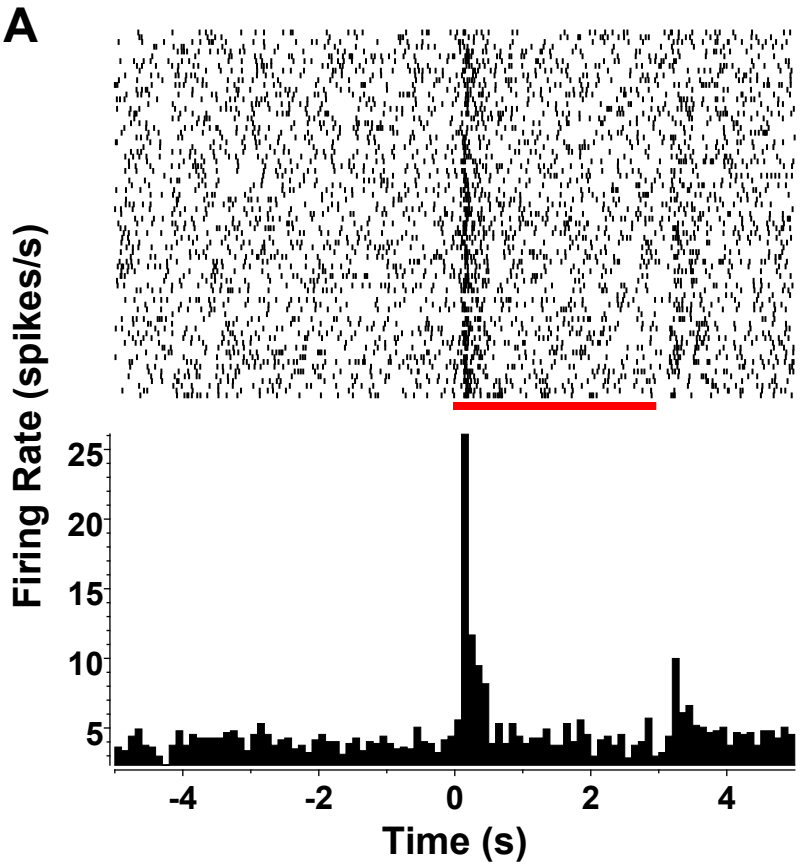
Conditioned responding on all training days, centered around the day on which the learning criterion was met (Learned, represented by red filled circle at x=0). Values reflect the mean (± SEM) of the percentage of cue presentations during which a port entry response was made normalized to port entries during a 3-s pre-cue baseline period. Blue numbers above data points indicate the number of animals with a training session on that day. Day 1 of training ranged from 7 to 13 days before the learning criterion was met.

Unpaired Day 9. Surprisingly, the percent of cells with phasic CS excitations (21/39 cells, 53.8%; p=0.835, Chi-square; Figure 2-3B) and inhibitions (8/39 cells, 20.5%; p=0.297, Chi-square) in Paired rats did not change as they learned the CS-US association. However, on Day 9 Unpaired animals had fewer cue-excited cells (9/39 cells, 23.1%) than Paired animals after learning ($p<0.05$, Chi-square; Figure 2-3B). The percent of Unpaired cue-inhibited cells was also lower on Day 9 (2/39 cells, 5.1%), although not significantly, than in the Paired group after learning (p=0.09, Chi-square). Hence we found that, rather than developing with learning, phasic excitations were present to a salient cue in naïve rats, but were found less often over time if the cue was not paired with a reward.

Although it is possible that we sampled from different populations of cue-excited VTA cells on different days, these cells had similar waveform lengths and baseline firing rates in all groups on all days (Table 2-1, firing rate, p=0.116; waveform length, p=0.785, One-Way ANOVA on Ranks), which argues against the appearance of a new cell population over time.

While CS-excited units in well-trained Paired animals had longer mean waveforms (0.43±0.03 ms) than other cells (0.29±0.03; $p<0.001$, t-test), the populations were overlapping (Table 2-1; Figure 2-5A). Also, CS-excited cells did not have significantly longer waveforms than other units on Day 1 (p=0.449, t-test) or after extinction (p=0.172, t-test). Unpaired cue-excited cells on Day 1 and Day 9 had longer waveforms (median=0.5 ms) than other cells (median=0.25 ms; Day 1: $p<0.005$, Day 9: $p<0.001$; Mann-Whitney Rank Sum test), although the populations were overlapping (Table 2-1; Figure 2-5B). No differences were seen in baseline firing rate between cue-

Figure 2-3. Phasic Excitations to Cue in Paired and Unpaired Animals



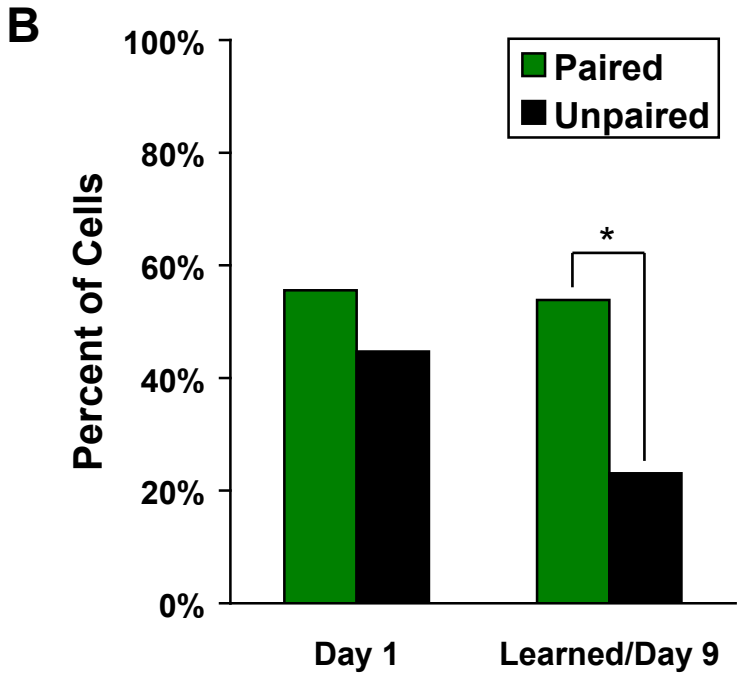


Figure 2-3. Phasic Excitations to Cue in Paired and Unpaired Animals

(A) Peri-event raster (top) and histogram (bottom, 100 ms bins) of a representative neuron with a phasic excitatory response to the cue from a well-trained animal (cue onset at time=0 s; red line indicates duration of cue) (B) Percent of VTA neurons excited by CS and Unpaired Cue. In the Paired group, over half of the neurons had an excitatory response to the CS on Day 1 and after Learning. In Unpaired controls, the proportion of neurons with an excitatory response to the Cue on Day 1 is similar to Paired animals, but on Day 9, Unpaired animals have fewer excitatory cue responses than Paired animals (*$p<0.05$).

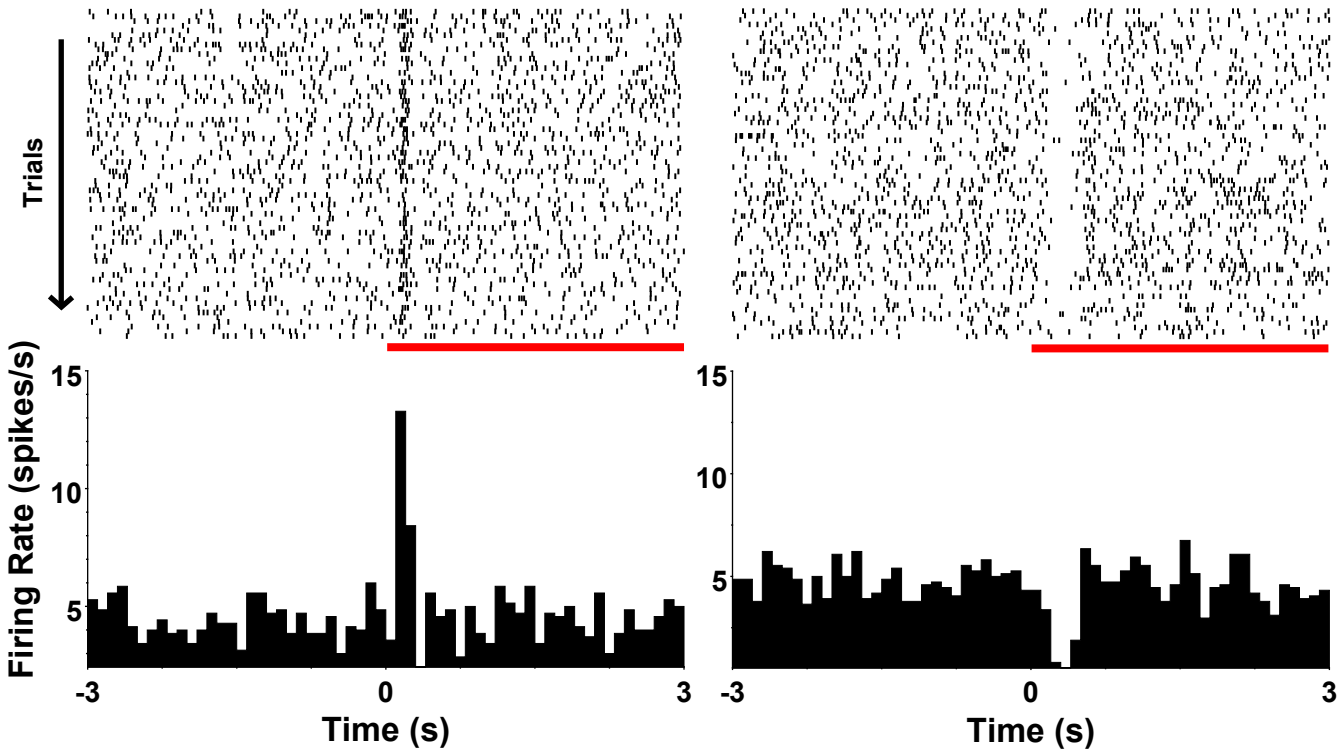


Figure 2-4. Phasic excitatory and inhibitory cue responses on Day 1

Peri-event rasters (top) and histograms (bottom, 100 ms bins) of representative neurons recorded on Day 1 showing phasic excitatory (A) and inhibitory (B) responses to the cue (cue onset at time=0 s; red line indicates duration of cue). Note that, rather than developing over the session, phasic responses are apparent from the very first trial.

Table 2-1. Electrophysiological Properties of Cue-Excited and Other VTA Neurons

Cue-Excited Cells							
	Paired (N=8 rats)					Unpaired (N=8 rats)	
	Day 1	Learned	Omission	Within-session Extinction	Extinction	Day 1	Day 9
Number of cells	20	21	19	10	11	21	9
Firing Rate (spikes/s)							
Mean	6.85±1.86	7.98±2.74	5.82±1.55	4.32±0.80	3.53±0.55	3.12±0.23	4.44±0.98
Median	4.37	4.29	3.79	3.57	3.07	3.07	3.60
Range	1.78 - 36.34	1.08 - 56.02	0.54 – 29.12	1.67 - 10.07	1.52 - 8.42	1.46 - 4.59	1.28 - 11.41
Waveform Length (ms)							
Mean	0.47±0.03	0.43±0.03	0.46±0.02	0.47±0.03	0.44±0.04	0.49±0.04	0.45±0.03
Median	0.50	0.45	0.50	0.50	0.50	0.50	0.50
Range	0.20 - 0.65	0.20 - 0.60	0.20 – 0.60	0.30 – 0.60	0.20 - 0.60	0.10 - 0.90	0.30 - 0.55

Other Cells							
	Paired (N=8 rats)					Unpaired (N=8 rats)	
	Day 1	Learned	Omission	Within-session Extinction	Extinction	Day 1	Day 9
Number of cells	16	18	5	12	9	26	30
Firing Rate (spikes/s)							
Mean	5.77±1.49	7.58±1.46	2.24±0.56	7.23±2.46	5.96±2.05	9.55±2.30	10.75±0.98
Median	3.74	5.83	1.84	4.63	3.51	4.05	4.89
Range	0.54 - 21.92	0.76 - 19.91	0.60 – 3.68	0.13 - 25.00	0.25 - 18.01	0.23 - 45.94	0.26 - 46.67
Waveform Length (ms)							
Mean	0.42±0.06	0.29±0.03	0.41±0.06	0.40±0.04	0.34±0.05	0.31±0.03	0.27±0.02
Median	0.43	0.25	0.50	0.43	0.33	0.25	0.25
Range	0.10 - 1.10	0.10 - 0.50	0.25 – 0.50	0.10 – 0.60	0.10 - 0.65	0.15 - 0.65	0.10 - 0.50

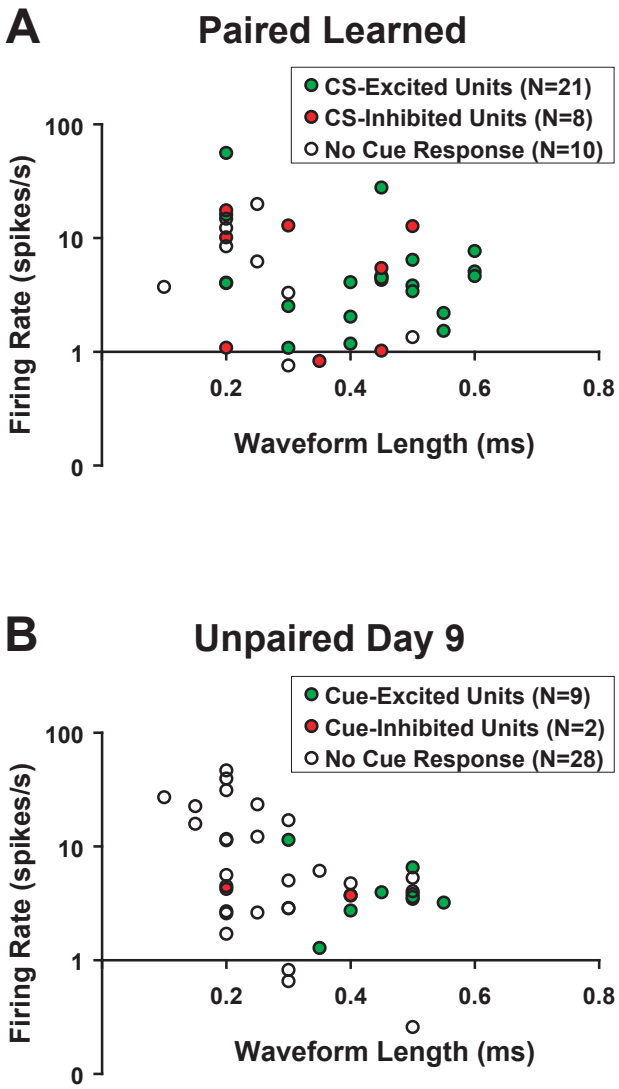


Figure 2-5. Cue-excited units have longer waveforms than other units

Electrophysiological measures of waveform length and baseline firing rate (see methods) of cue-excited, -inhibited, and -unresponsive units in (A) Paired animals after Learning and (B) Unpaired animals on Day 9.

excited and other cells in either the Paired or the Unpaired group (Table 2-1; Figure 2-5). Therefore, cue-excited neurons cannot be identified *a priori* using these electrophysiological characteristics *in vivo*.

The strength and duration of phasic cue excitations increase with learning

Although the percent of cells with excitatory CS responses did not change with conditioning, learning could be encoded in the strength or other aspects of the cue response. The mean normalized magnitude of phasic excitation in cue-excited cells increased over days in Paired ($p<0.05$, t-test), but not Unpaired, rats (Figure 2-6A). It also appeared that phasic response length increased over days in the Paired group (Figure 2-6B). To examine this possibility in further detail, we analyzed cue responses using 10-ms bins (Figures 2-6C and E). When Paired responses were compared on Day 1 and after learning using a 2-way repeated measures ANOVA, there was a main effect of stage of training [$F(1, 29)=11.9$, $p<0.005$], in accordance with the overall change in response magnitude (Figure 2-6A), as well as a significant interaction of stage of training with the time course of the neural response [$F(39, 1131)=2.46$, $p<0.001$; Figure 2-6C]. For Unpaired animals, there was no main effect of training day [$F(1, 26)=0.6$, $p=0.44$], but there was a significant interaction of stage of training with the neural response time course [$F(39, 1014)=2.02$, $p<0.001$; Figure 2-6E]. These data reflect the fact that the mean length of the cue response increased significantly over days in Paired (from 90.6±9.9 to 139.3±20.4 ms, $p<0.05$, t-test; Figure 2-6D), but not Unpaired units (from 68.5±9.7 to 46.3±9.6 ms; Figure 2-6F), while the mean onset latency of the response decreased over days in both groups (Paired: from 145.3±5.5 to 126.4±6.5 ms; Unpaired:

Figure 2-6. Magnitude and Length of CS Excitation Increases with Learning

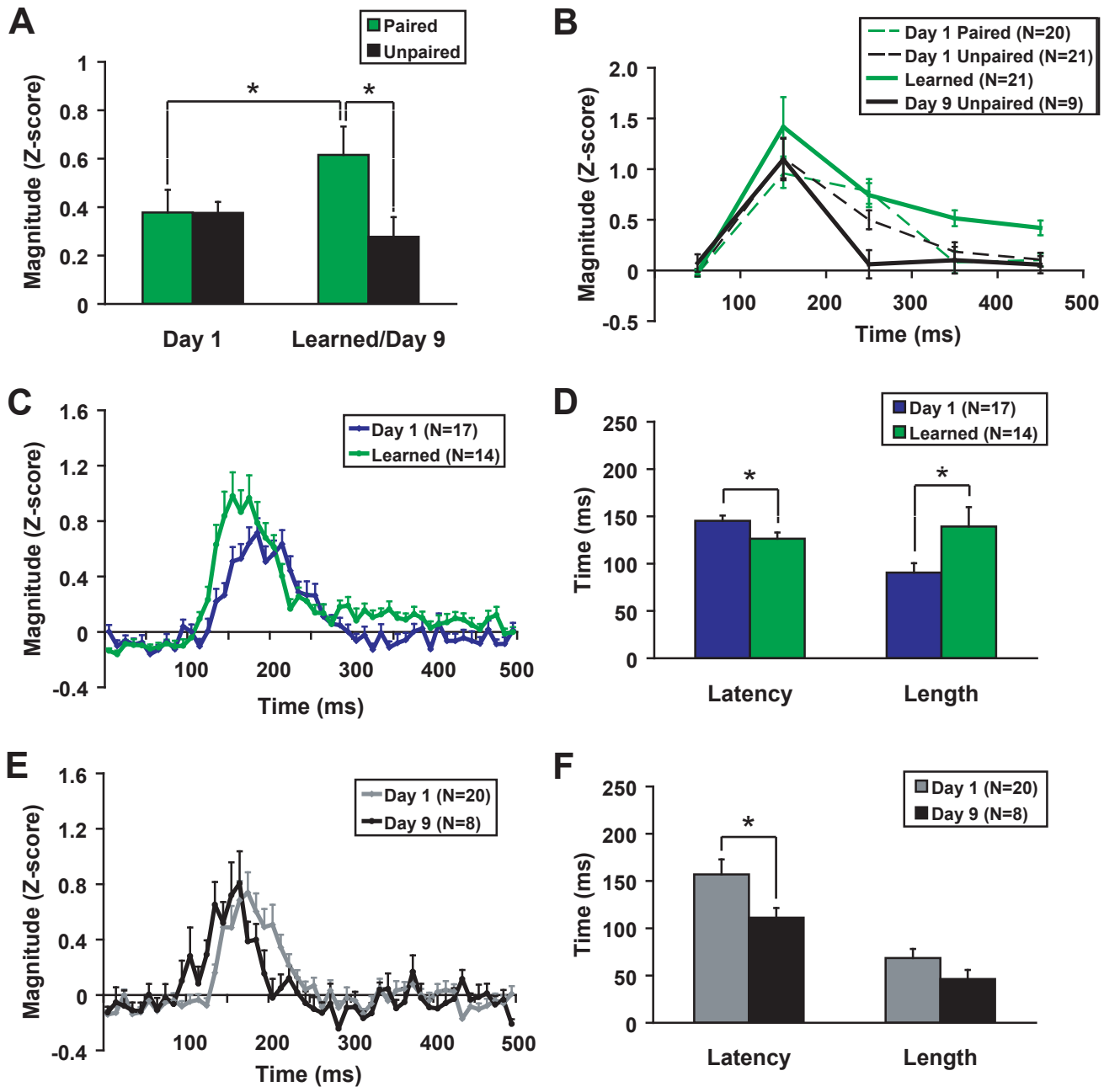


Figure 2-6. Magnitude and Length of CS Excitation Increases with Learning

(A) Magnitude of phasic cue excitation is larger in Paired animals after learning as compared to Day 1 (*$p<0.05$) and as compared to Unpaired controls trained for many days (*$p<0.05$). Values depict mean (±SEM) magnitude during the first 500 ms after cue onset, normalized to baseline firing rate. (B) Population histograms (100 ms bins, cue onset at time=0 ms) of excitatory cue responses normalized to baseline firing rate (mean±SEM). (C-F) Mean length of excitatory cue response increased over days in Paired, but not Unpaired animals, while the average onset latency of cue excitations decreased over days in both groups. (C and E) Population histograms (10 ms bins, cue onset at time=0 ms) of mean (±SEM) excitatory cue responses normalized to baseline firing rate. (D and F) Mean (±SEM) onset latency (*$p<0.05$) and length (*$p<0.05$) of excitatory cue responses.

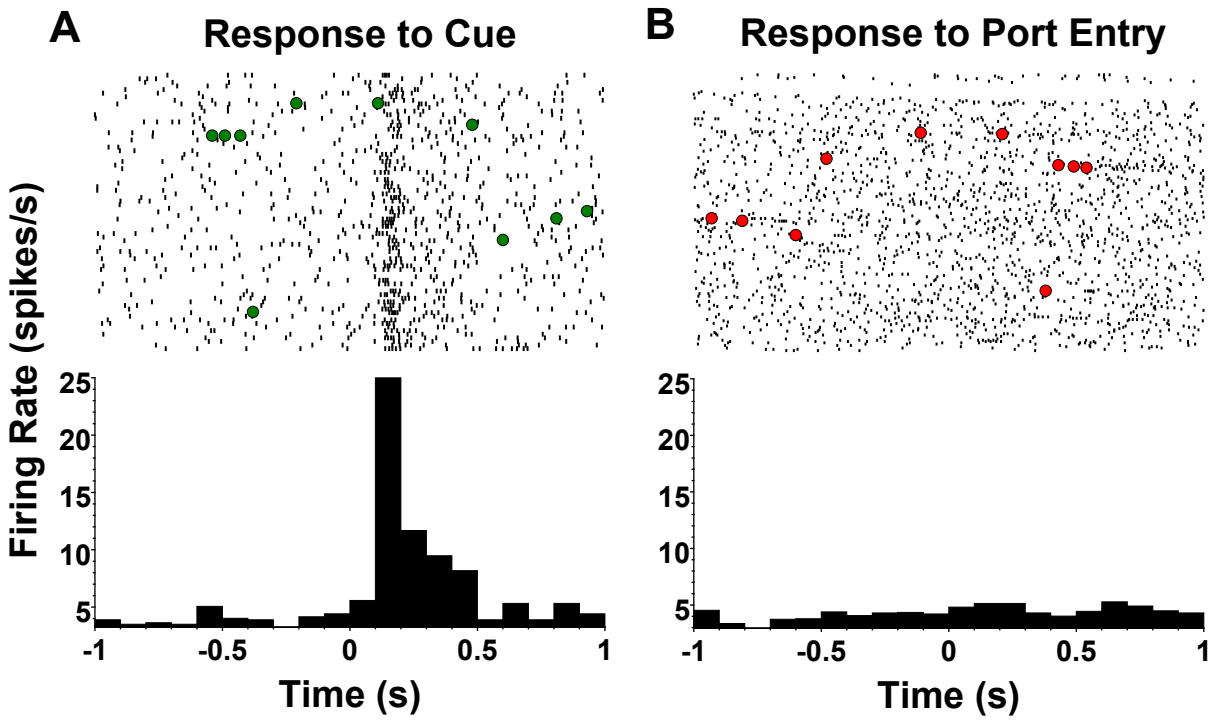


Figure 2-7. Lengthening of excitatory cue response with learning is not attributable to an increase in port entry responding
(A) Representative peri-event raster (top) and histogram (bottom, 100 ms bins) of a phasic excitatory response to cue onset in a well-trained Paired animal (cue onset at time=0 s). Green filled circles indicate times at which the animal entered the reward port. (B) Peri-event raster (top) and histogram (bottom, 100 ms bins) of the neural activity relative to port entry of the same neuron shown in (A) (port entry at time=0 s). Red filled circles indicate onset of cue presentation.

from 157±15.9 to 111.3±10.3 ms; $p<0.05$, Mann Whitney Rank Sum test; Figures 2-6D and F). The increased response length in the Paired group was not attributable to increased port entry responding, as port entries rarely occurred within 500 ms after cue onset and showed no clear temporal relationship with the sustained neural excitation (Figure 2-7). The increase in the magnitude and length of the excitatory response to a salient cue paired with reward, but not in the unpaired condition, suggests that these aspects of the neural response encode learning about the reward-predictive properties of the cue, while the latency decrease in both groups may reflect enhanced neural responding to stimuli in a reward-paired context, as it was not contingent upon pairing the discrete cue with reward.

Learning-related changes in cue excitations reverse with extinction

Do VTA responses to reward-predictive cues degrade as the cue-reward association is extinguished? We investigated this question by recording neural activity in well-trained Paired animals during 2-3 daily extinction sessions in which the cue was presented in the absence of reward. Extinction of the CS-US association decreased conditioned behavioral responding during the cue to 3.0±1.5%, compared to 42.6±2.3% prior to extinction ($p<0.001$, t-test). Just as the percent of CS-excited cells did not change with learning, Paired rats had similar proportions of CS-excited cells after extinction (11/20 cells, 55.0%) as when the CS-US association was well-learned (21/39 cells, 53.8%; $p=0.848$, Chi-square). However, when Paired responses were compared after learning and after extinction using 10 ms bins (Figure 2-8A), we found a decrease in the magnitude of the cue response: [main effect of stage of training ($F(1, 21)=12.0$, $p<0.005$); no interaction of

stage of training with neural response time course ($F(39, 819)=1.19$, $P=0.2$); 2-way repeated measures ANOVA]. The mean onset latency of excitatory cue responses increased with extinction relative to the Learned session (Figure 2-8B, $p<0.01$, Mann Whitney Rank Sum Test). As sucrose was absent in extinction, these data are consistent with the idea that the decrease in onset latency over days in both the Paired and Unpaired groups (above) is due to associative conditioning of the context with sucrose, rather than a change in the population of neurons sampled. Mean cue response length was shorter after extinction (62.2±8.5 ms, $p<0.01$, t-test) than after learning (Figure 2-8B), and there was also a trend that response length after extinction was shorter than on Day 1 ($p<0.1$, t-test).

Between-session analyses allow only population comparisons because of the uncertainty inherent in confirming that the same single unit is recorded over days. To investigate the cue responses of the same VTA neurons prior to and after extinction, 4 rats were run in within-session extinction, in which the first 30 CSs were paired with sucrose, while the remaining ~70 trials consisted of unrewarded cues. We examined behavior and neural activity in the first 30 trials (Pre-extinction) and the last 30 trials of the session (Post-extinction). The CS-US association was successfully extinguished, as demonstrated by a decrease in conditioned port entries over the session (Figure 2-8C, $p<0.005$, paired t-test). Ten of 22 total units (45.5%) displayed a phasic cue excitation in the Pre-extinction period, and while most (8/10, 80%) continued to show a significant cue response in the Post-extinction period, cue response magnitude decreased in parallel with conditioned behavior (Figures 2-8C and D, $p<0.01$, paired t-test). Taken together, the decreases in magnitude and length of excitatory cue responses with extinction of the CS-

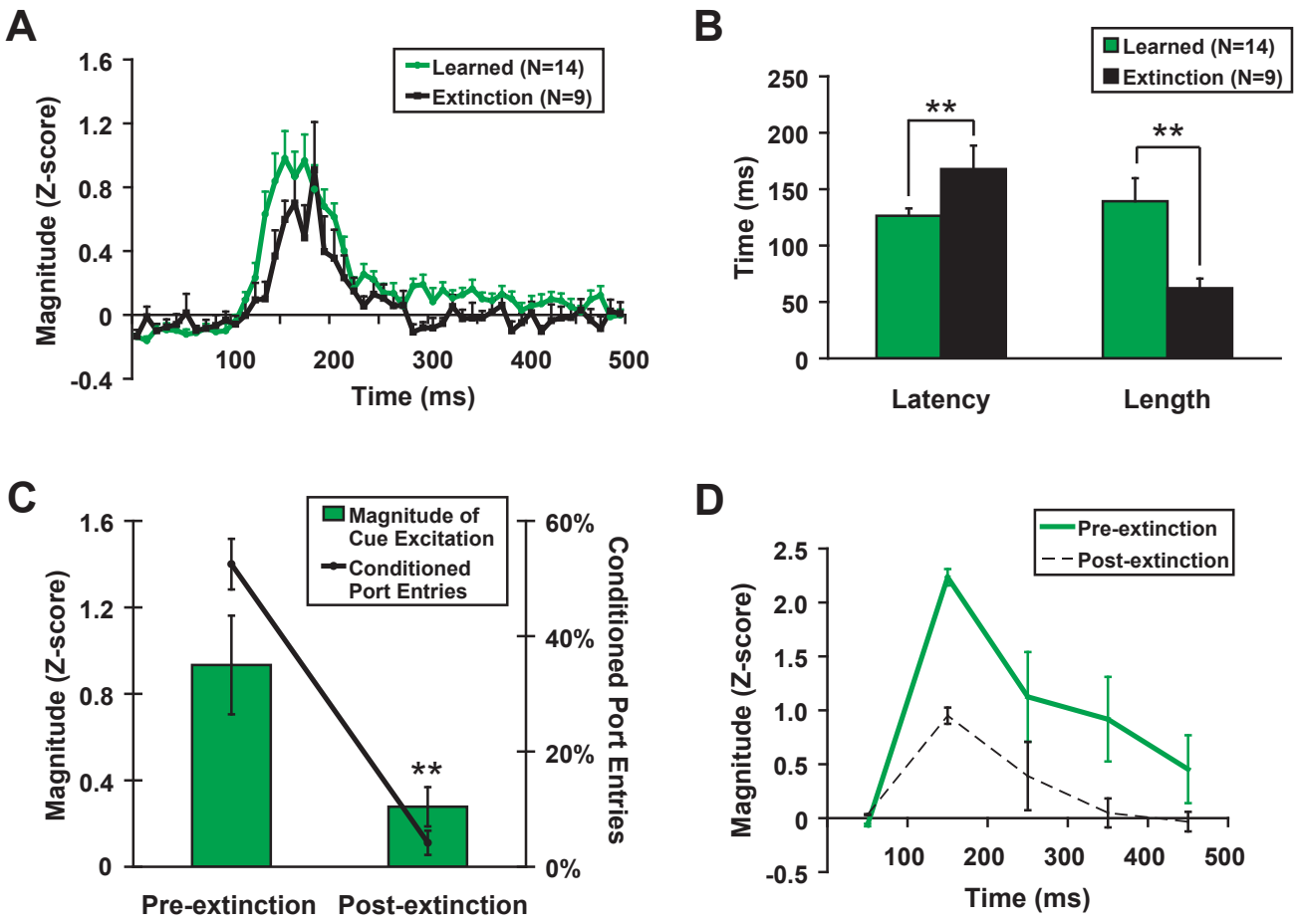


Figure 2-8. Learning-Related Changes in Cue Excitations Reverse with Extinction

(A) Population histograms of the magnitude of the cue response (normalized to baseline firing rate, mean±SEM, 10 ms bins, cue onset at time=0 ms). Excitatory cue responses degraded with between-session extinction of the CS-US association. (B) Mean (± SEM) onset latency of excitatory cue responses increased (**p<0.01) and mean (± SEM) length of excitatory cue responses decreased (**p<0.01) with between-session extinction of the CS-US association. (C) The magnitude of phasic cue excitation (normalized to baseline firing rate, mean±SEM) in cue-excited cells (N=10) decreased with within-session extinction (**p<0.01) in parallel with a decrease in conditioned port entries (mean±SEM) (**p<0.005). (D) Population histograms (mean±SEM, 100 ms bins, cue onset at time=0 ms) of excitatory cue response (normalized to baseline firing rate) in the first 30 (Pre-extinction) and the last 30 (Post-extinction) trials of within-session extinction.

US association support the hypothesis that these features of the neural response encode the reward-predictive properties of the cue.

Subpopulations of cue-excited VTA neurons show Congruent and Incongruent responses to primary reward

VTA neurons excited by reward-predictive cues are reported to show excitatory responses to unpredicted reward, which degrade as the neurons develop responses to the cue (Schultz, 1998). We found that ~half of cue-excited units were excited during sucrose consumption (termed "Congruent" cells), but that these sucrose responses were prevalent both before and after learning (Day 1: 11/20 cells, 55%; Learned: 10/21 cells, 47.6%). However, over a third of cue-excited units (Day 1: 7/20 cells, 35%; Learned: 8/21 cells, 38.1%) were inhibited during sucrose consumption. This second class of neurons was termed "Incongruent", as the neural cue response (excitation) was in the opposite direction of the sucrose response (inhibition). Figure 2-9 shows representative responses from individual neurons (A and B) and population histograms (C and D) of these subpopulations after learning (Congruent, left; Incongruent, right). Cue-excited cells with both Congruent (Day 1: 11/21 cells, 52.4%; Day 9: 6/9 cells, 66.7%) and Incongruent (Day 1: 5/21 cells, 23.8%; Day 9: 2/9 cells, 22.2%) reward responses were also seen in Unpaired rats. Baseline firing rates and waveform lengths (Figures 2-9E and F) of these two cell types were not significantly different in either Paired or Unpaired rats (Figure 2-9G; Table 2-2), suggesting that they cannot be discriminated by these criteria.

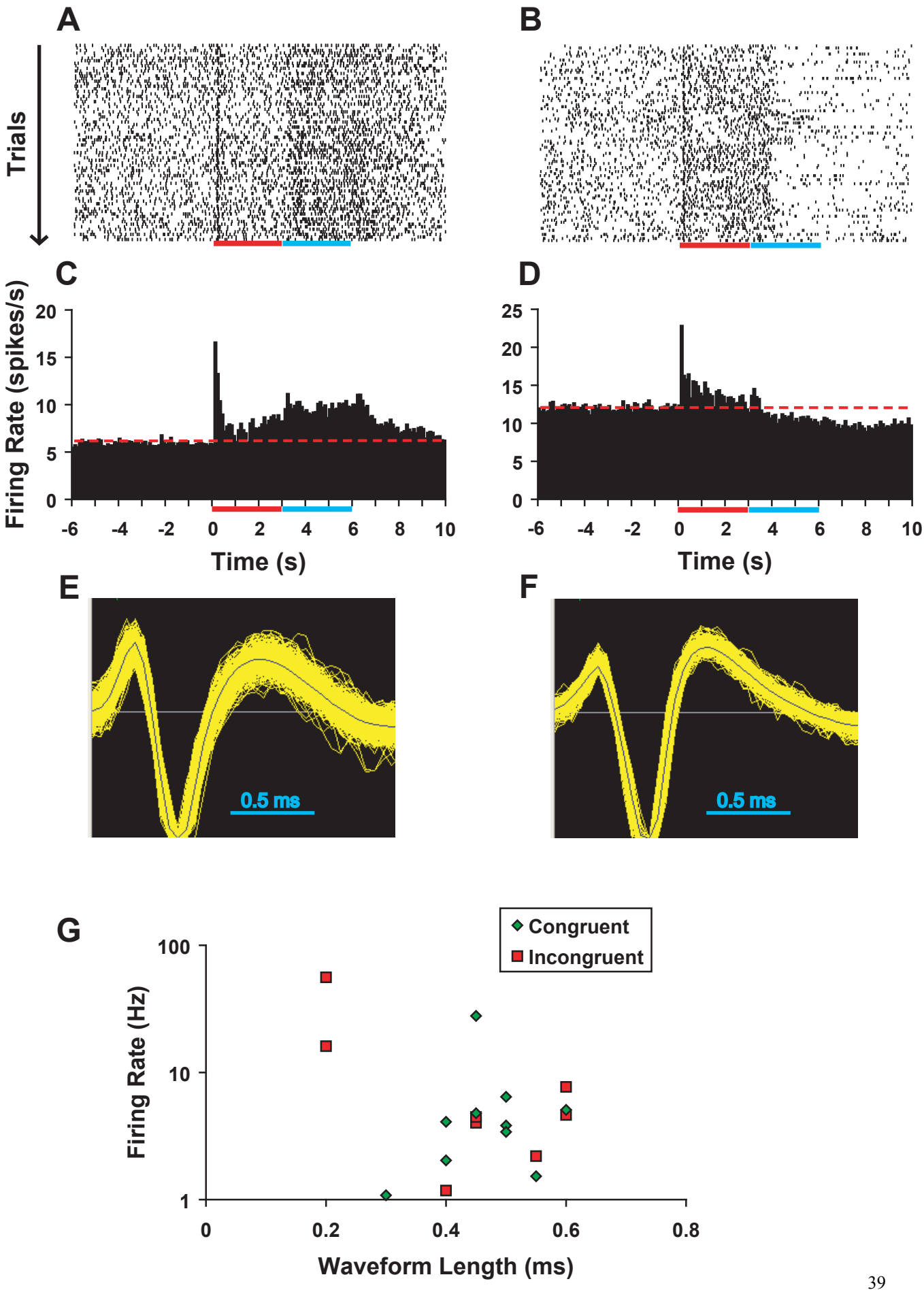

A
B
Trials
C
D
Firing Rate (spikes/s)
20
15
10
5
0
25
-6
-4
-2
0
2
4
6
8
10
Time (s)
E
F
0.5 ms
G
Congruent
Incongruent
100
10
1
Firing Rate (Hz)
0
0.2
0.4
0.6
0.8
Waveform Length (ms)

Figure 2-9. Cue-Excited VTA Neurons Show Congruent and Incongruent Responses to Primary Reward

Representative responses from individual neurons (A and B) and population histograms (C and D) of subpopulations of cue-excited neurons after learning (Left, Congruent, N=10 cells; Right, Incongruent, N=8 cells). Cue onset is at time=0 s. Solid red line indicates the duration of cue presentation, blue line indicates the duration of sucrose delivery. 100 ms bins were used for population histograms, red dotted line indicates mean baseline firing rate of the population. (E) and (F) show waveforms of neurons whose responses are shown in (A) and (B). (G) The electrophysiological measures of action potential waveform length and baseline firing rate (see methods) are indistinguishable in these subpopulations.

Table 2-2. Electrophysiological Properties of Congruent and Incongruent Cue-Excited VTA Neurons

Congruent Cells						
	Paired (N=8 rats)				Unpaired (N=8 rats)	
	Day 1	Learned	Omission	Within-session Extinction	Day 1	Day 9
Number of cells	11	10	10	5	11	6
Firing Rate (spikes/s)						
Mean	5.88±1.83	5.98± 2.48	4.05±0.60	3.41±0.87	3.34±0.28	3.95±0.55
Median	4.31	3.96	3.67	2.93	3.80	3.64
Range	1.78 - 23.64	1.08 - 27.82	1.55 - 6.76	1.67 - 6.53	1.91 - 4.59	2.73 - 6.55
Waveform Length (ms)						
Mean	0.50±0.02	0.47± 0.03	0.50±0.02	0.43±0.03	0.56±0.04	0.48±0.02
Median	0.50	0.48	0.50	0.40	0.50	0.50
Range	0.35 - 0.65	0.30 - 0.60	0.40 – 0.60	0.35 - 0.50	0.40 - 0.90	0.40 - 0.55

Incongruent Cells						
	Paired (N=8 rats)				Unpaired (N=8 rats)	
	Day 1	Learned	Omission	Within-session Extinction	Day 1	Day 9
Number of cells	7	8	6	2	5	2
Firing Rate (spikes/s)						
Mean	8.99±4.61	12.07± 6.49	10.28±4.48	3.57±0.03	2.98±0.57	2.44±1.16
Median	4.20	4.55	5.50	3.57	2.71	2.44
Range	2.56 - 36.34	1.18 - 54.84	1.54 – 29.12	3.53 - 3.60	1.46 - 4.39	1.28 - 3.60
Waveform Length (ms)						
Mean	0.41±0.05	0.43± 0.06	0.41±0.06	0.60	0.37±0.09	0.43±0.08
Median	0.40	0.45	0.43	0.60	0.45	0.43
Range	0.20 - 0.65	0.20 - 0.60	0.20 – 0.60	0.60	0.10 - 0.60	0.35 - 0.50

The strength of Congruent cue responses encodes associative conditioning of the cue with reward

Different neural reward responses imply functional differences in these two populations of cue-excited VTA cells. Figures 2-10A and B display the mean response of each population to cue presentation and sucrose consumption on Day 1 and after learning. Normalized cue response magnitude increased with learning in Congruent ($p<0.005$, t-test), but not Incongruent, cells ($p=0.77$, t-test; Figure 2-10C). Additionally, mean cue response length increased reliably in Congruent, but not Incongruent, cells (Figure 2-10D; Congruent: 84 to 152 ms, $p<0.05$, t-test; Incongruent: 104 to 147.5 ms, $p=0.324$, t-test). There was a small, non-significant decrease in onset latency from Day 1 to Learned in both populations (Congruent: 144 to 132.5 ms, $p=0.311$, t-test; Incongruent: 138 to 122.5 ms, $p=0.188$, t-test). Congruent VTA neurons therefore preferentially encode the reward-predictive properties of a cue in the strength (magnitude and length) of their cue response.

Congruent neurons display a negative prediction error to omitted reward

VTA neurons (n=24) were recorded in 4 well-trained Paired animals during a session in which sucrose delivery was omitted after CS presentation in 1/3 of trials. Most (8/10) Congruent units showed an inhibition in firing at the time of expected sucrose delivery on omission trials (Figures 2-11A and B), and the magnitude of the excitatory response of this population to sucrose delivery was significantly different from the magnitude of its inhibition to sucrose omission (Figure 2-12, $p<0.001$, paired t-test). In the 6 Incongruent units examined, no prediction error response was observed on omission trials, and there

Figure 2-10. Congruent CS Responses Strengthen with Learning

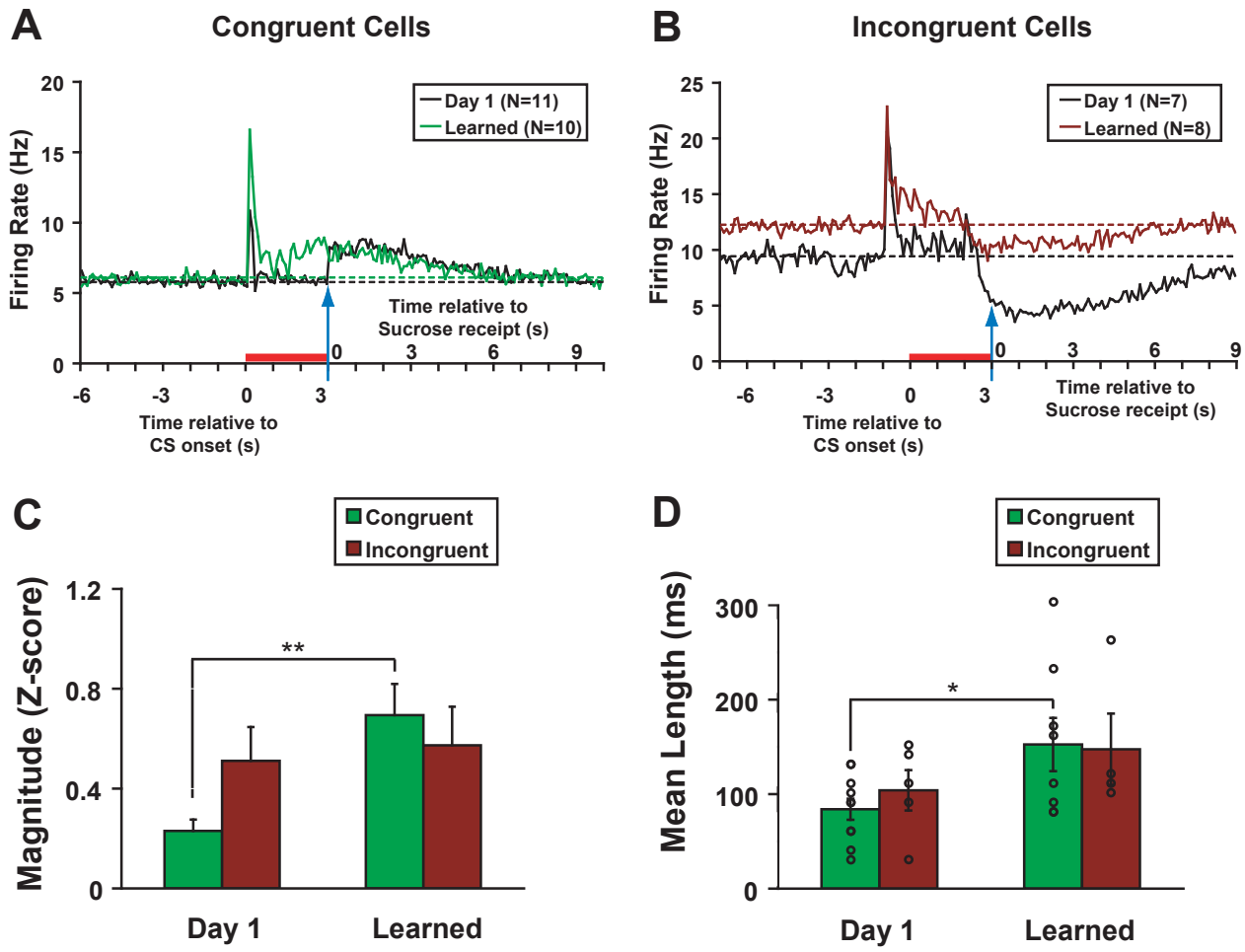


Figure 2-10. Congruent CS Responses Strengthen with Learning

Population histograms of firing rate (100 ms bins) of Congruent (A) and Incongruent (B) subpopulations on Day 1 and after learning. Solid red line indicates duration of cue presentation, blue arrow indicates beginning of sucrose consumption. Dotted lines indicate the mean baseline firing rate of the neural population of the same color. (C) The magnitude of phasic cue excitation (normalized to baseline firing rate, mean±SEM) increased with learning in Congruent (**$p<0.005$, t-test; Day 1: N=11 cells; Learned: N=10 cells), but not Incongruent ($p=0.77$, t-test; Day 1: N= 7 cells; Learned: N=8 cells) cells. (D) The mean (±SEM) cue response length increased with learning in Congruent (*$p<0.05$, t-test; Day 1: N=10 cells; Learned: N=8 cells), but not Incongruent cells ($p=0.324$, t-test; Day 1: N=5 cells; Learned: N=4 cells). Small circles represent the length of cue responses in individual cells, included here to better describe the distribution of responses within these small sample sizes. The number of visible circles is smaller than the number of cells because there were some pairs of cells with identical response lengths in the Congruent group on Day 1 (N=3 pairs) and Learned (N=1 pair).

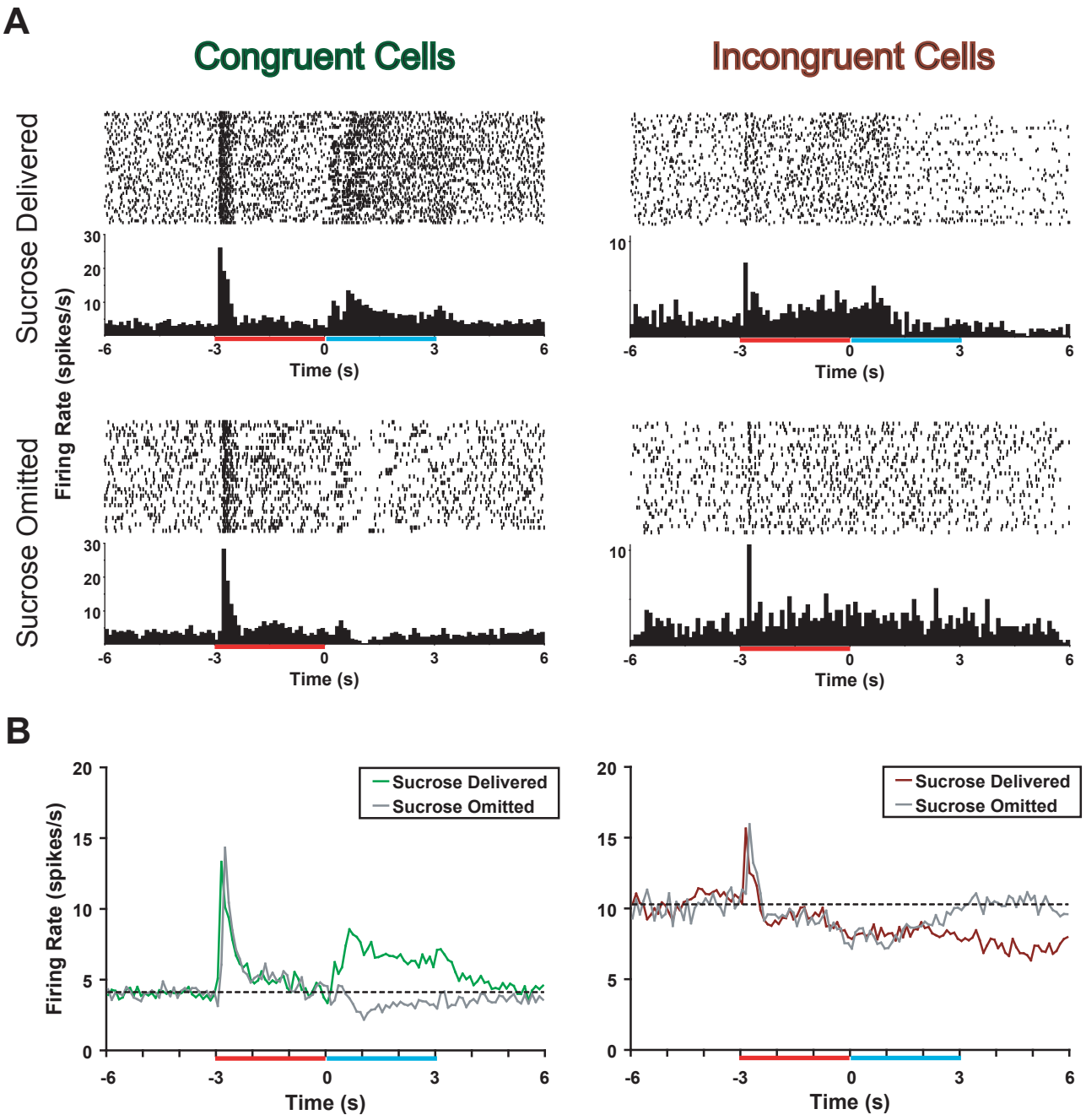


Figure 2-11. Congruent Cells Exhibit a Negative Prediction Error Signal to Reward Omission

(A) Perievent rasters and cumulative histograms showing the activity of representative Congruent (left) and Incongruent (right) neurons on reward delivery trials (top) and reward omission trials (bottom) in a sucrose omission session. (B) Population histograms (firing rate, mean±SEM, 100 ms bins) of cue-excited neurons in the sucrose omission session (Left, Congruent, N=10 cells; Right, Incongruent, N=6 cells). Black dotted lines indicate mean baseline firing rates. Solid red line indicates duration of cue presentation, blue line indicates duration of sucrose delivery. For all rasters and histograms, the time of expected sucrose delivery is at 0 s.

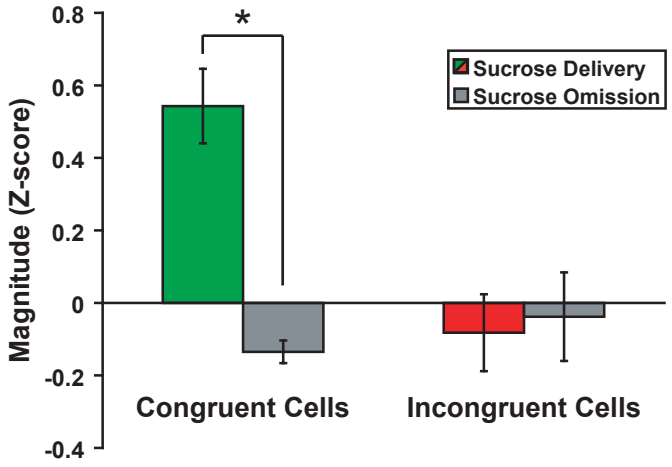


Figure 2-12. Negative reward prediction error in Congruent cells

The magnitude of the neural response at the time of expected sucrose delivery (normalized to baseline firing rate, mean ± SEM) in Congruent neurons (N=10 cells) on trials in which sucrose was delivered was significantly different from trials in which reward delivery was omitted (**p<0.005, paired t-test), while there was no significant difference in response magnitude on sucrose delivery and omission trials in the Incongruent population (N=6 cells, p=0.284, paired t-test).

was no significant difference in the magnitude of the response of the Incongruent population when sucrose was delivered or omitted (Figure 2-12, $p=0.284$, paired t-test). That a prediction error signal to omitted reward exists only in the Congruent subpopulation of cue-excited neurons further suggests that it, and not the Incongruent subpopulation, encodes reward prediction.

Congruent cue excitations are stronger on trials with conditioned approach

Even when well-trained, Paired rats did not make an anticipatory port entry during every presentation of the cue, likely due to the large trial number per session and the moderate level of water-restriction. In the Learned session, Paired animals received a mean of 78.4±4.4 (mean±SEM) cue presentations, and made conditioned port entries (during the 3 s cue) on 42.3±3.7 trials (54.1±3.9% of trials); these trials were defined as Conditioned Response (CR) trials. Port entries were often made in the several seconds after cue offset; these trials were excluded from the analysis, as they could not be reliably interpreted as responses to the cue rather than to the sound or smell of sucrose delivery. We defined No Response (NR) trials as those with no port entry within 10 seconds after cue onset, by which time the post-CS burst of port entry responding had ended in all rats. Across rats, there was a mean of 14.6±2.3 NR trials (18.3±2.3% of total trials) in the Learned session. To investigate a possible relationship between neural response strength and conditioned port entries, we compared cue response magnitude in all units excited by the cue after learning (n=21 cells) on NR trials to a randomly-selected matched number of CR trials. As shown in Figure 2-13A, the mean magnitude of cue excitation was larger on trials in which animals made a CR ($p<0.005$, paired t-test). The weaker cue excitation on NR

trials cannot be attributed to sucrose satiety, as these trials were well distributed over the entire session (Figure 2-13B).

Just as the increase in cue excitation magnitude with learning was seen only in the Congruent subpopulation, the cue response on CR trials was also stronger primarily in these cells (Figures 2-13C and D). In 4 rats for which video recordings allowed us to observe detailed behavior, we found that, on NR trials, rats usually were engaged in other behavior (grooming, exploring the box) when the cue was presented, which they did not interrupt to retrieve the sucrose. In these animals, cue excitation magnitude was significantly larger on CR than NR trials in Congruent ($p<0.005$, paired t-test), but not Incongruent, cells (Figure 2-13E). This suggests that excitatory responses to a reward-predictive cue in VTA cells, particularly those cells excited by reward, are modulated by how motivated the animal is to respond behaviorally to the cue.

Figure 2-13. Cue Excitations are Stronger on Trials in which Animals Show a Conditioned Behavioral Response

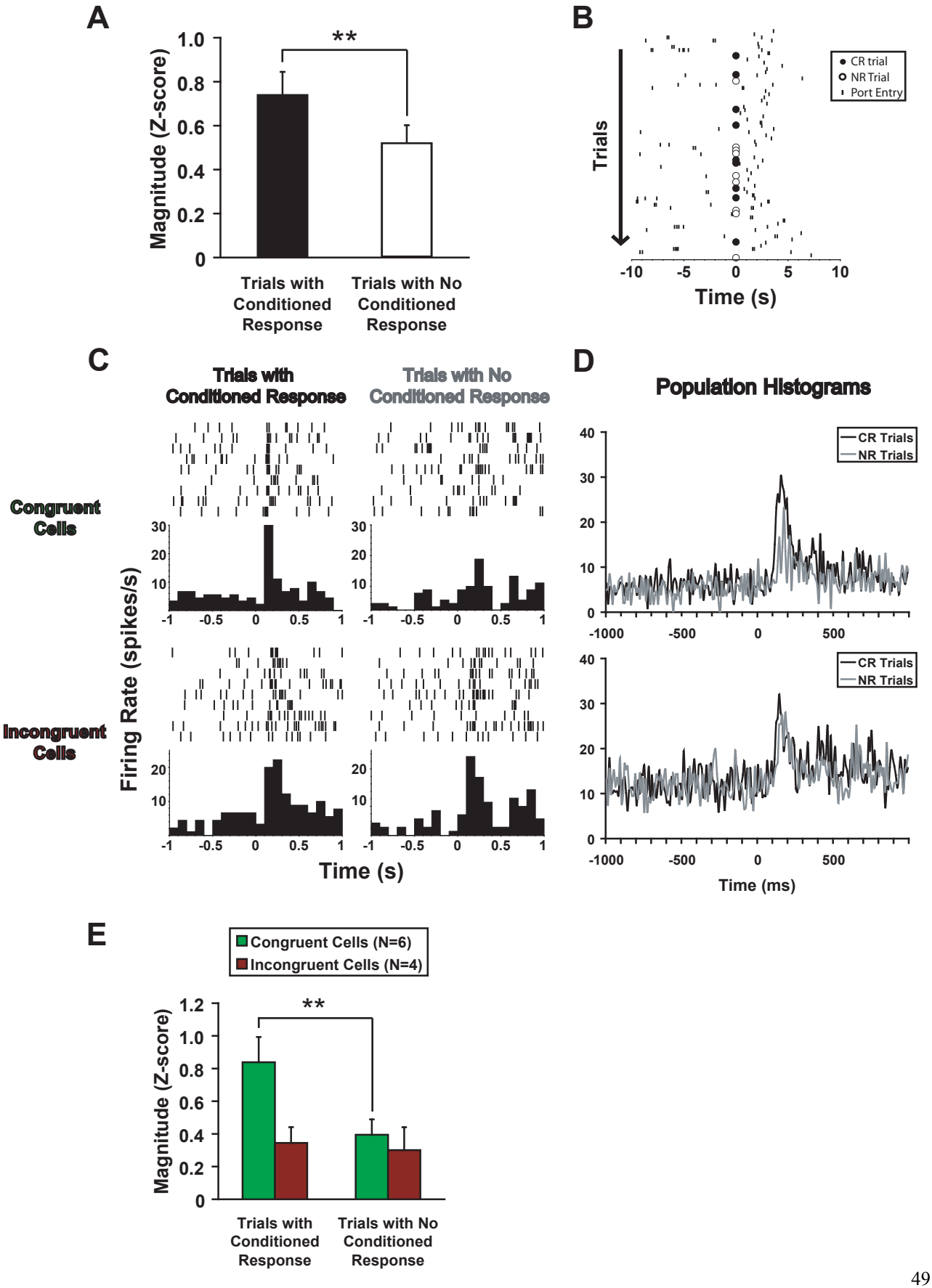


Figure 2-13. Cue Excitations are Stronger on Trials in which Animals Show a Conditioned Behavioral Response

(A) The magnitude of the neural response (normalized to baseline firing rate, mean±SEM) in CS-excited neurons (N=21 cells) in well-trained Paired animals was larger on CR trials in which animals showed a port entry response during CS presentation than on NR trials (**p<0.005). (B) Example from one animal showing behavioral responding to the CS (cue onset at time=0 s) throughout the Learned session. Note that CR and NR trials are well-distributed through the session. (C) Neural response rasters and cumulative histograms (100 ms bins) from a Congruent neuron (top) and a Incongruent neuron (bottom) from the same animal on the same CR (left) and NR (right) trials (cue onset at time=0 s). (D) Population histograms of the cue responses of all Congruent (top, N=10) and Incongruent (bottom, N=8) neurons on CR (black) and NR (grey) trials (10 ms bins, cue onset at time=0 ms). (E) In a subset of animals (N=4, see text), neural cue response magnitude (normalized to baseline firing rate, mean±SEM) was larger on CR trials than NR trials in Congruent (N=6, **p<0.005) but not Incongruent (N=4) neurons.

Discussion

We recorded VTA neural responses to visual cues during two procedures: one in which rats learned that the cue predicted the availability of sucrose reward, and one in which the cue had no reward-predictive value. Using an unbiased approach that included all neurons isolated in our analysis, we classified neurons functionally, by their responses to the cue and reward. We confirmed that there is a large population (~50%) of VTA neurons that show phasic excitatory responses to reward-predictive cues, as reported previously in primates (Schultz, 1998) and rats (Pan et al., 2005; Roesch et al., 2007), as well as a much smaller population (<20%) inhibited by such cues (Pan et al., 2005). Unlike previous studies, we found that such responses exist in similar proportions prior to and after conditioning, as well as after extinction. The cue-excited neural population is functionally heterogeneous; we identified a substantial subpopulation of Incongruent cells excited by the reward-predictive cue, but inhibited by the reward itself, in addition to the subpopulation of cells excited by both rewards and reward-predictive cues, termed Congruent cells. The strength, rather than the presence, of cue responses in Congruent neurons appears to encode the degree of associative conditioning to the cue, as well as the animal's motivation to respond behaviorally to the cue. The Incongruent subpopulation did not show any enhancement of cue response strength with learning, and may encode attentional or sensory properties of the cue.

That the percent of VTA units with cue excitations did not change with learning or extinction is surprising in light of previous findings that such responses in VTA neurons appeared as the animal learned that the cue predicted reward (Schultz, 1998; Pan et al., 2005). How can we explain this discrepancy? Perhaps neural responses on the first

day of training reflected learning about the reward-predictive properties of the cue many days before learning criteria were met. This is highly unlikely, as conditioned behavior during the cue was completely absent in the first session (Figure 2-1B). Even if this were the case, neural excitations and inhibitions to the cue did not develop over the first session but were seen from the session beginning (Figure 2-4). More importantly, Unpaired rats, for which the cue did not predict reward, show the same proportion of cue-excited neurons on Day 1 as Paired rats, as well as identical response magnitude. These findings strongly suggest that excitatory cue responses are not encoding reward prediction in this first session. Alternatively, the cue responses we observed in the first session could be novelty responses, which have been reported in VTA neurons. These responses are thought to degrade rapidly, within a few trials (Schultz, 1998); however, cat VTA neurons do not habituate to sensory stimuli for up to 2000 trials (Steinfels et al., 1983; Horvitz et al., 1997). The stimulus used in our study is very salient (multiple light flashes) and is presented in a temporally unpredictable manner (with a 1-minute variable inter-trial interval), so it is possible that novelty responses to such a stimulus could be elicited longer than for less salient stimuli used in other studies, and could be maintained until a conditioned neural response is also seen. In our procedure, pairing of the cue with reward is required for the maintenance of the neural response over many days, as is demonstrated by the lower percentage of cue-excited neurons in the Unpaired group on Day 9. Therefore, phasic excitatory responses to visual cues in VTA neurons may initially encode salient sensory properties of the cue, but are strengthened over time to encode the reward-predictive properties of the cue, or degraded over time to reflect the motivational irrelevance of the cue if it has no reward-predictive value. In sum, the cue-

reward association is not encoded in the mere presence of such responses, although it is required for their maintenance. This finding highlights an interaction between unconditioned and conditioned factors in jointly regulating cue responsiveness of individual units in the VTA.

Although an increase in the number of cue-excited units did not occur during acquisition of the cue-reward association, the magnitude and length of the cue response increased with learning and decreased with extinction in the Paired group, but did not change in Unpaired controls. Hence, the strength (magnitude and duration) of excitatory neural responses may encode the cue-reward association. Interestingly, we did not find learning-related changes in all types of cue-excited cells. The strength of cue responses in Congruent, but not Incongruent, neurons increased with learning. The different firing properties of Congruent and Incongruent cue-excited cells could result from distinct patterns of afferent input, varying neurochemical identities, or both. It may be tempting to call Congruent cells DAergic as the cue and reward responses of this population correspond closely with neurons presumed to be DAergic in other *in vivo* recording studies (Schultz, 1998, 2002; Pan et al., 2005; Pan and Hyland, 2005; Roesch et al., 2007). In addition, a majority of Congruent cells were inhibited during reward omission, a response characteristic often seen in presumed DA neurons (Mirenowicz and Schultz, 1994; Pan et al., 2005; Roesch et al., 2007). However, a major caveat of all studies in which VTA neural activity has been recorded in awake animals is that the neurochemical identity of the neurons was not demonstrated, and DA content was instead usually inferred from electrophysiological measures such as long triphasic action potential durations (>1.5 or 2.0 ms) and a low firing rate (<10 Hz), or D2 DA receptor agonist

inhibition. None of the Congruent cells we recorded fulfill the long action potential duration criterion used in most of the above studies, although their waveforms may be more similar to the putative DA units recorded by Roesch et al. (2007). We also observed Congruent cue responses in some cells with high baseline firing rates (>10 Hz). There are therefore two possibilities: one is that the classical identification criteria are correct, and none of the neurons we recorded are DAergic, and the other is that at least some of our Congruent neurons are DAergic, and the classical criteria are unreliable in this preparation. Importantly, a recent report has demonstrated that DA neurons which project to the PFC, the NAc core and medial shell, and the basolateral amygdala (BLA) have higher firing rates, and potentially shorter, biphasic action potential waveforms *in vivo*, than classical DAergic neurons (Lammel et al., 2008). These atypical DA neurons are also not inhibited by D2 receptor agonists. Voltammetric studies in rats trained on operant (Roitman et al., 2004) and Pavlovian (Day et al., 2007) tasks find that cue-induced DA release in the NAc is greater when rats have learned that the cue predicts reward. The learning-related strengthening of the cue response in the Congruent population suggests that it is a functional group with properties that could explain the changes in DA release demonstrated in these studies. However, the interesting possibility that non-DAergic VTA neurons contribute to the learning-related changes that we observe in the Congruent population cannot be ruled out, and deserves further investigation. Importantly, Congruent and Incongruent populations had completely overlapping and statistically indistinguishable action potential durations and baseline firing rates, suggesting that the delineation of classes of VTA neurons based on these electrophysiological measures, with the implication of functional segregation, is

unjustified. We propose, in agreement with Fields et al. (2007), that a more judicious approach to recording VTA neurons *in vivo* is to analyze the responses of all neurons isolated, and to classify populations functionally without attempting to infer their neurochemical content.

In addition to reflecting learning, the strength of excitatory cue responses was also correlated with motivation, as it was greater on trials in which well-trained rats approached the reward port during cue presentation. Specifically, Congruent cells fired more to the cue on conditioned response trials. In primates, the response magnitude of presumed DA neurons to an operant cue in an instrumental conditioning task is correlated with behavioral reaction time to the cue (Satoh et al., 2003). However, it has been argued that cue responses in presumed DA neurons encode the probability of reward (Fiorillo et al., 2003). In our task, the probability of reward was always 100% (except during extinction) and was equally predicted by the cue on every trial, but the magnitude of the Congruent cue response varied in a manner correlated with conditioned approach. Recent studies have found that presumed DA neurons encode the subjective value of rewards and action choices, rather than their absolute value (Bayer and Glimcher, 2005; Tobler et al., 2005; Morris et al., 2006; Roesch et al., 2007). It is reasonable to assume that the subjective value of the sucrose reward in our task was greater on trials in which an animal entered the port during or immediately after cue presentation, so the neural response strength may encode the value of the cue (as a predictor of sucrose) relative to the value of other behaviors (exploratory behaviors or grooming) available to the animal in the task. Importantly, the excitatory cue response is not tightly correlated in time with the production of the conditioned port entry on a trial-by-trial basis. Therefore, the

information encoded by Congruent cells is unlikely to reflect a specific motor response, but rather is consistent with a role in incentive motivation, as has been proposed for VTA DA neurons (Berridge and Robinson, 1998; Ikemoto and Panksepp, 1999).

It has been argued that the onset of neural excitations to reward-predictive cues in VTA DA neurons occurs at a latency too short to reflect reward per se (Redgrave and Gurney, 1999). In our study, it is interesting that the enhancement of cue excitation in Congruent neurons over learning is also reflected in an increase in the length of the excitation. Additionally, it is striking that a decrease in onset latency of neural cue excitations is seen in both Paired and Unpaired groups, further suggesting that the initial component of the excitation is unrelated to the reward-predictive properties of the cue. An attractive possibility is that the short-latency increase in cue-evoked firing encodes the sensory properties of the salient cue, perhaps enhanced in a reward-paired context, while the sustained excitation to the cue in Congruent neurons encodes its discrete reward-predictive properties. Perhaps multiple inputs arriving at slightly different times sum to produce the learned cue response: there are afferent projections to the VTA from brain regions involved in sensory perception and motivation that could provide such information. For example, the pedunculopontine nucleus (PPT) projects to the VTA (reviewed in Winn, 2006) and is excited by auditory and visual stimuli (Pan and Hyland, 2005). When this area is inactivated, excitatory responses to reward-predictive tone and light cues in VTA neurons are decreased but not abolished (Pan and Hyland, 2005), suggesting that afferents from other brain regions may also contribute to these responses. It has also been demonstrated that the superior colliculus (SC) provides input required for short-latency responses to visual stimuli in the VTA (Coizet et al., 2003; Comoli et al.,

2003; Dommett et al., 2005). An interesting hypothesis for future study is that sensory inputs such as the PPT and the SC induce short-latency cue excitations in Congruent neurons, while the enhancement of the cue excitation with learning, and on trials in which the animal chooses to respond, is mediated by inputs from motivation-related regions such as the NAc, PFC, and the amygdala.

Inhibitions to drugs of abuse, brain stimulation, food reward, and sexual stimulation are seen in VTA neurons in instrumental conditioning tasks, sometimes accompanied by excitations prior to responding for such rewards (Nishino et al., 1987; Kosobud et al., 1994; Kiyatkin and Rebec, 2001; Steffensen et al., 2001, 2006). These neurons are generally reported to be a homogenous short-waveform, high firing rate population and presumed GABAergic (Steffensen et al., 2001, 2006; but see Nishino et al., 1987; Kosobud et al., 1994; Kiyatkin and Rebec, 2001). In our study, the Incongruent neural population had waveform lengths and firing rates statistically indistinguishable from the Congruent population, with only a small number fulfilling previously described electrophysiological criteria for VTA GABA neurons (Steffensen et al., 1998). Incongruent cue responses were not modulated by learning or the choice to make a behavioral response to the cue. It is possible that phasic cue activity of these neurons reflects attention or sensory salience, perhaps also transmitted via PPT or SC afferents. The neurochemical identity of the Incongruent population, as well as its causal role in reward-directed behavior, are open questions and present an exciting opportunity for future research.

In summary, we have demonstrated the existence of two populations of cue-excited VTA neurons in a Pavlovian conditioning procedure: a Congruent population

excited by reward, as well as an Incongruent population inhibited by reward. The Congruent population, while displaying cue response characteristics often ascribed to presumed DA neurons, such as learning-related enhancement, extinction-related decrement, correlation with motivation, and a negative reward prediction error signal, did not meet classical criteria used to identify these neurons *in vivo*. The Incongruent population, which to our knowledge has not been characterized in this behavioral paradigm, does not display these cue response characteristics, although the response is maintained through the various stages of training examined. There are therefore two populations of neurons excited by reward-predictive cues in the VTA; both encode sensory or attentional properties of the cue, while only Congruent cells encode reward-prediction and motivation.

Chapter 3

Neuronal Responses to Aversive Pavlovian Cues in the Ventral Tegmental Area

Introduction

While the VTA (and its DA projections) are often thought to be selectively involved in reward learning, there is substantial evidence that they are important for the processing of aversive events as well. Direct stimulation of the VTA increases the amount of fear expressed (measured by the amplitude of a startle response) to a naturally aversive auditory stimulus (Borowski and Kokkinidis, 1996). However, the VTA does not seem to be critical for the basic expression of natural (unconditioned) fear responses; rather it may be involved in the development or expression of conditioned fear. Lesions of the VTA or inhibition of its activity, while sparing the unconditioned startle response, block the expression of conditioned potentiation of the startle response (Borowski and Kokkinidis, 1996; Munro and Kokkinidis, 1997; Greba et al., 2000b; Gifkins et al., 2002). DAergic signaling may be involved in this form of conditioned fear, as systemic administration of DA agonist drugs enhances its expression (Borowski and Kokkinidis, 1998), while a systemic antagonist blocks acquisition (Greba et al., 2000a). Amygdalar activity is required for fear-potentiated startle (van Nobelen and Kokkinidis, 2006), and DA release in the amygdala appears to be critical for fear conditioning in this paradigm; in the amygdala, but not in the NAc, dopamine D1 receptor activation is required for expression and D2 receptor activation for acquisition of fear-potentiation of the startle response (Lamont and Kokkinidis, 1998; Greba et al., 2000a; Greba et al., 2001).

Freezing is a natural response of the rat when a fearful stimulus is detected; this decreases the chances of it being seen by a predator. In the laboratory setting, rats will develop freezing responses to cues paired with the delivery of aversive stimuli such as footshock (Blanchard and Blanchard, 1969; Fanselow and Bolles, 1979). The DAergic projection to the amygdala appears to be activated by primary aversive stimuli such as predator odor (Morrow et al., 2000a) and footshock (Coco et al., 1992). This pathway may also be important for conditioned fear to cues, as systemic and intra-VTA quinpirole (presumed to inhibit the activity of DA neurons) or infusions of dopamine antagonists into the BLA interfere with the retrieval of conditioned fear (Guaracci et al., 1999; 2000; Nader and LeDoux 1999a,b). Amygdalar DA release may be involved in contextual as well as cue-induced fear, as an environment paired with footshock increased levels of a DA metabolite in the amygdala, and this effect was attenuated by pretreatment with the anxiolytic drug diazepam (Coco et al., 1992). However, DA is also released in the NAc, dorsal striatum, and PFC to footshock and tail-shock (Claustre et al., 1986; Abercrombie et al., 1989), and to predator odor and swim stress in the PFC (Claustre et al., 1986; Morrow et al., 2000b). DA release is also seen in the NAc (Pezze et al., 2001) and the PFC (Yoshioka et al., 1996; but see Feenstra et al., 1999) to a context paired with footshock, and in the NAc (Pezze et al., 2001) and the PFC (Feenstra et al., 2001) to a discrete cue paired with shock. One group found that not only was DA released in the NAc to a tone paired with shock, but also to a neutral light preconditioned w/ the tone (Young et al., 1998). Another study found that initial stimulus-footshock pairing induced PFC DA release, while a shift in DA release to the NAc was seen with subsequent pairings (Wilkinson et al., 1998). Interestingly, infusion of a DA agonist *or* an antagonist

into the mPFC has been shown to inhibit the expression and retrieval, but not the acquisition, of conditioned fear to a shock-paired tone (Pezze et al., 2003), suggesting that there may be an optimal level of DA in the PFC required for this behavior, similar to the inverted U-shaped function seen for the relationship of PFC DA levels to working memory (Arnsten et al., 1994; Williams and Goldman-Rakic, 1995). DA may be important for generating behavior to avoid aversive events, as the amount released in the NAc correlates with the number of behavioral avoidance responses emitted during an operant shock avoidance task, and DA-depleted rats freeze rather than exhibit escape responses during shock presentation (McCullough et al., 1993)

While DA is therefore clearly released in fearful or stressful situations, it has been argued that DA is released to aversive stimuli with a prolonged time course as the consequence of an opponent process system, unlike phasic increases in its release to rewarding stimuli (Daw et al., 2002; Ungless, 2004). In this model, DA release is briefly inhibited by aversive stimuli, but then slowly increases and slowly decreases, resulting in a tonic increase outlasting the stimulus. While it is suggested that this increase in DA release could encode a "relief" response to stimulus offset, it is unclear how or why the rewarding signal of relief should be conveyed prior to the offset of the painful stimulus. For example, even aversive stimuli that were present throughout the dialysate collection period induced DA release (Jackson and Moghaddam, 2004; Young, 2004). A more appealing hypothesis, supported by the abundant literature on DA release during stress, is that tonic DA release may play a role in stress-induced analgesia. Mild footshock stress increases the release of the opioid enkephalin into the medial VTA (Kalivas and Abhold, 1987) and intra-VTA administration of the opioid antagonist naloxone decreases, and a

mu opioid agonist increases, PFC and NAc DA metabolism and release (Kalivas and Abhold, 1987; Latimer et al., 1987; Kalivas and Duffy, 1990). It has been shown that endogenous opioids and Substance P act in the VTA to mediate stress-induced analgesia by increasing the activity of mesocorticolimbic DA neurons (Altier and Stewart, 1996; 1999a, b).

Unfortunately, data from microdialysis studies cannot provide the temporal resolution necessary to prove or disprove that the increases seen in DA release to aversive stimuli are tonic rather than phasic. Some traces in earlier voltammetry studies suggest the rapid onset of DA release in the NAc and PFC to restraint and tailpinch, but data were summarized on the time scale of minutes, and there is some uncertainty about whether only DA release was measured (Doherty and Gratton, 1992; 1996; Schultz, 2007). In addition, one voltammetric study failed to find increased DA release to a noxious tailpinch (Williams and Millar, 1990). Corticotrophin-releasing factor (CRF), which is released in the VTA in response to acute stress and plays a role in stress-activation of drug-seeking behavior (Wang et al., 2005), increases the firing rate of I_h+ (presumed DAergic) VTA neurons in a slice preparation on a timescale consistent with a tonic, rather than a phasic, increase (Wanat et al., 2008). However, phasic excitations to aversive events have been seen in some VTA recording studies in awake behaving animals. In primates, a small proportion (11-15%) of presumed DA neurons show phasic excitations to mild aversive stimuli (Mirenowicz and Schultz, 1996), and restraint stress increases the burst firing of presumed DA neurons in awake rats (Anstrom and Woodward, 2005). In one electrophysiological study conducted in rabbits *in vivo* (Guaracci and Kapp, 1999), approximately half of presumed DA neurons showed either

an excitatory or an inhibitory response to an auditory stimulus previously paired with shock. These neurons showed differential responding to the CS+ and CS-, most with greater excitation to the CS+. Using juxtacellular labeling in the VTA of anaesthetized rats, Ungless and colleagues found that confirmed DA neurons were typically inhibited by footpinch, while other neurons considered DAergic by classical criteria, which were excited by footpinch, were in fact non-DAergic (Ungless et al., 2004). However, these recording studies only analyzed the responses of neurons identified as DAergic using classical electrophysiological criteria, which exclude some tyrosine hydroxylase-positive neurons and include some tyrosine hydroxylase-negative neurons (Margolis et al., 2006b; Fields et al., 2007; Lammel et al., 2008; Luo et al., 2008). Recently-discovered high firing rate DA neurons, which project to the PFC, the NAc core and medial shell, and the basolateral amygdala (BLA) (Lammel et al., 2008) were not examined. This is particularly important since these target areas, especially the amygdala, may be critical for the role of DA in aversive conditioning. Additionally, it is unclear how to interpret responses to a painful stimulus in an anesthetized animal; perhaps motivational or analgesia-related neural responses were not elicited in this preparation (and in the similar study of Coizet et al., 2006) because no pain was experienced. Perhaps more importantly, the possibility does not seem to have been considered that, regardless of dopamine content, VTA neurons excited by aversive stimuli may play a role in fear conditioning.

We therefore recorded single-unit activity in the rat VTA without *a priori* selection criteria to characterize the responses of all types of VTA neurons to visual and auditory cues paired with inescapable footshock during the acquisition and expression of

fear conditioning followed by reversal fear conditioning using the same cues. We found many phasic excitatory (13/29 cells; 44.8%) and some inhibitory (3/29 cells; 10.3%) neural responses to footshock. Many cells (13/29; 44.8%) responded to both sucrose consumption and shock delivery, most commonly with excitations to both stimuli. We found phasic excitations (up to 45%) and inhibitions (up to 31%) to both the visual and auditory cues at all stages in conditioning. The percent of cue-inhibited cells correlated with the degree of fear expressed to the cue. While the percent of cue-excited units did not change significantly with learning, excitations were significantly stronger when the cue was the CS+ than when it was the CS-, and the strength of cue excitations was significantly correlated with the amount of fear expressed to the cue. Importantly, most (10/13) cue-excited units were also excited during reward consumption, while only one was inhibited. Therefore, VTA neurons excited by fear-predictive cues are not selectively excited by aversive stimuli, and are instead generally excited by reward. Taken together, our results suggest that VTA neurons phasically encode the fear-predictive properties of visual and auditory cues.

Materials and Methods

Animals and Surgery

Male Long-Evans rats (Harlan, Indianapolis, IN) weighing 400-500 g were implanted with microelectrode arrays directed unilaterally at the VTA (AP -5.4; ML +2.0; DV -8.0) under isoflurane anesthesia. The arrays, consisting of eight 50 μm diameter insulated tungsten electrodes (NeuroBiological Laboratories, Denison, TX), were affixed to the skull with dental cement. Animals were allowed to recover for 7-10 days after surgery

with *ad libitum* food and water. After the first magazine training session, subjects were water-restricted until the beginning of recording on the following day (23 hours). Weight was monitored daily, and all procedures were approved by the Gallo Center Institutional Animal Care and Use Committee and were in accordance with National Institutes of Health guidelines.

Behavioral Testing

Behavior and neural activity were measured in a behavioral chamber in which there was a small cue light on the wall opposite a reward delivery port equipped with a photobeam emitter and detector to record port entries. Footshocks were delivered through a removable metal grid floor, which was replaced by a solid plastic floor only during trial blocks testing conditioned freezing to the cues. A recording cable attached to a freely rotating commutator at the top of the chamber was plugged into the rat's headstage, allowing the rat unhindered access to all areas of the chamber. Unless otherwise specified, a houselight was on and white noise was present throughout all recording procedures. To shape reward port behavior, all rats were first given 1 day of magazine training, a 1 hr session in which unpredicted 10% liquid sucrose was delivered in the reward port with a variable intertrial interval (ITI; mean ITI= 30 s). On the next day, neural activity was recorded continuously during the reward delivery, habituation, and fear conditioning and reversal procedures described below. First, to assess neural responding to unpredicted reward, subjects received a 1-hr trial block in which 10% liquid sucrose was delivered in the reward port randomly, with a variable interval (VI) mean of 30s. Reward ports were then covered for the remainder of recording. Next, to

allow for behavioral and neural habituation to the visual and auditory stimuli which would later be used as conditioned stimuli paired with shock, subjects were given 10 presentations each of a flashing light (0.1 s on, 0.4 s off; 6 flashes over 3 s) and a tone (3 kHz, 3 s) on a VI schedule, mean 45 s. For habituation, as well as all other blocks, lights and tones were presented in a random order. This was followed by the initial fear conditioning block, in which either the light or the tone (CS+) was presented 15 times, with cue offset being coincident with the onset of a pulsatile footshock (0.5 s on, 0.5 s off; 3 pulses over 3 s; 0.4 mA), and the other cue (CS-) was also presented 15 times, never followed by the shock. Trials in the conditioning block were presented on a VI schedule, with a mean ITI of 90 s. Animals were then allowed to rest in the recording chamber with the houselight and white noise off for 30 minutes before the initial fear test block, in which the CS+ and CS- were each presented 25 times, in the absence of footshock. Trials in the test block were presented on a VI schedule, with a mean ITI of 60 s. For the fear test, the grid floor was replaced with a novel (solid plastic) floor, and small bowls of lab chow and water were placed in the recording chamber to allow observation of fear-induced cessation of feeding and drinking and to facilitate the observation of freezing to the cues, as well as to minimize the subject's deprivation. After the initial fear conditioning and test, fear conditioning, rest, and fear test blocks were repeated, but with the stimuli initially assigned as the CS+ and the CS- reversed.

The amount of time (in s) each subject spent freezing on each trial during the habituation block and each fear test block was recorded by observation of digital video recordings, and learned fear of the cue was determined by a paired t-test ($p<0.01$) comparing freezing during the cue to a 3 second baseline immediately prior to the cue.

To quantify the subject's fear of each cue, a fear index was computed by summing the time spent freezing during all presentations of that cue in a given test block, from which the time spent freezing during all pre-cue baselines was subtracted. To measure each subject's able to discriminate between the CS+ and the CS- in each test block, a discrimination index was computed by subtracting the CS- fear index from the CS+ fear index.

Histology

After completion of the final recording session, rats were deeply anesthetized with isoflurane after small electrolytic lesions (19μA current for 10 s) were made at the end of each electrode from which single units were recorded. Brains were removed and fixed with 10% formalin with 3% potassium ferrocyanide and then submerged in 0.01M phosphate buffer with 25% sucrose and 3% potassium ferrocyanide. 50 μm coronal sections were taken through the extent of the VTA and processed immunohistochemically for tyrosine hydroxylase reactivity as follows: free-floating sections were incubated in 3% hydrogen peroxide (10 min), rinsed, incubated in 50% alcohol (20 min), rinsed, and then incubated in normal donkey serum (10% in PBS, 30 min), before being incubated overnight in the primary antibody (rabbit anti-tyrosine hydroxylase polyclonal antibody, AB152, Chemicon, 1: 700 in PBS with 0.1% Triton-X100). Incubation with secondary antibody (biotinylated donkey anti-rabbit antibody, Jackson ImmunoResearch, 1:300) for 2 hr was followed by incubation in ExtrAvidin-peroxidase complex (Sigma; 1:2500 in PBS) for 2 hr. Peroxidase was histochemically visualized with diaminobenzidine (DAB, Fisher). Sections were examined under a light

microscope to verify that electrode placements (lesions marked with potassium ferrocyanide) were within the borders of the VTA as defined by the rat stereotaxis atlas (Paxinos and Watson, 1998).

Single-unit discrimination and characterization

Neural recordings were amplified, filtered (0.4 kHz low-cut and 5 kHz high-cut), and digitized using commercial acquisition software and hardware (Plexon Instruments, Dallas, TX). Single units were isolated based on consistent waveform shape and principal component analysis (Plexon Instruments), with autocorrelograms and interspike interval histograms used for additional rectification. Action potential waveform lengths were measured from initial inflection to first trough, and baseline firing rate was measured in the 10 s pre-cue (including both lights and tones) baseline period over the entire session. To compare baseline firing rates and waveform lengths of different neural populations, t-tests ($p<0.05$) were used.

Analysis of neural responses

Neural data were analyzed from a total of 4 rats with electrode placements in VTA and identifiable single units. A phasic cue response was considered significant if one or more 100 ms bins in the 500 ms after cue onset were statistically significant relative to a 500 ms pre-cue baseline ($p<0.01$ using the nonparametric Wilcoxon signed-rank test; MatLab, Mathworks, Natick, MA), and the direction of the response was determined by examining perievent histograms in NeuroExplorer (Plexon Instruments). Some phasic excitations (~15%) were followed by inhibitions; these mixed responses were classified

as excitations. To assess the neural response to sucrose, a variable was first created in NeuroExplorer to mark the time at which the animal received the sucrose, which was at the time of sucrose delivery itself only if the animal was already in the reward port, and was otherwise at the time of the first port entry after sucrose delivery. Because some units displayed phasic neural responses to port entry within 1 s of entry, a sucrose response was considered significant if one or more 1 s bins in the 2-5 s after sucrose receipt were significantly different from a baseline period -15 to -10 s before sucrose receipt ($p<0.01$, Wilcoxon signed-rank test). Because neural activity cannot be recorded while shock is delivered, we used a pulsatile shock (0.5 s on, 0.5 s off; 3 pulses over 3 s; 0.4 mA), and the neural response to shock was considered significant if one or more 100 ms bins in the 500 ms immediately after the first pulse were statistically significant relative to a 500 ms baseline prior to shock onset ($p<0.01$, Wilcoxon signed-rank test).

The magnitude of cue excitation was determined for each neuron by normalizing firing rate (FR) in 100 ms bins trial-by-trial using z-scores as follows:

$$Z=\frac{\text{FR in given bin} - \text{mean FR within an interval from -0.5 to +0.5 s around cue onset}}{\text{SD of FR within an interval from -0.5 to +0.5 s around cue onset}}$$

The normalized magnitude of the neural response was compared between test blocks using a paired t-test ($p<0.05$).

Results

We recorded the activity of 29 VTA neurons in 4 rats during the acquisition of fear conditioning to visual and auditory stimuli paired with inescapable footshock. The day before fear conditioning, animals were given one magazine training session to shape reward port behavior. On the day of recording (Figure 3-1A), animals were first presented with unpredicted deliveries of liquid sucrose solution in a port within the operant chamber (82.3 ± 8.1 trials, Mean ± SEM), to assess neural reward responses, and then with 10 presentations each of a 3 s flashing light and a 3 s pure tone, to allow animals to habituate to the cues. Fear conditioning consisted of 15 presentations of each cue, with the CS+ always immediately followed by footshock and the CS- never followed by footshock (Figure 3-1B). Conditioned behavioral and neural responses to the cues were then measured during 25 presentations of each cue, with no footshocks delivered. For the initial conditioning and test block, the light was used as the CS+ and the tone was used as the CS-; this was then followed by another conditioning and test block in which the cues were reversed.

Significant freezing to the light and tone cues was not seen during the habituation block in any of the subjects (Figure 3-1C; Fear Index: Light = -1 ± 1; Tone = 2 ± 1; Mean ± SEM). After the first fear conditioning block, all 4 rats displayed conditioned fear to the initial light CS+ (Figure 3-1C; Fear Index = 40 ± 2, Mean ± SEM), without exhibiting fear of the tone CS- (Fear Index = 3 ± 2, Mean ± SEM). Freezing to the CS+ in this first test block was highly significant in all animals relative to baseline freezing ($p<0.00005$, paired t-test), as well as relative to the tone CS- (Figure 3-1C; $p<0.001$, paired t-test of Fear Indices) and presentations of the light during habituation (Figure 3-1C; $p<0.0005$,

Figure 3-1. Experimental Paradigm and Behavioral Results

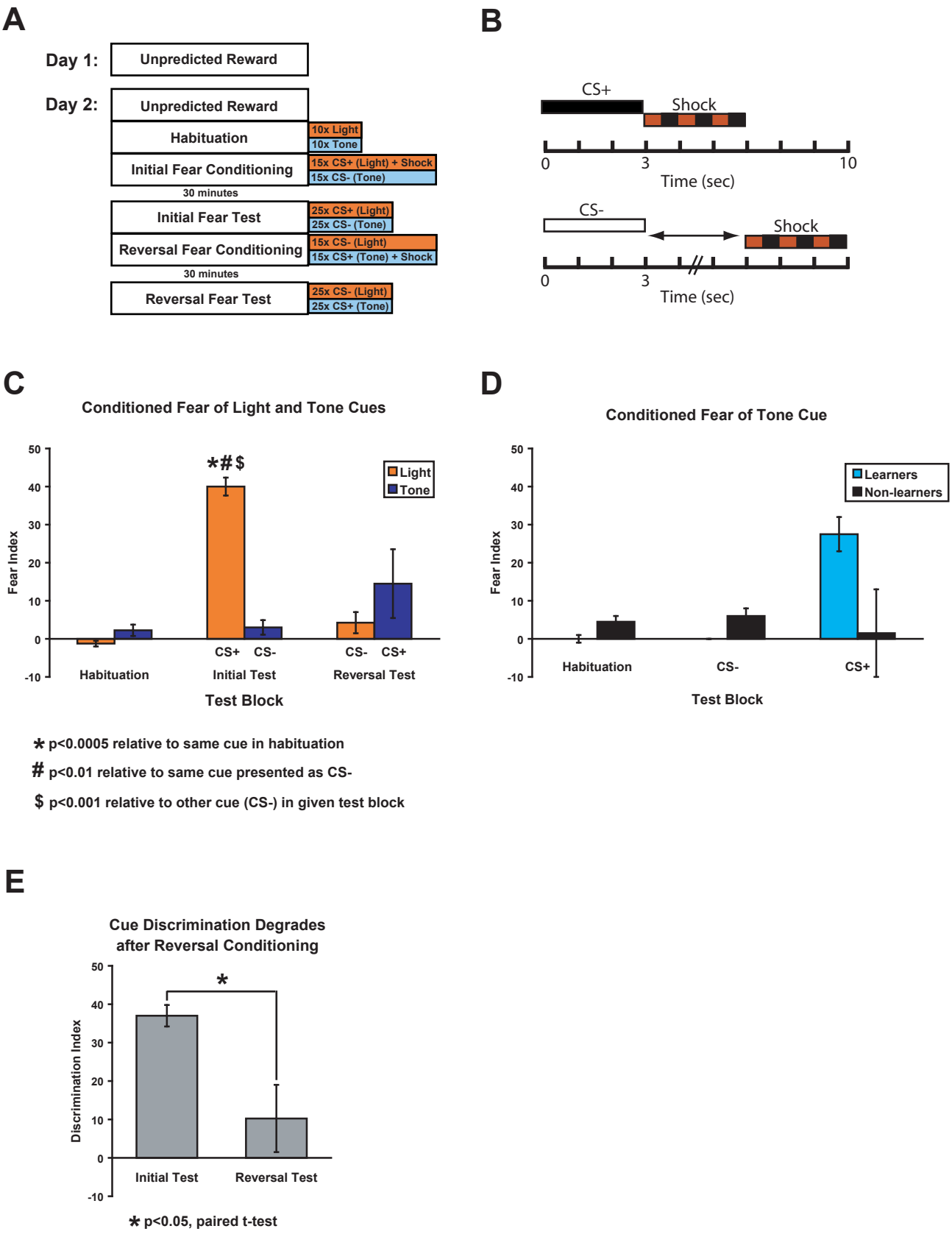


Figure 3-1. Behavioral Procedure and Electrode Placements

(A) Procedural training and test blocks (B) US delivery (a pulsatile shock, 0.5 s on, 0.5 s off; 3 pulses over 3 s; 0.4 mA) immediately followed each presentation of the CS (flashing light: 0.1 s on, 0.4 s off, 6 flashes in 3 s; or tone: pure 3 kHz tone for 3 s). US delivery had a variable temporal relationship with the CS-, such that it had no shock-predictive value. (C) Fear index for all animals to each cue during habituation, the initial test (light CS+), and the reversal test (tone CS+). Values reflect mean (±SEM) amount of time spent freezing (in s) during the 25 cue presentations, normalized to the amount of time spent freezing during a 3 s pre-cue baseline period. (D) Two animals showed significant conditioning to the tone CS+ in the reversal test (Learners) and two animals did not (Non-learners). Shown here are fear indices for the two groups to each cue during habituation, the initial test (light CS+), and the reversal test (tone CS+). Values reflect mean (±SEM) amount of time spent freezing (in s) during the 25 cue presentations, normalized to the amount of time spent freezing during a 3 s pre-cue baseline period. (E) Animals discriminated better between the CS+ and the CS- during the initial test than during the reversal test. Values reflect mean (±SEM) discrimination indices (CS+ fear index minus CS- fear index).

paired t-test of Fear Indices). However, after the second fear conditioning block, in which the cues used as the CS+ and CS- were reversed, subjects did not freeze significantly more to the tone during the reversal test as a group (Figure 3-1C; Fear Index for all subjects = 15 ± 9, Mean ± SEM) than during habituation ($p=0.32$, paired t-test of Fear Indices) or during the initial test ($p=0.37$, paired t-test of Fear Indices). Learning of reversal fear conditioning varied greatly between animals; freezing to the tone CS+ was highly significant (freezing relative to baseline, $p<0.0005$, paired t-test) in 2 of the 4 subjects (Figure 3-1D; "Learner" animals; Fear Index = 32 and 23), but did not reach significance in one "Non-learner" animal (Fear Index = 13; freezing relative to baseline, $p=0.07$, paired t-test), and was completely absent in the other "Non-learner" (Fear Index = -10; freezing relative to baseline, $p=0.21$, paired t-test). Overall, animals discriminated better (Figure 3-1E; $p<0.05$, paired t-test of Discrimination Indices) between the CS+ and the CS- in the first test block (Fear Index for light > tone, $p<0.001$, paired t-test; Discrimination Index = 37 ± 3, Mean ± SEM), than in the second test block (Fear Index for light vs. tone, $p=0.33$, paired t-test; Discrimination Index = 10 ± 9, Mean ± SEM). The lower level of fear displayed to the tone CS+ than to the light CS+ is not surprising, as fear conditioning to the tone was likely blocked due to its previous presentations unpaired with shock. This interpretation is supported by the maintenance of a trend towards conditioned fear of the light in 2 animals after reversal conditioning (Fear Index = 8 and 10; freezing relative to baseline, $p=0.07$ and 0.06, paired t-test), although all subjects did exhibit less fear during light presentations after reversal conditioning than during the initial test (Figure 3-1C; $p<0.01$, paired t-test of Fear Indices).

Neural Responses to Unconditioned Stimuli: Sucrose and Footshock

Both excitations (in 16/29 cells; 55.2%) and inhibitions (in 8/29 cells; 27.6%) in the activity of VTA neurons were seen during sucrose consumption. These responses were often preceded by excitatory responding at the time of port entry, and generally were maintained for the duration of entry into the reward port, but were not due to port entry alone (Figure 3-2). Neural activity cannot be recorded during the delivery of footshock, so we used pulsatile shock delivery (0.5 s on, 0.5 s off; 3 pulses over 3 s) and looked for changes in neural activity in the 0.5 s immediately following the first shock pulse (which should not reflect neural activity encoding relief, as there are two more shock pulses in each presentation). We found many excitatory (13/29 cells; 44.8%) and some inhibitory (3/29 cells; 10.3%) neural responses during this aversive event.

Figure 3-3 shows representative response profiles to sucrose and shock in individual neurons. As shown in Tables 3-1 and 3-2, many cells (13/29; 44.8%) responded to both sucrose consumption and shock delivery, representing all possible combinations, with the exception of a shock inhibition with no response to sucrose. Although VTA neurons display varied responses to both rewarding and aversive primary (unconditioned) stimuli, excitation to both shock and sucrose (in 6/29 cells; 20.6%) was the most common profile in neurons responsive to both stimuli. This suggests that VTA activity is not dominated by neurons excited by primary rewarding events and inhibited by primary aversive events.

Neural Responses to Visual and Auditory Cues Predicting Footshock

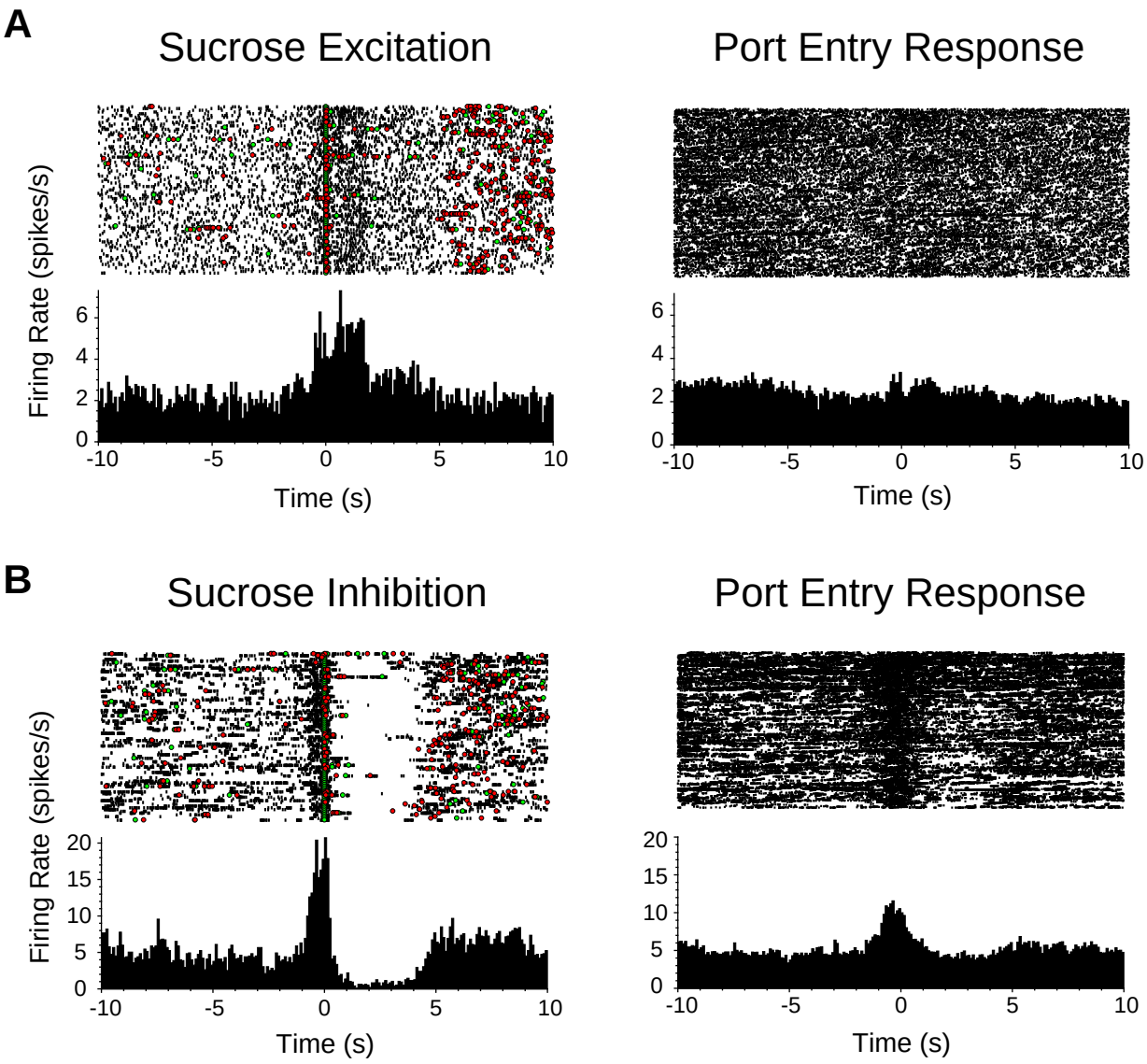


Figure 3-2. Neural responses to sucrose and port entry

Peri-event rasters (top) and histograms (bottom, 100 ms bins) of representative neurons with (A) excitatory and (B) inhibitory responses to sucrose delivery (left; onset of sucrose consumption at time=0 s; green dots indicate port entries and red dots indicate port exits). Responses to port entry (right) account for the initial excitation in both examples, but not for the response during consumption.

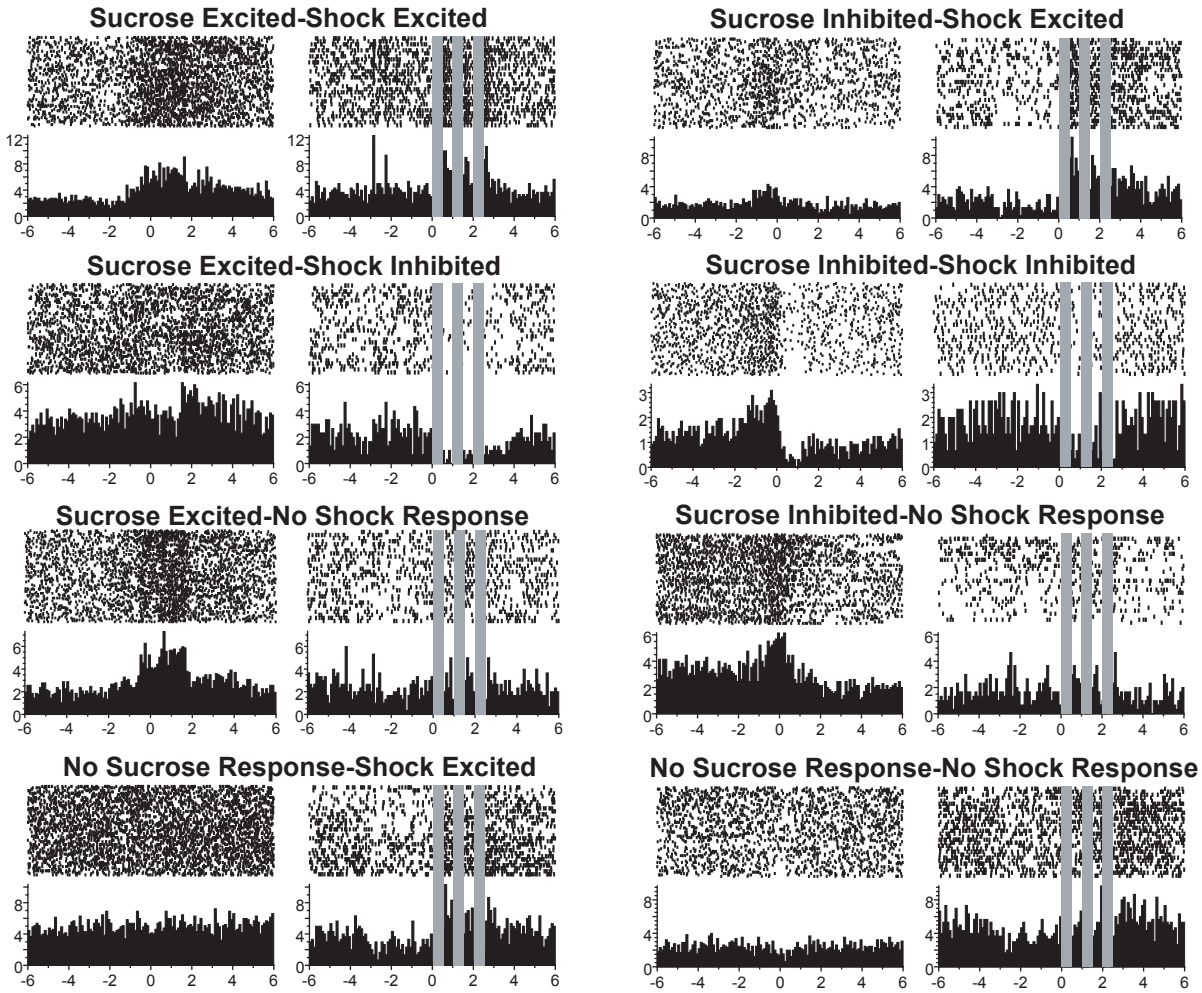


Figure 3-3. Neural responses to sucrose and shock

Peri-event rasters (top) and histograms (bottom, 100 ms bins) of representative neurons with varying responses to sucrose and shock (left side of each pair of raster, onset of sucrose consumption at time=0 s; right side, onset of shock at time=0 s). The grey bars indicate shock deliveries, during which neural activity could not be recorded.

Table 3-1. Number of cells responsive to sucrose and shock

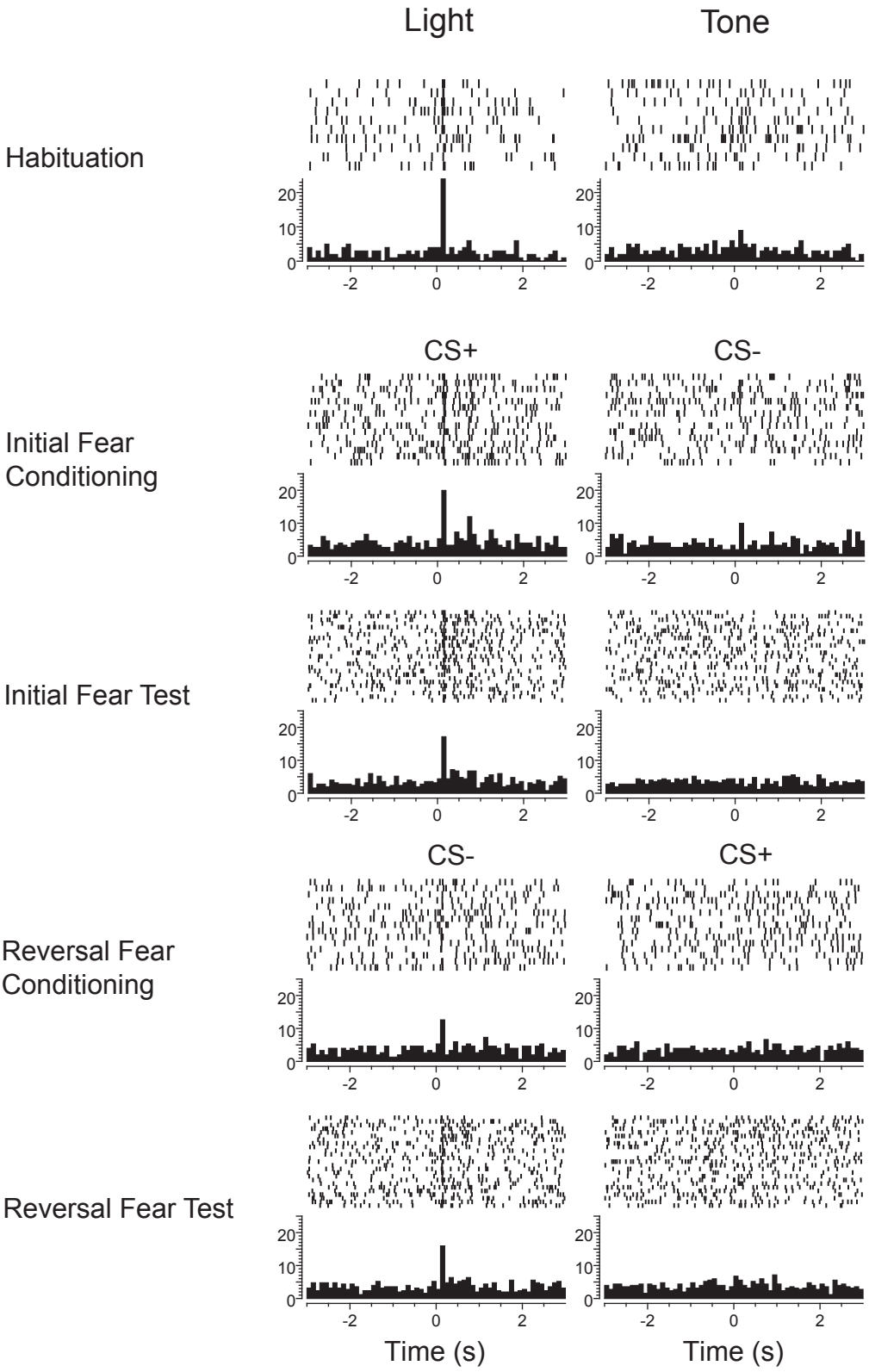
		Sucrose			
		Total	Excited	Inhibited	No Response
Shock	Total cells	**29**	**16**	**8**	**5**
	Excited	**13**	6	4	3
	Inhibited	**3**	2	1	0
	No Response	**13**	8	3	2

Table 3-2. Percent of cells responsive to sucrose and shock

		Sucrose		
		Excited	Inhibited	No Response
		55.2%	**27.6%**	**17.2%**
Shock Excited	**44.8%**	20.7%	13.8%	10.3%
Shock Inhibited	**10.3%**	6.9%	3.4%	0.0%
Shock No Response	**44.8%**	27.6%	10.3%	6.9%

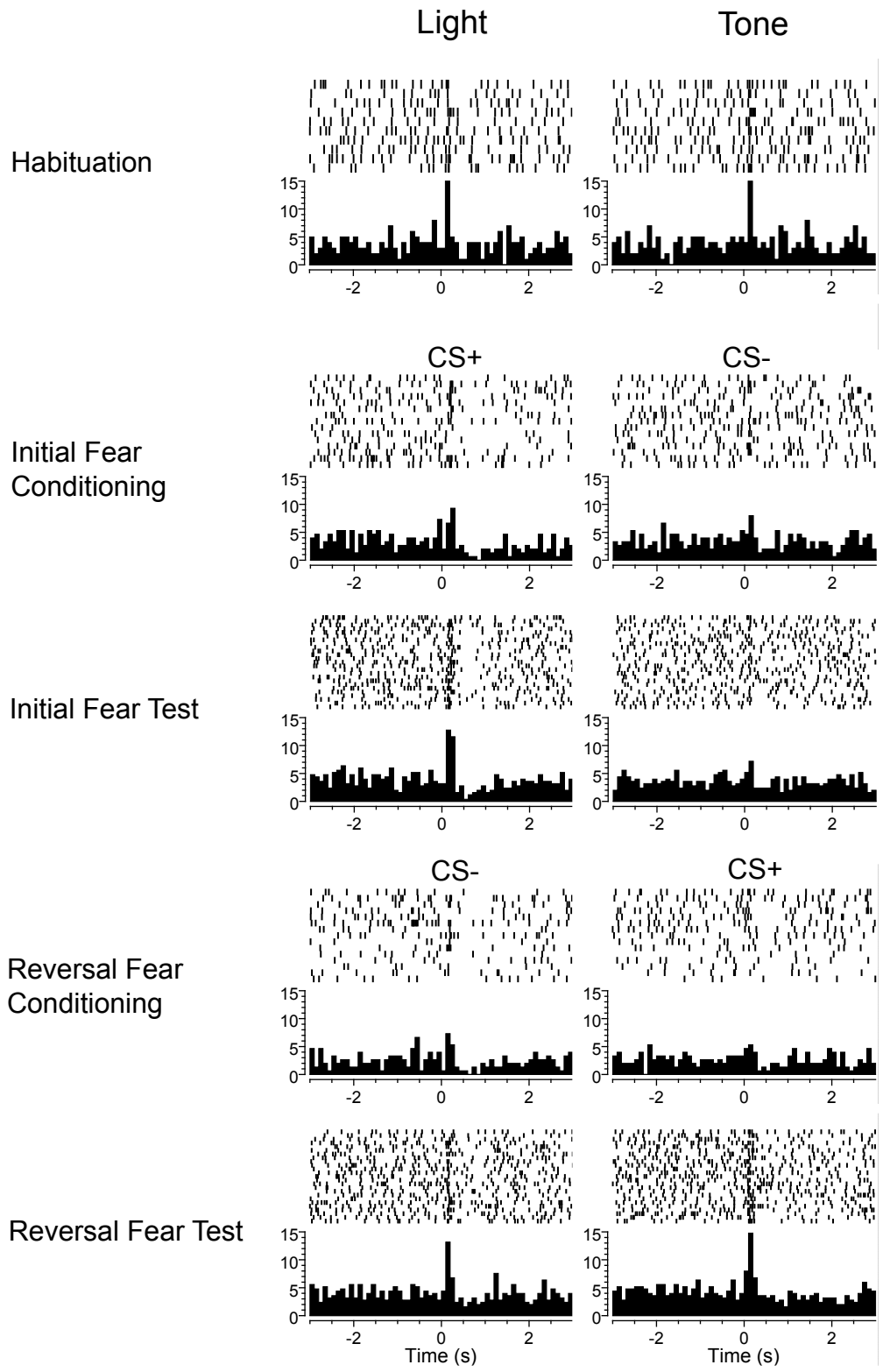
Our procedure allowed us to observe the responses of the same neurons to visual and auditory cues when they were novel (in Habituation), and during and after the acquisition of fear conditioning using each cue as a CS+ and a CS-. The light was assigned as the CS+ in initial conditioning and as the CS- in reversal conditioning, while the tone was first the CS- and then the CS+. Phasic excitations and inhibitions, within 500 ms of cue onset, were seen to both cues in every stage of training; 24/29 cells (82.8%) were light-responsive in at least one test block, while 17/29 cells (58.6%) were tone-responsive. Representative examples of the responses of eight individual VTA neurons to the cues through all stages of training are shown in Figure 3-4A-H. As these figures show, cue excitations were generally more robust than inhibitions; Figure 3-4G shows the clearest available example of inhibitory cue responses. The percent of total cells with significant cue responses in each test block is shown in Figure 3-5A and B. In habituation, 11/29 VTA neurons (37.9%) were excited by the novel light cue, while 3/29 cells (10.3%) were inhibited. There were fewer neural responses to the novel tone, which excited only 3/29 (10.3%) and inhibited 2/29 (6.9%) cells. This is likely due to the greater salience of the flashing light than the pure tone, but may also reflect preferential responding of VTA cells to visual, rather than auditory, cues. In the initial fear conditioning test, approximately the same proportion of cells were excited by the light (CS+) (12/29; 41.4%) as in habituation, but the proportion of cells inhibited was somewhat larger (9/29; 31.0%). In this first test block, the tone (CS-) excited only 1 cell (3.4%) and inhibited 3 cells (10.3%). After reversal fear conditioning, the light (CS-) continued to elicit neural excitations, in 13/29 (44.8%) cells, and inhibitions, in 5/29 (17.2%) cells. The tone, as

Figure 3-4A



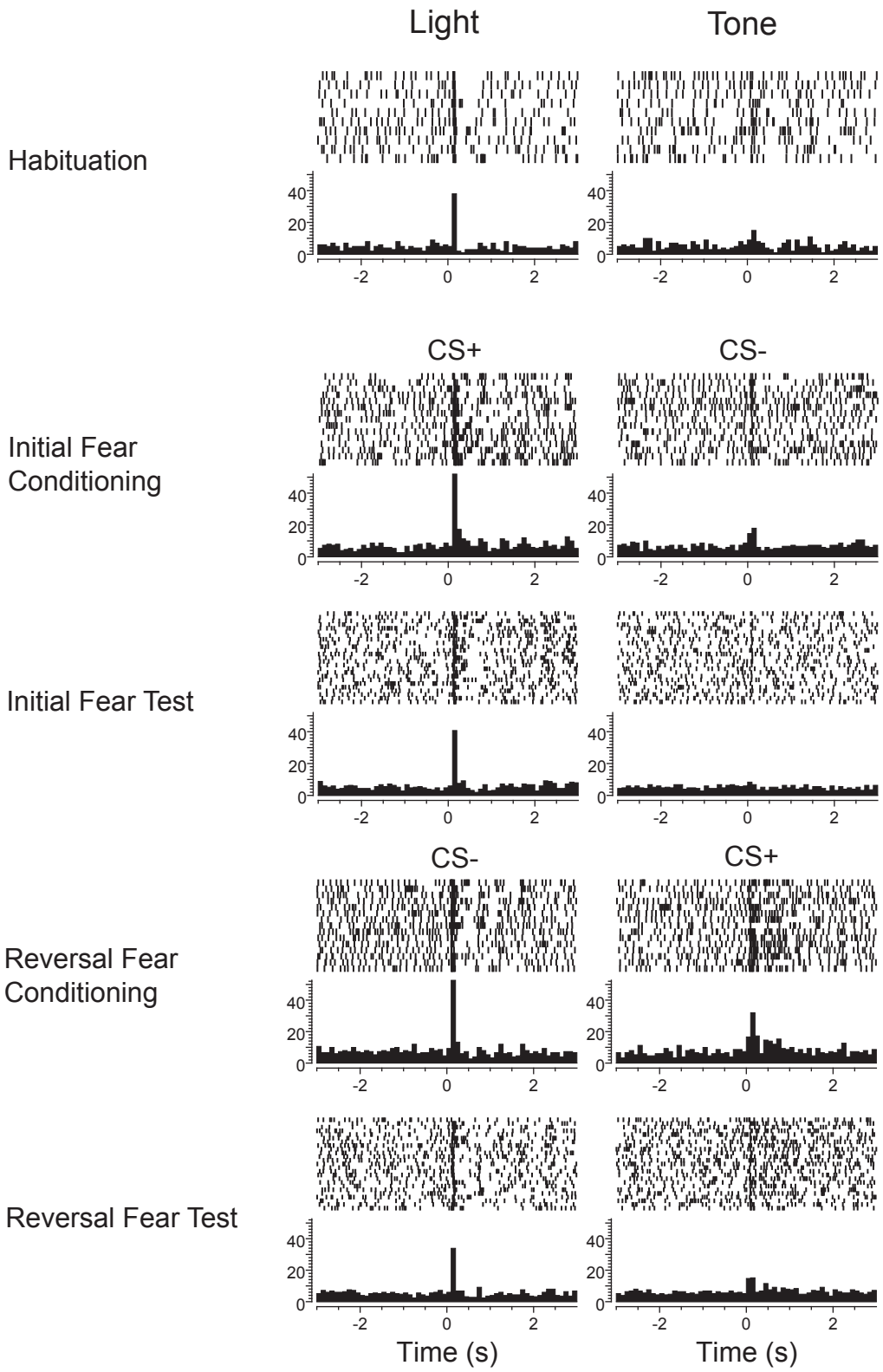
Unit F3.2a

Figure 3-4B



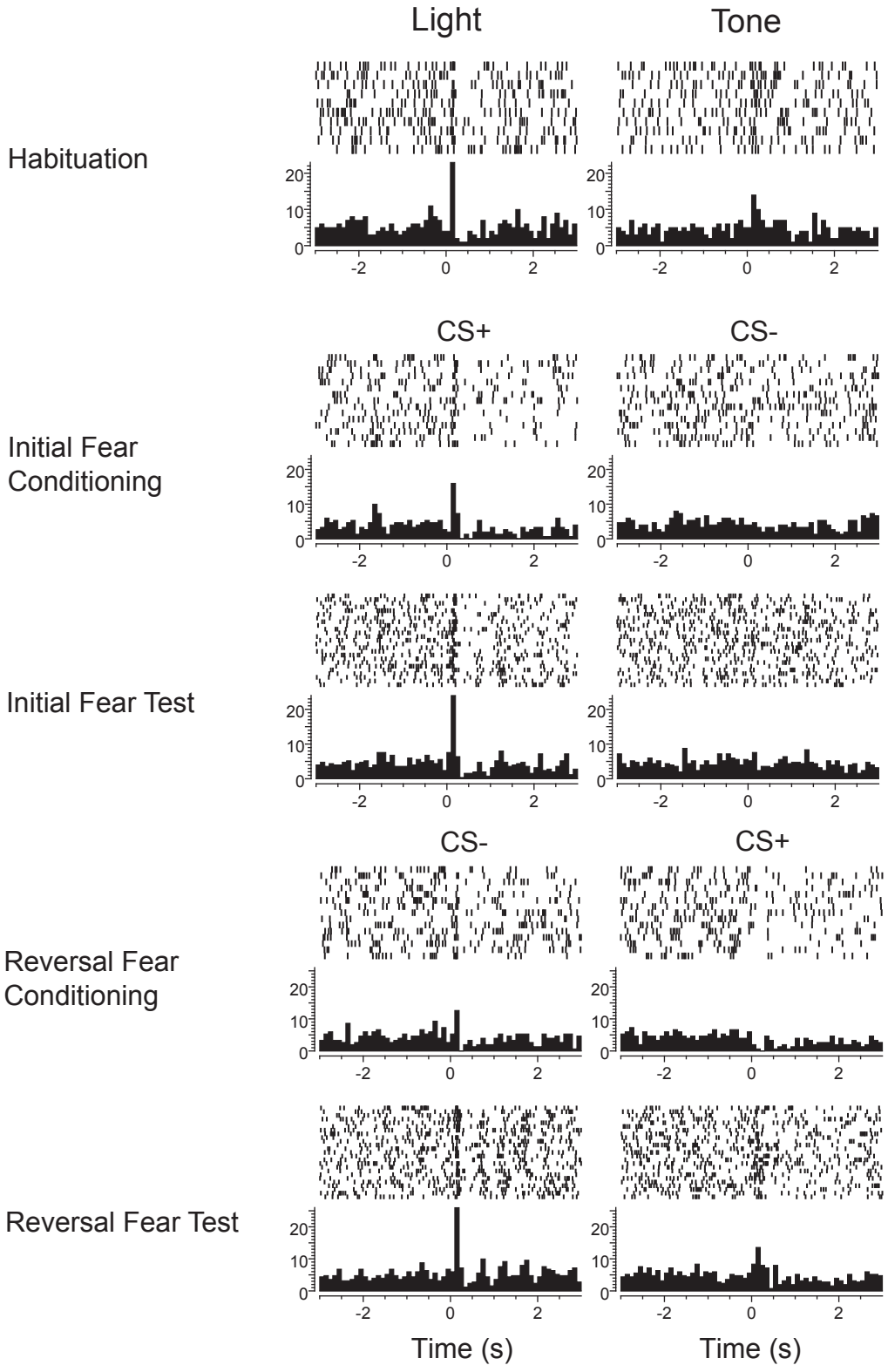
Unit F3.2c

Figure 3-4C



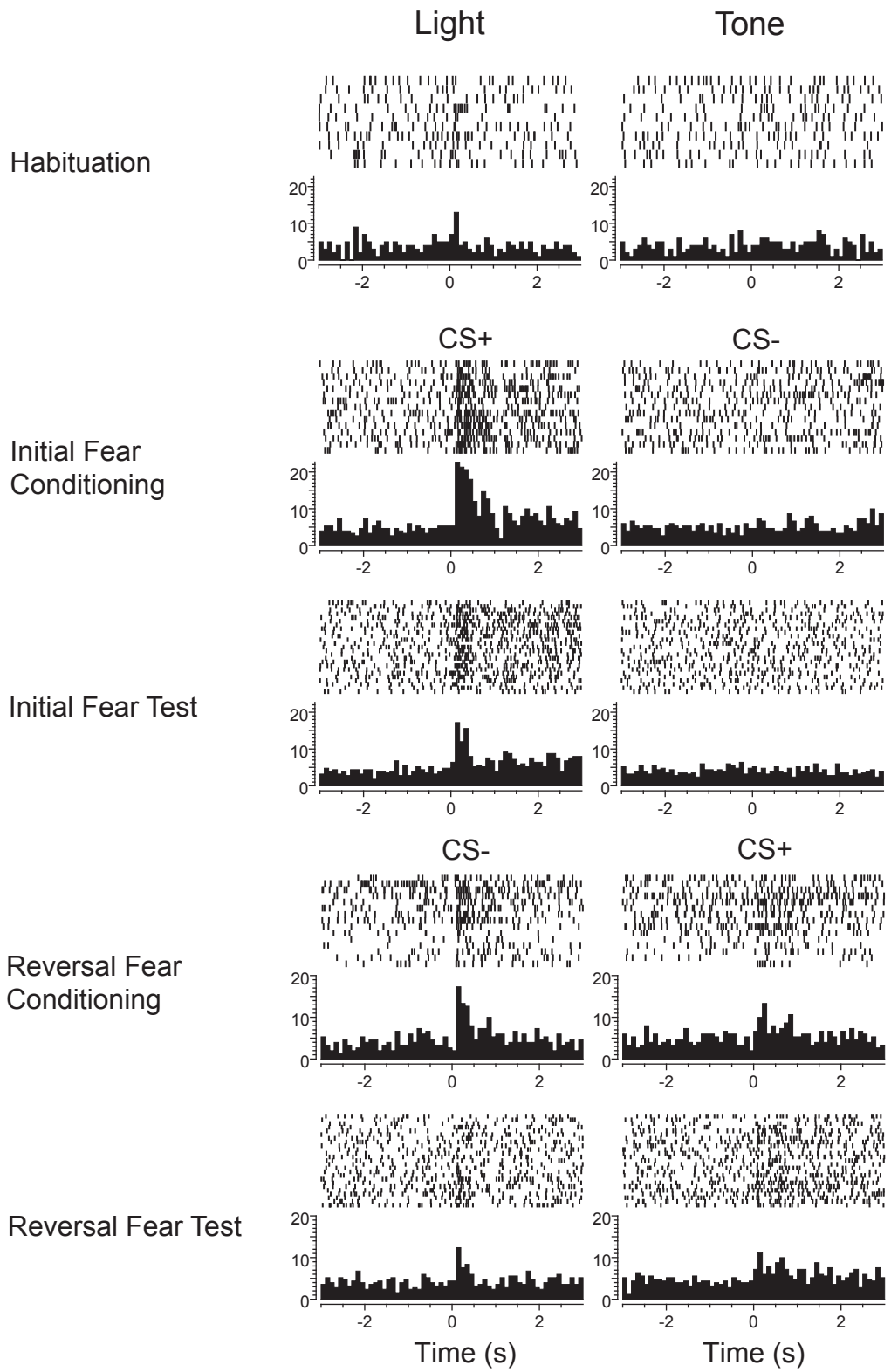
Unit F3.5b

Figure 3-4D



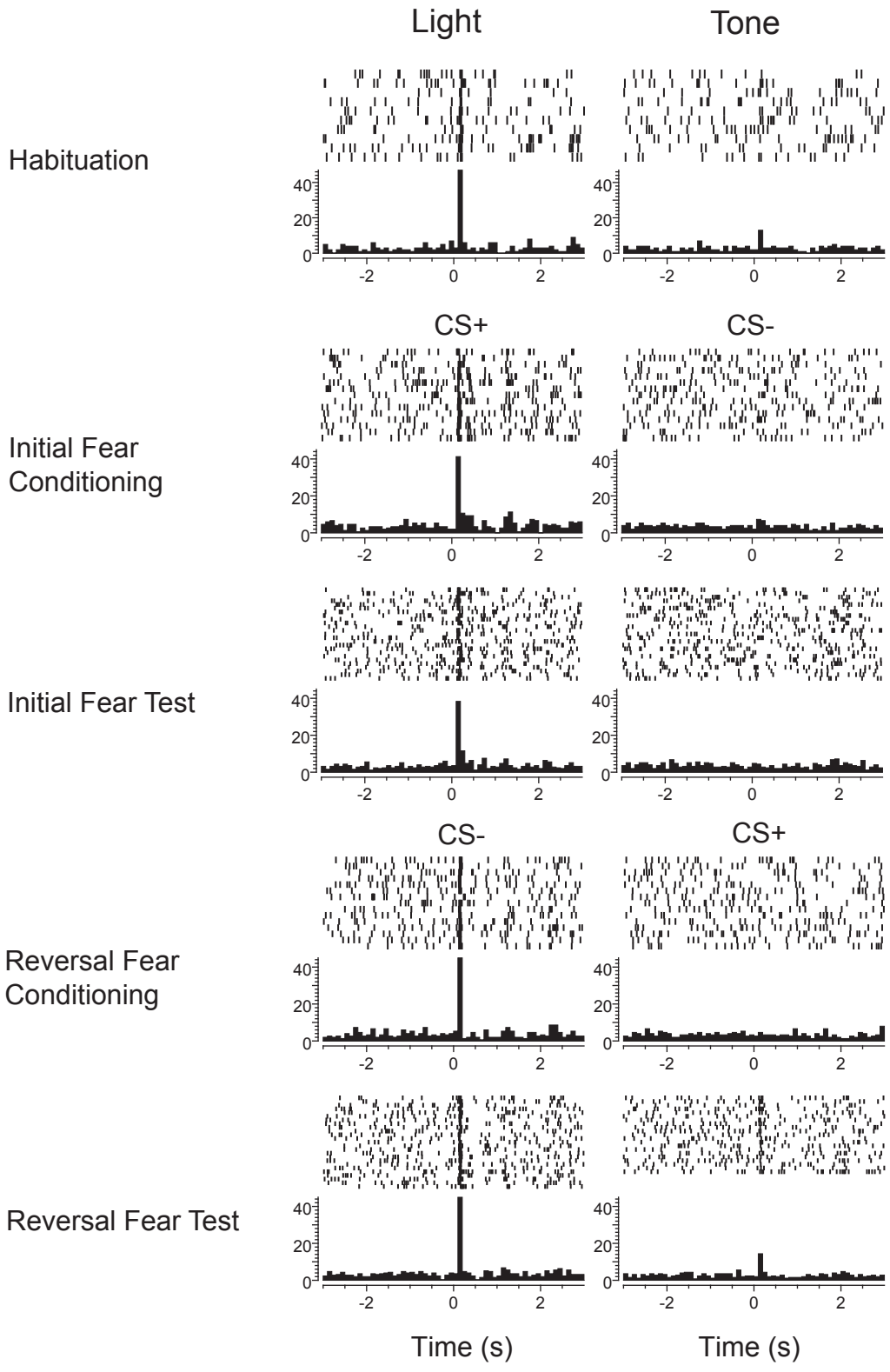
Unit F3.7a

Figure 3-4E



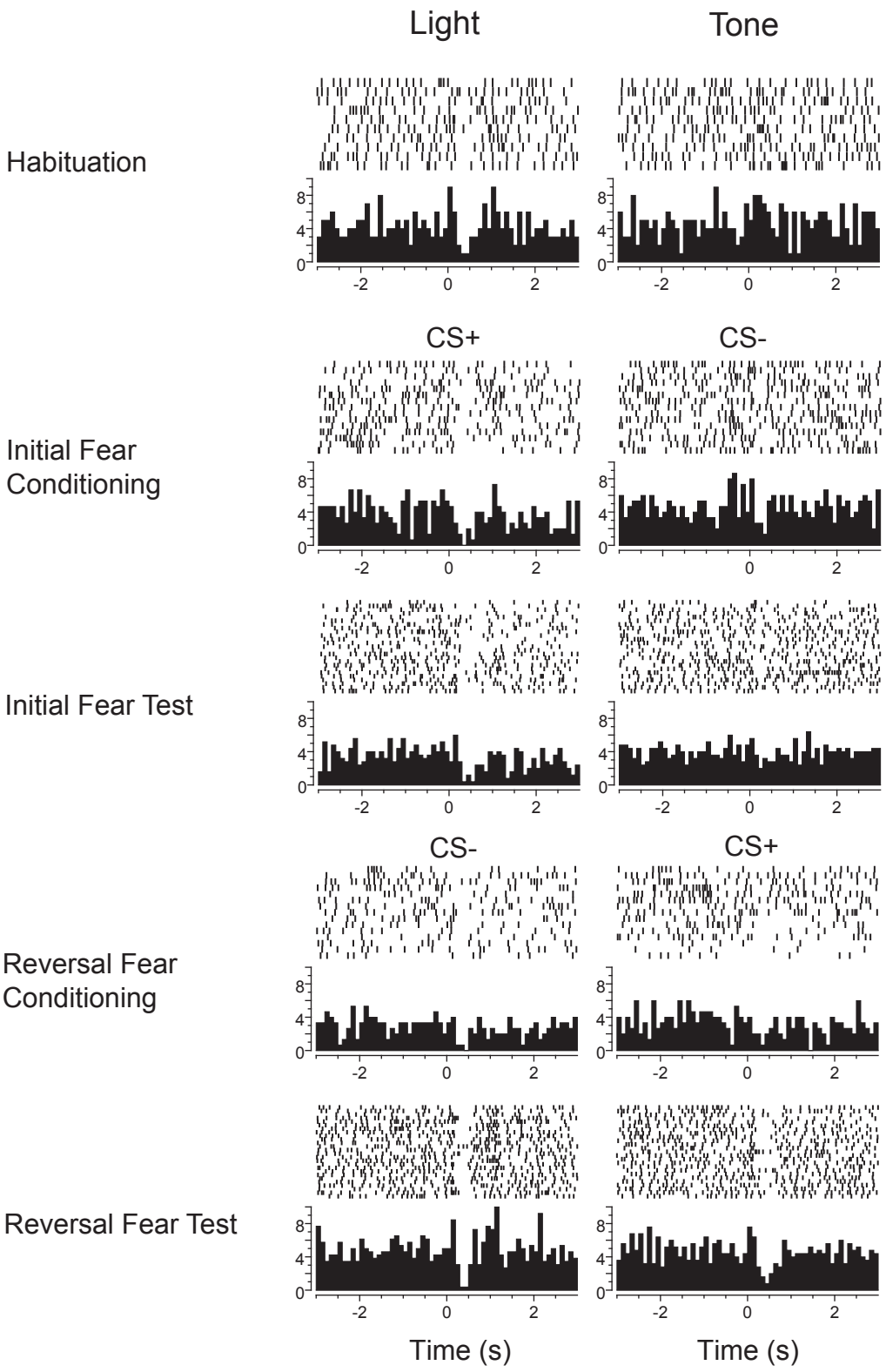
Unit F3.7c

Figure 3-4F



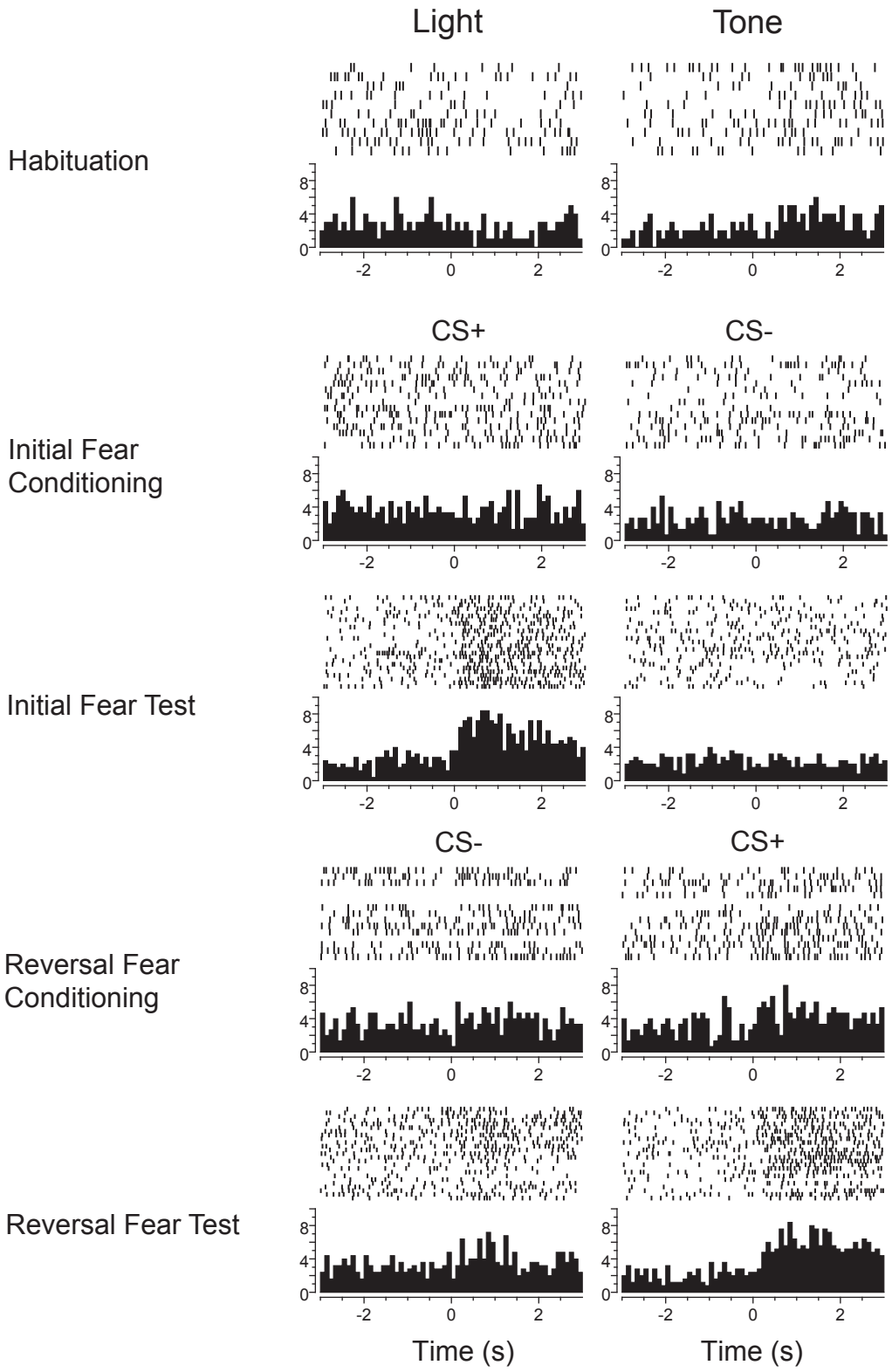
Unit F4.7b

Figure 3-4G



Unit F5.8c

Figure 3-4H



Unit F6.6b

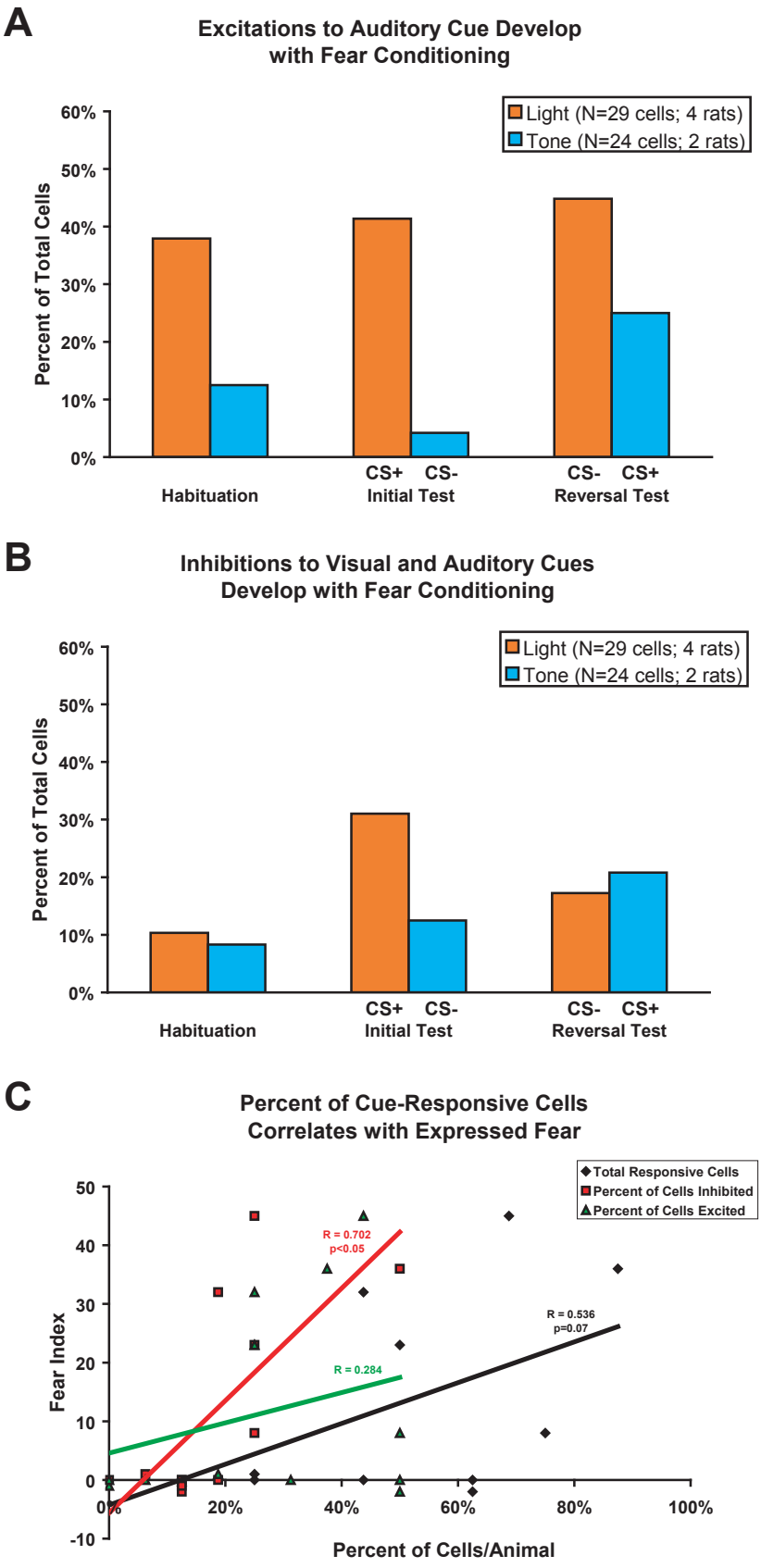
Figure 3-4. Examples of cue responses of individual cells

Peri-event rasters (top) and histograms (bottom, 100 ms bins) of representative neurons with phasic responses to light and tone cues through all training and test blocks (cue onset at time=0 s; y-axis represents firing rate in spikes/s).

Figure 3-5. Proportion of cue-responsive cells

Percent of total neurons with phasic excitations (A) and inhibitions (B) to light and tone cues in each test block. For tone responses, only neurons from Learner rats were included. (C) For two rats with ≥ 8 cells, linear regressions comparing the fear index for each cue in each block with the percent of cue-responsive, cue-excited, and cue-inhibited cells in that block. Each colored line represents the best fit for the individual data points of that color.

Figure 3-5. Proportion of Cue-Responsive Cells



the CS+ in the reversal block, elicited more excitations (8/29 cells; 27.6%) and inhibitions (5/29 cells; 17.2%) than when novel or as the CS-.

VTA neurons do not appear to be particularly selective for the sensory modality of cues; while there were more light-responsive than tone-responsive units, 7 of the 8 cells excited by the tone CS+, and 3 of the 5 cells inhibited by the tone CS+, showed similar responses to the light CS+. Five of the 13 neurons excited by one or both CS+s (38.4%) were excited by shock delivery, while one was inhibited. Interestingly, 76.9% of CS+-excited neurons (10 of 13) were also excited during sucrose consumption, while only 1 was inhibited, suggesting that these cue-responsive units are not selectively responding to aversive events. CS+-inhibited units (n=9) showed mixed responses to sucrose consumption (3 were excited, 3 were inhibited, 1 showed a mixed excitation-inhibition response). CS+-excited neurons did not have significantly different baseline firing rates (3.8 ± 0.4 spikes/s, Mean ± SEM) or action potential waveform lengths (0.56 ± 0.03 ms, Mean ± SEM) than other cells (6.9 ± 2.03 spikes/s; 0.51 ± 0.04 ms, Mean ± SEM; $p>0.1$, t-test).

As shown in Figures 3-5A and B, the proportion of tone-responsive (both excited and inhibited) and light-inhibited cells reflected fear conditioning better than the proportion of light-excited cells, as phasic excitations to the light cue were seen in similar proportions regardless of its pairing with shock. To examine this in more detail, we investigated a possible relationship between the proportion of cue-responsive cells and expressed fear in each test block in 2 subjects with ≥ 8 cells, which were also the 2 animals who displayed fear of the tone after reversal conditioning. The percent of cells per subject with an inhibitory response to each cue was significantly correlated with the

fear index for the cue in that test block (Figure 3-5C, linear regression, $R=0.702$, $p<0.05$). There was also a trend for such a correlation using the total percent of cue-responsive cells per subject ($R=0.536$, $p=0.07$), but no relationship with the percent of cue-excited cells ($R=0.284$, $p=0.37$). While these results are preliminary, from only 2 subjects, they suggest that some VTA neurons develop inhibitory responses to a cue as the animal learns the cue is predictive of an aversive event.

The strength of phasic excitations to visual and auditory cues encodes fear

Although the proportion of VTA neurons that were excited by the cues was not correlated with expressed fear, excitations were often stronger when the cue was the CS+ than when it was the CS-. Examples of such responses in two individual neurons can be seen in Figures 3-6A and B. Neurons excited by the light CS+ (n=12 neurons from 4 rats) showed weaker responses to the light in habituation and in the reversal test, when the light was the CS- (Figure 3-6C; $p<0.05$, paired t-test). Interestingly, in all 6 neurons excited by the tone CS+ in the two subjects which learned to fear the tone, the normalized magnitude of the tone CS- response was slightly negative, although only one of them displayed a significant inhibitory response. In these cells, the magnitude of the tone CS- response was significantly different from the CS+ response (Figure 3-6D; $p<0.0001$, paired t-test) and the tone response during habituation ($p<0.05$, paired t-test). Across all CS+ excited neurons, the magnitude of the neural response in a given test block was significantly correlated with the fear index for that cue in that block (linear regression, $R=0.422$, $p<0.005$). As shown in Figure 3-6E, this was true for both the light ($R=0.342$, $p<0.05$) and tone ($R=0.525$, $p<0.01$) cues.

Figure 3-6. Cue Excitation Strength Encodes Fear

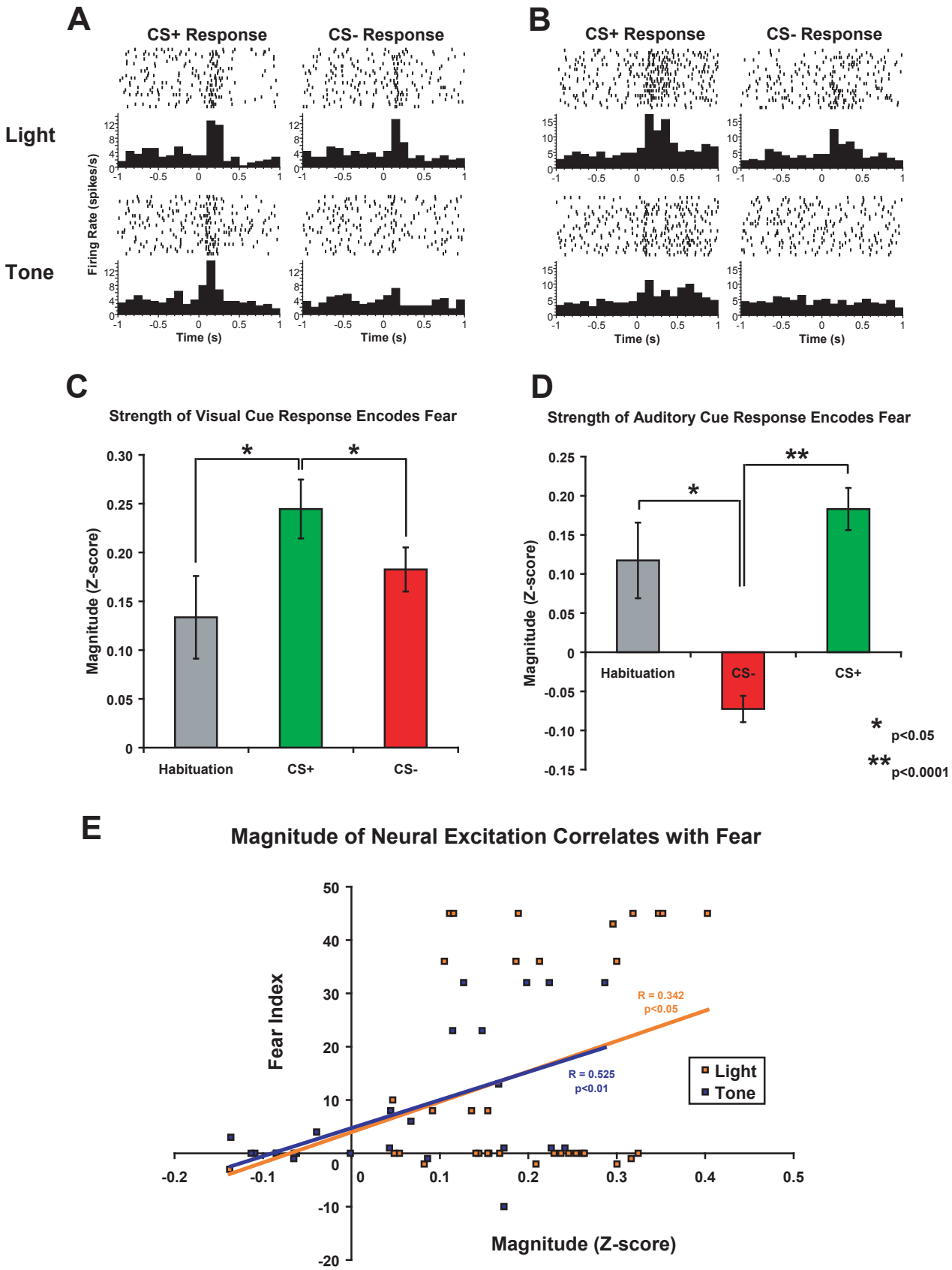


Figure 3-6. Cue excitation strength encodes fear

(A and B) Peri-event rasters (top) and histograms (bottom, 100 ms bins) of two representative neurons with phasic excitations to both light and tone cues (cue onset at time=0 s); the neural response to each cue was stronger when it was the CS+ than when it was the CS- . (C) The magnitude of phasic excitation to the light (normalized to baseline firing rate, mean±SEM) is larger when it was the CS+ than when it was the CS- or during habituation (paired t-test). (D) The magnitude of phasic excitation to the tone (normalized to baseline firing rate, mean±SEM) is larger during habituation and when it was the CS+ than when it was the CS- (paired t-test). (E) For all CS+-excited neurons, linear regressions comparing the fear index for each cue in each block with the magnitude of phasic excitation (normalized to baseline firing rate, mean±SEM) to that cue in that block. Each colored line represents the best fit for the individual data points of that color.

Discussion

We examined the activity of 29 VTA neurons using extracellar recording in 4 rats during the acquisition of fear conditioning to visual and auditory stimuli paired with inescapable footshock. We found that VTA neurons display varied responses to both rewarding and aversive primary (unconditioned) stimuli, with excitation to both shock and sucrose being the most common profile in neurons responsive to both stimuli. Phasic responses were seen to both visual and auditory cues in every stage of training. Excitations to the light cue were seen in similar proportions regardless of its pairing with shock; however, these responses were stronger when the light was a conditioned predictor of shock. A learning-related strengthening of the cue response was also seen for excitations to the tone. Unconditioned excitations to the tone were less common and weaker than for the light, but most cells excited by one conditioned cue (13/29; 44.8%) showed similar responses to the other, suggesting that these cells are not modality-selective. Regardless of the stage of training, the strength of both light and tone excitations was correlated with the amount of fear (freezing) expressed by the animal to that cue.

Could the phasic excitations seen to fear-associated cues be due to the excitatory actions of stress-induced release of endogenous opioids, Substance P, or CRF? A direct role is unlikely, as these have all been shown to increase tonic, rather than phasic, firing of VTA DA neurons (Altier and Stewart, 1996; 1999a, b; Wanat et al., 2007). While it has been suggested that increases in tonic DA activity decrease the likelihood of phasic responses (Grace, 2000), others have hypothesized that "high tonic DA levels will predispose to less excitable but more powerful mesolimbic-DA network influences" (Alcaro et al., 2007). In light of this claim, it is interesting that opioids inhibit the activity

of presumed GABA interneurons in the VTA (Johnson and North, 1992), and block long-term potentiation (LTP) of inhibitory synapses (Nugent et al., 2007), which could increase the likelihood of other inputs eliciting an excitatory phasic response.

We found that the proportion of cue-inhibited cells correlated with conditioned fear. While these results should be interpreted with caution as they were obtained from only 2 subjects, it is worth considering whether these cells represent the same population as the VTA confirmed DA cells inhibited by tailpinch in the anesthetized preparation of Ungless et al. (2004). It appears that this is unlikely, as only one of the cue-inhibited cells in our study was inhibited by footshock, and many were instead excited. The neurochemical identity of this population could not be ascertained in this study, as it has been recently shown that classical identification criteria for VTA DA neurons are insufficient (Margolis et al., 2006b; Fields et al., 2007; Lammel et al., 2008; Luo et al., 2008; but see Grace et al., 2007), and this presents an important question for future study.

It is often assumed that the VTA is part of a reward pathway, or one selective for learning about rewards. However, lesion and inactivation studies have shown that the VTA is required for conditioned fear (Borowski and Kokkinidis, 1996; Munro and Kokkinidis, 1997; Greba et al., 2000a; Gifkins et al., 2002) and phasic responses to aversive events have been observed in the VTA (Mirenowicz and Schultz, 1996; Guaracci and Kapp, 1999; Ungless et al., 2004; Anstrom and Woodward, 2005). The cells excited by fear-predictive cues in our study were not selective for aversive stimuli, as most of them were also excited by sucrose. This suggests that at least some VTA cells excited during reward processing are involved in learning about negative outcomes, and points to a general role in associative conditioning rather than one specific to hedonic

valence (i.e., whether the event predicted is “good” or “bad”). Future studies will determine whether VTA neurons encoding conditioned fear to cues are the same cells as those encoding reward prediction.

In summary, we recorded the activity of VTA neurons during a Pavlovian fear conditioning procedure, and found phasic responses to cues predicting footshock delivery in ~40% of isolated single units. The proportion of cue-inhibited cells, and the strength of cue responses in cue-excited cells, correlated with the fear expressed behaviorally by subjects to the cues. These phasic responses appear very similar to those seen in VTA neurons to reward-predictive cues, and most cells excited by the fear-conditioned cue were excited during reward consumption, suggesting that these VTA neurons are not generally selective for the hedonic valence of events.

Chapter 4

Conclusions and Future Directions

The studies presented in this thesis demonstrate that there are at least two functionally distinct neural populations in the VTA which show excitatory responses to Pavlovian appetitive and aversive conditioned cues. A novel population, Incongruent cells, is inhibited by reward, and shows no changes in its cue excitation with reward learning; it may therefore encode sensory aspects of the cue. Another population, Congruent cells, displays response properties similar to those found in presumed DAergic neurons in other studies: it is excited by reward, its cue excitation encodes reward prediction and motivation, and it shows an inhibitory response when expected reward is omitted. However, in a separate study, a population of cells was found with excitatory responses to fear-predictive cues which look strikingly similar to Congruent responses to reward-predictive cues, and which also strengthened with learning. Most neurons in this conditioned fear-encoding population were also excited by reward, further suggesting they may include Congruent cells. While a directly comparative study must be completed for certainty, it is likely that Congruent VTA neurons encode the fear-predictive as well as the reward-predictive properties of cues. What roles might these two neural populations play in learning about rewards and punishments?

Let us return to our original formulation: "To survive in natural environments, organisms must learn to approach stimuli which predict positive outcomes (food, water, sex, shelter) and avoid stimuli which predict negative outcomes (pain, hunger, thirst, attack from predators). This requires animals to learn (a) *salience* - which stimuli in an

environment deserve attention and are behaviorally relevant, and (b) *valence* - whether these stimuli predict "good" or "bad" events." Although often described as part of the "reward pathway" of the brain, the VTA does not appear to encode the hedonic valence of cues, as similar neural responses were seen to cues predicting sucrose or shock. Rather, these neurons appear to encode two separate aspects of salience: *sensory salience* ("something is happening!") and *motivational salience* ("you need to do something about it!").

Why should there be neural encoding of sensory salience at all, if such a signal communicates no information about obtaining reward or avoiding pain? Let us say that every morning when you wake up on the savannah, you see a wildebeest running quickly and an antelope sitting quietly, chewing some grass. Nothing particularly good or bad ever happens right after you see these animals. You will probably ignore both, because they lack *motivational salience*. The first time you saw them, you paid attention, but you have since learned that you do not need to do anything to get something good or avoid something bad when you see them, so you have habituated to their presence. However, while you may now barely notice the antelope, you will likely always notice the wildebeest because of its *sensory salience*; it is a large object in fast motion. Adaptively, it makes sense that we should pay more attention to things with a high degree of sensory salience (very loud, bright, or quickly moving objects), because they are more likely to be followed by motivationally relevant events. For example, it is much more likely that, one day, you will see a lion running after the running wildebeest than that you will see a lion next to the sitting antelope. It is therefore unsurprising that an area of the brain which encodes motivational salience also encodes sensory salience, and it is an exciting

possibility that these distinct aspects of a cue's salience may be mediated by the activity of two separate neural populations in the VTA. There is a current debate in the field about whether phasic responses in VTA neurons occur too quickly to encode the rewarding or reward-predictive properties of a stimulus (most recently elaborated in Redgrave et al., 2007). These authors suggest that short-latency (70-100 ms), short-duration (100-200 ms) phasic excitations in presumed DA neurons cannot encode reward prediction errors because they end prior to visual saccades (>200 ms after cue onset), so the animal cannot have yet identified the cue as rewarding. In light of this argument, it is important to note that associative conditioning was reflected in a lengthening of the cue response in Congruent neurons. Therefore, while the initial phase of the cue response may not reflect motivational salience, the extended excitation does, and occurs at a timescale well-suited for a distinction between meaningful and meaningless salient events.

Similarities between VTA responses to rewarding and aversive events

At first glance, similar responses to appetitive and aversive events in a brain region so strongly implicated in reward processing may seem puzzling. However, we should remember that reward and punishment can be thought of as two ends of a continuum. The absence of reward (hunger or thirst, for example) is aversive, and the absence of punishment (safety) is rewarding, and these hedonic effects are amplified when an outcome which is expected does not occur (as in disappointment or relief). But if it is true that VTA neurons, perhaps including DA neurons, are similarly excited by cues predicting both reward and punishment, how can this brain region appropriately modulate

approach and avoidance behavior? There is evidence that the posterior VTA may be more important in mediating drug reward than the anterior VTA (reviewed in Ikemoto, 2007); however, we found many individual VTA cells excited by both reward and shock. One interesting view is that mesolimbic DA "promotes the emergence of the *seeking* emotional disposition…an affective urge that characterizes all motivated behaviors" (Alcaro et al., 2007). These authors suggest that DA, by affecting large-scale rhythms in brain activity, may release "neurodynamic instinctual sequences". These sequences, while they have a genetic component, are very adaptable to the environment, and include exploratory behaviors such as orienting, sniffing, and visual saccades. If DA simply releases these sequences rather than choosing them according to valence, increased firing of DA neurons could elicit the seeking of either rewards or safety. Hedonic valence may be encoded in other brain regions interconnected with the VTA; for example, there appear to be specific "hot spots" in the NAc and ventral pallidum in which opioids enhance orofacial "liking" reactions (Pecina and Berridge, 2005; Tindell et al., 2006; Pecina et al., 2006). A major proponent of the incentive salience hypothesis, although he suggests that DA has specific contributions to reward, states that "it is beyond dispute that dopamine manipulations affect the performance strength of action patterns" (Berridge, 2007). While many aspects of DA functioning are not addressed completely by the "seeking" hypothesis, the idea that DA release does not explicitly assign valence, but instead allows for the expression of behaviors which are otherwise determined to be appropriate for the situation, fits well with our finding of similar VTA responses to appetitive and aversive cues.

Cell types in the VTA

It has been recently demonstrated that the classical criteria generally used to identify DA neurons *in vivo* exclude some tyrosine hydroxylase-positive neurons and include some tyrosine hydroxylase-negative neurons (Margolis et al., 2006b; Fields et al., 2007; Lammel et al., 2008; Luo et al., 2008; but see Grace et al., 2007). Although VTA function is often equated with VTA DAergic function, it has been known for quite some time that VTA projections to different targets contain varying amounts of DA neurons (Swanson, 1982; Fields et al., 2007). There exists a large population of non-DAergic VTA neurons (up to 45%; Swanson, 1982; Margolis et al., 2006b), some of which are likely to be GABAergic VTA neurons which project to the PFC and NAc (Van Bockstaele and Pickel, 1995; Steffensen and Henriksen, 1998; Carr and Sesack, 2000, Margolis et al., 2006b). Additionally, some VTA neurons are glutamatergic (Kawano et al., 2006; Yamaguchi et al., 2007) and VTA stimulation induces glutamate release in the PFC and NAc, which some authors suggest is due to the co-release of glutamate by DA neurons (Sulzer et al., 1998; Chuhma et al., 2004; Lavin et al., 2005; Lapish et al., 2007).

There is some pharmacological evidence that non-DAergic VTA neurons may play a role in mediating reward processing (Nader and van der Kooy, 1997; Laviolette and van der Kooy, 2001). However, because there has been very little examination of the responses of non-DA VTA neurons during appetitive or aversive events, aside from that which assumes they are interneurons and that their responses are only important insofar as they affect DA function, we are here comparing our data against DA-specific theories of VTA function. This may be appropriate given the similarity of the responses seen in our Congruent cells (negative reward prediction error, strengthening of cue response with

learning) with those seen in other recording studies of presumed DA neurons, as well as because any claim to have identified neurons as DAergic in an *in vivo* recording study is unsubstantiated (with the exception of those studies which utilize juxtacellular labeling, which has only been attempted in anesthetized preparations). However, as we cannot determine the neurochemical identity of the neurons we recorded, speculation about their function can and should take into account the possibility that any of them could be DAergic, GABAergic, glutamatergic, some combination thereof, or even none of the above. Certainly a complete understanding of the VTA requires that the neurotransmitter content of our functionally-defined neural populations be determined, and this presents an important, although technically very difficult, question for future studies.

VTA Inputs

There are afferent projections to the VTA from various brain regions important for sensory perception. For example, the superior colliculus (SC) is a critical source of visual input to midbrain DA neurons (Comoli et al., 2003; Dommett et al., 2005; Coizet et al., 2006). In addition, the pedunculopontine nucleus, an area which contains glutamatergic, GABAergic and cholinergic cells that project to the VTA (reviewed in Winn, 2006), shows excitatory responses to auditory, and, to a lesser degree, visual stimuli (Pan and Hyland, 2005). When this area was inactivated, excitatory responses to reward-predictive tone and light cues in presumed DA VTA neurons were decreased but not completely abolished (Pan and Hyland, 2005), suggesting that afferents from other brain regions may also contribute to excitatory conditioned cue responses in presumed DA VTA neurons. It has also been shown that the laterodorsal tegmentum, a brain region implicated in

attention and alerting responses, is essential for burst firing in VTA DA neurons (Lodge and Grace, 2006).

There are also VTA inputs from many brain regions known to be important in motivation and goal-directed behavior. For example, there is a direct excitatory input to the VTA from the PFC onto DA and non-DA cells (Sesack and Pickel, 1992), which stimulates burst firing in DA neurons (Overton and Clark, 1997). The central nucleus of the amygdala (CeA) also projects directly to the VTA (Fudge and Haber, 2000) and CeA-lesioned rats are impaired in the acquisition of conditioned approach to both visual and auditory CSs (Gallagher et al., 1990; Cardinal et al., 2002). It has been suggested that the pathway from the CeA to the VTA to the PFC mediates footshock-induced reinstatement of cocaine-seeking (McFarland et al., 2004). The basolateral nucleus of the amygdala (BLA) is required for CS-potentiated feeding (Holland and Gallagher, 2003) and encodes motivation and reinforcement (Tye and Janak, 2007), and its projection to the lateral hypothalamus is activated by food-predictive cues (Petrovich et al., 2005). The lateral hypothalamus, in turn, contains VTA-projecting orexin neurons known to be important for reward-seeking (Harris et al., 2005) and synaptic plasticity (Borgland et al., 2006) in the VTA. Other inputs not considered here include the NAc, the ventral pallidum, the locus coeruleus, the dorsal raphe, and the bed nucleus of the stria terminalis. Recent work has also shown that VTA inputs selectively target DAergic and GABAergic neurons projecting to certain areas but not others, suggesting the existence of segregated neural circuits through the VTA (Carr and Sesack, 2000; Omelchenko and Sesack, 2005; 2006; Balcita-Pedicino and Sesack, 2007). It remains to be seen whether activation of sensory inputs such as the pedunculopontine nucleus and the superior colliculus induces

only short-latency, short-duration cue excitations in VTA neurons, while the enhancement of the strength and duration of cue excitations with learning and on trials in which the animal chooses to respond is mediated by inputs from motivationally important regions such as the amygdala.

VTA Projections

Perhaps the VTA exerts differential effects in rewarding and aversive situations via differential activation of its projection targets. VTA neurons project to a variety of brain regions, including, but not limited to, the NAc, the mPFC, the amygdala, and the olfactory tubercle (Swanson, 1982), and investigations of VTA responses to behavioral correlates suggest that these neurons are a functionally heterogeneous population (Guaracci and Kapp, 1999; Ljungberg et al., 1992; Mirenowicz and Schultz, 1994, 1996; Schultz, 2002). This raises the possibility that some of the conflicting results discussed above could be explained by differential DA release in its target areas. However, the available data show no clear differences in target-specific DA release in appetitive and aversive paradigms. Aversive events or CSs cause DA release in the mPFC (Feenstra et al., 2001; Yoshioka et al., 1996), the NAc (McCullough et al., 1993; Pezze et al., 2001; Young et al., 1998), and the amygdala (Morrow et al., 2000a; Coco et al., 1992). Similarly, appetitive events or CSs also cause DA release in the mPFC (Richardson and Gratton, 1998, but see Mingote et al., 2004), the NAc (Cheng et al., 2003; Datla et al., 2002; Di Ciano et al., 1998; Ito et al., 2000; Phillips et al., 2003; Richardson and Gratton, 1996; Roitman et al., 2004, Day et al., 2006), and the amygdala (Harmer and Phillips, 1999; Nomura et al., 2004; Fallon et al., 2007). Most of these studies examining DA

release in appetitive and aversive procedures have found release in multiple brain regions (Abercrombie et al., 1989; Bassareo and Di Chiara, 1997; Wilkinson et al., 1998; Jackson and Moghaddam, 2004; Fallon et al., 2007). Few studies have specifically examined differential firing in VTA neurons projecting to different brain areas. Noxious tailpinch in anesthetized rats elicits responses (2/3 excitations, 1/3 inhibitions) in PFC-projecting, but not NAc-projecting, VTA neurons (Mantz et al., 1989). VTA DA neurons projecting to the NAc and the BLA are differentially inhibited by opioids (Ford et al., 2006), which may contribute to different responses during painful events. Interestingly, conventional slow-firing VTA DA neurons (the neurons selected for recording in most *in vivo* studies) only project to the NAc shell and the dorsolateral striatum, while newly-discovered fast-firing VTA DA neurons project to the NAc core and medial shell, the PFC, and the BLA (Lammel et al., 2008). It is also possible that DA differentially affects other inputs into areas in which it is released. For example, it has been suggested that phasic DA release in the NAc selectively facilitates hippocampal inputs (via D1 receptor activation) while increases in tonic DA release attenuate, and decreases facilitate, PFC input (via D2 receptor activation) (Goto and Grace, 2005). While the results presented in this thesis cannot speak to the projection targets of the neurons recorded, future work may be able to determine, using antidromic stimulation from projection targets, whether conditioned strengthening of reward-predictive and fear-predictive cue responses occurs selectively in VTA neurons projecting to different brain regions.

Post-synaptic effects

In slice preparations, DA increases the excitability of neurons in the NAc (Hopf et al., 2003) and the basolateral amygdala (Kroner et al., 2005) via cooperative direct actions on dopamine D1 and D2 receptors. In addition, while DA inhibits release of both GABA and glutamate in the NAc, only inhibition of GABA is sustained through stimulus trains, resulting in a net excitatory effect on post-synaptic cells (Hjelmstad, 2004). In the lateral amygdala, DA gates the induction of long-term potentiation (LTP, a measure of synaptic strength often thought of as a neural substrate of learning) by suppressing feedforward inhibition from local interneurons (Bissiere et al., 2003). It has also been suggested that DA release in the BLA suppresses the inhibitory influence of PFC inputs, which "is expected to maximize affective responses to sensory stimuli, as well as plasticity" (Grace and Rosenkranz, 2002). DA-dependent LTP has been found in the NAc in some studies (Kerr and Wickens, 2001; Schotanus and Chergui, 2008a) but not others (Pennartz et al., 1993). However, LTP was only absent when experiments were conducted in the presence of GABA antagonists, and therefore did not reflect the tonic inhibition that is removed by DA release, resulting in a net excitation (Hjelmstad, 2004; Nicola et al., 2004). That LTP may be related to NAc-dependent learning is suggested by the fact that both LTP (Kerr and Wickens, 2001; Schotanus and Chergui, 2008a,b) and appetitive instrumental learning (Smith-Roe and Kelley, 2000) involve activation of both DA and NMDA glutamate receptors in the NAc.

Congruent cue excitations are stronger to motivationally-relevant appetitive and aversive cues, while Incongruent excitations appear to encode mainly the sensory properties of the cue. A proper discussion of post-synaptic effects would require knowledge of the neurochemical identity of these neurons, but unfortunately this

information cannot be obtained in freely moving animals with currently available techniques. But for the sake of argument, let us assume for a moment that Congruent cells are DAergic and Incongruent cells are GABAergic. At the very least, Congruent responses to both cues and rewards, and Incongruent responses to rewards, are consistent with those reported in other studies for presumed DA and GABA VTA neurons, respectively. Now let us also assume that these two classes of cells project to the same target regions. In this model, when a cue is important (i.e. has motivational salience), the cue responses in Congruent cells will be stronger (larger and more sustained) than the ones in Incongruent cells. If DA is capable of directly suppressing inhibitory synaptic transmission in the given target area, as has been suggested in the NAc and amygdala (Hjelmstad, 2004; Bissiere et al., 2003), then the net signal of cue onset received by the target area will be permissive for the induction of LTP. However, if a cue is unimportant (lacks motivational salience), the Congruent cue response will be weaker than the Incongruent response, the target area will not only remain under tonic inhibition but will also be further inhibited by the cue-induced GABA release, and LTP will not be induced. In terms of how this would modulate the effects of other inputs to the target area, it is relevant to note the suggestion by Hjelmstad (2004) that NAc inputs exhibiting "longer, higher-frequency bursts, presumably encoding more salient information, will be excited [by DA] to a greater degree than shorter, slower (and less salient) bursts." This view is similarly expressed by Nicola et al. (2000; 2004) and Horvitz (2002); namely, that NAc responses to strong excitatory inputs are strengthened, and those to weak excitatory inputs are weakened, in the presence of DA, improving the "signal-to-noise ratio" of processing in this circuit. A similar role for DA in the PFC has been postulated, although

here it appears to be dependent on which activation state the post-synaptic neuron is in ("up" – relatively depolarized, and therefore close to spike threshold, or "down" – relatively hyperpolarized from spike threshold) (Seamans et al., 2001; Durstewitz and Seamans, 2002; Peters et al., 2004). In PFC neurons, it has been found that VTA stimulation inhibits spontaneous firing but enhances evoked firing (Lavin et al., 2005), and, interestingly, the short onset latency of this effect led the authors to suggest that it was mediated by glutamate co-released with DA. While we must emphasize that the neurochemical content of the neurons we recorded is unknown, neural responses which encode motivational salience are consistent with a role in contrast enhancement if they reflect the release of DA and with an increase in general excitability if they reflect the release of glutamate.

Inhibition to omitted reward

If attribution of motivational salience, mediated by an increase in signal-to-noise ratio, is the function of excitatory responses to reward- and fear-predictive cues in a subset of VTA neurons (which likely includes some DA neurons), what information is then conveyed by an inhibition at the time of expected reward when it is omitted? An interesting possibility is that it induces a pause in contrast enhancement (which favors only strong inputs) to allow weaker excitatory inputs to affect the neural circuit. Behaviorally, this may contribute to the flexibility of adaptive behavior, as follows: When an expected outcome fails to occur, what the animal has already learned about this environment is not valid in this particular instance, and there may be stimuli present which provide information about why this is the case. The animal's attention should

therefore be broadened from the cue with which it already has a learned association, to all stimuli available, to promote a more accurate understanding of the relationships of these stimuli to important events. If VTA cue-excited neurons encode motivational salience regardless of hedonic value, this should be equally true in situations when an expected aversive outcome fails to occur, and an upcoming study will directly examine the possibility that VTA neurons inhibited by reward omission are also inhibited by the omission of an expected shock.

A modulatory or causal role in learning?

There is evidence that reward learning may become DA-independent with extended training (Choi et al., 2005). If DA serves mainly to increase the signal-to-noise ratio of other inputs to target areas, then it may not be required for behaviors in response to conditioned cues so well-learned (or so instinctual) that these inputs already have very strong excitatory effects. This may explain why DA is not required for unconditioned consummatory (Salamone et al., 1997; 2003) and fear (McCullough et al., 1993; Borowski and Kokkinidis, 1996) responses, as primary stimuli may induce sufficiently strong excitatory effects to elicit behavior on their own. This is also consistent with the finding in presumed DA neurons that excitatory responses to reward-predictive cues will diminish with very extended training (Ljungberg et al., 1992), and with observations that Parkinson's patients, while finding it difficult to initiate movement in response to internal or weak external cues, have much less difficulty in responding normally to highly salient cues such as fire alarms.

It has been argued that DA may not be required for simple reward learning (Berridge and Robinson, 1998; Cannon and Palmiter, 2003; Hnasko et al., 2005; Robinson et al., 2005), but others have shown that it is required for behavioral responses and NAc firing to reward-predictive cues (Yun et al., 2004a,b), as well as for the acquisition of conditioned fear (Greba et al., 2000b). It is quite possible that the learning-related changes seen in DA release and the firing of presumed DA neurons are "a consequence, and not a cause, of activity in other neural systems that are more directly responsible for learning computations" (Berridge, 2007). This provides an additional means of understanding the significance of the encoding of both appetitive and aversive associative conditioning in the VTA; perhaps this activity simply imbues cues with motivational salience generally, increasing the likelihood of innate and learned responses to such stimuli, while activity in other interconnected neural circuits encodes their hedonic valence, and elicits the specific approach or avoidance behaviors which are relevant to the situation. Transient inactivation of the VTA during the acquisition, but not the expression, of appetitive and aversive conditioned behavior may be able to answer the question of whether the neural changes required for learning take place in the VTA itself or elsewhere.

In summary, we recorded the activity of VTA neurons during appetitive and aversive Pavlovian conditioning in awake, behaving rats. We found two populations of VTA neurons excited by a visual reward-predictive cue in the appetitive Pavlovian conditioning procedure: a Congruent population excited by reward, as well as an Incongruent population inhibited by reward. The Congruent population displayed cue response characteristics often ascribed to presumed DA neurons, such as learning-related

enhancement, extinction-related decrement, correlation with motivation, and a negative reward prediction error signal. The Incongruent population did not display these cue response characteristics, and may encode the sensory salience of the cue. In the Pavlovian fear conditioning procedure, we found phasic responses to visual and auditory cues predicting footshock delivery. The proportion of cue inhibitions, and the strength of cue excitations, encoded behaviorally expressed cue-elicited fear. Conditioned phasic responses to appetitive and aversive cues were very similar, and most cells excited by the fear-conditioned cue were excited during reward consumption. Taken together, our findings suggest that VTA neurons whose responses change with learning are not generally selective for the hedonic valence of events, but may instead reflect the motivational salience of both rewarding and aversive events.

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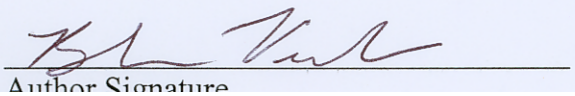

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