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Neural and Behavioral Regulation of Defensive Response Strategies

A dissertation submitted in partial satisfaction of the requirements for the degree

Doctor of Philosophy in Psychology

by

Lindsay Renee Halladay

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ABSTRACT OF THE DISSERTATION

Neural and Behavioral Regulation of Defensive Response Strategies

by

Lindsay Renee Halladay

Doctor of Philosophy in Psychology

University of California, Los Angeles, 2013

Professor Hugh T. Blair, Chair

Fear and defensive behaviors are regulated by complex interactions among many different brain regions. Pathways for individual responses to fear such as freezing and startle potentiation have been well defined, but mechanisms for selecting between competing defensive responses are not fully understood. Research presented here has focused on elucidating the neural mechanisms modulating defensive responding. Pharmacological experiments investigating how neuromodulatory signaling in the periaqueductal gray (PAG) mediates conditional and unconditional responses to aversive stimuli showed that mu-opioid receptors may selectively regulate descending supraspinal motor pathways that drive active movement behaviors. Additional pharmacological experiments investigated the role of medial prefrontal cortex (mPFC) in defensive action selection. Results suggested that mPFC might bidirectionally modulate active defensive responding. Electrophysiological

recording experiments identified individual cell populations in areas of mPFC that seem to mediate conditional freezing and flight, indicative of a more diverse role for mPFC in regulating defensive expression than other past studies have shown. Finally, behavioral experiments examining the nature of fear reinstatement showed for the first time that defensive freezing can be reinstated by a "reminder" stimulus rather than an aversive event. Findings presented here provide evidence for a novel, hypothetical model of how fear and defensive behaviors are mediated by neural circuits in the amygdala (AMG), PAG, mPFC.

The dissertation of Lindsay Renee Halladay is approved.

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2013

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Halladay LR, Blair HT (submitted) Medial prefrontal cortex and midbrain periaqueductal gray regulate transitions between competing defensive response strategies.

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locomotor activity in the young rat: possible role of the indirect pathway. Neuroscience 155:603-612.

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Halladay LR, Zelikowsky M, Blair HT, Fanselow MS. *Vicarious reinstatement of extinguished fear responses.* Poster presented at the Society for Neuroscience 41st Annual Meeting, Washington, D.C., 2011

Halladay LR, Blair HT. *Impaired expression of active but not passive defensive responding after infusions of morphine into the dPAG.* Poster presented at the Society for Neuroscience 40th annual meeting, San Diego, CA, 2010

Halladay LR, Shlifer IG., Blair HT. *mPFC, amygdala, and periaqueductal gray: a model for defensive turning behavior in rats.* Poster presented at the 4th Annual Neural Microcircuits Training Program Symposium, UCLA, 2009

Halladay LR, Shlifer IG, Tarpley JW, Blair HT. *Role of medial prefrontal cortex in defensive action selection.* Poster presented at the Society for Neuroscience 38th Annual Meeting, Washington, D.C., 2008

Halladay LR, Shlifer IG, Tarpley JW, Blair HT. *The role of mPFC in defensive turning behavior in rats.* Poster presented at the 6th FENS Forum of European Neuroscience, Geneva, Switzerland, 2008

Halladay LR, Tarpley JW, Shlifer IG, Blair HT. *The role of medial prefrontal cortex in defensive turning behavior in rats.* Poster presented at the 3rd Annual Neural Microcircuits Training Program Symposium, UCLA, 2008

Chapter 1

General Introduction

1.1 OVERVIEW

Fear and defensive behaviors are regulated by complex interactions among many different brain regions. Dysfunction in any of these brain regions can result in inappropriate defensive behaviors, as seen in cases of anxiety disorders like posttraumatic stress disorder (PTSD) (Gueze et al., 2008; Kasai et al., 2008), specific phobia (Straube et al., 2007), social anxiety disorder (Evans et al., 2008), obsessive-compulsive disorder (OCD) (Millet et al., 2013), and generalized anxiety disorder (Krain et al., 2008) (for reviews see Davidson et al., 1999; Kent and Rauch, 2003). Pathways for individual responses to fear such as freezing and startle potentiation have been well defined, but mechanisms for selecting between competing defensive responses are not fully understood. My doctoral dissertation research has focused on elucidating the neural mechanisms modulating defensive responding. Findings presented here provide evidence for a novel, hypothetical model of how fear and defensive behaviors are mediated by neural circuits in the amygdala (AMG), periaqueductal gray (PAG), and medial prefrontal cortex (mPFC). Understanding how activity within the brain's "fear" circuitry determines which defensive responses are appropriate for specific situations will provide important new insights into the neural mechanisms regulating both normal and pathological fear states.

1.2 PAVLOVIAN FEAR CONDITIONING

A widely used procedure employed in animal models of fear and anxiety is Pavlovian fear conditioning, in which animals come to fear a neutral conditional stimulus (CS), such as an auditory tone, by pairing it with a previously non-associated, aversive stimulus

(unconditional stimulus, or US), such as an electrical shock. The animal quickly learns that the CS predicts the US, and as a result, the CS evokes conditional fear responses in the absence of the US. In rodent fear conditioning experiments, some of the most commonly measured conditional fear responses are freezing (Blanchard and Blanchard, 1969a; Fanselow, 1980), startle potentiation (Davis and Astrachan, 1978; Hitchcock and Davis, 1986), and ultrasonic vocalizations (Blanchard and Blanchard, 1990; Lee et al., 2000; Borszcz and Leaton, 2003; Kim et al., 2010). It is commonly assumed that expression of one of these defensive behaviors during an experiment is a monotonic function of fear (more of the behavior indicates more fear, and less of the behavior indicates less fear). But this assumption can be problematic because animals can display a range of defensive behaviors based upon the perception of threat. Stages of threat vary along a "predatory imminence continuum," spanning from low-risk (pre-encounter) to high-risk (interaction) threats (Blanchard and Blanchard, 1969b; Bolles, 1970; Fanselow and Lester, 1988). As threat levels increase, defensive response strategies change, often transitioning from a passive defense such as freezing ("post-encounter") to a more active defense such as flight or even attack ("circa strike") (Fanselow and Lester, 1988; Fanselow, 1989, 1994; Blanchard and Blanchard, 1990). Thus, assuming that fear levels can be accurately measured by a single behavior may sometimes lead to an inaccurate, or incomplete, measure of fear. To address this problem, our lab has developed a novel Pavlovian fear conditioning paradigm which permits measuring more than one behavioral defense, so that fear can be measured more accurately over a broader range of intensities (Tarpley et al., 2010; Halladay and Blair, 2012). Rats are trained to fear an auditory CS (white noise pips; 20 sec) by pairing it with a

shock US (2 sec train) to one of their eyelids (see Figure 1.1A). Each training day is comprised of 6 "test" trials, during which rats receive 6 presentations of the CS alone, followed immediately by 16 "training" trials, which consist of CS-US pairings. On a given experiment day, we define 'pre-shock trials' as the 7 trials during which the CS and CX (20 sec prior to CS onset) periods both preceded the day's first US presentation (all 6 CS alone trials, plus the first CS-US pairing trial), whereas 'post-shock trials' are defined as the remaining 15 CS-US pairing trials (Figure 1.1A). The conditional response elicited in our rats is dependent upon how recently they encountered the US. Each day, during the initial CS alone test trials (>24h since last encounter with US), trained rats will freeze in response to the CS. But when CS-US pairing trials are given during the training session, shock delivery causes the rat to start performing a specific flight behavior during the CS: turning away from the trained eyelid in anticipation of the shock. Importantly, trained rats will turn away from the trained eyelid even if they have recently been shocked on the untrained eyelid. Hence, tone-evoked turning away from the anticipated eyelid shock appears to be a state-dependent conditional response that depends not only upon the contingency between the CS and US, but also upon the rat's momentary level of "anxiety" or "predatory imminence" at the time when the CS is presented.

In accordance with the assumptions of the predatory imminence continuum, (Fanselow and Lester, 1988; Fanselow, 1989, 1994) our rats appear to express maximum freezing during an intermediate level of fear (see Figure 1.1B). Higher levels of fear elicit a switch in defensive strategy; conditional behavior changes from freezing to flight (which we measure as avoidant turning). Because freezing and flight are mutually exclusive defensive

strategies, defensive freezing observed in our animals often decreases while defensive turning increases. As indicated by the figure, as an animal's predatory imminence level changes from intermediate to high (due to US exposure, akin to predatory interaction), a rat is more likely to exhibit CS-evoked turning (circa-strike) in addition to freezing (post-encounter) after a recent encounter with the US.

Although much is known about the brain circuitry underlying fear learning and expression, it is not fully understood how high levels of fear result in defensive strategy switching. Insight into the neural mechanisms mediating transitions between passive and active defenses can help elucidate the dysfunctional mechanisms that lead to the inappropriate fear behaviors seen in many anxiety disorders. Thus, the focal point of my dissertation research is examining how connections between the medial prefrontal cortex (mPFC), amygdala (AMG), and periaqueductal gray (PAG) mediate the transition between defensive strategies as fear along the predatory imminence continuum changes.

1.3 FEAR CIRCUITRY

Three primary structures mediating the learning and expression of fear are AMG, PAG, and mPFC. Figure 1.2 illustrates a recently proposed neural circuit for fear, based partly upon a recently published review by Sotres-Bayon and Quirk (2010).

1.3.1 Amygdala

Numerous past studies have implicated the AMG as a mediator of fear acquisition and expression (Klüver and Bucy, 1937; Kalin et al., 2001; LeDoux et al., 1990a, 1990b; Davis,

1992; Maren, 2001). It is believed that the AMG is important for attaching aversive motivational value to states and stimuli that predict danger (Weiskrantz, 1956; LeDoux, 2000; Blair et al., 2005b; Paton et al., 2006; Seymour and Dolan, 2008). Fear researchers often divide the amygdaloid nuclei into three functionally distinct areas: the basolateral complex (BLA), the central nucleus (Ce) and the intercalated cells (ITC).

1.3.1.1 Anatomical organization and connections

The BLA is generally considered to be the amygdalar input region. It is often divided into lateral (LA), and basal (B) nuclei. Projection neurons in BLA are glutamatergic (excitatory), multipolar cells with multi-collateral axons and spiny dendritic trees, whereas many of the local interneurons in BLA are GABAergic (inhibitory), with short axons and aspiny dendrites (Hall, 1972; McDonald, 1992). The Ce is subdivided into lateral Ce (CeL) and medial (CeM) nuclei. Efferent projections from the Ce consist almost entirely of GABAergic neurons. A majority of the cells resemble spiny striatal projection neurons, while a much smaller amount are aspiny local circuit neurons (McDonald, 1985; Pare and Smith, 1993). Recent studies by Haubensak et al. (2010) and Ciocchi et al. (2010) outlined the inhibitory circuits in the AMG. They propose that the BLA and possibly auditory thalamus excite inhibitory "CeL_{on}" cells, whose projections inhibit "CeL_{off}" neurons that normally exert inhibitory control over CeM neurons that drive freezing behavior (see below). In this way, input to CeL activates CeM through CeL_{on}- CeL_{off} disinhibition. Haubensak et al. (2010) also propose that CeM is composed of two types of inhibitory neurons, one of which controls PAG-mediated defensive behaviors (see below) via disinhibition from CeL.

The ITC are small and densely packed clusters of neurons abutting the external (lateral ITC) and internal (medial ITC) capsule, of which the internal are the fiber tracts separating BLA from Ce. Hence, ITC cells lie in between BLA and Ce. Most ITC neurons are GABAergic and have flat dendritic trees with high spine density (McDonald, 1985; Nitecka and Ben-Ari, 1987; Pare and Smith, 1993a). These neurons contain a high density of dopamine D1 and mu-opioid receptors. The small percentage of non-GABAergic ITC neurons are presumed to be acetylcholinergic (Nitecka and Frotscher, 1989).

Nuclei within the AMG interact via a variety of connections. The BLA sends direct excitatory connections to the Ce, as well as indirect connections through the ITC which appear to exert a feed-forward inhibitory influence on Ce neurons. B projects to both CeL and CeM, while LA projects solely to CeL (Krettek and Price, 1978b; Pare and Smith 1993b; Savander et al., 1995; Royer et al., 1999). Within the BLA, the LA sends excitatory glutamatergic projections to B, which in turn sends weak feedback projections back to LA.

The AMG interacts with a variety of other structures. It receives sensory input from a variety of cortical and thalamic areas. BLA sends output projections mainly to cortex and the striatum (STR), while the Ce accounts for the output projections to the brain stem. Both BLA and Ce have projections terminating in acetylcholinergic forebrain areas, regions in the thalamus and hypothalamus, and the bed nucleus of the stria terminalis (BNST). Nearly all ITC cells have projections only to other amygdalar areas (Krettek and Price, 1977; Hopkins and Holstege, 1978; Krettek and Price, 1978a; Pare and Smith, 1994).

Output from the AMG influences excitability within the brain. Ce projections can trigger release of brain stem neuromodulators such as acetylcholine (Ach), norepinephrine

(NE), and dopamine (DA). Among other roles, these neuromodulators are believed to regulate plasticity in the AMG and other structures (Hasselmo and Giocomo, 2006; Abraham, 2008).

1.3.1.2 Functional role in fear and defensive behaviors

Lesion studies involving the AMG have shown that it mediates behavioral expression of fear and awareness of threat (Weiskrantz, 1956; Klüver and Bucy, 1937; Davis, 1990; Tarpley et al., 2010), suggesting that perception of imminent threat in particular situations is highly dependent upon actions of the AMG. In fear conditioning models, the AMG encodes the emotional state of fear that is elicited by the CS after it has become associated with the US (Davis and Lee, 1998; Fanselow and LeDoux 1999; LeDoux 2000). Synaptic plasticity has been reported to occur in the AMG (LA) both in vitro (Chapman et al., 1990; McKernan and Shinnick-Gallagher, 1997) and in vivo during fear conditioning (Maren and Fanselow, 1995; Quirk et al., 1995; Rogan and LeDoux, 1997; Blair et al., 2001; 2005b; Goosens and Maren, 2002), which seems to be the mechanism by which CS-US associations are formed. This is thought to be why acquisition and expression of fear conditioning is greatly impaired after AMG damage (LeDoux et al., 1990a,b; Davis, 1992; Amorapanth et al., 2000; Nader et al., 2001; Goosens and Maren, 2001; Maren, 2001; Gale et al., 2004; Blair et al., 2005a).

Much has been learned about the functional organization of fear circuits in the AMG. In the case of an auditory CS, it is believed that sensory inputs from the inferior colliculus (IC) or thalamic relays synapse in the medial geniculate nucleus (MGN) or the posterior

intralaminar nucleus (PIN), and that projections from these neurons first arrive in the LA of the AMG, which then send axons to CeL (LeDoux et al., 1990a, 1990b; Shinonaga et al., 1994; Woodsen et al., 2000; and see Davis, 2000). The Ce then projects to neurons in PAG and other midbrain structures, brainstem, and cortex, where behavioral and endocrine responses to fear are induced (LeDoux 2000; Davis and Whalen, 2001). AMG-dependent fear can lead to many different kinds of defensive responses, including both active and passive avoidance behavior. It is possible that different nuclei within the AMG may differentially be responsible for these distinct behaviors. Some evidence suggests that outputs from LA may modulate active defensive responding, whereas those from Ce modulate passive defense responding (Killcross et al., 1997; Amorapanth et al., 2000).

1.3.2 Periaqueductal Gray

The midbrain periaqueductal gray (PAG) is an important target of AMG output, which is believed to mediate behavioral expression of conditioned and unconditioned defensive responses to aversive stimuli (Fanselow, 1991; Carrive, 1993). Projections from the AMG to the PAG are necessary for the expression of conditioned freezing (LeDoux et al., 1988; Fanselow, 1991; De Oca et al., 1998). Whereas the AMG is thought to assign aversive value to stimuli and situations, the PAG seems to be important for converting that emotional value into a specific behavioral action.

1.3.2.1 Anatomical organization and connections

The midbrain PAG is known to be involved in nociception and sensory processing of aversive stimuli (Yaksh et al., 1976; Basbaum and Fields, 1984). Anatomically, the PAG is divided into four columns that lay along the cerebral aqueduct. The columns are dorosmedial (DMPAG), dorsolateral (DLPAG), lateral (LPAG), and ventrolateral (VLPAG). At a more gross level, the PAG is commonly subdivided into dorsal and ventral regions (dPAG and vPAG, respectively), where dPAG is comprised of DMPAG, DLPAG, and the dorsal part of LPAG, and vPAG is comprised of the ventral LPAG and VLPAG. Evidence from lesion and stimulation studies suggests that dPAG may control flight-related defensive behaviors (Halpern, 1968; Liebman et al., 1970; DiScala et al., 1984; Bandler and DePaulis, 1991; Fanselow, 1991; DePaulis et al., 1992; De Oca et al., 1998; Morgan et al., 1998; Keay and Bandler, 2001; Tarpley et al., 2010; Halladay and Blair, 2012), and activation of serotonergic receptors in rodent dPAG can induce the fight or flight response which may manifest as flight behavior and/or aggression in defensive response to imminent danger (Deakin and Graeff, 1991; Beckett and Marsden, 1997; Jacob et al., 2002). In contrast to dPAG, vPAG mediates opioid analgesia and defensive freezing behavior (Fanselow and Helmstetter, 1988; De Oca et al., 1998; Morgan et al., 1998; Thorn et al., 1989; Vianna et al., 2001a, 2001b). Stimulation of serotonergic receptors in rodent vPAG results in a passive, inhibitory type of defensive behavior (Bago and Dean, 2001). It is not clear how these behaviorally opposing divisions of PAG "compete" for defensive response manifestation, but it has been suggested that serotonergic projections from vPAG to the dorsal raphe nucleus (DRN) may normally inhibit dPAG in order to prevent flight behavior until necessary (i.e., when distal

threat becomes imminent) (Graeff et al., 1997). Competing defensive behaviors may also be modulated by areas outside of PAG, such as the AMG or medial prefrontal cortex (mPFC) (Halladay and Blair, submitted).

1.3.2.2 Functional role in fear and defensive behaviors

Projections from the AMG to the PAG appear to be necessary for the expression of learned fear (Carrive et al., 2000). The AMG has direct, reciprocal connections with PAG, but these primarily involve vPAG and not dPAG (Rizvi et al., 1991). As mentioned above, several lines of evidence suggest that vPAG mediates movement suppression behaviors such as freezing, whereas dPAG drives active avoidance behaviors like flight (Depaulis et al., 1992; Kim et al., 1993; De Oca et al., 1998). Lesions to the vPAG but not dPAG attenuate freezing (Kim et al., 1993; De Oca et al., 1998; Vianna et al., 2001; Leman et al., 2003), and lesions of dPAG impair active avoidance behavior as seen during footshock (Fanselow, 1991). Additionally, recording studies show that neurons in dPAG are positively correlated with movement (which occurs during active avoidance behaviors), whereas neurons in vPAG are negatively correlated with movement (so that neuronal firing is highest when an animal is freezing, as during passive avoidance) (Tarpley et al., 2010; Halladay and Blair, submitted). It is important to note that pharmacological inactivation of PAG can block the expression of CS-evoked freezing and turning, as well as US-evoked turning (Tarpley et al., 2010), indicating that both CS- and US-evoked fear responses involve the PAG.

1.3.3 Medial prefrontal cortex

Evidence suggests that the medial prefrontal cortex (mPFC) plays a key role in modulating the expression of fear responses, by way of its projections to AMG and PAG (Quirk et al., 2003; Maren and Quirk, 2004; Laviolette et al., 2005; Maier et al., 2006; Corcoran and Quirk, 2007; Burgos-Robles et al., 2009; Halladay and Blair, submitted). There is evidence that the various contributions mPFC provides to the fear circuit are anatomically arranged within a dorsal/ventral gradient. Dorsal regions of mPFC seem to modulate motor behaviors, while more ventral regions modulate the cognitive and emotional properties of fear. The most ventral regions of mPFC seem to be involved in the visceral components of fear (Vertes, 2006; Euston et al., 2012).

1.3.3.1 Anatomical organization and connections

mPFC has many reciprocal connections with the AMG; projection neurons from mPFC innervate the BLA, where sensory information is evaluated for emotional and arousal affect (Rosen and Schulkin, 1998; Vertes, 2006; Sotres-Bayon and Quirk, 2010). Different subregions within mPFC send different projections to BLA; the prelimibic cortex (PL) sends excitatory connections to BLA, the area critical for conditioned fear expression (Vertes, 2004). Since BLA excites CE, activity in PL ultimately leads to an increase in Ce output, which generates fear via projections to lower midbrain and brainstem areas (Hopkins and Holstege, 1978). The infralimbic (IL) region of mPFC sends its excitatory projects to CeL and ITC. mPFC-ITC projections are thought to be important for fear extinction by activating ITC-

Ce feed forward inhibition (Berretta et al., 2005). Projections from mPFC also synapse in areas of PAG. The rostroventral portion of PL and IL projects to vPAG, whereas the caudodorsal portion of PL and IL projects to the dPAG (Floyd et al., 2000). Direct connections from PAG to mPFC are not known.

1.3.3.2 Functional role in fear and defensive behavior

Several recent studies have provided a substantial amount of evidence that mPFC modulates the expression of learned fear (Morgan et al., 1993; Quirk et al., 2000, 2006; Santini et al., 2001; Milad et al., 2004; Sierra-Mercado et al., 2006, 2011; Corcoran and Quirk, 2007; Quinn et al., 2008; Sotres-Bayon and Quirk, 2010). It has been suggested that some projections from mPFC inhibit output from CE, thereby acting as a gate for expression of CS-evoked fear responses (Quirk et al., 2003; Likhtik et al., 2005). This view is supported by evidence that mPFC stimulation reduces Ce responsiveness to inputs from BLA, leading to inhibition of the expression of learned fear (Milad and Quirk, 2002; Quirk et al., 2003; Likhtik et al., 2005). But conversely, other studies have found that inactivating (rather than stimulating) mPFC attenuates the expression of conditioned fear (Sierra-Mercado et al., 2006; Corcoran and Quirk, 2007; Halladay and Blair, submitted) and can have antidepressant-like effects in rats (Scophino et al., 2010). It has been argued that these conflicting results might be better clarified by examining the roles of distinct subregions in mPFC separately, since areas like PL and IL project to different brain regions and target different nuclei in AMG.

Studies that have attempted to differentiate the modulatory effects PL and IL contribute to fear learning have suggested broad roles for each region; a common consensus is that PL seems to mainly modulate the expression of fear, while IL has an opposing role – to inhibit fear (Morgan and LeDoux, 1995; Vidal-Gonzales et al., 2006; Corcoran and Quirk 2007; Hikind and Maroun, 2008; Sotres-Bayon and Quirk, 2010; Sierra-Mercado et al., 2011). The idea that PL and IL cortices bidirectionally modulate fear comes from a variety of sources showing that: 1. Microstimulation of PL and IL enhances and attenuates fear expression, respectively (Vidal-Gonzalez et al, 2006), 2. Inactivating PL disrupts fear expression but not extinction memory, while inactivating IL impairs extinction memory but not fear expression (Sotres-Bayon and Quirk, 2010; Sierra-Mercado et al., 2011), and 3. Extinction training causes PL neurons that had acquired a response to conditional stimuli to decrease their firing rates (Burgos-Robles et al., 2009) while activity is potentiated in IL neurons that initially had become depressed during fear acquisition (Gilmartin and McEchron, 2005; Quirk et al., 2006; Santini, et al, 2008).

The idea that PL and IL have opposing roles is somewhat simplistic however, since authors of studies examining mPFC-AMG connections commonly divide ventral mPFC into PL and IL subregions using dorsoventral boundaries. Others, however, split the mPFC rostrocaudally, which is more appropriate for examining its output to PAG because rostroventral PL-IL projects to vPAG, while dorsocaudal PL-IL projects to dPAG (Floyd et al., 2000). These direct projections from mPFC to PAG may also be responsible for regulating defensive responses to threatening stimuli (Floyd et al., 2000; Gabbott et al., 2005; Maier and Watkins, 2005; Price, 2005; and see Mobbs et al., 2007, 2009). Since vPAG and dPAG

are thought to mediate passive and active defense behaviors, respectively, it is possible that rostroventral mPFC may modulate freezing responses, while dorsocaudal mPFC may modulate flight. Recently, it was shown in humans that depending on proximity of threat, neuronal activity shifts from PFC to PAG, with more imminent or inescapable threat evoking activity in PAG and inhibiting activity in PFC (Mobbs et al., 2007, 2009; see also McNaughton and Corr, 2004). Mobbs et al. (2007, 2009) suggest that distal threat may involve complex planning of defensive strategies, thereby necessitating mPFC involvement (Miller, 2000; Miller and Cohen, 2001; Amat et al., 2005; Shackman, 2011), whereas more proximal threats evoke reflexive activity produced by actions of the PAG during threatening situations (Fanselow, 1991). This raises the possibility that dysfunctional activity in mPFC can lead to excessive fear expression. Human studies tend to support this possibility, since imaging studies have shown that excessive anxiety is often accompanied by abnormalities in mPFC and AMG activity (see Etkin and Wager, 2007). It has been proposed that disorders involving panic may be associated with underactivity of mPFC, and disorders involving generalized anxiety may be characterized by overactivity of mPFC (Berkowitz et al, 2007).

1.4 Outline of Chapters

Although much is known about brain circuitry underlying fear learning and the expression of freezing, many questions regarding the nature of conditional responding remain unanswered. Both freezing and flight have been used as indices of fear in past studies, but little is known about neural circuitry mediating the switch between the two behaviors. Elucidating the circuitry responsible for this transition is necessary to understand

dysfunctional mechanisms underlying anxiety in both normal and pathological instances. The purpose of this research was to determine the neural mechanisms mediating the control of strategic defensive responding. Findings are outlined below.

Chapter 2: The role of mu-opioid receptor signaling in the dorsolateral periaqueductal gray on conditional and unconditional responding to threatening and aversive stimuli

Experiment 1: Fear conditioned rats received intra-dPAG infusions of drugs targeting mu-opioid or serotonergic receptors to assess the effects of opioid or serotonin receptor stimulation upon defensive responding.

Chapter 3: The role of medial prefrontal cortex in the selection of defensive response strategies

Experiment 2: Infusions of muscimol to inactivate or picrotoxin to hyperactivate mPFC were administered to fear conditioned rats in order to investigate mPFC's role in the transition from conditional freezing to flight.

Chapter 4: Electrophysiological recordings of medial prefrontal cortex and midbrain periaqueductal gray: regulation of competing defense response strategies

Experiment 3: Single neurons were recorded in areas of mPFC and dPAG during fear conditioning to examine whether activity from distinct populations of cells corresponded with different defensive behaviors.

Chapter 5: Reinstatement of extinguished fear by an unextinguished conditional stimulus

Experiment 4: After extinction to one of two concurrently fear-conditioned stimuli, rats were presented with the unextinguished stimulus to test whether a reminder of the US could reinstate conditional fear to the extinguished stimulus.

Figure 1.1

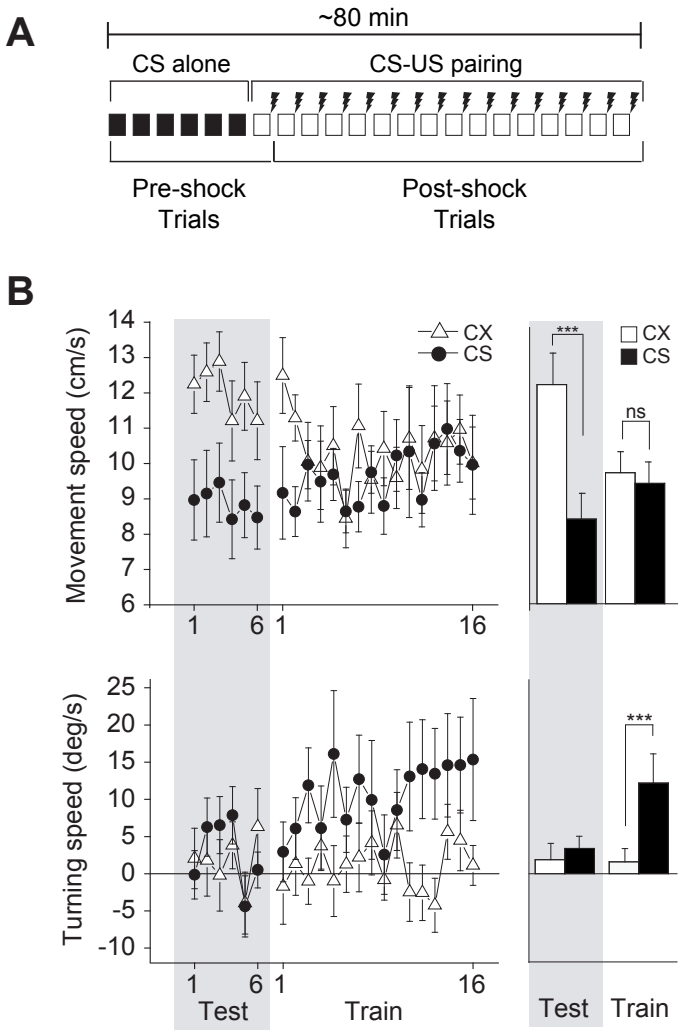

Figure 1.1. *Fear conditioning design and example behavior.* ***A***, Each session consisted of 6 CS alone trials followed by 16 CS-US pairings, separated by uniformly random intertrial intervals of 180-240 s. ***B*****,** Line graphs at left show averaged movement speed (y-axis) (top) or turning velocity (bottom; positive values indicate turning in the direction away from the trained eyelid) during the CX and CS periods in trained rats (n=30) for individual trials (x-axis) of test (shaded area) and training (unshaded area) sessions. Bar graphs at right show movement speed (top) and turning velocity (bottom) averages for test trials versus training trials, with symbols indicating significance levels for Newman-Keuls posthoc comparisons. Symbol legend: ***=p<.001, **=p<.01, ns= not significant. (Data from Halladay and Blair, 2012)

Figure 1.2

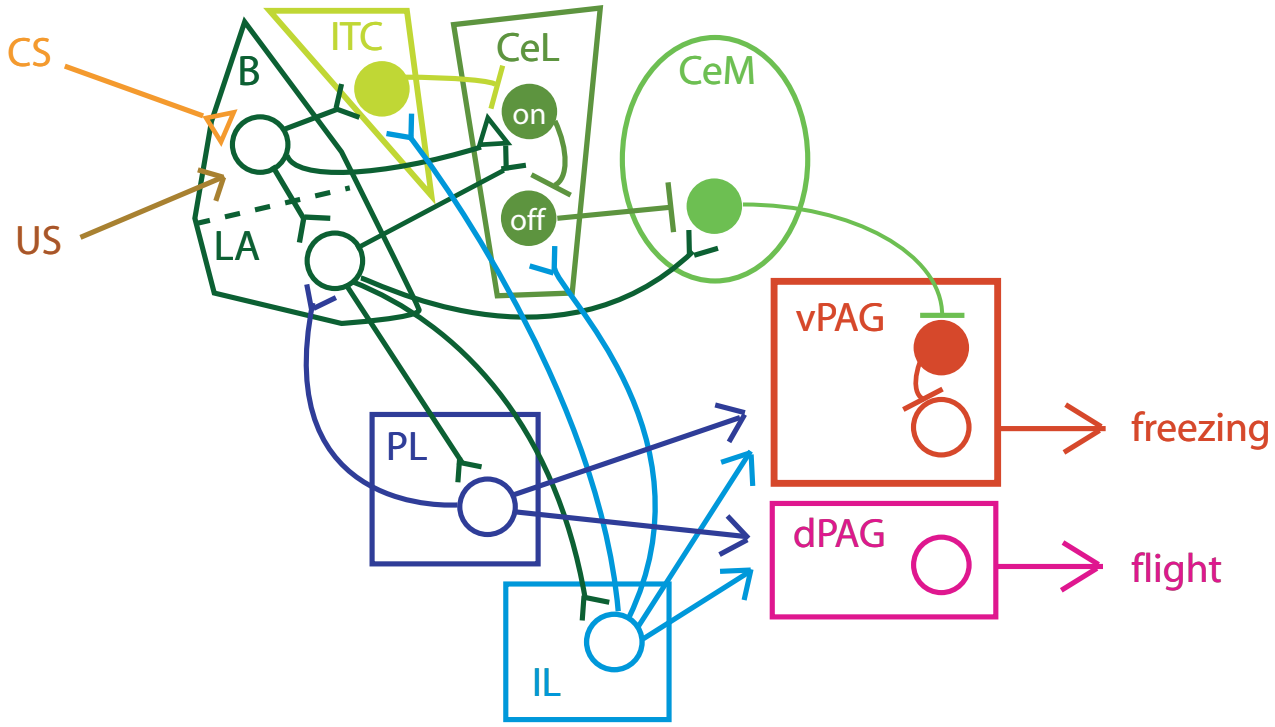

Figure 1.2. *Schematic diagram of a proposed fear circuit.* An auditory fear-conditioned CS excites the basolateral nucleus (BLA) of the AMG. Efferent projections from the central medial nucleus (CeM) of the AMG to ventral PAG (vPAG) induce freezing behavior. While CeM doesn't project to dorsal PAG (dPAG), CS-evoked flight behavior may be mediated by indirect projections through prelimbic (PL) or infralimbic (IL) cortices. Both PL and IL have known projections to dPAG, which could be the mechanism underlying transitions between freezing and flight behavior. Additionally, PL has excitatory reciprocal connections with BLA which are thought to facilitate freezing behavior, while IL inhibits CeM ouput by its projections to ITC and CeL, and thus inhibits freezing expression.

Chapter 2

Examining the effects of opioidergic and serotonergic drugs in dlPAG on conditional and unconditional responding to threatening and aversive stimuli

2.1 INTRODUCTION

When faced with threat, an animal's defensive responses are triggered so that it can escape from or ward off the danger (Bolles and Fanselow, 1980; Fanselow and Sigmundi, 1986; Fanselow et al., 1994). The midbrain periaqueductal gray (PAG) mediates antinociceptive responses that are triggered by encounters with painful or threatening stimuli (Basbaum and Fields, 1984; Watkins and Mayer, 1986; Fanselow, 1991), as well as behavioral defenses such as flight and freezing (Fanselow, 1991; Depaulis et al., 1992; Kim et al., 1993; De Oca et al., 1998; Morgan et al., 1998; Vianna et al., 2001; Leman et al., 2003). Evidence suggests that ventral PAG (vPAG) mediates conditional freezing, whereas dorsal (dPAG) drives active defensive behaviors, such as flight (Fanselow, 1991; Depaulis et al., 1992; Kim et al., 1993; De Oca et al., 1998; Vianna et al., 2001; Leman et al., 2003). Since these two defensive behaviors are mutually exclusive, it has been proposed that activity shifts from vPAG to dPAG when increasing levels of threat cause a shift in defensive strategies from freezing to flight behavior (Tarpley et al., 2010; see Walker et al., 1997). In our particular paradigm (see Chapter 1), animals will express either freezing or flight behavior depending upon whether they have recently encountered a shock US. Although the neural mechanism mediating the switch between freezing and flight behavior has yet to be fully understood, it has been suggested that neuromodulators known to regulate activity in PAG, such as opioids or serotonin (5-HT), may mediate behavioral defenses (Deakin and Graeff, 1991). Neuromodulators in PAG seem like a likely candidate to modulate the

balance between activation of vPAG versus dPAG, thereby regulating the switch between active and passive defensive responding.

Among the many receptor subtypes in PAG, it has been well established that mu-opioid receptors are high in density (Goodman et al., 1980; Mansour et al., 1988; Brodsky et al., 1995) and play a significant role in pain modulation (Reynolds, 1969; Mayer et al., 1971; Pert and Yaksh, 1975; Yaksh et al., 1976; Smith et al., 1988; Manning et al., 1994), including analgesic responding to conditional fear stimuli (Helmstetter and Fanselow, 1987; Fanselow and Helmstetter, 1988; Helmstetter and Landeira-Fernandez, 1990). Opioids in PAG also contribute to defensive responding and aversion (Motta and Brandão, 1993; Morgan et al., 1998; Anseloni et al., 1999; Monassi et al., 1999; but see De Luca-Vinhas et al., 2006). Since painful stimuli like shock can trigger endogenous opioid release in PAG (Bolles and Fanselow, 1982), and shock exposure leads to the transition from freezing to flight behavior in our fear conditioning paradigm, it is possible that opioidergic mechanisms might trigger the behavioral transition from passive to active defense responding.

Another neuromodulator with the potential for mediating defensive strategy shifts is 5-HT. Firstly, both clinical and animal studies have provided evidence that serotonergic mechanisms mediate anxiolytic and anxiogenic effects depending on which receptor subtype is stimulated (Hoehn-Saric, 1982; Norman et al., 1989; Graeff 1990, 1996; Salzman et al., 1993; Griebel 1995; Gray and McNaughton 2000; Nutt, 2001; Amat et al., 2004; Maier and Watkins, 2005; Graeff, 2012). Second, studies have also shown that 5-HT in PAG modulates both conditional and unconditional fear-evoked responses, including, but not limited to, defensive

avoidance (Graeff et al., 1990, 1997), and antinociception (Baptista et al., 2012). Since 5-HT seems to modulate both emotional and motor aspects of fear and defensive responding, it seems possible that serotonergic mechanisms within PAG might regulate the switch between defensive freezing and flight.

Here, we investigated how mu-opioid and 5-HT receptor signaling in PAG mediates conditional and unconditional responses to aversive stimuli during a Pavlovian fear conditioning task. Freely behaving rats were trained to fear an auditory conditional stimulus (CS) by pairing it with a shock unconditional stimulus (US) delivered to one of their eyelids. When the CS was subsequently presented to trained rats, they exhibited movement suppression responses (indicative of "freezing") when they had not encountered a shock within the past 24 h and flight behavior (i.e., turning away from the eyelid where the shock US was anticipated) during the CS after recent shock exposure. Hence, the recent encounter with shock induced a change in the mode of the rat's defensive responses to the CS, as reported previously (Tarpley et al., 2010).

To investigate the role of PAG mu-opioid and 5-HT receptors in these distinct modes of defensive behavior, we analyzed responses to the CS and US in trained rats following infusions of the selective serotonin reuptake inhibitor (SSRI) fluoxetine (FLU), the mu-opioid agonist morphine (MOR) and/or the partially mu-selective antagonist naltrexone (NAL) into the dorsal and lateral columns of PAG (dlPAG). Only opioidergic manipulations produced observable effects upon defensive responding. Results are discussed in the context of

clinical and pharmacological evidence that interactions between MOR and NAL may be more complex than simple competition for binding at the mu-receptor.

2.2 EXPERIMENTAL PROCEDURES

2.2.1 Subjects and surgery

Male Long-Evans rats weighing 350-400 g were housed singly and reduced to 85% of ad-lib weight through limited daily feeding. While it is possible that housing rats singly could influence their selection of defensive strategies, it has been shown that cage-mate number does not significantly affect physiological variables that would indicate changes in anxiety (Giralt and Armario, 1989; Brown and Grunberg, 1995). Under deep isoflurane anaesthesia, all rats were implanted with a pair of insulated stainless steel wires (75 um diameter) threaded into the skin of each eyelid for delivering the periorbital shock US. Rats were also implanted with a pair of 26 gauge microinjector guide cannulae (Plastics One, Roanoake, VA) targeted bilaterally in the PAG (7.5 mm posterior, 0.75 mm lateral and 5.2 mm ventral to bregma). A total of 39 rats underwent surgery, of which 9 were later dropped from the study because of misplacement of cannula tips identified during histology (n=4), or failure to reach behavioral criterion for progressing to the drug delivery phase of the study (n=5, see section 2.2.3 below). The remaining 30 rats were included in the data analysis.

2.2.2 Fear conditioning experiments

Throughout pre-exposure and fear conditioning sessions, rats constantly foraged on a 70x70 cm platform for 20 mg purified food pellets (Bioserv, Frenchtown, NJ) dropped from an overhead dispenser at ~30 s intervals, to provide a baseline of motor activity against which stimulus-evoked movement and turning behavior could be measured. The CS for fear conditioning was a train of 70 dB white noise pips, each lasting 250 ms, delivered at 1 Hz for 20 s through an overhead speaker. The US was a train of 2.0 mA shock pulses, each lasting 2.0 ms, delivered to one eyelid at a rate of 6.66 Hz for 2 s. During CS-US pairing trials, the first shock pulse was always delivered 300 ms after the offset of the final (20^{th}) CS pip. The inter-trial interval was uniformly random between 180 and 240 s.

2.2.3 Experimental Design

After recovery from surgery, rats were pre-exposed (i.e., habituated) for 5 days (20 min/day) to the experimental platform before any fear conditioning sessions were conducted. During this time, the animals learned to chase food pellets and became habituated to the test environment. Following pre-exposure, rats received 4-7 consecutive days of training, each consisting of 6 CS-alone presentations (test sessions) and 16 CS-US paired presentations (training sessions). The first intracranial infusion was administered one day after the second consecutive training session during which the rat's turning bias exceeded 4 degrees/sec in the direction away from the trained eyelid (5 rats that failed to meet this criterion by the 7^{th} day of training were dropped from the study and did not

receive drug infusions). For rats that reached behavioral criterion, different drugs were infused over multiple days, with a rest day given after each infusion day, followed by a day of drug-free retraining before the next infusion. Infusion conditions included fluoxetine (FLU), morphine (MOR) alone, naltrexone (NAL) alone, combination of MOR and NAL, and vehicle (VEH). The order of drug infusions was counterbalanced across rats. This repeated measures design was beneficial because it reduced the number of animals required for the study, and because it eliminated variability of injection sites among drug conditions, since all drugs were infused at the same set of injection sites.

2.2.4 Behavioral Scoring

The rat's moment-to-moment position on the platform was sampled at 30 Hz by an overhead video tracking system (Neuralynx Corporation, Bozeman, MT), which monitored the location of three light-emitting diodes (red, blue, green) attached to the animal's headstage for automated scoring of freezing, movement, and turning behavior using software developed in our laboratory. The algorithm for scoring freezing behavior has been described elsewhere (Moita et al., 2003, 2004). The algorithm for scoring movement and turning behavior first performed one iteration of smoothing (5-point adjacent averaging) upon the position data for each of the three colored LED's. The center point of the three LEDs was obtained by averaging their x and y coordinates, and the displacement distance of this center position between each successive video frame gave the rat's linear movement speed. The angles of the axes passing through each pair of tracking LED's (red-green, red-

blue, blue-green) was measured with respect to the horizontal axis of the video screen. Two of the angles (red-blue and blue-green) were rotated by the appropriate amount to align them with the third angle (red-green), and the mean of these transformed angular measurements was computed using circular averaging to obtain the rat's directional heading for each video frame (if one of the LEDs was occluded, then only one of the three color axes was used to estimate the directional heading). The change in directional heading angle between each successive video frame gave the rat's angular head-turning velocity.

2.2.5 Infusions

All drugs were dissolved in a vehicle solution of 0.9% sterile saline containing 2% Tween. Drugs were infused intracranially into both hemispheres of PAG at a volume of 0.25 µl/ side, through 33-gauge injectors at a rate of 0.2 µl /min. FLU was dissolved at a concentration of 25 nmol, and MOR was dissolved at 30 nmol. A low dosage of NAL (NAL1x) was dissolved at 26 nmol, and a high dosage of NAL (NAL10x) was dissolved at 260 nmol. Cocktail drugs (MOR+NAL1x and MOR+NAL10x) were also infused at a volume of 0.25 µl per side. Prior to drug infusion, dummy cannulae (which were in place at all times except during infusions to prevent clogging of the guide cannulae) were removed and injector cannulae were inserted in their place. After drug infusions, the injectors were left in place for an additional 2 min to allow diffusion of the drug away from the cannulae tip, after which the injectors were removed and replaced with dummy cannulae. Throughout the infusion process, the animal was held gently on the experimenter's lap. After the infusion was

complete, the rat was returned to its home cage for 15 min to allow time for the drug to take effect before the experiment resumed.

2.2.6 Histological Procedures

For histological verification of cannula placements, rats received bilateral infusions (0.25 µl per side) of a red BODIPY fluorophore to mark the injection site. Fifteen minutes later, rats were given intraperitoneal injections of a lethal dose of sodium pentobarbital (100 mg/kg) and perfused intracardially with 10% formalin in phosphate buffered saline (PBS). Brains were extracted and cryoprotected in a sucrose formalin solution. Fixed brains were sectioned (40 µm) using a cryostat, at coordinates ranging from -5.3 to -8.8 mm posterior to Bregma. Tissue sections were mounted onto slides, counterstained with the blue-fluorescent DAPI, and coverslipped. Sections were digitally photographed at 4x magnification under fluorescence microscopy, and anatomical boundaries were determined by overlaying the images with templates from the atlas of Paxinos and Watson, (1998).

2.3 RESULTS

The first intracranial drug infusion was administered after each rat reached a criterion for conditioned responding, with additional infusions of different drugs given on subsequent days in counterbalanced order (see section 2.2.3). This repeated measures design made it possible to perform within-subject comparisons to analyze the behavioral

effects of different drugs. A total of 30 rats reached criterion and received drug infusions. Fourteen rats received 3 infusions each (i.e., VEH, MOR, and NAL), and 13 rats received 6 infusions each (i.e., VEH, MOR, NAL, NAL10x, MOR+NAL, and MOR+NAL10x). The remaining 3 rats were only included in the VEH-only analyses (section 2.3.1) because they did not receive all infusions necessary to be included in the repeated measures design of the other analyses.

2.3.1 Conditioned responses: movement suppression and turning behavior

Figure 2.1A plots movement speed data on the day of VEH infusions during the CX and CS periods. Results were analyzed using a 2×2 ANOVA with stimulus (CX vs. CS) and trial type (test vs. train) as repeated factors. The two-way interaction was significant ($F_{1,29}$=14.69, p=.0006), indicating that both stimulus and trial type influenced movement speed. Newman-Keuls post hoc comparisons revealed that during test sessions, rats exhibited significant suppression of their movement speed during the CS when compared against the CX (p=.0001). This result is consistent with many prior studies showing that a fear-conditioned CS elicits movement suppression when it is presented to rats that have not recently encountered the US (Blanchard and Blanchard, 1969; Fendt and Fanselow, 1999; Blair et al., 2005; Tarpley et al., 2010). However, during training trials, movement speeds during the CX were significantly lower than during test trials (p=.0002), so that there was no longer a significant difference between CX and CS movement speeds (p=.14). This decrease

in movement during the CX appeared to be an indicator of post-shock fear, which caused the rats to move less after they had been shocked (training trials) than before they had been shocked (test trials) on each experiment day. Hence, two different types of movement suppression were measurable in the behavioral data: CS-evoked and US-evoked movement suppression.

To quantify these two different movement suppression effects, we computed two suppression ratios denoted by R_{CS} and R_{US} (see Methods). R_{CS} measured suppression of movement by the CS relative to the CX on each trial, and this was computed separately for test (R_{CS}^{Test}) versus training (R_{CS}^{Train}) trials. Figure 1B shows that R_{CS}^{Test} was significantly less than 0.5 (z_{29}=5.4, p<.00001), reflecting the presence of CS-evoked movement suppression during test sessions. However, R_{CS}^{Train} was significantly larger than R_{CS}^{Test} (paired t_{29}=3.16, p<.004), and R_{CS}^{Train} was not significantly less than 0.5 (z_{29}=1.8, p<.09), reflecting the absence of CS-evoked movement suppression during training trials. A likely explanation for this result is that CS-evoked movement suppression was abolished by US-evoked movement suppression, which reduced CX movement during training trials to a level where the CS could no longer produce additional movement suppression. To test this interpretation, R_{US} was computed to measure suppression of movement during the CX period of training trials (which occurred after shock delivery) versus test trials (which occurred before shock delivery). Figure 2.1B shows that R_{US} was significantly less than 0.5 (z_{29}=4.6, p=.00007), indicating that the shock US did indeed suppress movement during the CX period of

subsequent training trials. Moreover, there was a significant negative correlation between R_{CS}^{Train} and R_{US} across rats (r_{30}=-.42, p=.02), indicating that rats which showed greater US-evoked movement suppression also showed greater impairment of CS-evoked movement suppression during training trials.

Figure 2.1C plots mean turning velocities for all rats on the day of their VEH infusions. Since turning velocity is a signed quantity, a mean velocity of zero does not indicate the absence of turning behavior during the trials, but instead indicates that rats turned equally in both directions, and thus showed no bias for turning toward versus away from the trained eyelid. Throughout this paper, we shall adopt the convention that positive angular velocities indicate a bias for turning away from the trained eyelid, whereas negative angular velocities indicate a bias for turning toward the trained eyelid (recall that the trained eyelid was counterbalanced over rats). For this reason, we refer to averaged turning velocity scores as the "turning bias."

Turning bias data was analyzed using the same 2 $\times$ 2 ANOVA design described above for the movement speed analysis. There was a significant interaction between stimulus and session ($F_{1,29}$=30.90, p<.0001), indicating that both factors influenced turning bias. Post hoc comparisons revealed that during test trials, turning bias did not differ during the CS versus CX (p=.47), and it can be seen from Figure 1C that this was because rats exhibited no turning bias at all during test trials. But during training trials, rats exhibited robust turning behavior during the CS when compared against the CX (p=.0002). These results replicate findings by Tarpley et al. (2010), which showed that after recent shock delivery, rats did not freeze to

the CS, but instead responded by turning in the direction away from the trained eyelid. Hence, CS-evoked movement suppression is no longer observed after recent shock delivery (apparently because baseline fear has increased to the point where movement is equally suppressed during both the CX and CS), but CS-evoked turning behavior emerges in its place. This behavioral transition can be seen clearly in the line graphs of Figure 1, where it is shown that during the first and second training trials, a decrease in movement speed during the CX (Figure 1A) occurs in tandem with an increase in turning bias during the CS (Figure 2.1C).

2.3.2 MOR and NAL infusions

Cannula placements for all rats included in the study (n=30) are shown in Figure 2.2. All placements were in the dorsolateral or lateral columns of PAG. Drug solutions tend to diffuse dorsally along the cannulae path, with comparatively less diffusion in the ventral direction. It is thus likely that injections may have diffused dorsally to affect the overlying superior colliculus, but comparatively less likely that drugs diffused ventrocaudally to affect the ventral PAG. Lateral diffusion into the dorsomedial and mesencephlic nuclei may have occurred as well.

Most rats (n=27) that reached behavioral criterion received bilateral infusions of MOR (0.25 µl per side, 30nmol) and NAL (0.25 µl per side, 26 nmol) into dlPAG. After a 15 min delay in the home cage, infusions were followed by a standard experimental session on

the platform, and data from these sessions were analyzed to assess the behavioral effects of infusing and NAL into dlPAG. Figure 2.3A shows movement speeds during the CX and CS periods, averaged over test trials for each drug treatment. Drug effects were compared by performing a 3 × 2 ANOVA on movement speed with drug (VEH vs. MOR vs. NAL) and stimulus (CX vs. CS) as repeated factors. The main effect of stimulus was significant ($F_{1,26}$=48.61, p<.0001) but the drug × stimulus interaction was not ($F_{2,52}$=0.41, p=.66), indicating that drugs did not impair conditioned suppression of movement by the CS. Confirming this, post hoc comparisons showed that movement during the CS was significantly lower than during the CX for all three drug treatments (VEH: p=.0002; MOR: p=.0001; NAL: p=.0001), indicating that neither MOR nor NAL impaired conditioned movement suppression during test sessions. However, there was a significant main effect of drug ($F_{2,52}$=7.87, p=.001), and post hoc comparisons revealed that compared to the VEH treatment, MOR attenuated movement speeds during both the CX (p=.0001) and CS (p=.0001), whereas NAL did not affect movement speeds during the CX (p=.40) or CS (p=.84). Hence, MOR reduced baseline movement speed without affecting CS-evoked movement suppression during test trials.

Figure 2.3B plots movement speeds during training trials. Effects of drug infusions upon movement speed during training trials were analyzed using the same 3 × 2 ANOVA design described above for test trials. There was a significant main effect of drug ($F_{2,52}$=5.72, p=.006), mainly because in comparison with VEH, MOR strongly attenuated movement speed during both the CX (p=.0004) and CS (p=.0001). The main effect of stimulus

($F_{1,26}$=19.78, p=.0001) and drug × stimulus interaction ($F_{2,52}$=4.27, p=.019) were also significant. Post hoc comparisons revealed that during training trials, movement during the CS was significantly lower than during the CX after MOR infusions (p=.0001), but not after VEH (p=.40) or NAL (p=.16) infusions. These results indicate that CS-evoked movement suppression persists during training trials after MOR (but not NAL) infusions, in marked contrast with the VEH treatment, for which rats no longer showed any CS-evoked movement suppression during training trials (see above, Figures 2-1A,B).

To further analyze the effects of MOR and NAL upon movement suppression by the CS, a 3 × 2 ANOVA was performed on R_{CS} values with drug (VEH vs. MOR vs. NAL) and session (R_{CS}^{Test} vs. R_{CS}^{Train}) as repeated factors (Figure 2.3E). There was a significant main effect of session ($F_{2,52}$=13.87, p=.001) because $R_{CS}^{Test} < R_{CS}^{Train}$ for the VEH (p=.0009) and NAL (p=.02) conditions. By contrast, R_{CS}^{Test} did not differ from R_{CS}^{Train} in the MOR condition (p=.85), and this gave rise to a significant main effect of drug ($F_{2,52}$=5.38, p=.008) as well as a significant interaction between drug and session ($F_{2,52}$=3.49, p=.04). Supporting the idea that MOR 'rescued' CS-evoked movement suppression by blocking US-evoked movement suppression that normally occurred during training trials and was responsible for the observation that $R_{CS}^{Test} < R_{CS}^{Train}$ in the VEH and NAL groups, R_{US} was significantly less than 0.5 for the VEH (z_{26}=4.6, p<.00001) and NAL (z_{26}=5.5, p<.00001) conditions, but not the MOR (z_{26}=1.05, p=.30) condition. Moreover, there was a significant negative correlation between R_{CS}^{Train} and R_{US} in the VEH (r_{27}=-.42, p=.03) and NAL (r_{27}=-.58, p=.001) groups, but not in the

MOR group (r_{27}=-.10, p=.61), which further supports the conclusion that in individual rats, US-evoked movement suppression partially accounted for the loss of CS-evoked movement suppression during training trials.

Figure 2.3C plots turning bias during test trials. Effects of drug infusions upon turning bias were assessed using the same 3 × 2 ANOVA design that was used for analyses of movement speed. As explained above, turning bias was minimal during test trials for the VEH treatment because CS-evoked turning responses did not emerge until rats began receiving shocks during training trials (see Figure 2.1C). Turning bias during test trials remained low after MOR and NAL infusions. Confirming this, there was no significant main effect of drug ($F_{2,52}$=0.22, p=.81) or stimulus ($F_{1,26}$=1.43, p=.24), and also no stimulus × drug interaction ($F_{2,52}$=2.95, p=.06) for test trials. Figure 2.3D plots turning bias during training trials. It was explained above that in the VEH treatment, the transition from test to training trials triggered an emergence of CS-evoked turning away from the eyelid where shock delivery was anticipated (see Figure 2.1B). Confirming this, analysis of turning bias during training trials (Figure 2.3D) yielded a significant main effect of stimulus ($F_{1,26}$=20.23, p=.0001), and post hoc comparisons revealed that turning bias was significantly greater during the CS than CX in all three drug treatments (VEH: p=.0001; MOR: p=.004; NAL: p=.0001). The main effect of drug ($F_{2,52}$=3.32, p=.04) and stimulus × drug interaction ($F_{2,526}$=3.30, p=.04) were also significant. Post hoc comparisons revealed that compared to the VEH treatment, CS-evoked turning was significantly reduced after infusions of MOR (p=.0003), but not NAL (p=.18).

Drug effects upon unconditioned responses to the US were assessed by performing a 3 × 2 ANOVA on US-evoked movement speed with drug (VEH vs. MOR vs. NAL) and stimulus (CX vs. US) as repeated factors (Figure 2.3F). Shocks evoked bursts of motor activity from the rats, yielding a significant main effect of stimulus ($F_{1,26}$=32.57, p<.0001). Post hoc comparisons confirmed that movement speed during the US was significantly higher than during the CX for all three drug treatments (VEH: p=.0001; MOR: p=.0002; NAL: p=.0001), so US-evoked motor responses were not abolished after infusions of MOR or NAL. There was no significant main effect of drug ($F_{2,52}$=2.37, p=.10), and the drug × stimulus interaction was not significant ($F_{2,52}$ =0.50, p=.61). However, post hoc comparisons revealed that MOR attenuated movement speeds during the US compared to the VEH treatment (p=.02) whereas NAL did not (p=.84).

2.3.3 NAL dosage response and interactions with MOR

Results presented above indicate that NAL had no discernable effects upon behavior, raising questions about whether NAL was pharmacologically active in dlPAG at the dosage we administered. To test this, a subset of the rats (n=13) was bilaterally infused with NAL at a dosage 10 times higher (NAL10x: 25 µg in .25 µl/ side) than the dosage used in the above experiments (NAL1x: 2.5 µg in .25 µl/ side). Both dosages of NAL were also infused (on different experiment days) concurrently with MOR in cocktail solutions containing both MOR and NAL. MOR was always administered at the same dosage used above (5.75 µg

in .25 µl/ side), and NAL was administered at either the low dosage (MOR+NAL1x) or high dosage (MOR+NAL10x). The NAL10x, MOR+NAL1x, and MOR+NAL10x cocktails were given on different days, in an order that was counterbalanced and interspersed with infusions of other drugs.

Figure 2.4A shows movement speeds during the CX and CS periods averaged over test trials for each drug treatment. A 2 × 3 ANOVA was conducted upon these movement speeds with MOR (- vs. +) and NAL (0 vs. 1x vs. 10x) as repeated factors. Data from the CX and CS periods were collapsed together for this analysis (the CX versus CS periods are compared against one another below, in the analysis of suppression ratios). The main effect of MOR was significant ($F_{1,12}$=10.08, p=.008), replicating the result reported above that there is a general reduction in baseline motor movement after infusions of MOR. There was no significant main effect of NAL ($F_{2,24}$=0.26, p=.78) or MOR × NAL interaction ($F_{2,24}$=0.36, p=.70), indicating that MOR reduced baseline motor movement regardless of whether NAL was infused in combination with the drug. Hence, neither dosage of NAL appeared to be effective at reversing MOR's movement suppression effects.

Drug effects upon CS-evoked movement suppression were analyzed by performing 2 × 3 ANOVAs separately upon R_{CS}^{Test} and R_{CS}^{Train} (Figure 2.4B,C), with MOR (VEH vs. MOR) and NAL (VEH, NAL1x, or NAL10x) as repeated factors in both cases. For the analysis of R_{CS}^{Test}, there was no significant main effect of MOR ($F_{1,12}$=0.003, p=.96), but the main effect of NAL was significant ($F_{2,24}$=5.26, p=.01). Planned comparisons (collapsing across the MOR

factor) revealed that the cause of this main effect was a significant impairment of CS movement suppression by the high dosage of NAL (VEH vs. NAL10x: $F_{1,12}$=8.0, p=.015); the low dosage of NAL had no effect upon CS-induced movement suppression (VEH vs. NAL1x: $F_{1,12}$=1.85, p=.20), in agreement with findings reported above. Impairment of CS-induced movement suppression by NAL10x did not appear to be affected by infusions of MOR, since there was no MOR x NAL interaction ($F_{2,24}$=0.33, p=.72). Analysis of R_{CS}^{Train} yielded similar results: there was no significant main effect of MOR ($F_{1,12}$=2.96, p=.11), but the main effect of NAL was significant ($F_{2,24}$=4.03, p=.03), with no MOR $\times$ NAL interaction ($F_{2,24}$=1.02, p=.38). Consistent with findings reported above, CS-evoked movement suppression was not observed during training trails for the VEH condition (z_{12}=1.71, p=.11), but was observed for the MOR condition (z_{12}=2.7, p=.02). Post-hoc comparisons revealed that CS-evoked movement suppression under MOR was abolished by co-infusion of NAL at the high (MOR vs. MOR+NAL10x: p=.03) but not the low (MOR vs. MOR+NAL1x: p=.60) dosage.

Figure 2.5A plots turning bias during training trials (test trials are not shown, since turning behavior was not observed during test trials as reported above). Effects of drug infusions upon turning bias were assessed using the same 2 $\times$ 3 ANOVA design employed in the above analyses. This analysis yielded a significant main effect of MOR ($F_{1,12}$=6.31, p=.03), but not NAL ($F_{2,24}$=2.28, p=.12), with no significant interaction ($F_{2,24}$=0.78, p=.47). Post hoc comparisons confirmed that MOR attenuated CS-evoked turning under all dosages of NAL (MOR: p=.03; MOR+NAL1x: p=.02; MOR+NAL10x: p=.04). Consistent with findings described above, CS-evoked turning bias remained intact after infusions of NAL alone at either dose,

but there was a trend toward attenuation of CS-evoked turning by the low dosage (NAL1x: p=.07) that was not as pronounced at the higher dosage (NAL10x: p=.26).

As reported above, the US had two measurable behavioral effects: it acutely evoked reflexive motor responses, and it also produced a long-lasting suppression of movement throughout subsequent training trials within a given session. Effects of drugs upon both of the responses to the US were analyzed. Figure 2.5B shows averaged reflex movement speeds during US delivery for training trials. Analysis of drug effects upon reflexive US-evoked movement yielded a main effect of both drug ($F_{1,12}$=7.29, p=.02) and NAL dosage ($F_{2,24}$=4.39, p=.02), with no significant interaction ($F_{2,24}$=1.81, p=.18). Post hoc comparisons revealed that only the MOR+NAL1x treatment significantly reduced US-evoked responses compared to VEH (p=.004), suggesting that the low dose (but not the high dose) of NAL may have interacted with MOR to enhance suppression of movement following shock delivery. A synergistic effect between MOR and NAL1x was also suggested by results from an analysis of drug effects upon US-evoked movement suppression (Figure 2.5C). A 2×3 ANOVA upon R_{US} yielded no significant main effect of MOR ($F_{1,12}$=0.04, p=.85), but there was a significant main effect of NAL ($F_{2,24}$=7.05, p=.004), with no significant interaction ($F_{2,24}$=1.99, p=.16). Post-hoc comparisons revealed that R_{US} was significantly lower in the MOR+NAL1x than the MOR alone condition (p=.006), suggesting that the low dose of NAL may have interacted with MOR to enhance suppression of movement during the CX period following shock delivery.

2.3.4 FLU infusions

Nineteen rats received infusions of FLU (0.25 µl per side, 25 nmol). Figure 2.6A shows movement speeds during the CX and CS periods, averaged over test trials for each drug treatment. Drug effects were compared by performing a 2 × 2 ANOVA on movement speed with drug (VEH vs. FLU) and stimulus (CX vs. CS) as repeated factors. The main effect of stimulus was significant ($F_{1,18}$=18.28, p=.0005) but not the main effect of drug ($F_{1,18}$=1.46, p=.24) or drug × stimulus interaction ($F_{1,18}$=0.70, p=.41), indicating that drugs did not impair conditioned suppression of movement by the CS. Confirming this, post hoc comparisons showed that although CS movement speeds were high for FLU than VEH (p=.03), movement during the CS was significantly lower than during the CX for both drug treatments (VEH: p=.0002; FLU: p=.0002), indicating that FLU did not impaired conditioned movement suppression during test sessions.

Figure 2.6B plots movement speeds during training trials. Effects of drug infusions upon movement speed during training trials were analyzed using the same 2 × 2 ANOVA design described above for test trials. There was no significant main effect of drug ($F_{1,18}$=0.02, p=.90), main effect of stimulus ($F_{1,18}$=0.17, p=.68), or drug × stimulus interaction ($F_{1,18}$=0.61, p=.45). Post hoc comparisons revealed that during training trials, there was not a difference between movement during the CS versus CX in VEH (p=.80) or FLU (p=.85) treatments. These results indicate that like VEH rats, FLU infused rats no longer showed any CS-evoked movement suppression during training trials (see above, Figures 2.1A,B).

Figure 2.6C plots turning bias during test trials. Effects of drug infusions upon turning bias were assessed using the same 2 × 2 ANOVA design that was used for analyses of movement speed. As explained above, turning bias was minimal during test trials for the VEH treatment because CS-evoked turning responses did not emerge until rats began receiving shocks during training trials (see Figure 2.1C). Turning bias during test trials remained low after FLU infusions. Confirming this, there was no significant main effect of drug ($F_{1,18}$=0.84, p=.37) or stimulus ($F_{1,18}$=0.09, p=.76), and also no stimulus × drug interaction ($F_{1,18}$=0.43, p=.52) for test trials. Figure 2.6D plots turning bias during training trials. As explained above, the transition from test to training trials triggered an emergence of CS-evoked turning in VEH rats (see Figure 2.1B). Confirming this, analysis of turning bias during training trials (Figure 2.6D) yielded a significant main effect of stimulus ($F_{1,18}$=13.93, p=.002), and post hoc comparisons revealed that turning bias was significantly greater during the CS than CX in both drug treatments (VEH: p=.0003; FLU: p=.0002). But the main effect of drug ($F_{1,18}$=2.71, p=.12) and stimulus × drug interaction ($F_{1,18}$=0.37, p=.55) were not significant, indicating that FLU did not affect turning bias in training trials.

Drug effects upon unconditioned responses to the US were assessed by performing a 2 × 2 ANOVA on US-evoked movement speed with drug (VEH vs. FLU) and stimulus (CX vs. US) as repeated factors (Figure 2.6F). Shocks evoked bursts of motor activity from the rats, yielding a significant main effect of stimulus ($F_{1,18}$=26.19, p<.0001). Post hoc comparisons confirmed that movement speed during the US was significantly higher than during the CX for both VEH (p=.0001) and FLU (p=.0001) treatments, so US-evoked motor responses were

not abolished after infusions of FLU. There was no significant main effect of drug ($F_{1,18}$=0.11, p=.74), and the drug × stimulus interaction was not significant ($F_{1,18}$ =0.02, p=.90), suggesting that FLU did not affect US reflex responses.

2.4 DISCUSSION

Here we investigated how passive and active defensive responses were affected by infusions of FLU, MOR and NAL into dlPAG during a fear conditioning task. We assessed effects of these drugs upon several behaviors, including conditional movement suppression evoked by a fear-conditioned auditory CS, conditional turning responses evoked by the same CS after rats had recently re-encountered the shock US, unconditional reflex responses to the shock stimulus, and unconditional suppression of baseline movement activity after recent shock delivery. Infusions of FLU had no observable effect on defensive responding. We found that MOR infusions spared CS-evoked movement suppression but impaired CS-evoked flight behaviors, while leaving US-evoked reflex movements largely intact. NAL infusions had no effect on CS or US-evoked responses, except at very high dosages that may have affected molecular targets other than the mu receptors. When rats were infused with a cocktail containing both MOR and NAL, the two drugs appeared to interact independently or synergistically rather than competitively, suggesting that interactions between MOR and NAL may be more complex than simple competition for binding at the mu receptor.

2.4.1 Effects of MOR infusions

We found that MOR infusions tended to decrease baseline movement speeds during the CX and CS periods of both test and training sessions (Figures 2.3A,B). These results agree with previous data showing that MOR can attenuate motor activity, either when administered systemically (Vanderschuren et al., 1999; Kalinichev et al., 2004; Craft et al., 2006; Halladay et al., 2009) or when infused into the PAG (Morgan and Clayton, 2005). However, unconditioned movement responses to the US were only slightly attenuated by infusions of MOR into dlPAG (Figure 2.3F), indicating that spinal motor reflexes were largely unaffected. Such findings are consistent with the notion that PAG may be a component of a mesencephalic locomotor network, which relays descending motor commands to the spinal cord (Morgan et al., 1998). If so, then infusions of MOR into dlPAG may inhibit motor output during the CX and CS periods by attenuating transmission of descending motor commands, without affecting spino-trigeminal motor reflexes such as those engaged by the shock US.

Both VEH and MOR-infused rats exhibited similar conditioned suppression of movement during the CS in test sessions (Figures 2.3A), indicating that MOR did not impair conditioned fear of the CS. During training sessions (after recent shock), control rats no longer exhibited CS-evoked movement suppression (Figures 2.1A,B and 2.3B,E), but instead exhibited CS-evoked turning responses away from the trained eyelid (Figures 2.1C and 2.3D). By contrast, MOR-infused rats still exhibited movement suppression to the CS during training sessions (Figures 2.3B,E), and the emergence of CS-evoked turning responses was significantly attenuated but not completely abolished (Figure 2.3D). There are two plausible interpretations (which are not

mutually exclusive) for why MOR infusions impaired the emergence of CS-evoked turning responses (while preserving CS-evoked movement suppression) during training sessions. First, since turning behavior involves movement, impaired turning may have been a secondary effect of MOR's tendency to reduce overall motor activity. Second, since CS-evoked turning responses emerge in conjunction with shock delivery during training trials (Figure 2.1), impaired turning may have resulted from MOR-induced antinociception which attenuated the aversiveness of the shock and rendered it less able to induce a transition to CS-evoked turning responses. In support of this latter interpretation, MOR impaired unconditional post-shock movement suppression (Figure 2.3E), an effect which is also consistent with the idea that MOR rendered the US less aversive, and thus less able to suppress baseline movement activity. In opposition to this explanation, it might be argued that the lack of post-shock movement suppression following MOR was a floor effect, given that MOR suppressed movement even during test sessions, before the shock had been delivered (Figure 2.3A). But since CS-evoked movement suppression remained intact following MOR (Figure 2.3E), it was clearly possible to measure additional suppression below the CX baseline levels that were observed following MOR. Hence, it seems likely that impairment of post-shock movement suppression may have resulted at least in part from MOR's antinociceptive properties, and not simply from a floor effect upon movement speeds.

In summary, we found that infusions of MOR into dlPAG reduced overall movement, as well as CS-evoked turning responses, but did not abolish conditional fear responding and had minimal effects upon US-evoked reflex responses. These results agree with previous data

showing that intra-PAG MOR can attenuate motor activity (Morgan and Clayton, 2005) and promote antiaversive effects in fearful situations (Anseloni et al., 1999), while not affecting unconditioned reflexes to painful stimuli (Borges et al., 1988; Brandão et al., 1990).

2.4.2 Effects of NAL infusions

Prior studies have reported that intra-PAG infusions of NAL do not impair expression of conditioned freezing responses (Helmstetter and Landeira-Fernandez, 1990; Castilho et al., 2002; see McNally et al., 2004) or post-shock freezing (Hammer and Kapp, 1986; De Luca-Vinhas et al., 2006). Consistent with these prior findings, we found that CS-evoked movement suppression during test sessions was not significantly affected by NAL infusions at the 1x dosage (Figures 2.3A and 2.3E), which was similar to the dosage used in previous studies. However, we found that CS-evoked movement suppression during test sessions was impaired by infusions of NAL at the 10x dosage (Figure 2.4C), without impairing the expression of CS-evoked turning responses during training sessions (Figure 2.5A), unconditional reflex responses (Figure 2.5B), or post-shock movement suppression during training trials (Figure 2.5C). Hence, it appears that NAL10x did not impair the ability of the CS to induce conditional fear, but instead impaired ability of the CS to elicit a specific fear response—conditional movement suppression—while sparing its ability to elicit other conditional fear responses, such as turning, and unconditional responses to the US.

NAL impaired CS-evoked movement suppression only at the 10x but not the 1x dosage, and three explanations (which are not mutually exclusive) for these results merit consideration. First, since the 10x dosage was more concentrated, it may have allowed NAL to spread farther from the injection site and thereby act non-locally at distant brain regions that were not affected by the 1x dosage. Second, it is known that NAL binds different opiate receptors in vivo with varying affinities, and evidence suggests that higher doses of NAL are required to antagonize kappa receptors than those required to antagonize mu, delta, and epsilon receptors (Parsons et al., 1989; Girodano et al., 1990). Hence, impairment of CS-evoked movement suppression by NAL may have resulted from blockade of kappa opiate receptors, which remained functional at the 1x but not the 10x dosage. Supporting this possibility, past evidence has shown that intra-PAG administration of kappa-opioid antagonists produces anti-aversive effects, which manifest as increased thresholds for unconditioned freezing and escape behaviors (De Ross et al., 2009) as well as reduced acquisition of place aversion (Sante et al., 2000). Third, naloxone has been shown to antagonize GABA receptors at high doses (Breuker et al., 1976; Dingledine et al., 1978), and this may have caused excitation that contributed to impairments of CS-evoked movement suppression, since pharmacological excitation of dPAG has been shown to result in increases in flight behavior and other active, defensive-like escape reactions (Brandão et al., 1982; Bandler et al., 1985; Depaulis et al., 1986; Borelli et al., 2005; see Brandão et al., 1999, 2005).

2.4.3 Effects of combined MOR and NAL infusions

Since MOR and NAL are known to interact competitively at mu receptors (Simantov and Snyder, 1976; Kosterlitz and Leslie, 1978), we co-infused both drugs together to test whether they would interfere with one another's effects. We expected NAL to block or reverse of MOR's behavioral effects, as has been observed in prior studies (Anseloni et al., 1999). But surprisingly, the behavioral effects of MOR and NAL seemed largely independent of one another, because most effects induced by MOR were not reversed by NAL at either the 1x or 10x dosage, and impairment of CS-evoked movement suppression by the high dosage of NAL was similar in both the presence and absence of MOR.

Co-infusing NAL and MOR did not reverse MOR's ability to suppress movement speeds (Figures 2.4A,B), nor its ability to impair conditioned turning responses (Figure 2.5A). However, MOR's ability to block post-shock movement suppression was reversed by NAL (Figure 2.5C). Parodoxically, attenuation of US-evoked reflexes by MOR was enhanced by NAL at the 1x dosage (Figure 2.5A). This result is reminiscent of clinical findings which have shown that systemic co-administration of MOR with low doses of NAL (but not high doses) can paradoxically enhance MOR-induced analgesia and reduce MOR tolerance (Crane and Shen, 1995, 1998; Shen and Crane, 1997; Powell et al., 2002; Terner et al., 2006).

Another behavioral effect that was observed after MOR infusions was the perseverance of CS-evoked movement suppression during training sessions, which differed from the VEH condition where CS-evoked movement suppression was seen only during test

but not training sessions (Figures 2.3E and 2.4D). This perseverance of conditioned movement suppression was no longer seen when MOR was co-infused with NAL10x (Figure 2.4D). However, this cannot simply be interpreted as reversal of the MOR effect by NAL10x, because NAL10x impaired CS-evoked movement suppression during test sessions in the VEH condition as well (Figure 2.4C). The most parsimonious explanation for this pattern of results is that conditioned movement suppression was consistently impaired by NAL at the 10x dosage, regardless of whether it was co-infused with MOR or VEH alone.

In summary, NAL did not reverse (and in some cases appeared to enhance) suppression of motor activity by MOR, suggesting that interactions between MOR and NAL were more complex than simple competition for binding at the mu receptor. Although these results were unexpected, they are not incompatible with current knowledge of MOR and NAL pharmacology. Recent clinical findings have shown that systemic co-administration of MOR with low doses of NAL can paradoxically enhance MOR-induced analgesia and reduce MOR tolerance (Crane and Shen, 1995, 1998; Shen and Crane, 1997; Gan et al., 1997; Joshi et al., 1999; Powell et al., 2002; Terner et al., 2006). It has also been found that NAL can bind the scaffolding protein filamin A with a higher affinity than the mu-opioid receptor (Wang et al., 2008), and since filamin A normally interacts with opioid receptors, NAL's actions upon this scaffolding protein may be partly responsible for its exertion of pharmacological effects that do not antagonize the effects of MOR.

2.4.4 Effects of FLU infusions

Serotonergic mechanisms in the PAG have been proposed to play a role in conditioned and unconditioned fear expression (Nogueira and Graeff, 1995; Graeff et al., 1996; Hamalainen and Lovick, 1997; Brandão et al., 1999), so we examined whether inhibition of 5-HT reuptake by fluoxetine in dPAG would influence fear behaviors in our task. Infusions of FLU resulted in a slight increase in movement speed during the CS in test sessions (see Figure 2.6D), but aside from this small effect, intra-PAG FLU did not alter defensive behavior in any observable way. Although FLU is very commonly prescribed to patients as an anxiolytic (Sadock and Sadock, 2007), likely due to its ability to sensitize 5-HT_{1A} and 5-HT_{2A} receptors that normally inhibit the dPAG (Zanoveli et al., 2007), perhaps the drug's efficacy in human patients is not readily observable in rodent models of anxiety. It may also be the case that modulation of defensive responding in dPAG occurs through actions of one or more of the many additional serotonergic receptors not tested here. Future studies will be necessary to determine what role, if any, 5-HT receptors in dPAG have in strategic defensive responding.

Acknowledgments

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Figure 2.1

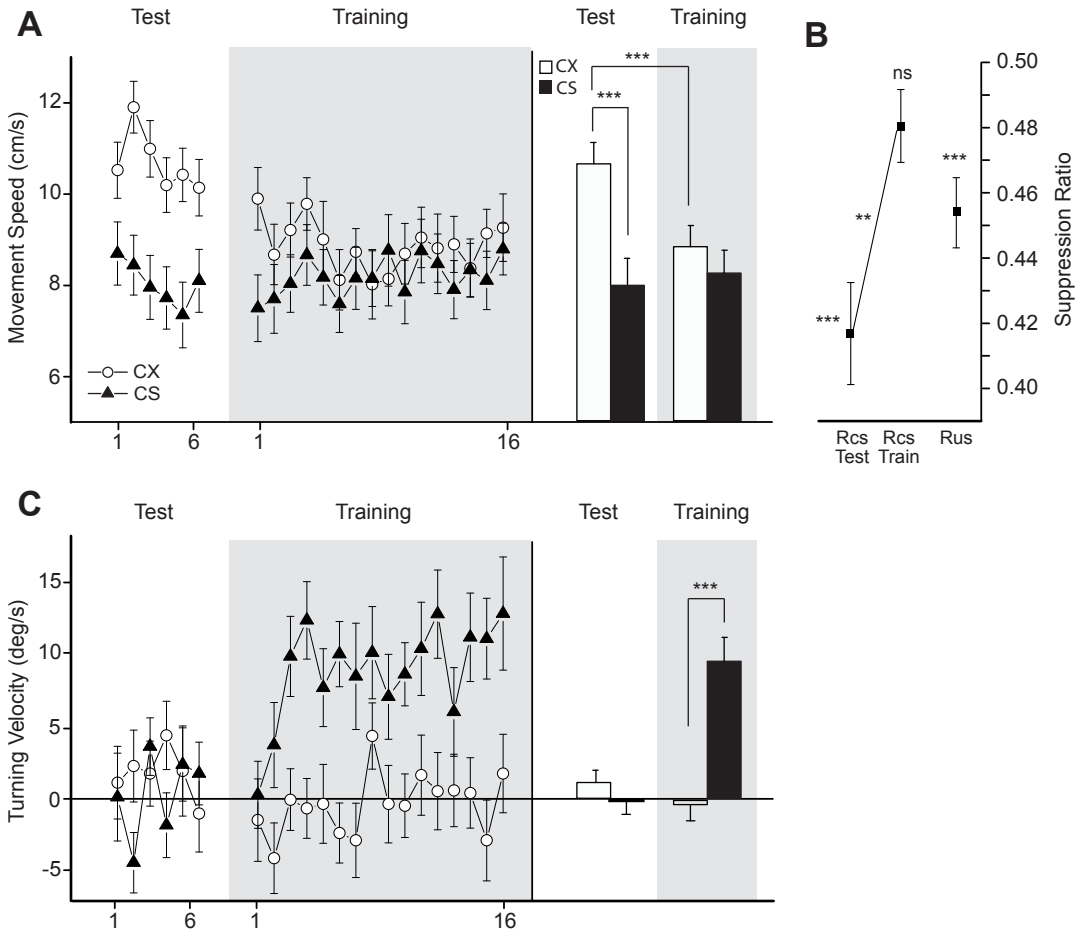

Figure 2.1. *Conditioned defensive behavior in VEH-infused rats.* A) Line graphs at left show averaged movement speed (y-axis) during the CX and CS periods in trained rats (n=30) for individual trials (x-axis) of test (unshaded area) and training (shaded area) sessions after VEH infusions. Bar graphs at right show movement speed averages for test trials versus training trials, with symbols indicating significance levels for Newman-Keuls posthoc comparisons. B) Mean CS suppression ratio (R_{cs}) is shown for test and training trials, and mean US suppression ratio (R_{us}) is shown for training trials (symbols next to each point indicate significance of comparison against the neutral suppression ratio of 0.5, whereas the symbol marking the line indicates significance of the comparison between R_{cs}test and R_{cs}train). C) Same as 'A' exceptthe y-axis plots turning velocity in deg/s, with positive values indicating turning in the direction away from the trained eyelid. Symbol legend: ***=p<.001, **=p<.01, ns= not significant.

Figure 2.2

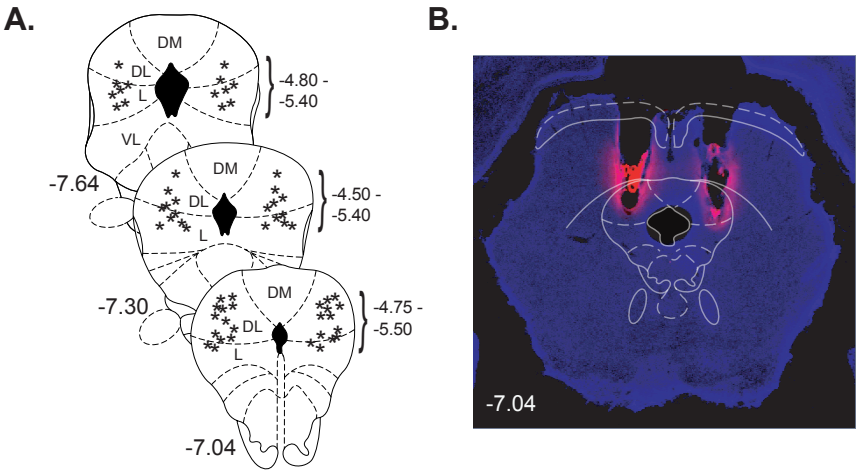

Figure 2.2. *Distribution of cannula placements in dlPAG.* A) Reconstruction of cannula tip placements are shown for rats that received infusions of MOR and/or NAL (n=30), using coronal section diagrams from the atlas of Paxinos and Watson (1998). Anteroposterior coordinates relative to bregma are given below each diagram, and columns of PAG are labeled as follows: DM=dorsomedial, DL=dorsolateral, L=lateral. B) Example image of red BODIPY fluorophore against blue DAPI counterstain to illustrate drug diffusion away from dlPAG injection sites.

Figure 2.3

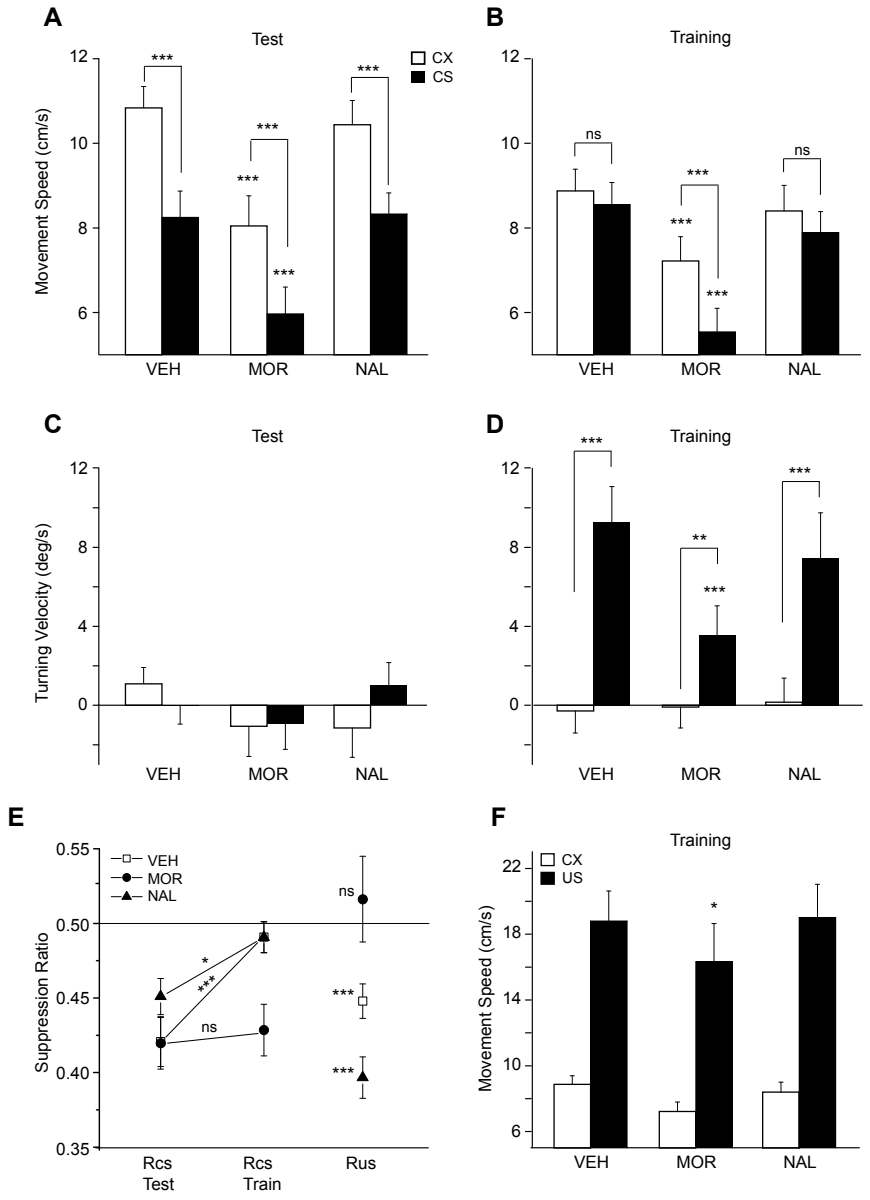

Figure 2.3. *Movement suppression and turning responses in MOR- and NAL-infused rats.* Bar and line graphs show behavior data averaged over trials, then over rats (n=27). Movement speed during CX and CS are shown for test (panel A) and training (panel B) trials. Turning bias during CX and CS are shown for test (panel C) and training (panel D) trials. Panel E shows a comparison of R_{cstest} versus $R_{cstrain}$ (significance of Neuman-Keuls posthoc comparisons shown by symbols decorating the line for each drug condition) alongside mean R_{us} values (symbols beside each point indicate significance of comparison against the neutral suppression ratio of 0.5). Panel F shows movement speed during CX and US for training trials. Symbol legend: ***=p<.001, **=p<.01, *=p<.05, ns= not significant.

Figure 2.4

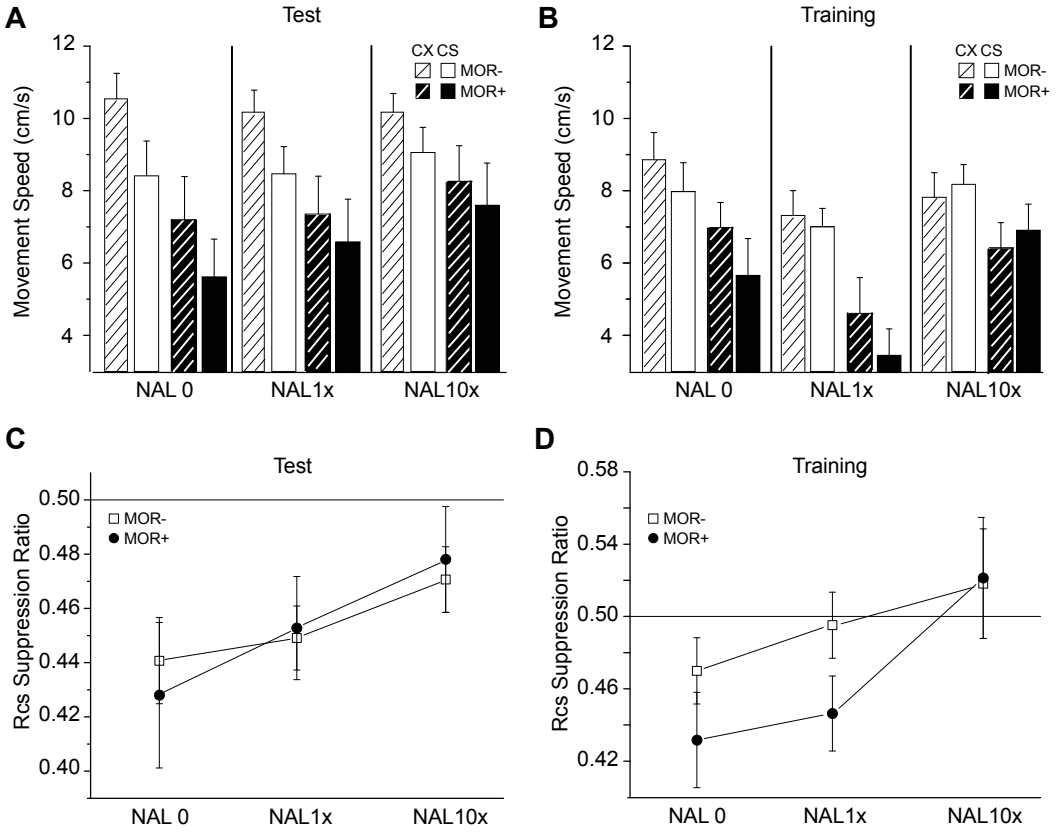

Figure 2.4. *Dosage response to NAL in the presence versus absence of MOR.* Behavioral responses of rats (n=13) after infusions of NAL0, NAL1x or NAL10x were compared in the presence versus absence of MOR. Averaged movement speeds during the CX and CS are shown for test (panel A) and training (panel B) sessions. CS-evoked movement suppression ratios are shown for test (panel C) and training (panel D) sessions.

Figure 2.5

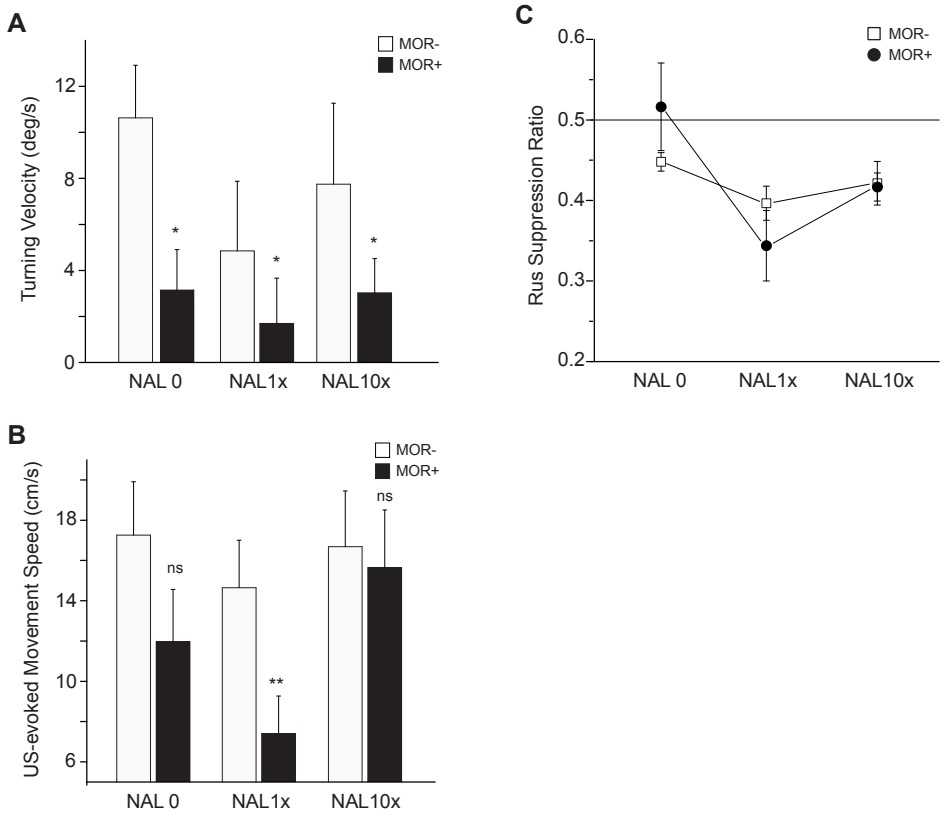

Figure 2.5. *Dosage response to NAL for conditioned turning, unconditioned reflexes, and US-evoked movement suppression.* Behavioral responses of rats (n=13) after infusions of NAL0, NAL1x or NAL10x were compared against those that were combined with MOR infusions. A) Mean turning bias during the CS for training trials (symbols indicate significance of comparison between MOR- and MOR+). B) Mean movement speed during the US for training trials (symbols indicate significance of comparison between MOR- and MOR+). C) Mean US-evoked movement suppression ratios (R_{us}) for test versus training sessions. Symbol legend: **=p<.01, *=p<.05, ns= not significant.

Figure 2.6

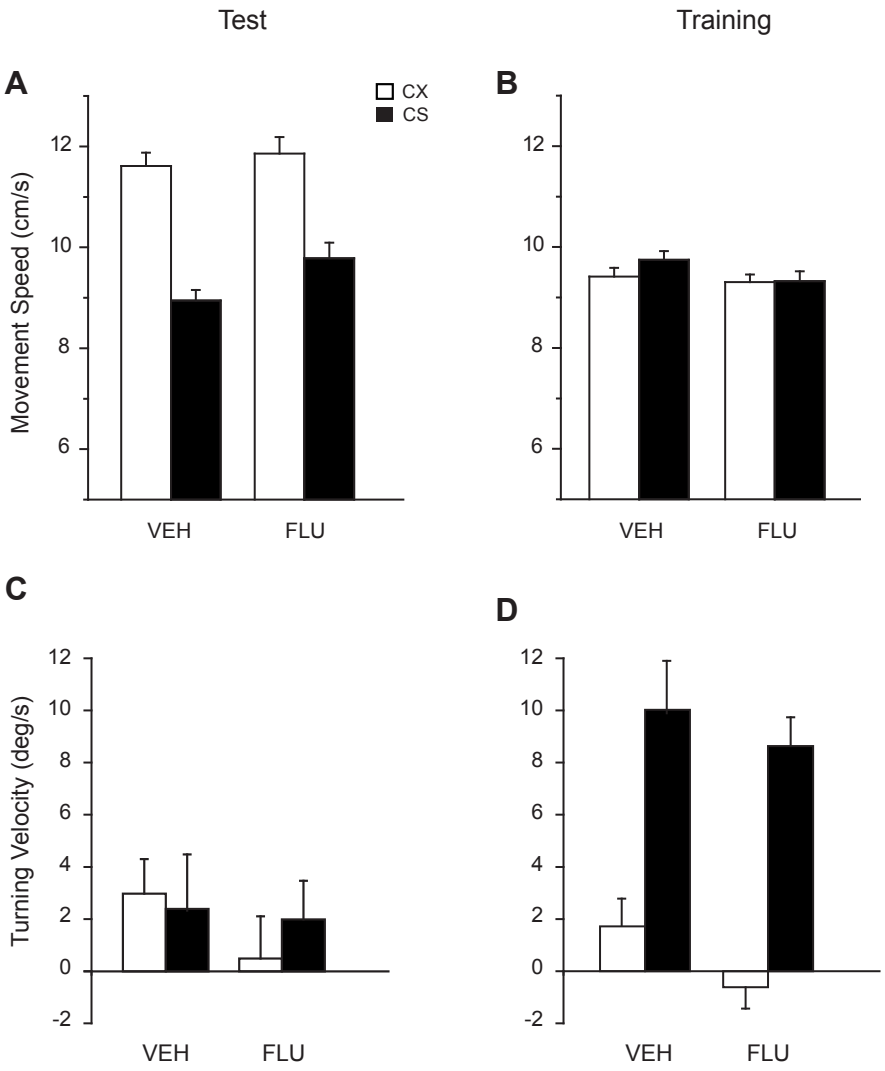

Figure 2.6. *Movement speed and turning data in FLU-infused rats.* Bar and line graphs show behavior data averaged over trials, then over rats (n=19). Movement speed during CX and CS are shown for test (panel A) and training (panel B) trials. Turning bias during CX and CS are shown for test (panel C) and training (panel D) trials.

Chapter 3

The role of medial prefrontal cortex in the selection of defensive response strategies

3.1 INTRODUCTION

Decades of research have identified key structures in distinct neural circuits mediating the expression of fear. Numerous studies have provided evidence that the amygdala (AMG) mediates fear expression by its ability to assign aversive value to states and stimuli that predict aversive outcomes (Klüver and Bucy, 1937; Weiskrantz, 1956; Davis, 1992; Killcross et al., 1997; LeDoux, 2000; Maren, 2001; Blair et al., 2005a,b; Paton et al., 2006; Seymour and Dolan, 2008). The AMG sends projections to areas of the periaqueductal gray (PAG), which translate that aversive value into behavioral defensive responding (Bandler and DePaulis, 1988; Fanselow, 1991; De Oca et al., 1998; Vianna et al., 2001b). While interactions between AMG and PAG that produce the expression of defensive behaviors have been extensively studied and are well understood, there are many modulatory mechanisms contributing to the expression of fear that are less clear. Once such source of modulatory input is the medial prefrontal cortex (mPFC), which in recent years has begun to garner a great deal of interest due to its purported influence on the fear system.

The mPFC is generally considered to be important for decision making and executive control (Holroyd et al., 2002; Ridderinkhof et al., 2004; Bechara and Damasio, 2005), both of which involve working memory processes. mPFC also seems to mediate some aspects of memory storage, but there is some debate to whether mPFC is primarily necessary for "recent" (Corcoran and Quirk, 2007) or "remote" (Bontempiet et al., 1999; Frankland et al., 2004) long term memories, or for short term memory (Seamans et al., 1995; Narayanan et al., 2006). Regardless, mPFC is regarded as a critical source of "top-down" control. And

importantly, mPFC activity seems to be heavily modulated by emotional events (Baeg et al., 2001; Bechara and Damasio, 2005; Fellows, 2007; Rushworth et al., 2011; Shackman et al., 2011), raising the likelihood that mPFC may be a critical modulator of fear memory.

In rodent mPFC, there seems to be a dorsal/ventral gradient in which more dorsal regions control actions and motor responding, while ventral regions mediate emotion and autonomic functions (for review see Euston et al., 2012). Much evidence has implicated ventral regions of mPFC in the modulation of fear (Morgan and LeDoux, 1995; Quirk et al., 2006; Sierra-Mercado et al., 2006; Peters et al., 2008; Sotres-Bayon et al., 2009; Sotres-Bayon and Quirk, 2010), namely two anatomically distinct regions known as the prelimbic (PL) and infralimbic (IL) cortex. A common consensus is that PL and IL have opposing roles in the modulation of fear. There is evidence that PL mediates the expression of fear (Vidal-Gonzalez et al., 2006; Sierra-Mercado et al., 2011); inactivating PL reduces fear evoked by conditioned contextual and auditory stimuli, but does not does not affect the acquisition of fear or retrieval of fear extinction (Corcoran and Quirk, 2007; Laurent and Westbrook, 2009; Sierra-Mercado et al., 2011). Recording studies have also shown that neurons in PL exhibit sustained activity that is correlated with the expression of fear elicited from a conditional auditory stimulus (Burgos-Robles et al., 2009).

IL cortex, on the other hand, is believed by many to be a source of fear inhibition. Lesion and inactivation studies have shown that IL is crucial for consolidation and recall of extinction (Quirk and Mueller, 2008; Laurent and Westbrook, 2009; Sierra-Mercado et al., 2011). Additionally, post-extinction training activity bursts in some IL neurons is found to be correlated with later extinction performance (Burgos-Robles et al., 2007), presumably due

to extinction-related plasticity in IL. Furthermore, the intrinsic excitability of neurons in IL fluctuates based upon whether an animal is undergoing conditioning or extinction procedures, with more enhanced activity following extinction and decreased activity after conditioning (Santini et al., 2008).

It has been speculated that the opposing roles of PL and IL may be attributable to their differing efferent projections to AMG (Vidal-Gonzalez et al., 2006). While PL projects to the basolateral AMG (BLA) and excites neurons there, IL inhibits output from AMG by its direct and indirect (via intercalated cells) projections to the central nucleus of AMG (CeA) (Morgan and LeDoux, 1995; Fuster, 1997; Vertes, 2004; Berretta et al., 2005; ; Vidal-Gonzalez et al, 2006; Corcoran and Quirk, 2007; Hikind and Maroun, 2008). PL and IL's ability to modulate activity in AMG suggests that mPFC mediates aspects of emotional processing (Sotres-Bayon and Quirk, 2010), whereby excitation from PL to BLA enhances fear, and inhibition due to IL's actions on CeA inhibits fear. There seems to be evidence for this assumption, but it must be noted that PL and IL both project directly to PAG (Floyd et al., 2000). It is possible that direct input to PAG from mPFC mediates the behavioral output produced there, indicating a role for mPFC in defensive *action* modulation, rather than *emotional* modulation. Most of the evidence supporting the idea that mPFC influences AMG-mediated emotion relies on tasks which use freezing behavior as a measure for fear (Milad et al., 2004; Sotres-Bayon et al., 2004; Vidal-Gonzalez et al., 2006; Corcoran and Quirk, 2007; Laurent and Westbrook et al., 2008; Muigg et al., 2008; Burgos-Robles et al., 2009; Thompson et al., 2010; Orsini et al., 2011). While these studies have provided immeasurable insight to circuitry mediating fear, it is inherently impossible to discriminate

between the emotional and motor components of fear expression since freezing behavior in such studies is assumed to be concomitant with fear. Having the ability to measure two distinct fear behaviors may help elucidate this problem.

Our lab utilizes a fear conditioning paradigm that allows for the observation of two mutually exclusive defensive behaviors in the same animal during the same test session (Tarpley et al., 2010; Halladay and Blair, 2012). As described in Chapter 1, animals in our paradigm will exhibit one of two defensive strategies in response to the CS, depending upon whether they have recently encountered shock. Trained rats that have not been shocked recently will respond to the CS with defensive freezing, but after exposure to the US, the CS will evoke a conditional flight behavior. Importantly, our paradigm provides us the opportunity to measure a broader range of defenses defined by the "predatory imminence continuum" (Fanselow and Lester, 1988; Fanselow, 1989) including post-encounter freezing and circa-strike flight. This allows us to differentiate between the emotional and action-related components of fear expression more easily than a task with only one behavioral response would allow. For example, studies measuring only freezing behavior may assume that reductions in freezing are attributable to reduction in fear, but in our paradigm, reduction of freezing behavior can either be associated with reduced fear or enhanced fear (i.e., freezing is attenuated because flight is potentiated).

Both the freezing and turning behavior we observe rely upon activity in PAG and AMG (Tarpley et al., 2010). Although the neural pathway for mediating freezing behavior has been well-documented (LeDoux et al., 1988; Kim et al., 1993; De Oca et al., 1998; Carrive et al., 2000; Wilensky et al., 2006), the neural circuitry mediating conditional flight

responses is less understood. Evidence supports the idea that ventral PAG (vPAG) controls freezing behavior (Fanselow and Helmstetter, 1988; Thorn et al., 1989; De Oca et al., 1998; Morgan et al., 1998; Vianna et al., 2001a, 2001b), while dorsal PAG mediates flight (Halpern, 1968; Liebman et al., 1970; DiScala et al., 1984; Bandler and DePaulis, 1988; Fanselow, 1991; DePaulis et al., 1992; De Oca et al., 1998; Morgan et al., 1998; Keay and Bandler, 2001; Tarpley et al., 2010; Halladay and Blair, 2012). Since CeA preferentially projects to vPAG and not dPAG (Rizvi et al., 1991), it is unclear how emotional information from the AMG leads to the strategic "choice" of freezing versus flight. One possibility is that it does so indirectly through the mPFC. Since PL and IL areas of mPFC seem to have opposing roles in fear expression, they may have direct connections with PAG that mediate the opposing defensive strategies, freezing and flight.

To investigate whether mPFC can modulate the transition from freezing to flight behavior, rats trained in our paradigm received infusions of muscimol to inactivate, or picrotoxin to hyperactivate areas of mPFC that project directly to PAG (Floyd et al., 2000). We found that inactivation of these regions spared freezing and impaired conditional flight responses, whereas hyperactivation impaired freezing and enhanced flight. Based upon these findings, it seems possible that the transition from freezing to flight after shock delivery may be due to a persistent increase in mPFC responsiveness to the CS. The idea that mPFC mediates transitions between defensive strategies based upon present circumstances is consistent with theories that implicate mPFC in the regulation of decision policies and behavioral strategies by altering the manner in which stimuli are mapped onto behavioral responses (Miller, 2000; Miller and Cohen, 2001).

3.2 EXPERIMENTAL PROCEDURES

3.2.1 Subjects and Surgery

Adult male Long-Evans rats weighing 350-400 g were housed singly and reduced to 85% of *ad-lib* weight by daily limited feeding. All rats were deeply anaesthetized with isofluorane and surgically implanted with a pair of insulated stainless steel wires (75µm diameter) threaded into the skin of each eyelid for delivering the periorbital shock US. Rats (n=36, of which 30 were included in the study and 6 were excluded for misplaced infusion cannulae or faulty eyelid wires) were implanted with a pair of 22 gauge microinjector guide cannulae (Plastics One, Roanoake, VA) targeted bilaterally in mPFC (2.5 mm anterior, 0.5 mm lateral and 4.8 mm ventral to bregma) at an angle of 28 degrees to prevent drug diffusion into areas immediately dorsal to the target region. All experimental procedures were approved by the UCLA Animal Research Committee and were conducted in accordance with USA federal guidelines.

3.2.2 Fear conditioning

After recovery from surgery, rats were pre-exposed for 5 days (20 min/day) to the experimental context (70 x 70 cm platform) before any fear conditioning sessions were conducted. During this time, the animals learned to chase food pellets and became habituated to the test environment. On every day following pre-exposure, rats received an identical regimen of fear conditioning trials: 6 CS-alone presentations (test trials) followed by 16 CS-US pairings (training trials). To provide a baseline of motor activity against which

stimulus-evoked movement and turning behavior could be measured, rats constantly foraged for 20 mg purified food pellets (Bioserv, Frenchtown, NJ) dropped from an overhead dispenser at ~30 s intervals throughout all pre-exposure and fear conditioning sessions. The CS used for fear conditioning was a train of 70 dB white noise pips, each lasting 250 ms, delivered at 1 Hz for 20 s through an overhead speaker. The US was a train of 2.0 mA shock pulses, each lasting 2.0 ms, delivered to one eyelid at a rate of 6.66 Hz for 2 s. During CS-US pairing trials, the first shock pulse was always delivered 300 ms after the offset of the final (20^{th}) CS pip. The intertrial interval was uniformly random between 180 and 240 s.

3.2.3 Behavioral data acquisition and scoring

Each rat's position on the experimental platform was sampled at 30 Hz by an overhead video tracking system (Neuralynx Corporation, Bozeman, MT), which monitored the location of three light-emitting diodes (LEDs) of different colors (red, blue, and green) attached to the animal's headstage for automated scoring of movement and turning behavior using software developed in our laboratory. The algorithms for scoring movement and turning behavior have been described elsewhere (Tarpley et al., 2010; Halladay and Blair, 2012).

3.2.4 Intracranial drug infusions

Rats were given unilateral or bilateral injections of the GABA agonist MUS to inactivate mPFC, or the GABA antagonist PTX to hyperactivate mPFC. After five days of training in the fear conditioning task, rats received pre-session infusions of different drugs on different days, in a repeated measures design. A drug-free retraining session was always given in between each infusion session. Of the 30 rats that were included in the data analysis (see above, section 3.2.1), 15 rats only received infusions of MUS (into the left, right, or both hemispheres on different days in counterbalanced order), 14 only received infusion of PTX (bilaterally on the first day following the 5th training session), and 2 received infusions of both drugs (these two rats received bilateral MUS infusions on the first day following the 5th training session, then a drug free retraining day, followed by bilateral PTX infusions, and did not receive any unilateral MUS infusions). Hence, data was analyzed from a total of 17 bilateral MUS infusions, 15 unilateral MUS infusions, and 16 bilateral PTX infusions, in a total of 30 rats, with no rat receiving the same infusion twice. Both drugs were dissolved in 0.9% sterile saline, and a total volume of 0.4 µl per hemisphere was infused into mPFC through 26 gauge injectors at a rate of 0.2 µl/ min. MUS was dissolved at a concentration of 0.25 mg/mL, PTX was dissolved at a concentration of 0.20 mg/mL. Prior to drug infusion, dummy cannulae (which were in place at all times except during infusions to prevent clogging of the guide cannulae) were removed and injector cannulae were inserted in their place. After drug infusions, the injectors were left in place for an additional 2 min to allow diffusion of the drug away from the cannulae tip, after which the injectors were removed and replaced with dummy cannulae. Throughout the infusion process, the

animal was held gently on the experimenter's lap. After the infusion was complete, the rat was returned to its home cage for 15 min to allow time for the drug to take effect before the experiment resumed.

3.2.5 Histological Procedures

At the end of the experiment, rats were intraperitoneally injected with an overdose of pentobarbital (100 mg/kg) and perfused intracardially. Brains were extracted and fixed in a formalin sucrose solution. Tissue was later sectioned into 40 μm slices and mounted on slides for cannula and electrode placement verification. To assess the extent of drug diffusion, cannula-implanted rats that had previously received MUS injections were given a final infusion of fluorescent muscimol (tagged with Bodipy® TMR-X fluorophore, Invitrogen product #M2400) into mPFC, at the same volume, concentration, and rate that had been used for infusions of non-fluorescent muscimol in behavioural experiments (see above, section 3.2.4). Fluorescent muscimol was infused 30 min prior to the pentobarbital injection.

3.3 RESULTS

To investigate neural mechanisms underlying behavioral transitions between freezing and flight defenses, we pharmacologically inactivated or hyperactivated mPFC prior to experimental fear conditioning. Each experimental session consisted of 6 presentations of the CS alone followed by 16 CS-US pairings. For each trial, we define the CX period as the

20 s time window preceding onset of the first CS pip, the CS period as the 19.6 s time window between CS and US onset, and the US period as the 2 s period following US onset. On a given experiment day, we define 'pre-shock trials' as the 7 trials during which the CX and CS periods both preceded the day's first US presentation (all 6 CS alone trials, plus the first CS-US pairing trial), whereas 'post-shock trials' are defined as the remaining 15 CS-US pairing trials.

3.3.1 Injector placements and drug diffusion

To minimize tissue damage in the targeted mPFC subregions, cannula guides were bilaterally implanted at a lateral-to-medial angle of 28 degrees (Figure 3.1B), with injector tips targeted to the IL subregion (Figure 3.1A). To assess the extent of drug diffusion, rats that received MUS infusions (n=17) were given a final infusion of fluorescence-tagged MUS immediately prior to euthanasia, using the same volume (0.4 µl per hemisphere) and concentration (0.25 mg/ml) that had been previously used for standard (non-fluorescent) MUS on experiment days. Analysis of fluorescence distributions (Figure 3.1B) indicated that MUS concentrations were highest at the injector tip, but it was usually possible to detect backflow of fluorescence along the cannula track, as well as light radial diffusion of fluorescence away from the cannula track. This diffusion pattern of detectable fluorescence suggests that effective doses of the drugs are likely to have reached both IL and PL on the medial side of the cannula, and probably flowed into the white matter of the corpus callosum (where MUS and PTX were presumably inactive) on the lateral side of the cannula.

Hence, histological analysis indicates a likelihood that drugs were pharmacologically active within a region of mPFC (gray shading in Figure 3.1C) that includes portions of both IL and PL that have been shown to send direct projections to dorsal as well as ventral PAG (Floyd et al., 2000).

3.3.2 Behavioral measures of freezing and flight CRs

Figure 3.2 shows drug-free movement speed (Figure 3.2A) and turning (Figure 3.2B) data from all rats (n=30), averaged on the day prior to each rat's bilateral infusion of either MUS or PTX (pre-PTX data is shown only for rats that did not receive bilateral MUS, n=13). Movement speed data for pre- and post-shock trials (averaged first over trials, then over rats) were analyzed using a 2×2 ANOVA with stimulus (CX versus CS) and trial type (pre-shock versus post-shock) as repeated factors. There was a significant interaction between stimulus and trial type ($F_{1,29}$=21.70, p<.0001), indicating that both factors influenced movement speed. Posthoc comparisons revealed that during pre-shock trials, rats exhibited suppression of their movement speed during the CS when compared against the CX (p=.0002). However, during post-shock trials, movement speeds during the CX were lower (p=.0002) and during the CS were unchanged (p=.23) with respect to pre-shock trials, so that there was no longer a significant difference between CX and CS movement speeds during post-shock trials (p=.37). In addition, the US also evoked reflexive motor responses compared to the post-shock CX baseline (paired t_{29}=7.24, p<.0001).

It may seem counterintuitive that the mean movement speed did not increase during post-shock presentations of the CS, since data the CS elicits flight during post- but not pre-shock trials. However, while flight CRs are elicited by the CS almost exclusively during post-shock and not pre-shock trials, it is not conversely true that movement suppression is elicited only during pre-shock but not post-shock trials (Tarpley et al., 2010; Halladay and Blair, 2012). During the CS period of post-shock trials, rats commonly exhibit alternation between complete movement cessation (freezing) and vigorous turning in the direction away from the eyelid where shock is anticipated (flight). Depending upon the ratio of time spent freezing versus turning while the CS is on, the mean movement speed during the CS period can be similar to that during the CX period on a given trial. For this reason, movement speed is not a sensitive measure of flight CRs, especially after averaging data across trials and then rats.

A more sensitive measure of flight CRs is mean turning velocity, because flight CRs are specifically characterized by biased turning away from the eyelid where US delivery is anticipated (Tarpley et al., 2010). Figure 3.2B plots mean angular velocities for the drug-free condition, with positive velocities indicating a bias for turning away from the trained eyelid, and negative velocities indicating a bias for turning toward the trained eyelid. Since turning velocity is a signed quantity, a mean velocity of zero does not necessarily indicate the absence of turning behavior during the trials, but instead indicates that rats turned equally in both directions, and thus showed no bias for turning toward versus away from the trained eyelid.

Turning bias data for pre-shock and post-shock trials (averaged first over trials, then over rats) were analyzed using a 2×2 ANOVA with stimulus (CX versus CS) and trial type (pre-shock versus post-shock) as repeated factors. There was a significant interaction between stimulus and session ($F_{1,29}$=21.94, *p*<.0001), indicating that both factors influenced turning bias. Posthoc comparisons revealed that turning bias did not differ during the CS versus CX (p=.34) for pre-shock trials (rats exhibited no turning bias at all during pre-shock trials), but for post-shock trials, the CS evoked robust turning behavior compared to both the post-shock CX (*p*=.0001) and the pre-shock CS (p=.0002).

3.3.3 Bilateral infusions of MUS and PTX

3.3.3.1 Movement speed during pre-shock trials

Changes in movement speed during pre-shock trials following bilateral infusions of MUS (n=17) or PTX (n=16) were analyzed using 2 × 2 ANOVAs with drug (drug-free versus infusion) and stimulus (CX versus CS) as repeated factors (Figure 3.2C). The main effect of MUS was not significant ($F_{1,16}$=2.42, p=.14), but despite this, posthoc comparisons indicated that movement speed under MUS was greater than the drug-free condition during both the CS (p=.0002) and CX (p=.01) periods, suggesting that MUS produced some increase in baseline motor activity (this result is further corroborated by unilateral infusion data, see below). There was a strong main effect of stimulus ($F_{1,16}$=15.56, p=.001) and a trend in the interaction ($F_{1,16}$=2.42, p=.14). Posthoc comparisons showed that movement speed during

the CS was significantly less than during the CX period for both the drug-free (p=.0002) and MUS conditions (p=.02). Hence, bilateral MUS infusions did not impair CS-evoked movement suppression during pre-shock trials. Infusions of PTX, however, did affect movement suppression during pre-shock trials (see Figure 3.3A). There was a significant main effect of PTX ($F_{1,15}$=5.41, p=.03) but not stimulus ($F_{1,15}$=.14, p=.71), and no interaction ($F_{1,15}$=2.77, p=.12). PTX-infused rats no longer exhibited movement suppression during the CS, but instead showed a non-significant increase in movement speed during the CS versus CX (p=.15), and the movement speed during the CS for the PTX condition was significantly higher than for the drug-free condition (p=.04). This enhancement of movement speed during the CS was probably not due to a generalized increase in motor movement, since movement speed during the CX was not different for drug-free versus PTX conditions (p=.81). Instead, the higher CS-evoked movement speed under PTX may have reflected a shift in rats' defensive response strategy during the CS, specifically, a shift away from movement suppression (freezing) and toward movement enhancement (flight). This interpretation gains further support from analyzing turning bias data during post-shock trials.

3.3.3.2 Turning bias during post-shock trials

Changes in turning behavior following bilateral infusions of MUS or PTX were analyzed using 2×2 ANOVAs with drug (drug-free versus infusion) and stimulus (CX versus CS) as repeated measures (Figure 3.2C). There was a significant main effect of MUS ($F_{1,16}$=11.19, p=.004) and stimulus ($F_{1,16}$=5.07, p=.04), and a significant interaction

($F_{1,16}$=7.64, *p*=.01). Posthoc comparisons confirmed that turning was greater during the CS than CX for the drug-free (p=.03) but not the MUS (*p*=.55) condition. Moreover, during post-shock trials, CS-evoked turning was significantly reduced after MUS infusions compared to the drug-free condition (*p*=.01). Bilateral infusions of PTX had the opposite effect from MUS, enhancing rather than impairing turning responses during post-shock trials (Figure 3.3B). The 2×2 ANOVA yielded a significant main effect of PTX ($F_{1,15}$=8.96, *p*=.009) but not stimulus ($F_{1,15}$=7.54, *p*=.02), and an interaction between PTX and stimulus ($F_{1,15}$=2.73, *p*=.12). Posthoc comparisons revealed that not only was there significantly more turning during CS than CX for both both the drug-free (*p*=.004) and PTX (*p*=.0002) conditions, but PTX infusions also potentiated CS-evoked turning compared to the drug-free condition (*p*=.02), without affecting turning during the CS period (*p*=.75).

3.3.3.3 US-evoked reflexes and movement suppression

As stated previously, unconditioned motor reflex responses were elicited by the US. Figs. 2C and 3A show averaged reflex movement speeds during US delivery. Effects of MUS and PTX upon reflex responses were assessed by conducting 2×2 ANOVAs of movement speed with drug (drug-free versus infusions) and stimulus (CX versus US) as repeated factors. Both drug-free and infused rats exhibited reflexive responses to the US that were significantly elevated over CX (stimulus main effects, MUS: $F_{1,16}$=35.11, *p*<.0001; PTX: $F_{1,15}$=17.96, *p*=.0007). Posthoc comparisons showed that neither MUS nor PTX infusions affected US-evoked movements compared to the drug-free condition (MUS: *p*=.11; PTX: *p*=.16), so drug effects were not attributable to any obvious changes in sensitivity to the

shock. US delivery on the first training trial of each day produced long-lasting suppression of CX movement throughout subsequent trials on that day. Effects of MUS and PTX upon this post-shock freezing were assessed by analyzing movement speeds during the CX period using 2×2 ANOVAs with drug (drug-free versus infusions) and trial type (pre- versus post-shock) as repeated factors. For MUS infusions, there was no main effect of drug ($F_{1,16}$=1.44, *p*=.25) and no interaction ($F_{1,16}$=.06, *p*=.81), but there was a significant effect of trial type ($F_{1,16}$=10.05, *p*=.006); movement speeds during the CX were lower for post-shock than pre-shock trials in both the drug-free (p=.04) and MUS (p=.07) conditions. For PTX infusions, there was no main effect of drug ($F_{1,15}$=2.08, *p*=.17) and no interaction ($F_{1,15}$=.98, *p*=.34), but there was a significant effect of trial type ($F_{1,15}$=20.46, *p*=.0004) because CX movement speeds were lower during post-shock than pre-shock trials for both the drug-free (p=.001) and PTX (p=.02) conditions. This pattern of results indicates that neither MUS nor PTX had any effect upon reflexive reactions to the US, and also did not greatly alter the ability of the US to induce post-shock freezing. Hence, it appears that the aversiveness of the US was not greatly altered by bilateral infusions of MUS or PTX.

3.3.4 Unilateral infusions of MUS

3.3.4.1 Movement speed during pre-shock trials

Of the 17 rats that received bilateral infusions of MUS, 16 rats also received two unilateral infusions of MUS—one ipsilateral and the other contralateral from the eyelid

where the US was delivered—on different experiment days in counterbalanced order (see Methods; note that unilateral infusions of PTX were not given). Effects of unilateral MUS infusions upon movement speed during pre-shock trials was analyzed using 2×2 ANOVAs with drug (drug-free versus MUS) and stimulus (CX versus CS) as repeated factors (Figure 3.2D,E). For ipsilateral infusions, there was a significant main effect of MUS ($F_{1,15}$=17.45, *p*=.0008), and posthoc comparisons indicated that relative to the drug-free condition, movement speed under MUS was greater during both the CS (p=.0002) and CX (p=.0003) periods. There was also a significant main effect of stimulus ($F_{1,15}$=29.09, *p*=.00008), which was explained by the fact that movement during the CS was significantly less than during the CX period for both the drug-free (p=.0002) and MUS conditions (p=.0002). There was no interaction between MUS and stimulus for ipsilateral infusions ($F_{1,15}$=0.47, *p*=0.5). For contralateral infusions, the main effect of MUS was not quite significant ($F_{1,15}$=3.67, *p*=.07), but despite this, posthoc comparisons still indicated that movement speed under MUS was greater than under the drug free condition during both the CS (p=.002) and CX (p=.003) periods. The main effect of stimulus was significant ($F_{1,15}$=22.15, *p*=.0003), which again was explained by the fact that movement during the CS was significantly less than during the CX period for both the drug-free (p=.004) and MUS conditions (p=.004). The interaction between MUS and stimulus was not significant for contralateral inactivations ($F_{1,15}$=0.002, *p*=0.97). Taken together, these results indicate that unilateral inactivation of IL in either hemisphere did not abolish CS-evoked movement suppression, consistent with the lack of impairment following bilateral inactivations reported above.

3.3.4.2 Turning bias during post-shock trials

Changes in turning behavior following unilateral infusions of MUS were analyzed using 2×2 ANOVAs with drug (drug-free versus MUS) and stimulus (CX versus CS) as repeated measures (Figure 3.2D,E). For ipsilateral infusions, there was a significant main effect of both stimulus ($F_{1,15}$=33.0, p<.00001) and MUS ($F_{1,15}$=33.0, p=.03), but no significant interaction between stimulus and MUS ($F_{1,15}$=0.3, p=.59); posthoc comparisons confirmed that turning was greater during the CS than CX for both the drug-free (p=.0002) and MUS (p=.0002) conditions, but CS turning was reduced under MUS compared to drug free (p=.05). For contralateral infusions, there was a significant main effect of both stimulus ($F_{1,15}$=17.67, p=.0008) and MUS ($F_{1,15}$=0.11, p=.75), but no significant interaction between stimulus and MUS ($F_{1,15}$=23.25, p=.0002); posthoc comparisons again confirmed that turning was greater during the CS than CX for both the drug-free (p=.0002) and MUS (p=.0002) conditions, but CS turning was reduced under MUS compared to drug free (p=.001), and interestingly, CX turning was increased under MUS compared to drug free (p=.01).

3.3.4.3 US-evoked reflexes and movement suppression

Following MUS infusions, rats continued to exhibit unchanged reflexive responses to the US, as evidenced by movement speeds that were normally elevated during the US compared to the CX. The 2×2 ANOVA revealed that for ipsilateral infusions, there was no main effect of MUS ($F_{1,15}$=1.46, p=.25) but a strong main effect of stimulus (CX versus US: $F_{1,15}$=33.52, p<.00001), and a trend in the interaction ($F_{1,15}$=4.09, p=.06). Posthoc

comparisons showed that US-evoked movement speeds after ipsilateral MUS infusions were similar to the drug-free condition (p=.67). For contralateral infusions, there was a main effect of MUS ($F_{1,15}$=5.9, *p*=.03) and strong main effect of stimulus ($F_{1,15}$=29.54, *p*=.00007), but no significant interaction ($F_{1,15}$=.01, *p*=.92), and US-evoked movement speeds after contralateral MUS were similar to the drug-free condition (*p*=.09). Effects of unilateral infusions upon post-shock movement suppression were assessed by analyzing movement speeds during the CX period using 2×2 ANOVAs with drug (drug-free versus MUS) and trial type (pre- versus post-shock) as repeated factors. For ipsilateral infusions, there was a main effect of both MUS ($F_{1,15}$=18.15, *p*=.0007) and trial type ($F_{1,15}$=17.46, *p*=.0008), but there was no significant interaction ($F_{1,15}$=.29, *p*=.6). Posthoc comparisons revealed that this was because although CX movement speeds were lower during post-shock than pre-shock trials for both the drug-free (p=.002) and ipsilateral MUS (p=.01) conditions, MUS also generally elevated movement speeds compared to the drug free condition for both pre- (p=.0004) and post-shock (p=.0002) trials. Results were similar for contralateral infusions; there was a main effect of both MUS ($F_{1,15}$=6.54, *p*=.02) and trial type ($F_{1,15}$=14.89, *p*=.002), but no significant interaction ($F_{1,15}$=.03, *p*=.87), and CX movement speeds were lower during post-shock than pre-shock trials for both the drug-free (p=.05) and contralateral MUS (p=.07) conditions, but MUS also generally elevated movement speeds over the drug free condition for both pre- (p=.04) and post-shock (p=.02) trials. This pattern of findings indicates that unilateral MUS had no effect upon reflexive reactions to

the US, nor upon the ability of the US to suppress CX movement during post-shock trials, but did cause a non-specific increase in baseline locomotor activity.

3.4 DISCUSSION

To investigate the role of mPFC in mediating transitions between freezing and flight, we performed intracranial infusions to either inactivate or hyperactivate a region of mPFC—encompassing portions of the IL and PL subregions—that sends direct projections to both dorsal and ventral PAG in rodents (Floyd et al., 2000). Inactivation of mPFC by MUS did not impair conditioned movement suppression during pre-shock trials, but impaired expression of conditioned flight responses (defensive turning) during post-shock trials (Figure 3.2). Our injector tips were located in IL, so these findings could be viewed as consistent with prior evidence that inactivation of IL does not impair freezing (Sierra-Mercado et al., 2011). However, we used a larger infusion volume (0.4 μL/hemisphere) that may have affected both IL and PL (Figure 3.1B), so it must be noted that prior studies have reported that PL inactivation impairs freezing (Sierra-Mercado et al., 2006; 2011).

Hyperactivation of mPFC by PTX impaired expression of conditioned movement suppression during pre-shock trials, and enhanced expression of conditioned flight responses during post-shock trials (Figure 3.3). These findings bring to mind prior evidence that electrical stimulation of IL can disrupt conditioned freezing responses (Milad and Quirk, 2002), and PTX infusions into IL can prime fear extinction (Chang and Maren, 2011), but again, with the caveat that our infusions encroached upon PL as well as IL (Figure 3.1B). We

did not observe flight responses during pre-shock trials under PTX, so PTX alone was not sufficient to substitute for shock delivery as an inducer of transitions from freezing to flight. Instead, PTX seemed to enhance the emergence of post-shock flight responses. One possible explanation for this result could be that PTX augmented the aversiveness of the US, rendering it more effective at inducing the transition to flight responses. However, US-evoked motor reflexes and post-shock movement suppression were not enhanced by PTX (Figure 3.3), so by these measures, the aversiveness of the shock remained unchanged after PTX infusions.

It has been proposed that PL and IL subregions of mPFC may exert opposing influences on conditioned fear, based upon evidence that they promote and suppress, respectively, the expression of conditioned freezing responses (Sotres-Bayon and Quirk, 2010; Sierra-Mercado et al., 2011). An alternative explanation for such findings could be that different mPFC subregions exert opposing influences upon movement, rather than upon fear. Here, inactivation and hyperactivation at the same mPFC injection site exerted opposing influences on movement (freezing versus flight), but apparently left fear intact, since one of the two conditioned fear responses was always expressed. Further investigation is warranted to better dissociate the roles of different mPFC subregions in regulating emotions (such as fear) versus behavioral response strategies (such as freezing versus flight) in the presence of danger.

Figure 3.1

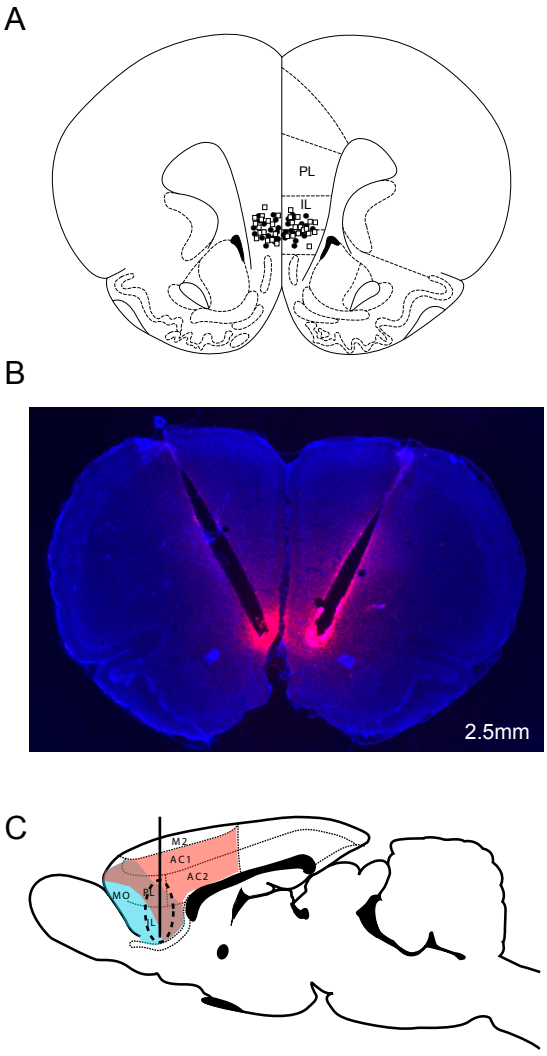

Figure 3.1. *Histological reconstruction of intracranial infusion sites.* ***A,*** Reconstructed cannula tip placements in mPFC (n=30 per hemisphere) at +2.5 mm anterior to bregma are overlaid on a coronal template from the atlas of Paxino and Watson (1997); symbols indicate rats infused with MUS (Ï‰or PTX (¡‰ ***B,*** Imaging of fluorescent muscimol (red) against DAPI counterstain (blue) shows diffusion pattern of the drug away from the injection site in mPFC. ***C,*** Midsaggital view (+0.4 mm lateral to midline) of the estimated drug diffusion radius (dashed oval) around the cannula path (straight line), overlaid on a diagram adapted from Floyd et al. (2000) showing regions of mPFC that project to dPAG (red), vPAG (blue), or both (gray). MO=medial orbital cortex; IL=infralimbic cortex; PL=prelimbic cortex; M2=secondary motor cortex; AC1, AC2=Cingulate cortex areas 1 and 2.

Figure 3.2

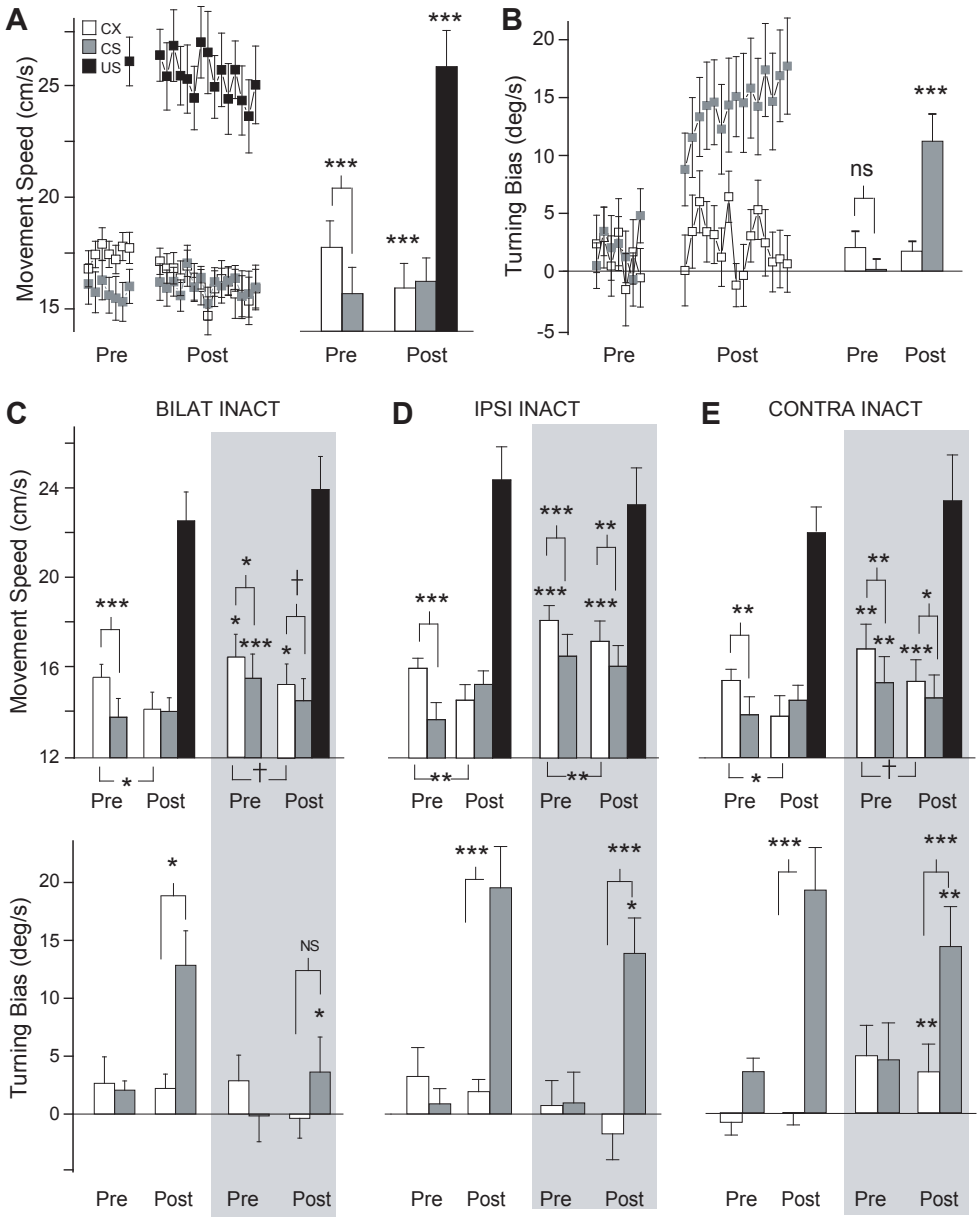

Figure 3.2. *Effects of MUS on defense responses.* ***A,*** Left graph shows trial-by-trial averages of movement speed data from pre- (Pre) and post-shock (Post) trials for all rats (n=30) on the drug-free day prior to their bilateral MUS or PTX infusion; bar graphs at right show mean movement speeds during the CX, CS and US periods. ***B***, Same as 'A' except that mean turning velocity is plotted on the ordinate (positive and negative velocities indicate turning away from or toward the trained eyelid, respectively). ***C***, Top graph shows averaged movement speeds for drug-free (unshaded) versus bilateral MUS (gray shaded) conditions; bottom graph shows the same comparison for averaged turning velocity data. ***D***, Same as 'C' except that data is shown for MUS infusions ipsilateral to the trained eyelid. ***E***, Same as 'C' except that data is shown for MUS infusions contralateral to the trained eyelid. In C-D, drug-free data is from the day immediately prior to each infusion condition. In all graphs, symbols indicate significance levels for Newman-Keuls posthoc comparisons; symbols directly above bars denote Pre vs. Post comparisons (***= p<.001, **=p<.01, *=p<.05, †p<.1, ns=not significant).

Figure 3.3

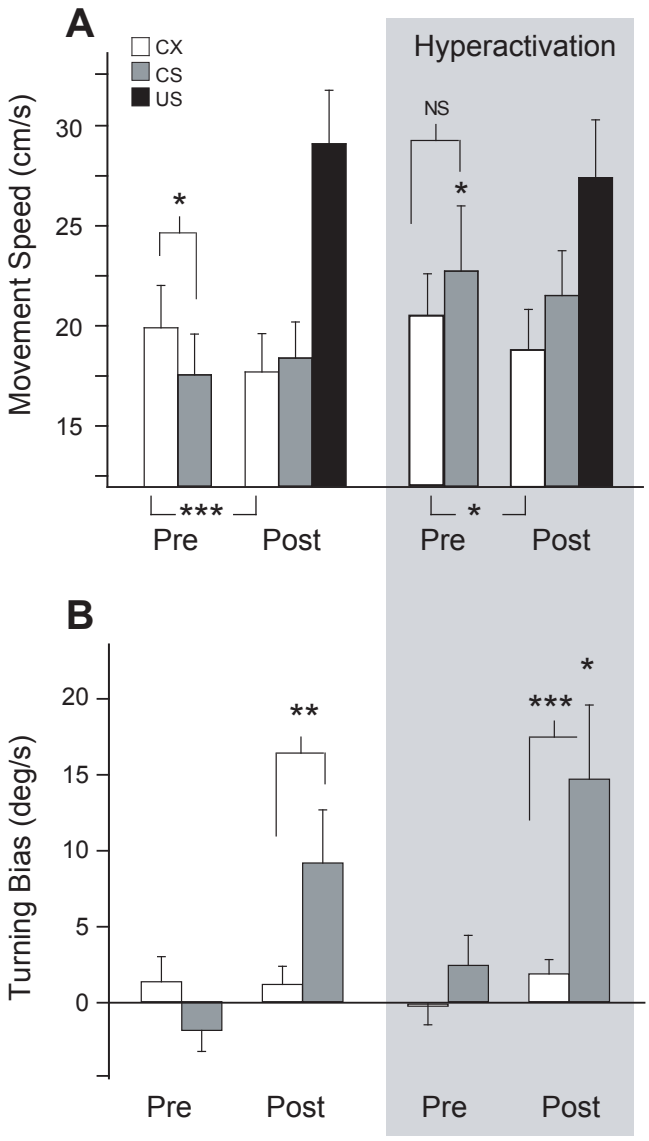

Figure 3.3. *Effects of PTX on defense responses.* ***A,*** Top graph shows averaged movement speeds for drug-free (unshaded) versus bilateral PTX (gray shaded) conditions; bottom graph shows the same comparison for averaged turning velocity data. Drug free data is from the day immediately prior to the PTX infusion. In all graphs, symbols indicate significance levels for Newman-Keuls posthoc comparisons; symbols directly above bars denote Pre vs. Post comparisons (***= p<.001, **=p<.01, *=p<.05, †p<.1, ns=not significant).

Chapter 4

Electrophysiological recordings of medial prefrontal cortex and midbrain periaqueductal gray: regulation of competing defense response strategies

4.1 INTRODUCTION

Animals express different defensive behaviors in the presence of danger depending upon the nature of the threat, and these behaviors can be organized along a spectrum referred to as the "predatory imminence continuum" (Blanchard and Blanchard, 1969a,b; Bolles, 1970; Fanselow and Lester, 1988; Mobbs et al., 2007, 2009). Low levels of threat are characterized by non-defensive behaviors (such as exploration or goal-seeking), intermediate threat levels elicit "post-encounter" responses such as freezing to avoid detection by predators, and high threat levels elicit "circa-strike" responses such as flight from danger or fighting back against the threat if no escape is possible. Evidence suggests that competing defensive response strategies may be regulated by distinct microcircuits within brain structures that mediate fear and anxiety, including the amygdala (LeDoux et al, 1988; Davis, 1992; Killcross et al., 1997; Amorapanth et al., 2000; LeDoux, 2000; Seymour and Dolan, 2008; Gozzi et al., 2010), midbrain periaqueductal gray (Bandler and DePaulis, 1988; LeDoux et al., 1988; Fanselow, 1991; De Oca et al., 1998; Walker et al., 1997; Vianna et al., 2001), and medial prefrontal cortex (Amat et al., 2005; Mobbs et al., 2009; Sotres-Bayon and Quirk, 2010; Shackman 2011; Sierra-Mercado et al., 2011).

Here we investigated how neural circuits in mPFC and dlPAG mediate transitions between two defensive responses: freezing and flight. Rats were fear conditioned by pairing an auditory CS (white noise pips) with an aversive US (brief trains of shock pulses delivered to one eyelid). We have previously shown that after rats are trained in this task, the CS elicits freezing behavior when the US has not recently been encountered, but begins to elicit flight responses after recent encounters with the shock US; both freezing and flight

are impaired by pharmacological disruptions of either the amygdala or PAG (Tarpley et al., 2010; Halladay and Blair, 2012). To investigate neurobiological mechanisms underlying behavioral transitions between freezing and flight, single-unit recordings were obtained from mPFC and dlPAG; more than half of dlPAG neurons and about one fifth of mPFC neurons responded to the CS selectively during flight conditional responses (CRs), and most mPFC neurons that responded to the CS did so in a manner that was contingent upon the rat's behavioral response (freezing versus flight).

Based on these results, we propose that shock delivery may persistently alter the responsiveness of neurons in mPFC and dlPAG to subsequent presentations of the CS, and these altered responses to the CS may underlie the generation of different CS-evoked defensive responses (freezing or flight) before versus after shock encounters.

4.2 EXPERIMENTAL PROCEDURES

4.2.1 Subjects and surgery

Adult male Long-Evans rats weighing 350-400 g were housed singly and reduced to 85% of ad-lib weight by daily limited feeding. All rats were deeply anaesthetized with isofluorane and surgically implanted with a pair of insulated stainless steel wires (75µm diameter) threaded into the skin of each eyelid for delivering the periorbital shock US. For mPFC infusion experiments, rats (n=36, of which 30 were included in the study and 6 were excluded for misplaced infusion cannulae or faulty eyelid wires) were implanted with a pair of 22 gauge microinjector guide cannulae (Plastics One, Roanoake, VA) targeted bilaterally

in mPFC (2.5 mm anterior, 0.5 mm lateral and 4.8 mm ventral to bregma) at an angle of 28 degrees to prevent drug diffusion into areas immediately dorsal to the target region. For physiological recording experiments, rats (n=5, of which 4 were included in the study and one was excluded because of failure to obtain recordings during the expression of freezing and flight CRs) were implanted with 16 moveable tetrodes which targeted mPFC and dlPAG. Four tetrodes per hemisphere were implanted in each structure of each rat. In mPFC, the four tetrodes were arrayed from 1.7 to 2.7 mm anterior to bregma at a lateral offset of 0.5-1.0 mm from the midline, and the tips were advanced over the course of the experiment from a depth of 2.0 to 5.0 mm dorsal to bregma. In dlPAG, the four tetrodes were arrayed from 6.8 to 7.8 mm posterior to bregma at a lateral offset of 0.5-1.0 mm from the midline, and the tips were advanced over the course of the experiment from a depth of 4.0 to 6.0 mm dorsal to bregma. Rats implanted with recording electrodes were also implanted with a pair of 22 gauge microinjection cannula targeted bilaterally in the basolateral amygdala (3.0 mm posterior, 5.3 mm lateral, and 8.0 mm ventral to bregma), which were used to inactivate the amygdala with muscimol during neural recordings on some experiment days. We did not obtain a sufficiently large sample of recorded neurons during amygdala inactivations to report conclusive results on the effects of amygdala disruption in this paper. Hence, inactivation sessions are excluded from the present analyses, and all data reported here is from drug-free recording sessions conducted at least 48 hours after the most recent amygdala inactivation. All experimental procedures were approved by the UCLA Animal Research Committee and were conducted in accordance with USA federal guidelines.

4.2.2 Fear conditioning

After recovery from surgery, rats were pre-exposed for 5 days (20 min/day) to the experimental context (70 x 70 cm platform for the inactivation experiments, 80 cm diameter cylindrical enclosure for electrophysiology experiments) before any fear conditioning sessions were conducted. During this time, the animals learned to chase food pellets and became habituated to the test environment. On every day following pre-exposure, rats received an identical regimen of fear conditioning trials: 6 CS-alone presentations (test trials) followed by 16 CS-US pairings (training trials). To provide a baseline of motor activity against which stimulus-evoked movement and turning behavior could be measured, rats constantly foraged for 20 mg purified food pellets (Bioserv, Frenchtown, NJ) dropped from an overhead dispenser at ~30 s intervals throughout all pre-exposure and fear conditioning sessions. The CS used for fear conditioning was a train of 70 dB white noise pips, each lasting 250 ms, delivered at 1 Hz for 20 s through an overhead speaker. The US was a train of 2.0 mA shock pulses, each lasting 2.0 ms, delivered to one eyelid at a rate of 6.66 Hz for 2 s. During CS-US pairing trials, the first shock pulse was always delivered 300 ms after the offset of the final (20^{th}) CS pip. The intertrial interval was uniformly random between 180 and 240 s.

4.2.3 Behavioral data acquisition and scoring

Each rat's position on the experimental platform was sampled at 30 Hz by an overhead video tracking system (Neuralynx Corporation, Bozeman, MT), which monitored

the location of three light-emitting diodes (LEDs) of different colors (red, blue, and green) attached to the animal's headstage for automated scoring of movement and turning behavior using software developed in our laboratory. The algorithms for scoring movement and turning behavior have been described elsewhere (Tarpley et al., 2010; Halladay and Blair, 2012). Each trial from neurophysiological recording experiments was classified as a freezing, flight, or no response trial based upon the rat's behavior during the trial. This classification was carried out by averaging the rat's movement speed during consecutive 1 s bins throughout the trial (30 video tracking samples per bin). An unpaired t-test was then performed to compare movement speeds in the 20 bins immediately preceding CS onset against the 19 bins immediately following CS onset, and the outcome of this comparison was use to classify the trial type as either freezing (CX>CS; $p<.1$), flight (CX<CS; $p<.1$), or no response ($p>.1$).

4.2.4 Single unit data acquisition

Single units in mPFC and dlPAG were recorded using a DigitalLynx S-series acquisition system (Neuralynx). Waveforms were isolated manually using Spikesort3D (Neuralynx) software. To be included in data analyses, spikes had to exceed a minimum amplitude threshold of 70 μV peak-to-peak, and exhibit a refractory period of at least 1 ms base upon analysis of interspike interval histograms. Some cells were recorded on more than one experiment day, and in these cases, data was collapsed across all recording days prior to analysis (see Figure 4.1). Spike trains recorded on different training days were

considered to be from the same cell if a) they were recorded from the same tetrode, b) the tetrode had been advanced <80 µm between recordings, and c) cluster boundaries and waveform shapes were visually similar on all tetrode channels for each session.

4.2.5 Analysis of single unit recording data

Neurophysiological data analysis only included cells that were recorded during at least 3 freezing and 3 flight trials across all days during which the cell was isolated.

4.2.5.1 Classification of cell types

Single units recorded in dlPAG and mPFC were classified according to how the cell's firing rate changed between the CX and CS periods during freezing versus flight trials. To measure this change, all of the data collected from a given cell (across all days on which the cell was recorded) during trials of a given type (freezing or flight) was used to generate a peristimulus histogram (PSTH) aligned to the onset of the first CS pip (bin size = 1 s). An unpaired t-test was then performed to compare the cell's firing rate during the 20 bins preceding CS onset (CX period) against the 19 bins following CS onset (CS period) for each trial type.

4.2.5.2 Population averaged PSTHs

To generate population-averaged PSTHs for freezing and flight trials, the firing rate in each histogram bin was converted to a z-score normalized to the mean and standard

deviation of the 20 firing rate bins from the CX period of freezing trials; the normalized histograms were then averaged across cells to generate population-averaged histograms.

4.2.5.3 Responses to pips and shocks

Responses to auditory pips were analyzed on a short time scale by plotting PSTHs aligned to the onset of each CS pip using a bin size of 2 ms. Confidence intervals on the spike counts in each bin were computed from a baseline period spanning 500 ms (250 bins) prior to the onset of each pip, based upon an assumption of Poisson spiking (Abeles, 1982). A neuron was considered to exhibit auditory evoked responses if there were one or more bins exceeding 99% confidence, or two or more bins exceeding 95% confidence, in the 100 ms (50 bins) time window following pip onset. The cell's response latency was computed as the time delay from pip onset to the first bin exceeding the 95% confidence threshold. Responses to shock pulses were analyzed in a similar manner, except that the baseline period for computing confidence intervals spanned 50 ms (25 bins) preceding each shock onset, and a neuron was considered to be shock responsive if there were one or more bins exceeding 99% confidence, or two or more bins exceeding 95% confidence, in time window spanning 10-100 ms (40 bins) following pip onset; the 0-10 ms time period was omitted because each shock pulse generated a brief stimulus artifact that occluded recording for <10 ms after the offset of the 2 ms shock pulse. The latency of shock-evoked responses was computed as the as the time delay from pip onset to the first bin in the analysis window exceeding the 95% confidence threshold.

4.2.6 Histological Procedures

At the end of the experiment, rats were intraperitoneally injected with an overdose of pentobarbital (100 mg/kg) and perfused intracardially. Brains were extracted and fixed in a formalin sucrose solution. Tissue was later sectioned into 40 μm slices and mounted on slides for electrode placement verification.

4.3 RESULTS

To investigate neural mechanisms underlying behavioral transitions between freezing and flight defenses, we conducted electrophysiological recording experiments while rats expressed conditioned fear responses. Each session consisted of 6 presentations of the CS alone followed by 16 CS-US pairings (Figure 4.1A). For each trial, we define the CX period as the 20 s time window preceding onset of the first CS pip, the CS period as the 19.6 s time window between CS and US onset, and the US period as the 2 s period following US onset (Figs. 1C,D). On a given experiment day, we define 'pre-shock trials' as the 7 trials during which the CX and CS periods both preceded the day's first US presentation (all 6 CS alone trials, plus the first CS-US pairing trial), whereas 'post-shock trials' are defined as the remaining 15 CS-US pairing trials (Figure 4.1A,B).

4.3.1 Behavioral classification of trial types

To analyze correlations between neural activity and defensive behavior, experimental trials were classified into one of three types according to the rat's behavior during the trial (see Methods): 1) 'freezing trials' were those during which the rat's movement speed was significantly lower during the CS than CX period, 2) 'flight trials' were those during which the rat's movement speed was significantly higher during the CS than CX period, and 3) 'no response' trials were those during which the rat's movement speed did not significantly differ during the CS versus CX period. Figure 4.2B shows movement speeds during freezing, flight, and no response trials, averaged over all trials of each type during which at least one neuron was recorded (total of 902 trials from 41 recording sessions in 4 rats). Pie graphs in Figure 4.2A show that of the pre-shock trials (n=287), 59.9% were freezing trials and only 6.3% were flight trials (the rest were no response trials). Conversely, of the post-shock trials (n=615), 66.4% were flight trials and only 6.8% were freezing trials (again, the rest were no response trials). These results are consistent with prior studies showing that the CS-evoked responding is dominated by freezing CRs during pre-shock trials, whereas flight CRs emerge during post-shock trials (Tarpley et al., 2010; Halladay and Blair, 2012). Mean movement speed during the CX period was lower for flight than freezing trials (t_{579}=15.0; p<.00001), which reflects post-shock movement suppression on trials given after the first US delivery on each experiment day.

Figure 4.2C shows the rats' mean turning velocity towards versus away from the shocked eyelid during each trial type. A 3×2 ANOVA of turning velocity with trial type

(freezing, moving, no response) and stimulus (CX, CS) as independent factors yielded a highly significant interaction effect ($F_{2,896}$=21.48; p<.00001), and Scheffe corrected posthoc comparisons revealed that turning during the CS for flight trials was greater than all other conditions (p<.00001 for all comparisons), while the other conditions did not different significantly from one another (p>.66 for all comparisons). These results show that during the CS period of flight trials, rats turned away from the eyelid where shock delivery was anticipated, as shown in prior studies (Tarpley et al., 2010). This turning behavior supports the interpretation that CS-evoked movements observed during flight trials were indeed flight CRs, directed away from the expected US.

4.3.2 Neural recordings in dlPAG

A total of 74 neurons were recorded from dlPAG, with a mean baseline firing rate of 2.96±.36 Hz and a mean peak-to-peak spike height of 93.6±1.0 □V. Most of the dlPAG cells (n=57) were recorded from either the dorsomedial or dorsolateral column (henceforth abbreviated dPAG), and the remaining cells (n=17) were recorded from the lateral column (henceforth abbreviated lPAG). Reconstructions of recording sites are shown in Figure 4.2.

4.3.2.1 Classification of cell types

Single units were classified according to how their firing rates changed between the CX and CS periods during freezing versus flight trials (Figure 4.2B). Cells that significantly changed their firing rate during freezing but not flight trials were classified either as FRZ+ or FRZ- cells, depending upon whether their firing rates increased or decreased, respectively, during the CS period. Similarly, cells that significantly changed their firing rate during flight but not freezing trials were classified either as FLT+ or FLT- cells. Cells that significantly changed their firing rate in the same direction during both freezing and flight trials were deemed to be 'CS responsive' and thus classified either as CSR+ or CSR- cells, depending upon whether their firing rates increased or decreased, respectively, during the CS period of both trial types. Cells that significantly changed their firing rate in opposing directions during freezing and flight trials were deemed to be 'movement correlated' (since movement speed also changed in opposing directions during freezing and flight trials), and were thus classified either as MOV+ or MOV- cells, depending upon whether their firing rates changed in the same or the opposite direction, respectively, from the rat's movement speed. Cells that did not significantly change their firing rate between the CX and CS period of either freezing or flight trials were deemed to be 'non-responsive' and were thus classified either as NR cells. Fifty-four percent of dlPAG cells (40 of 74) were classified as FLT+ cells (Figure 4.3C), twenty-seven percent (20 of 74) were classified as CSR+ cells (Figs. 1D, 3B), eleven percent (8 of 74) were classified as MOV+ cells (Figure 4.3A), and eight (5 of 74) were classified as NR cells. In addition, one dlPAG cell was classified as a FRZ- cell. Analysis with a

Fisher's exact test revealed that a neuron's type was significantly contingent upon whether it was recorded in dPAG or lPAG (p=.02), and binomial tests revealed that this was mainly because MOV+ cells were significantly more prevalent in lPAG than dPAG (p=. 038); by contrast, FLT+ (p=.31), CSR+ (p=.997), and NR cells (p=.65) were evenly distributed across recording sites in lPAG and dPAG.

4.3.2.2 Baseline firing rate changes

Baseline firing rates during the CX period (Table 1) were analyzed using a 4×2 ANOVA with cell type (MOV+, CSR+, FLT+, NR; the analysis included only cell types for which N≥7) as an independent factor and trial type (freezing, flight) as a repeated factor (Figure 4.4D). There was a significant main effect of trial type ($F_{1,69}$=5.28, p=.02) but not cell type ($F_{1,69}$=1.09, p=.36), and no interaction ($F_{3,69}$=1.53, p=.22). Posthoc comparisons revealed that only cells belonging to the MOV+ category showed significantly lower baseline firing rates during the CX period of flight versus freezing trials (p=.047), supporting the conclusion that movement speed was a primary determinant of firing rates for MOV+ cells (since movement speed was also lower during the CX for flight than freezing trials, as shown in Figure 4.1C). Baseline firing rates did not differ significantly between freezing versus flight trials for FLT+ (p=.85), CSR+ (p=.62), or NR (p=.87) cells, but firing rate decreases can difficult to detect in cells with low baseline spike rates, which may partly explain why MOV+ cells

(which had the highest baseline firing rate during freezing trials) were the only cell type to exhibit a baseline firing rate decrease during flight trials.

4.3.2.3 Pip-evoked responses

Motor activity was influenced by the CS on the same time scale at which firing rate comparisons were made to classify cell types (see Figure 4.1C), so it was difficult to dissociate how sensory versus motor influences contributed to CS-evoked firing rate changes. To address this confound, short-latency responses to the CS pips were analyzed on a fast time scale to assess whether dlPAG neurons exhibited sensory evoked responses to the auditory pips (see Methods). About half of the dlPAG cells (34 of 74, or 46%) exhibited excitatory responses that were time locked to pip onset, with a mean onset latency of 32.7 ± 1.7 ms; none of the dlPAG cells showed evidence of being inhibited by pip onset. None of the pip responsive cells belonged to the MOV+ category (Figure 4.4C), supporting the interpretation that motor (rather than sensory) influences were the primary determinant of firing rate increases and decreases for MOV+ cells. Among the remaining cells types, pip responses were observed for fifty-eight percent (23 of 40) of FLT+ cells (Figure 4.4A) and fifty percent (10 of 20) of CSR+ cells (Figure 4.4B); in addition, 1 of 5 NR cells was pip responsive (not shown). A 2×2 ANOVA with cell type (FLT+ versus CSR+, independent) and trial type (freezing versus flight, repeated) as factors yielded a main effect of trial type ($F_{1,30}$=16.52; p=.0003) but not of cell type ($F_{1,30}$=0.81; p=.38), with no

interaction ($F_{1,30}$=0.12; p=.83). Posthoc comparisons indicated that pip-evoked responses were larger during flight than freezing trials for both the FLT+ (p=.035) and CSR+ (p=.011) cell types. Hence, expression of flight CRs was correlated with an increase in the magnitude of pip-evoked responding by FLT+ and CSR+ neurons in dlPAG (Figure 4.4A,B).

4.3.2.4 Shock-evoked responses

Shock-evoked responses of dlPAG neurons were analyzed on a short time scale by plotting PSTHs triggered by individual shock pulses (see Methods). Forty-one dlPAG cells (55%) were excited by shock pulses (Figure 4.5A) with a mean onset latency of 15.4±1.1 ms. Seventeen cells (23%) were inhibited by shock pulses (Figure 4.5B). Shock responsiveness was not contingent upon a cell's type classification (Fisher's exact test, *p*=.66), but shock-responsiveness was found to be contingent upon whether a cell was recorded in dPAG versus lPAG (Fisher's exact test, *p*=.02). Binomial tests revealed that this was because cells inhibited by shock were found exclusively in dPAG and not in lPAG (*p*=.02), whereas cells excited by shock (*p*=.43) were evenly distributed between dPAG and lPAG, as were cells that were non-responsive to shocks (*p*=.60).

4.3.3. Neural recordings in mPFC

A total of 71 neurons were recorded from mPFC, with a mean baseline firing rate of 2.93±.32 Hz and a mean peak-to-peak spike height of 108.8±3.1 □V. These cells were distributed among three mPFC subregions: ACC (n=31), PL (n=29), and IL (n=11) subdivisions of mPFC (Figure 4.6). Recording sites in ACC were located within a region that projects mainly to dorsal PAG, whereas recording sites in IL and PL were located within a region that projects to both dorsal and ventral PAG (Floyd et al., 2000).

4.3.3.1 Classification of cell types

Neurons recorded in mPFC were classified into different types by comparing their firing rates during the CX versus CS periods of freezing and flight trials, as described above for dlPAG neurons. Thirty-two percent (23 of 71) of the cells in mPFC did not exhibit any significant difference in their firing rate during the CX versus CS periods for either freezing or flight trials, and were thus classified as NR cells. Twenty-three percent of the mPFC neurons (15 of 71) were classified as MOV+ cells (Figure 4.7B), and one neuron was classified as an inhibitory movement (MOV-) cell because its firing rate that was inversely related with movement speed. Twenty percent of cells recorded in mPFC (14 of 71) were classified as FLT+ cells (Figure 4.7C). One neuron had a firing rate that decreased during the CS for flight trials, but did not change for freezing trials, and was thus classified as an inhibitory flight (FLT-) cell. Eighteen percent of the cells (13 of 71) showed a response to

the CS only during freezing but not during flight trials (Figure 4.7D), and of these cells, 6 were excited (FRZ+) and 7 were inhibited (FRZ-) during freezing. Six percent of the mPFC cells (4 of 71) responded to the CS during both freezing and flight trials, and of these cells, 3 were excited (CSR+) and one was inhibited (CSR-) during the CS. Of these cell types observed in mPFC, only MOV+ cells were unevenly distributed across mPFC subregions ($\chi^2_{2,71} = 6.08$, $p = .048$), exhibiting higher concentrations in both ACC and IL than in PL. By contrast, there was no significant variability across subregions in the concentrations of FLT+ cells ($p = .77$), FRZ+ cells (p=.86), FRZ- cells (p=.42), or NR cells (p=.55). Interestingly, all of the CSR+ and CSR- cells were recorded in PL, but due to the small sample of such cells (n=4), it was not possible to determine whether this reflected a significantly higher concentration of CSR neurons in PL than in other subregions.

4.3.3.2 Baseline firing rates

Baseline firing rates during the CX period (Table 2) were analyzed using a 4×2 ANOVA with cell type (FLT+, MOV+, FRZ-, NR; the analysis included only cell types for which N≥7) as an independent factor and trial type (freezing, flight) as a repeated factor (Figure 4.8D). There was a significant main effect of trial type ($F_{1,55}$=5.44, p=.02) but not of cell type ($F_{1,55}$=0.54, p=.66), and no interaction ($F_{3,55}$=1.8, p=.16). Posthoc comparisons revealed that only cells belonging to the MOV+ category trended toward lower baseline firing rates during the CX period of flight versus freezing trials (p=.054), supporting the conclusion that

movement speed was a primary determinant of firing rates for MOV+ cells (since movement speed was also lower during the CX for flight than freezing trials, as shown in Figure 4.1C).

4.3.3.3 Pip-evoked responses

To analyze fast auditory responses of mPFC neurons, spikes evoked by the CS pips were analyzed on a short time scale in the manner described above for dlPAG neurons. In contrast with the dlPAG neurons (see Figure 4.4), none of the mPFC neurons exhibited significant CS-evoked responses that were time locked to the CS onset (Fig.8). This lack of pip-evoked responses suggests that firing rate changes of mPFC neurons during the CS period were probably not driven directly by sensory stimulation, but instead reflected slower modulation by the CS of ongoing processes such as motor activity, behavioral response strategies, or motivational states (see Discussion).

4.3.3.4 Shock-evoked responses

To assess whether mPFC neurons exhibited sensory responses to the shock US, responses to shock pulses were analyzed on a short time scale using a bin size of 2 ms (Figure 4.9). About one third of the cells in mPFC (24 of 71) exhibited significant US-evoked spike responses that were time-locked to the onset of periorbital shock pulses (Figure 4.9). A chi-square test indicated that the proportion of shock responsive neurons differed

significantly among mPFC subregions ($\chi^2_{2, 71} = 8.8$, $p = .01$). Approximately half of the cells in ACC (15 of 31) were excited by shock with an onset latency of 16.1 ± 1.5 ms, and half of the cells in IL (5 of 11) were excited by shock with an onset of 13.2 ± 2.3 ms. Fourteen percent (4 of 29) of the cells in PL were excited by shock, with a longer mean onset latency of 28.8 ± 9.3 ms.

4.4. DISCUSSION

Single-unit recording experiments were conducted to investigate the roles of mPFC and dlPAG in orchestrating freezing and flight responses to an auditory fear cue. Results indicate that these structures may interact with one another to regulate transitions between different defensive response strategies.

4.4.1 Single-unit recordings in dlPAG

A large proportion (more than half) of dlPAG neurons increased their firing rate during presentations of a fear-conditioned CS when it elicited flight responses, but not when it elicited freezing responses, and were thus classified as FLT+ cells (Figure 4.3C). This finding is consistent with a large body of evidence implicating dlPAG in the expression of defensive movements (Halpern, 1968; Liebman et al., 1970; DiScala et al., 1984; Bandler and DePaulis, 1991; Fanselow, 1991; DePaulis et al., 1992; De Oca et al., 1998; Keay and Bandler, 2001; Mobbs et al., 2007, 2009; Tarpley et al., 2010; Halladay and Blair, 2012). About one fourth of dlPAG neurons exhibited CS-evoked increases in their firing rate during

both freezing and flight trials (Figure 4.3B), and were thus classified as CSR+ cells. About one tenth of dlPAG cells were classified as MOV+ cells, because their firing rates increased during CS period of flight trials and decreased during CS period of freezing trials (Figure 4.3A), and thus always changed in the same direction as movement speed.

A large proportion of dlPAG neurons (notably excluding MOV+ cells) exhibited fast responses to the auditory CS pips, at a mean latency of ~32 ms from pip onset. It is unlikely that these pip-evoked responses were driven by direct inputs to dlPAG from mPFC, because mPFC neurons did not exhibit short-latency responses to the onset of auditory pips (Figure 4.8). Some mPFC neurons increased their firing rates during the CS, in agreement with prior studies (Baeg et al., 2001; Burgos-Robles et al., 2009; Sotres-Bayon et al., 2012), but these CS-evoked firing rate changes were too slow to drive the fast pip-evoked responses seen in dlPAG neurons. In rodents, neurons in the basolateral and central nuclei of the amygdala respond to a fear conditioned auditory CS with onset latencies ranging between 15-30 ms (Quirk et al., 1995; Goosens et al., 2003; Maren and Quirk, 2004; Ciocchi et al., 2010; Johansen et al., 2010), which would be an appropriate time window for driving the pip-evoked responses we observed in dlPAG, but our recordings were obtained from columns that do not receive direct input from the amygdala (Rizvi et al., 1991). If pip-evoked responses in dlPAG were driven by the amygdala, then it could have been via an intervening structure, such as the substantia nigra (Brandão et al., 2005). Another possibility is that pip-evoked responses could have been driven by direct inputs to dlPAG from auditory cortex (Newman et al., 1989), which exhibits associative plasticity during fear conditioning (Quirk et al., 1997; Letzkus et al., 2011). Pip-evoked responses in dlPAG were significantly larger

during flight than freezing trials (Figure 4.4), but many FLT+ neurons increased their firing rates on a slow time scale during the CS period of flight trials (similar to the non pip-responsive FLT+ neurons recorded in mPFC), without exhibiting pip-evoked responses at all (Figure 4.4A, bottom graph). Further study is needed to investigate whether fast CS-evoked responses of dlPAG neurons are functionally involved in generating conditioned flight responses.

4.4.2 Single-unit recordings in mPFC

In comparison with dlPAG neurons, mPFC neurons exhibited more diverse patterns of responding during the expression of conditioned fear. Fully one third of mPFC neurons were classified as NR cells that did not change their firing rate during the CS at all. Only about one fifth of mPFC neurons were classified as FLT+ cells, firing selectively to the CS during flight trials (Figure 4.7B). Another one fifth of mPFC cells were classified as FRZ+ or FRZ- cells (Figure 4.7C), because they increased or decreased (respectively) their firing rates during presentations of fear conditioned CS when it elicited freezing but not flight responses, in agreement with prior reports showing that some mPFC neurons fire in correlation with conditioned freezing (Burgos-Robles et al., 2009; Sotres-Bayon et al., 2012). Yet another one fifth of mPFC neurons were classified as MOV+ or MOV- cells, because their firing rates were correlated with the rats' movement speed. The firing rates of MOV+ cells increased during flight trials and decreased during freezing trials (Fig, 7A), and MOV- cells behave in the opposite manner. MOV+ and MOV- cells were most highly concentrated in the ACC subregion. Only a small proportion of mPFC cells (less than five percent) fired non-

selectively during the CS regardless of whether it elicited freezing or flight. Hence, the responses of most mPFC cells to the CS were contingent upon the rat's behavior (freezing or flight), suggesting that mPFC neurons may mediate defensive response strategies during encounters with threatening stimuli.

4.4.3 Shock-evoked responses

In both dlPAG (Figure 4.5) and mPFC (Figure 4.9), neurons that responded during the CS period were intermingled with, and partially overlapped with, neurons that responded to shocks (Figs. 2 and 6). These findings are consistent with prior studies showing that shock responsive neurons are found in both PAG and mPFC of rodents (Sanders et al., 1980; Heinricher et al., 1987; Sikes and Vogt, 1992; Yamamura et al., 1996; Johansen et al., 2010). The shock stimulus appeared to be a main trigger for behavioral transitions from freezing to flight, because on each experiment day, only conditioned freezing was observed during pre-shock trials, whereas CS-evoked flight did not emerge until post-shock trials (Tarpley et al., 2010; Halladay and Blair, 2012). These findings suggest the possibility that shock-evoked responses of neurons in mPFC and dlPAG might trigger persistent changes (lasting from seconds to minutes) in the responses of local microcircuits in these regions to presentations the CS, effectively storing a 'working memory' for the recent occurrence of shock, in concordance with theories that emphasize the role of mPFC in short-term memory functions (Fuster and Alexander, 1971; Fuster, 1973; Uylings et al., 2003). Persistent alterations in CS-evoked firing may in turn modulate the performance of CS-evoked defensive responses, mediating a transition from freezing to flight, in accordance

with theories that posit a role for mPFC and its descending projections in modulation of response strategies (Miller, 2000; Miller and Cohen, 2001) and defensive behaviors (Shackman, 2011).

Figure 4.1

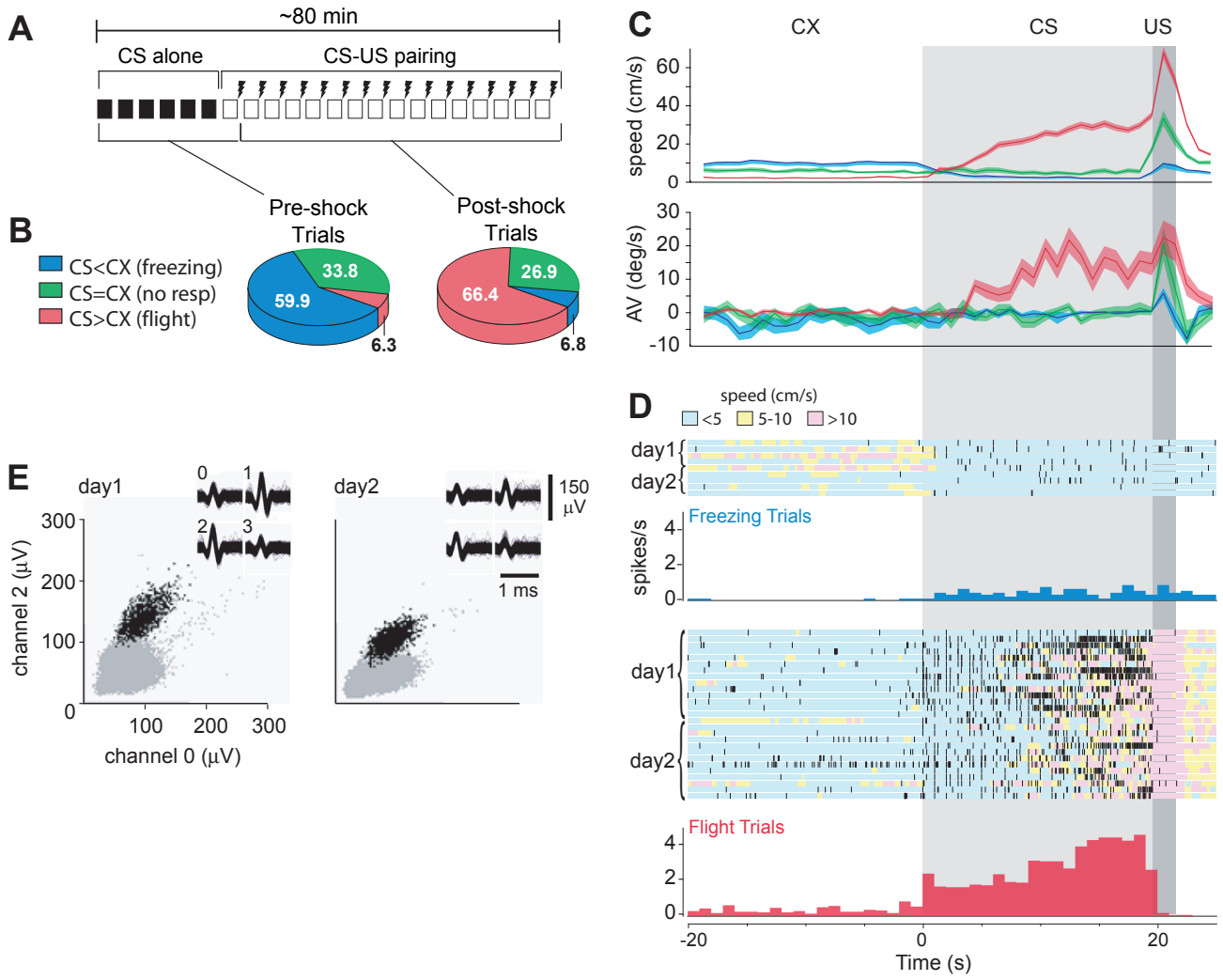

Figure 4.1. *Behavioral and neural activity during fear conditioning trials*. ***A***, Each session consisted of 6 CS alone trials followed by 16 CS-US pairings, separated by uniformly random intertrial intervals of 180-240 s. ***B***, Proportion of pre- and post-shock trials from neural recording sessions that were classified freezing, no response, or flight trials. ***C***, Movement speed (top) and turning bias (bottom) during the CX (unshaded area), CS (light shaded area), and US (dark shaded area) periods of each trial; data is averaged separately across sessions for freezing (blue), no response (green) and flight (red) trials. ***D***, Example of spike data from a dlPAG neuron recorded over two days; color of shading beneath spike rasters indicates the rat's movement speed during each trial, and rate histograms (1s bins) show the cell's mean firing rate averaged over all freezing (blue) and flight (red) trials. ***E***, Spike waveforms (upper right) and cluster analysis for the dlPAG neuron on both recording days.

Figure 4.2

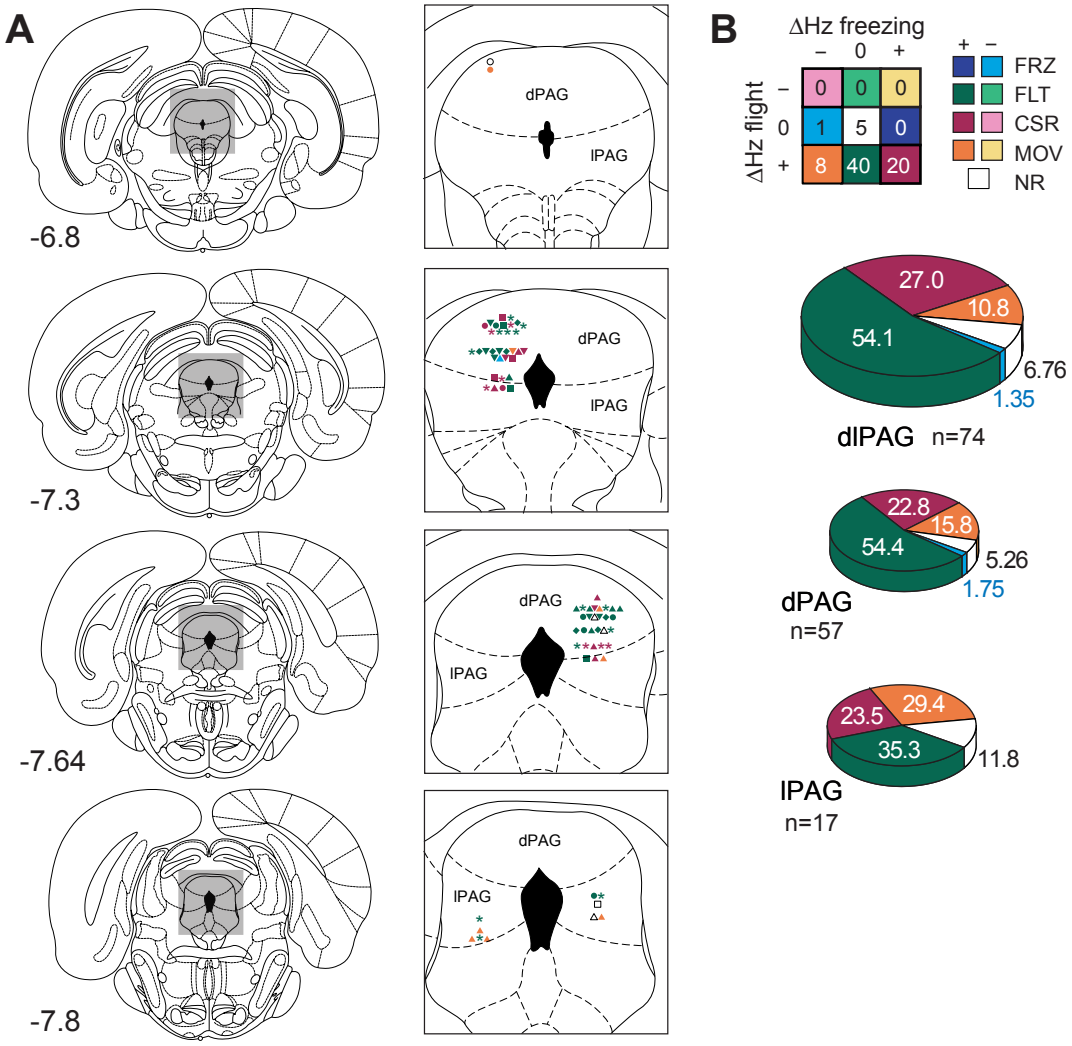

Figure 4.2. *Histological reconstruction of recording sites in dlPAG.* ***A***, Reconstructed recording sites of dlPAG neurons (n=74) are overlaid upon coronal templates (with coordinates in mm relative to bregma) from the atlas of Paxino and Watson (1997); colors indicate each cell's type classification (see panel B) and symbols indicate how the cell responded to CS pips or shock pulses (* excited by pips and shocks, ▲ excited by shocks but not pips, ■ excited by pips but not shocks, ◆ excited by pips and inhibited by shocks, ▼ no pip response and inhibited by shocks, ● no response to pips or shocks). B, Proportions and numbers of cells in dlPAG (and in dPAG vs. lPAG subregions) that were classified in each type category; table provides a color key for the type categories.

Figure 4.3

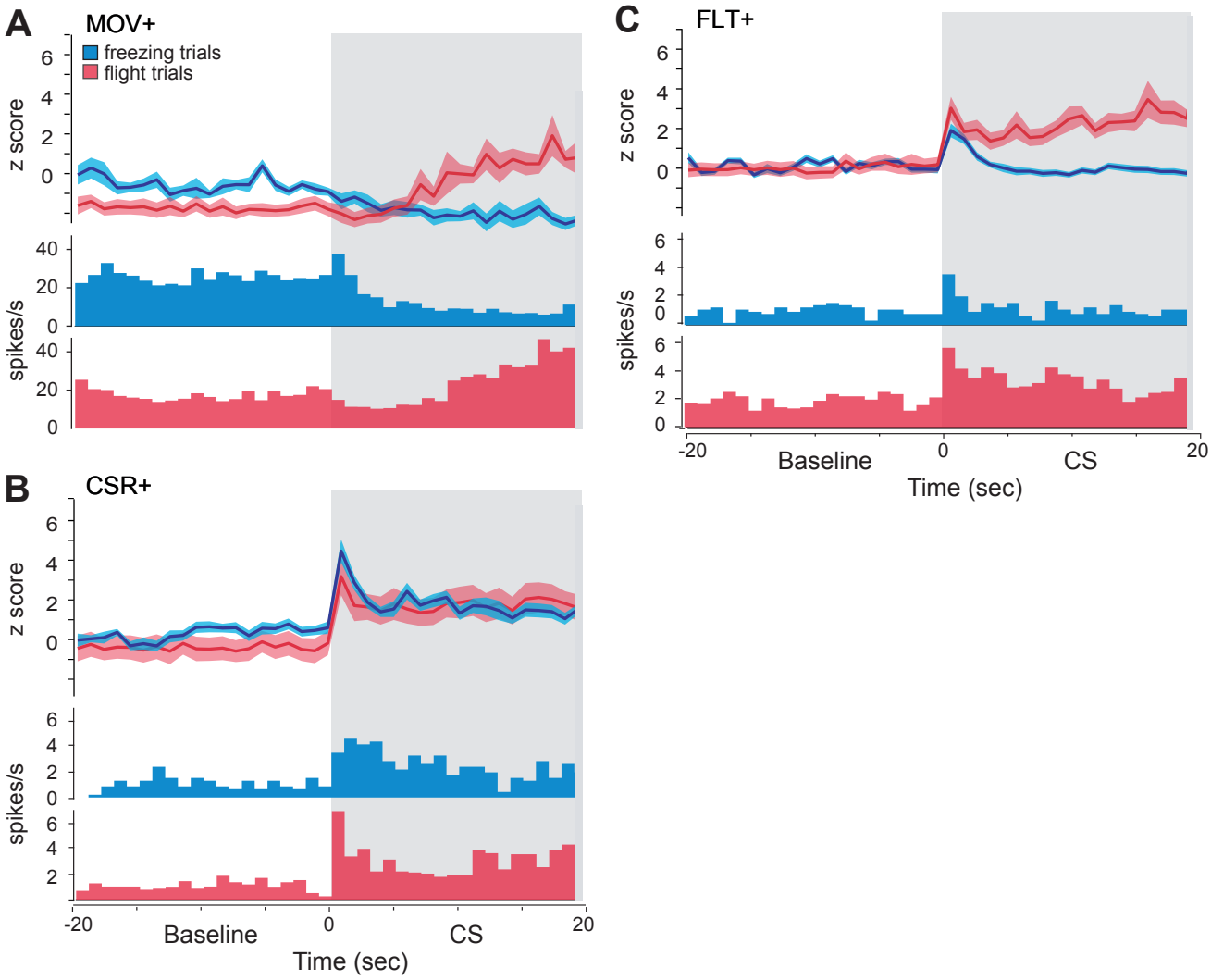

Figure 4.3. *Population activity of dlPAG neurons*. Each panel shows population averaged firing rates (top graph) and a PSTH for one example cell (bottom graphs) for three cell types recorded in dlPAG: ***A***, MOV+ cells (n=8); ***B***, CSR+ cells (n=20); ***C***, FLT+ cells (n=40). CS period is shaded in gray, CX period is unshaded.

Figure 4.4

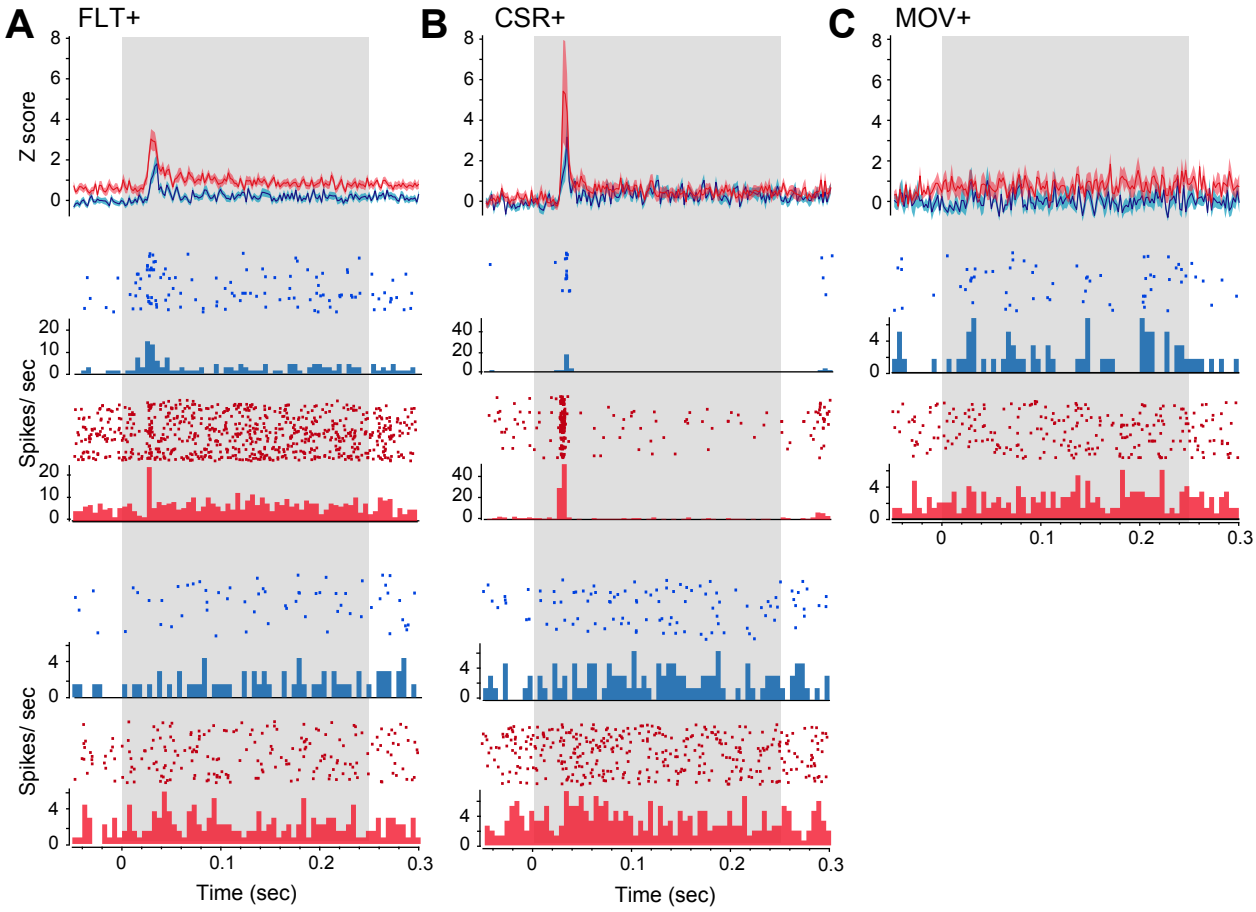

Figure 4.4. *Pip-evoked responses of dlPAG neurons.* ***A,*** Top graph shows population averaged firing rates (2 ms bins) for pip-evoked responses averaged across all FLT+ cells in dlPAG; middle and bottom graphs shows rasters and PSTHs for examples of pip responsive and non-pip responsive FLT+ cells, respectively. ***B,*** Same as 'A' except that data is from CSR+ cells. ***C,*** Same as 'C' except that data is from MOV+ cells (there were no examples of pip responsive MOV+ cells).

Figure 4.5

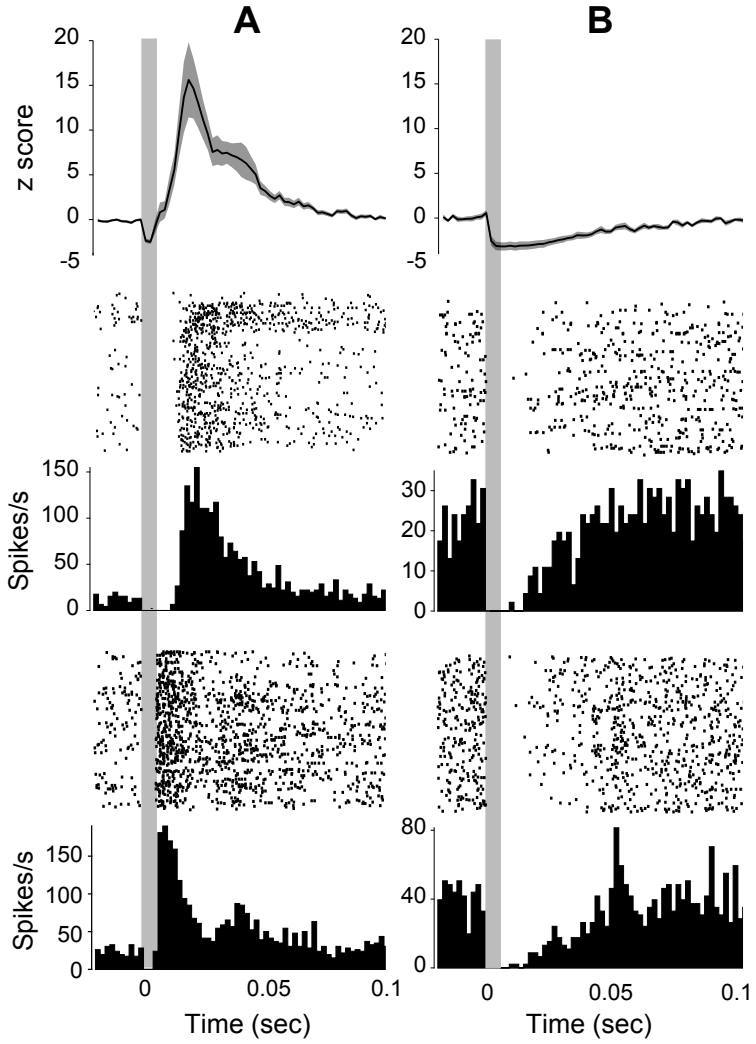

Figure 4.5. *Shock-evoked responses of dlPAG neurons*. ***A,*** Top graph shows population averaged firing rates for all dlPAG neurons that were excited by shocks (n=41), bottom graphs show raster plots and PSTHs for two examples of such cells. ***B,*** Same as 'A' except that data is shown for dlPAG neurons that were inhibited by shocks (n=17).

Figure 4.6

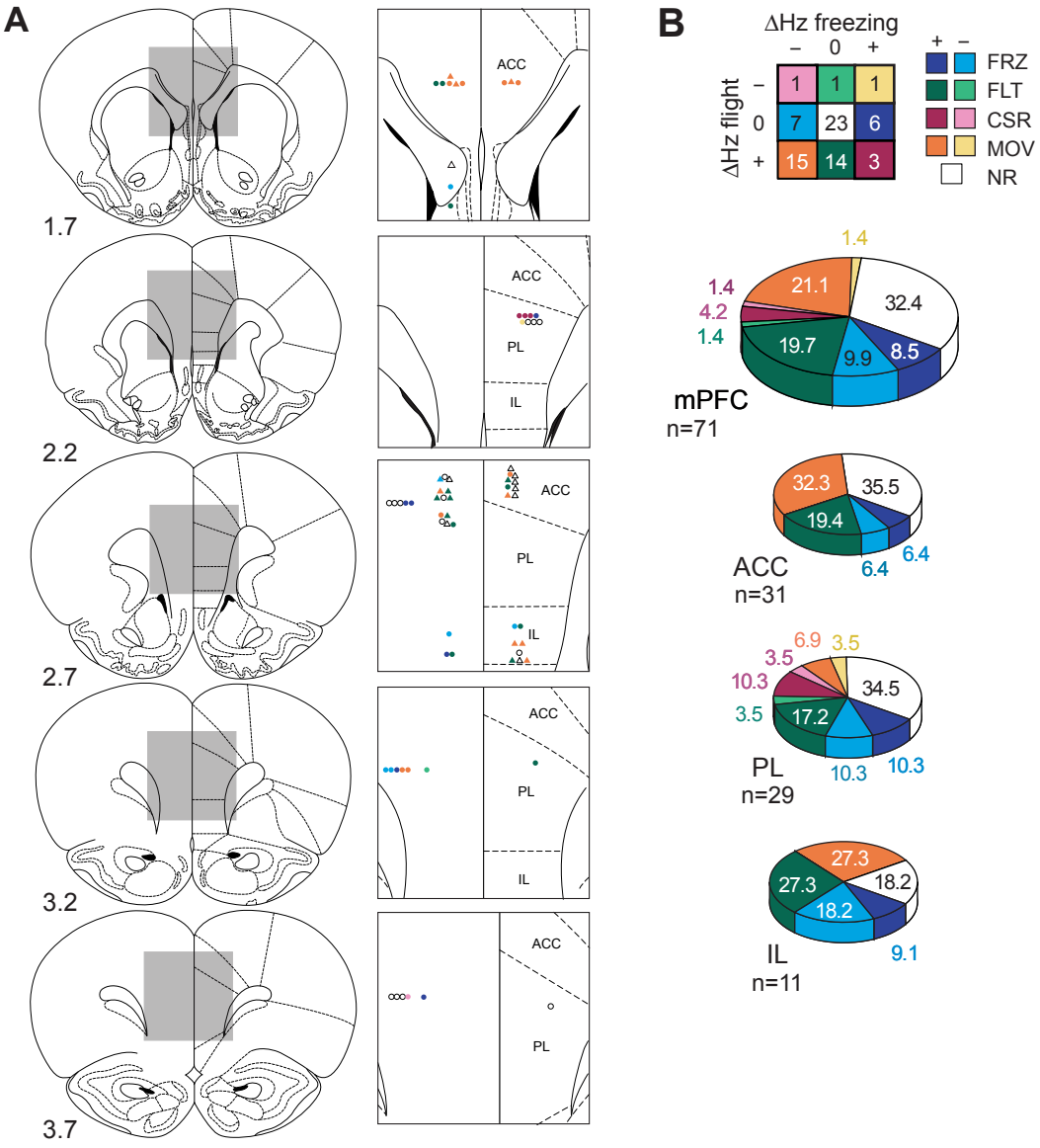

Figure 4.6. *Histological reconstruction of recording sites in mPFC*. ***A***, Reconstructed recording sites of mPFC neurons (n=71) are overlaid upon coronal templates (with coordinates in mm relative to bregma) from the atlas of Paxino and Watson (1997); colors indicate each cell's type classification (see panel B) and symbols indicate how the cell responded to CS pips or shock pulses ▲ excited by shocks but not pips; ● no response to pips or shocks). B, Proportions and numbers of cells in mPFC (and in IL, PL, and ACC subregions) that were classified in each type category; table provides a color key for the type categories.

Figure 4.7

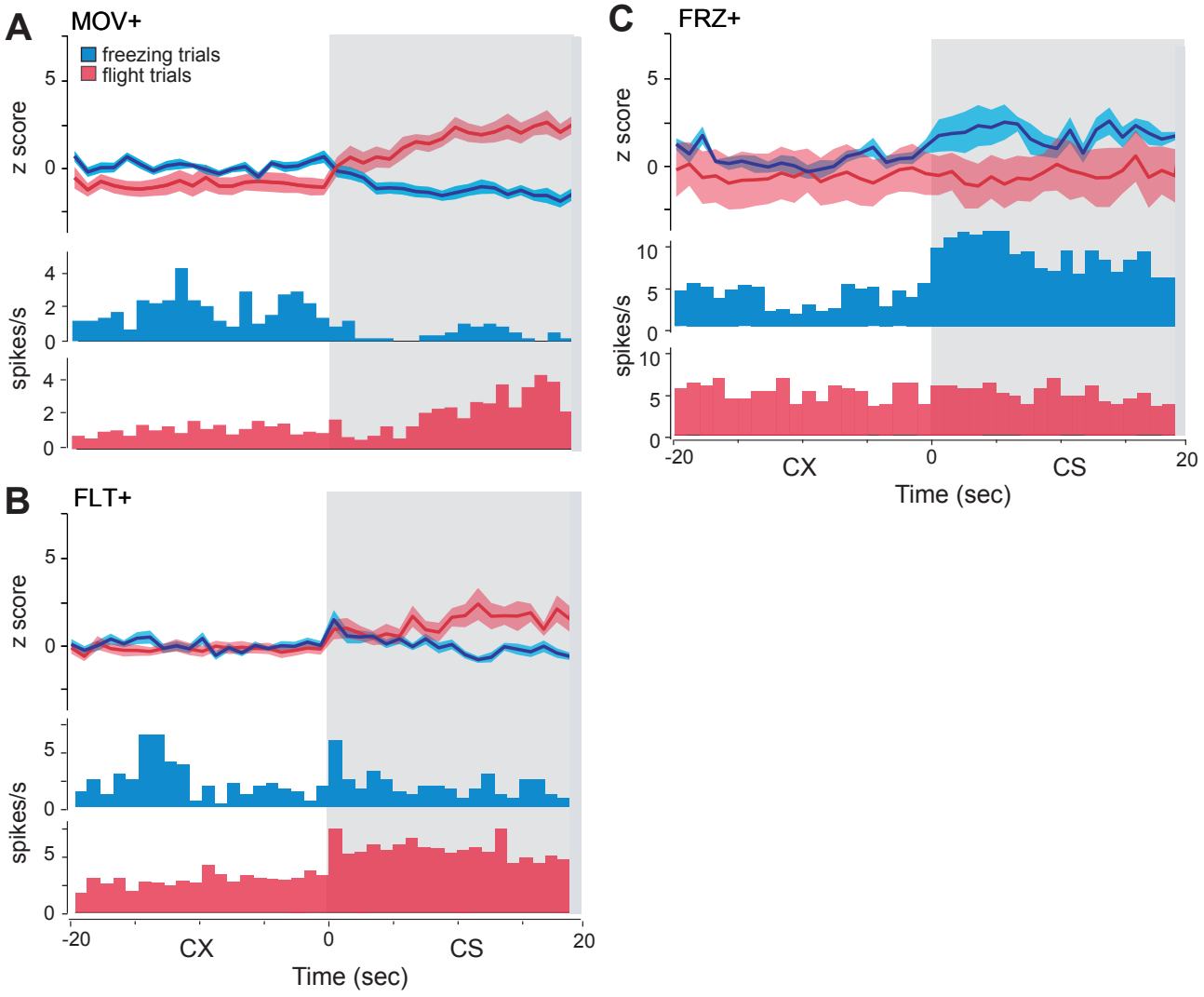

Figure 4.7. *Population activity of mPFC neurons.* Each panel shows population averaged firing rates (top graph) and a PSTH for one example cell (bottom graphs) three cell types recorded in mPFC: ***A***, MOV+ cells (n=15); ***B***, FLT+ cells (n=14); ***C***, FRZ+ cells (n=6). CS period is shaded in gray, CX period is unshaded.

Figure 4.8

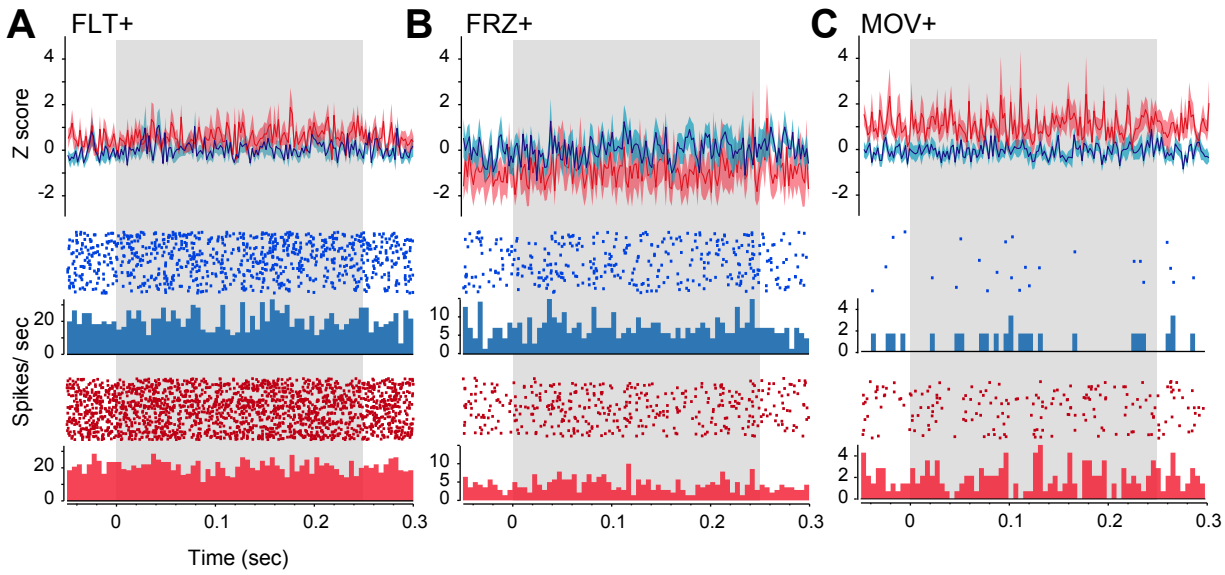

Figure 4.8. *Pip-evoked responses of mPFC neurons*. ***A,*** Top graph shows population averaged firing rates (2 ms bins) for pip-evoked responses averaged across all FLT+ cells in mPFC; bottom graph shows rasters and PSTHs for an example cell. ***B,*** Same as 'A' except that data is from FRZ+ cells. ***C,*** Same as 'C' except that data is from MOV+ cells.

Figure 4.9

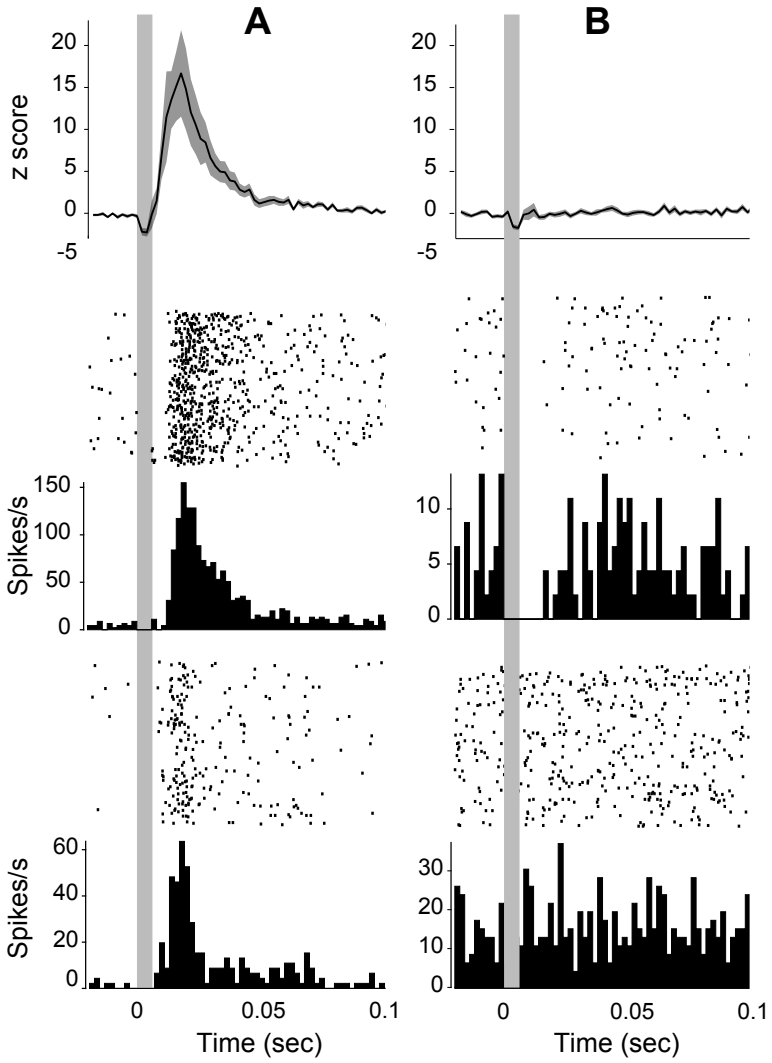

Figure 4.9. *Shock-evoked responses of mPFC neurons.* ***A,*** Top graph shows population averaged firing rates for all mPFC neurons that were excited by shocks (n=24), bottom graphs show raster plots and PSTHs for two examples of such cells. ***B,*** Same as 'A' except that data is shown for mPFC neurons that did not respond shocks (n=46).

Table 4.1

		TRIAL TYPE	
CELL TYPE	N	Freezing	Flight
FLT+	40	2.3 ± 0.3	2.2 ± 0.3
CSR+	20	4.4 ± 2.8	2.9 ± 1.7
MOV+	8	3.6 ± 0.7	3.1 ± 0.7*
NR	5	3.1 ± 1.3	2.9 ± 1.2

Table 4.1 Mean CX firing rates for cell types in dlPAG (includes only types for which N>1; *p<.05 for paired t-test comparing freezing versus flight trials).

Table 4.2

		TRIAL TYPE	
CELL TYPE	N	Freezing	Flight
NR	23	2.0 ± 0.7	1.7 ± 0.5
MOV+	15	3.5 ± 1.0	2.5 ± 0.8†
FLT+	14	1.9 ± 1.0	2.0 ± 1.0
FRZ-	7	3.3 ± 1.4	2.4 ± 0.9
FRZ+	6	5.7 ± 1.5	5.7 ± 2.6
CSR+	3	5.1 ± 3.8	6.5 ± 5.7

Table 4.2 Mean firing rates for cell types in mPFC (includes only types for which N>1; †=p<.1 for paired t-test comparing freezing versus flight trials).

Chapter 5

Reinstatement of extinguished fear by an unextinguished conditional stimulus

5.1 INTRODUCTION

In Pavlovian fear conditioning, subjects are trained to fear a previously neutral conditional stimulus (CS) by pairing it with an aversive unconditional stimulus (US) until the CS comes to evoke "emotional" fear responses, which in rodents are commonly measured by species-specific defense reactions such as freezing (Fanselow 1980; Sigmundi et al. 1980). Extinction of conditional fear occurs when the previously conditioned CS is repeatedly presented without the US, causing fear responding to diminish (Pavlov 1927; Rescorla and Heth 1975; Bouton and Bolles 1979). In humans, the phenomenon of fear extinction is exploited by clinical therapies for anxiety disorders, in which patients learn to reduce responses to fear-arousing stimuli by repeated unreinforced exposures (Marks 1979; de Silva and Rachman 1983; Mineka 1985; Davey 1997; Bouton et al. 2001; Eelen and Vervliet 2006; Craske et al. 2008).

Evidence indicates that fear extinction does not simply "erase" the previously learned CS-US association, but instead forms a new memory that the CS has become safe, which inhibits expression of the original fear memory (Pavlov 1927; Konorski 1948, for review see Lattal et al. 2006, but see also Kim et al., 2007). Supporting this, it is well established that after extinction, conditional fear responses to a CS can re-emerge without any additional CS-US pairings by the mere passage of time (spontaneous recovery) (Pavlov 1927; Robbins 1990), by presenting the extinguished CS in a context other than the extinction context (renewal) (Bouton and Bolles 1979; Bouton and King 1983), or by non-contingent presentations of the US (reinstatement) (Rescorla and Heth 1975; Bouton and

Bolles 1979). Hence, fear extinction is a rather fragile phenomenon, and this may limit the effectiveness of extinction-based therapies for anxiety disorders (Boschen et al. 2009).

Reinstatement of fear responding has been demonstrated both in animal (Rescorla and Heth 1975; Bouton and Bolles 1979; Kim and Richardson 2007) and human studies (Hermans et al. 2005; Dirikx et al. 2007). Reinstatement is typically induced by re-exposure to an aversive *unconditional* stimulus, but here we investigated whether exposure to a non-extinguished *conditional* stimulus would have the capability to reinstate conditional fear responding to an extinguished CS. Rats were trained to fear two different CSs, a light and tone, by pairing them with a footshock US. One of the CSs was then extinguished by repeatedly presenting it without the US. The unextinguished CS was then presented shortly after extinction, and it was found that this reinstated conditional fear to the extinguished CS, in much the same way that prior studies have shown reinstatement by an aversive US. Based upon these findings, we argue that standard reinstatement of fear by a US might more accurately be referred to as "unconditional reinstatement," whereas reinstatement of fear by a CS (as shown here) can be referred to as “conditional reinstatement.” Mechanisms which may underlie this conditional reinstatement phenomenon, as well as implications for the effectiveness of extinction therapy in human anxiety disorders, are discussed.

5.2. EXPERIMENTAL PROCEDURES

5.2.1 Subjects

Adult male Long-Evans rats (n=32 Harlan, Indianapolis, IN) weighing 270-300g were housed singly and maintained on a 12-hour light/dark cycle with access to food and water *ad libitum*. Prior to behavioral training, rats were handled daily (2 min per rat) for 7 days. All experimental procedures were approved by the UCLA Animal Research Committee and were conducted in accordance with USA federal guidelines.

5.2.2 Apparatus

All experimental equipment and software was manufactured by Med-Associates, Inc, (St. Albans, VT). Behavioral experiments were carried out in acoustically isolated fear conditioning chambers (30 x 25 x 25cm) with floors composed of stainless steel rods wired to a shock generator and scrambler operated by a programmable stimulus controller. Fear was indexed by defensive freezing, which was scored using the automated near infrared (NIR) video tracking program VideoFreeze™, which recorded behavior at 30 frames per s. Rats were considered to be freezing during episodes when the video image activity fell below a calibrated threshold during 30 or more continuous frames (Jacobs et al., 2010). Rats received fear conditioning sessions in one room (henceforth designated as Context A) and fear extinction and testing sessions in another room (henceforth, Context B). The experimental room corresponding to Contexts A versus B was counterbalanced across rats in each experimental group.

One context included chambers with white plastic inserts curving inward from the back of the chamber and a grid floor with stainless steel rods (4.8 mm thick) spaced 1.6 cm apart (center to center). Underlying pans were scented with 50% Simple Green and cleaned with 70% isopropyl alcohol. Chambers were illuminated with white house lights. Fans mounted above each chamber provided background noise (60dB). Rats were transported to the context in their home cages, hung on racks mounted to a portable cart and covered with a white sheet.

The alternative context included chambers with aluminum side walls, an opaque plastic rear wall, and a distinct grid floor pattern consisting of two planes of "staggered" stainless steel rods (4.8 mm thick) spaced 1.6 cm apart. Chambers were scented and cleaned with a 1% acetic acid solution. Chamber house lights and background fans were turned off. Rats were transported to the context in their home cages, placed adjacent to each other on a portable cart and covered with a metal grate.

5.2.3 Acquisition and extinction training

All rats were delay conditioned in Context A to fear two different CSs: a 30s tone (80 db, 2800 Hz) and house light flashing at 1Hz (0.5 s on / 0.5 s off) for 30 s. Rats were not pre-exposed to Context A prior to training. Over two days of training, rats were given 8 CS-US pairings per day (4 alternating presentations of each CS, with order of presentation counterbalanced across groups) for a total of 16 CS-US pairings (8 per CS). During paired trials, each 30 s CS co-terminated with a 2 s footshock (1.0 mA), with a constant intertrial interval (ITI) of 4 min (and a 3 min baseline period preceding the first pairing trial of each

training session). 24 h after the second training session, rats were pre-exposed to Context B for 38 min (approximately the length of an extinction session) to extinguish any contextual fear that might have generalized from Context A to Context B. The following day, rats began extinction training on one of the CSs (light or tone, counterbalanced across and within context assignments), henceforth designated as CS_{Ext}. Two extinction sessions were given on two consecutive days, each beginning with a 3-min baseline period, followed by 30 presentations of CS_{Ext} (30 s each, with a 1-min ITI). In total, rats received 60 extinction trials over 2 days.

5.2.4 Reinstatement and extinction testing

After the final presentation of CS_{Ext} on Extinction Day 2, rats remained in Context B for an additional 4 min, and then received differing treatments in each group (Figure 5.1). The conditional reinstatement (CRI) group received 8 unpaired "reminder" presentations of the unextinguished CS (CS_{Unext}) separated by a 1 min ITI, the novel (NOV) group received 8 unpaired presentations of a novel cue (CS_{Nov}; 73 dB white noise for 30 s), and the control (CTRL) and unextinguished (UNEXT) groups did not receive any additional stimulus, but remained in Context B for an equal amount of time as the other two groups. Rats were returned to Context B 24 h following Extinction Day 2 for testing of their fear responses to the extinguished CS_{Ext} (CTRL, NOV, CRI groups) or unextinguished CS_{Unext} (UNEXT group). The test session began with a 3-min baseline period followed by 8 CS presentations (lasting 30 s each with a 1 min ITI).

5.3 RESULTS

All rats (n=8 per group; 32 total) were conditioned to fear the light and tone CS during 2 days of acquisition training in Context A (Figure 5.2). CS-evoked freezing was averaged across all trials on Acquisition Day 2, and analyzed using a 2 x 4 repeated measures ANOVA with CS (CS_{Ext} vs CS_{Unext}) as a within-subject factor and group (CTRL vs UNEXT vs CRI vs NOV) as a between-subject factor. Freezing did not differ with group ($F_{3,21}<1$, $p=.81$) or CS type ($F_{1,7}<1$, $p=.95$), indicating that all rats acquired similar CS-evoked fear responses to both CSs. After acquisition, rats were given a day of pre-exposure to Context B, and then underwent extinction training to CS_{Ext} (light or tone, counterbalanced) by delivering 30 unpaired presentations of CS_{Ext} per day for 2 days (see section 2.2 and 2.3).

Figure 5.3 shows freezing responses during extinction and test sessions. Baseline freezing to Context B was analyzed by conducting a series of one-way ANOVAs (one for each day) with treatment group (CTRL vs UNEXT vs CRI vs NOV) as the independent factor. There were no group differences in baseline freezing on Extinction Day 1 ($F_{3,28}=.34$, $p=.793$; Figure 5.3A), Extinction Day 2 ($F_{3,28}=0.09$, $p=.968$; Figure 5.3B), or Test Day ($F_{3,28}=1.41$, $p=.260$; Figure 5.3C). Freezing to CS_{Ext} on each extinction day was analyzed using a 6x4 ANOVA with extinction block (1-6) as a within-subject factor and treatment group (CTRL vs UNEXT vs CRI vs NOV) as a between-group factor. The main effect of block was highly significant on both Day 1 ($F_{5,140}=7.56$, $p<.001$) and Day 2 ($F_{5,140}=6.57$, $p<.001$), indicating that, as expected, freezing decreased across extinction trials. There was no main effect of group ($F_{3,28}=2.25$,

p=.104) or block x group interaction ($F_{15,140}$=1.3, p=.21) on Day 2, indicating that by the end of extinction trials, all groups had similarly extinguished their responding to CS_{Ext}.

Immediately after extinction on Day 2, rats in the CRI group received 8 "reminder" trials during which CS_{Unext} was presented (Figure 5.3B, shaded area). In this group of rats, freezing to CS_{Unext} (averaged across all 8 reminder trials) was not significantly different from freezing to CS_{Ext} during the first trial block of Extinction Day 1 (paired t=0.12, p=.909), indicating that the rats were still afraid of CS_{Unext} after responding to CS_{Ext} had been extinguished. Further supporting this conclusion, freezing to CS_{Unext} during the reminder trials was significantly greater than during the last trial block of Extinction Day 2 (paired t=5.59, p=.0008). Hence, in the CRI group, extinction of conditional responding to CS_{Ext} did not generalize to CS_{Unext}. In addition, freezing to CS_{Unext} during reminder trials in the CRI group was significantly greater than freezing to post-extinction presentations of CS_{Nov} in the NOV group (independent t=2.73, p=.016). This provides additional evidence that freezing during the reminder trials in the CRI group was indeed a Pavlovian fear response to CS_{Unext}, rather than an unlearned response to the unexpected presentation of a stimulus other than CS_{Ext} at the end of extinction training.

CS-evoked freezing during the test session (see Figure 5.3C) was analyzed using a one-way ANOVA with group (CTRL vs UNEXT vs CRI vs NOV) as an independent factor. There was a significant main effect of group ($F_{3,28}$=3.72, p=.023). Planned comparisons revealed that freezing to CS_{Ext} was significantly greater in CRI rats than in CTRL (p=.021) or NOV (p=.016) rats, but not different from freezing in UNEXT rats (p=.770). However, freezing to CS_{Ext} in NOV rats was no different than in CTRL rats (p=.913), so reinstatement of fear

responding was only triggered by presentations of an unextinguished CS, and not a novel stimulus, during the reminder period following the last trial block of extinction on Day 2. These results indicate that presentations of CS_{Unext} triggered reinstatement of conditional fear responding to CS_{Ext}.

5.4 DISCUSSION

Here we have shown that in rats, conditional fear responding to an extinguished CS can be reinstated by the presentation of an unextinguished CS (but not a novel stimulus). This suggests that reinstatement of fear can be triggered not only by an aversive US (as in traditional "unconditional" reinstatement), but also by conditional aversive stimuli that have previously become associated with shock. This phenomenon of conditional reinstatement may have implications for clinical outcomes of extinction therapy in human anxiety disorders, because it implies that relapse might be triggered by a broader variety of stimuli than has previously been appreciated.

5.4.1 When does reinstatement occur?

“Reinstatement” typically refers to the ability of re-exposure to a US to cause re-emergence of conditional responding after extinction. However, conditional fear responding to a formerly contingent CS has been shown to re-emerge after encountering aversive stimuli relevant, but not identical, to a previously conditioned US, such as non-contingent presentations of a shock of differing intensity (Kim and Richardson 2007), or after presentation of a qualitatively different US, like a loud noise (Rescorla and Heth 1975).

Hence, reinstatement does not seem to depend upon the specific sensory properties of the stimulus, but rather upon its ability to activate a representation of the affective properties of the aversive US (Rescorla and Heth 1975). This idea is further supported by evidence that reinstatement can occur after biological manipulations triggering arousal, such as electrical stimulation of the amygdala (Kellett and Kokkinidis 2004), systemic epinephrine (Haroutunian and Riccio 1977; Morris et al. 2005b), administration of adrenocorticotropin (Richardson et al. 1984; Ahlers and Richardson 1985), or exposure to a "dangerous" context (Morris et al. 2005a).

Results presented here are consistent with these prior findings, in that they demonstrate how an aversively valent experience (i.e., encountering an unextinguished CS) can cause the re-emergence of conditional fear to a previously extinguished stimulus. This effect was not seen in animals presented with a novel stimulus rather than the unextinguished CS, suggesting that the emotional valence associated with the unextinguished CS was essential to the reinstatement of conditional responding. The extinguished and unextinguished CS were both paired with the same US during training in our study, but an interesting question for future studies is whether fear to an extinguished CS might also be reinstated by exposure to unextinguished CS that has been previously associated with a different aversive US. If so, then this would suggest that the unextinguished CS may act as a 'non-specific stressor' to reinstate conditioned fear, perhaps in a way that is similar to how an extinguished operant drug-seeking response can be reinstated by a non-specific stressor that has not previously been associated with the

operant response or its rewarding outcome (Shaham and Stewart, 1994; Ahmed and Koob, 1997).

In contrast with our present findings, Rescorla and Cunningham (1978) found that non-reinforced presentations of an unextinguished light CS did not reinstate conditioned fear responses to an auditory CS that had been extinguished five days earlier, but instead, selectively attenuated fear responses on the first (but not the second) extinguished CS trial. The failure of the unextinguished CS to reinstate conditioned fear in this prior study may be attributable to differences between the procedures of that study versus the present study. For example, Rescorla and Cunningham (1978) trained each CS on separate acquisition days, while our study used alternating presentations of each CS on two identical acquisition days. It is thus possible that concurrent training allowed the two CSs to serve as 'reminders' for one another in the present study, in contrast with the study of Rescorla and Cunningham (1978). Second, the CS_{Unext} reminder period occurred just minutes after the last extinction trial (and 24h before test) in the present study, while Rescorla and Cunningham (1978) did not present the unextinguished CS until just prior to test (more than five days after extinction training). It has been shown that fear memory traces and conditioned behavioral responses fluctuate with time (Kamin 1957; Glickman 1961; McGaugh 1966; Huppert and Deutsch 1969; DeVietti and Hopfer 1974; Holloway and Sturgis 1976; Nader et al. 2000; Myers et al. 2006; Monfils 2009). For example, presentations of a CS or US enhanced performance on a fear avoidance task if presented one hour after training, but not after a longer delay (i.e., three or 21 days) (Gisquet-Verrier et al.1989). If time interacts with

memory retrieval, the intervals used here between extinction, presentations of CS_{Unext}, and testing may have been one determinant of our findings.

5.4.2 Mechanisms for extinction and reinstatement

Delivering unreinforced presentations of a previously trained CS in an extinction context may cause the extinction context to form two competing associations with the US. First, the extinction context may become a conditioned inhibitor of the US (Pavlov 1927), because unreinforced presentations of the CS can trigger negative error signals that cause the extinction context to predict the absence of shock (Rescorla 1979). Second, extinction may result in second order conditioning of the context, whereby pairing the extinction context with the trained CS makes it a second-order predictor of the US (Rizley and Rescorla 1972). Which of these two processes dominates may depend upon the number of CS presentations given in the extinction context. It has been shown that when discrete (rather than contextual) cues are paired with non-reinforced presentations of a previously trained CS, small numbers of pairings tend to produce second order conditioning, whereas large numbers of pairings tend to produce conditioned inhibition (Yin et al. 1994). Extinction requires that many non-reinforced CS presentations take place in the extinction context, and this may cause the extinction context to become a conditioned inhibitor of the US. Indeed, it has been suggested that extinction itself may result from conditioned inhibition, whereby the extinction context forms an inhibitory association with the US that sums with the excitatory association between the CS and US, thereby canceling out conditioned responding (Rescorla and Cunningham 1977; Rescorla 1979).

A “pure” conditioned inhibitor should be able to inhibit any predictor of the US, so if the extinction context becomes a conditioned inhibitor, it follows that conditioned responding to any CS (not just the extinguished CS) should become attenuated in the extinction context. Supporting this prediction, some prior studies have yielded evidence for generalization of extinction across CSs (Pavlov 1927; Dubin and Levis 1974). However, other studies have reported that responding diminishes selectively to the extinguished CS and not other CSs (Bouton and King 1983; Holland and Lamare 1984; Richards and Sargent 1984). In our present study, rats in the CRI group showed robust freezing to the unextinguished CS_{Unext} when it was presented in the same extinction context where unpaired presentations of CS_{Ext} had been delivered, so we did not observe evidence for generalization of extinction across CSs as would be expected if the extinction context had become a conditioned inhibitor.

As an alternative to conditioned inhibition account of extinction, it has been proposed that unreinforced CS presentations may cause the extinction context to become a “negative occasion setter” that gates conditional responding to the CS (Bouton 1993, 1994; Brooks and Bouton 1993; Schmajuk and Holland 1998). In contrast to a conditioned inhibitor, an occasion setter modulates the ability of a specific CS to predict the US, rather than forming a direct inhibitory association with the US itself (Holland and Lamare 1984; Holland 1989). Under this view of extinction, reinstatement could be explained by assuming that encountering an aversive US in the extinction context alters the representation of the context in a way that prevents it from continuing to act as a negative occasion setter for the

extinguished CS (Bouton and Swartzentruber 1986). It has been reported that the spatial firing properties of hippocampal place cells in a context are altered after presentations of a shock in that context (Moita et al. 2003), and this could provide a mechanism by which reinstatement shocks could alter the neural representation of a context that has become a negative occasion setter. If so, then our present findings suggest that if negative occasion setting does occur during fear extinction, then the context representation may be altered not only by US presentations (as during standard unconditional reinstatement), but by presentations of an unextinguished CS as well (as in the conditional reinstatement observed here).

Reinstatement of fear conditioning may occur because presenting the US (or another aversive stimulus) in the extinction context results in contextual fear conditioning (Bouton and Bolles 1979), which subsequently counteracts the ability of the extinction context to inhibit conditional responding (and may even endow the context with an ability to excite conditioned responding). If reinstatement after extinction results from contextual fear conditioning, then reinstatement might be expected to cause an increase in baseline freezing to the extinction context after reinstatement. By extension, conditional reinstatement might result from second-order conditioning of the context by pairing it with the unextinguished CS (note that the unextinguished CS is only presented a few times in the extinction context, and this may favor second-order conditioning over conditioned inhibition). But in our study, rats in the CRI group showed no elevation of baseline freezing

to the context during the test session, and thus it does not appear that second-order context conditioning accounted for conditional reinstatement.

Alternatively, the unextinguished CS may reinstate responding to the extinguished CS by acting as a reminder of the acquisition context and thereby facilitating retrieval of the CS-US association over the CS-noUS association (Bouton, 1993; Brooks and Bouton, 1993). Recently, Winterbauer and Bouton (2010) reported that an extinguished leverpress response would "resurge" if a second unextinguished lever was presented. In this case, the unextinguished lever could have acted much as our unextinguished CS. However, in these experiments the reminder cue was administered during the test of the extinguished response. The reinstatement we observed occurred when the putative reminder cue was presented 24 h prior to test as part of the extinction session. Thus, presentation of an unextinguished cue appears to have both immediate and long-term consequences for a response that was previously extinguished.

If the unextinguished CS acted as a reminder cue, then the extinguished CS-US association might somehow have been recalled and reconsolidated after presentation of the unextinguished reminder cue. However, this would have required the unextinguished CS to trigger reconsolidation of the extinguished CS's association with the US. Prior results indicate that reconsolidation of one CS can be disrupted without affecting reconsolidation of another CS (Debiec, 2006), so it does not seem that reconsolidation generalizes easily between cues. Hence, it seems unlikely that the unextinguished CS triggered reconsolidation of the extinguished CS-US association.

5.4.3 Clinical Implications

Often, treatments for anxiety disorders such as specific phobias or PTSD rely upon fear extinction strategies to desensitize fearful stimuli (Mineka 1985; Davey 1997; Bouton et al. 2001; Eelen and Vervliet 2006). Conditional reinstatement of fear by unextinguished conditional stimuli could be one factor that limits the effectiveness of such exposure therapy. For example, a veteran suffering from PTSD may experience anxiety and panic when encountering cues associated with the trauma from combat. Experiences such as being in large crowds or rounding blind corners may remind the veteran of combat situations, triggering PTSD symptoms (Crowson et al. 1998). If the veteran successfully undergoes exposure therapy to extinguish fear responses to these cues, but then later is exposed to untreated trauma-associated cues like hearing the sound of a helicopter (Scurfield et al. 1992), seeing or reading war-related media (Hilton 1997), or learning about the enlistment of a family member (Nachshoni and Singer 2006), then it is possible that the conditioned anxiety triggered by these untreated cues may conditionally reinstate memories of the associated trauma (which the "treated" CS had also been associated with), thus resulting in the re-emergence of anxiety during future encounters with the treated cues of large crowds or blind corners (effectively "undoing" the exposure therapy by conditional reinstatement). A better understanding of the mechanisms underlying this conditional reinstatement phenomenon may prove useful for developing extinction therapies for anxiety disorders that are more resistant against relapse by reinstatement,

Acknowledgements

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Figure 5.1

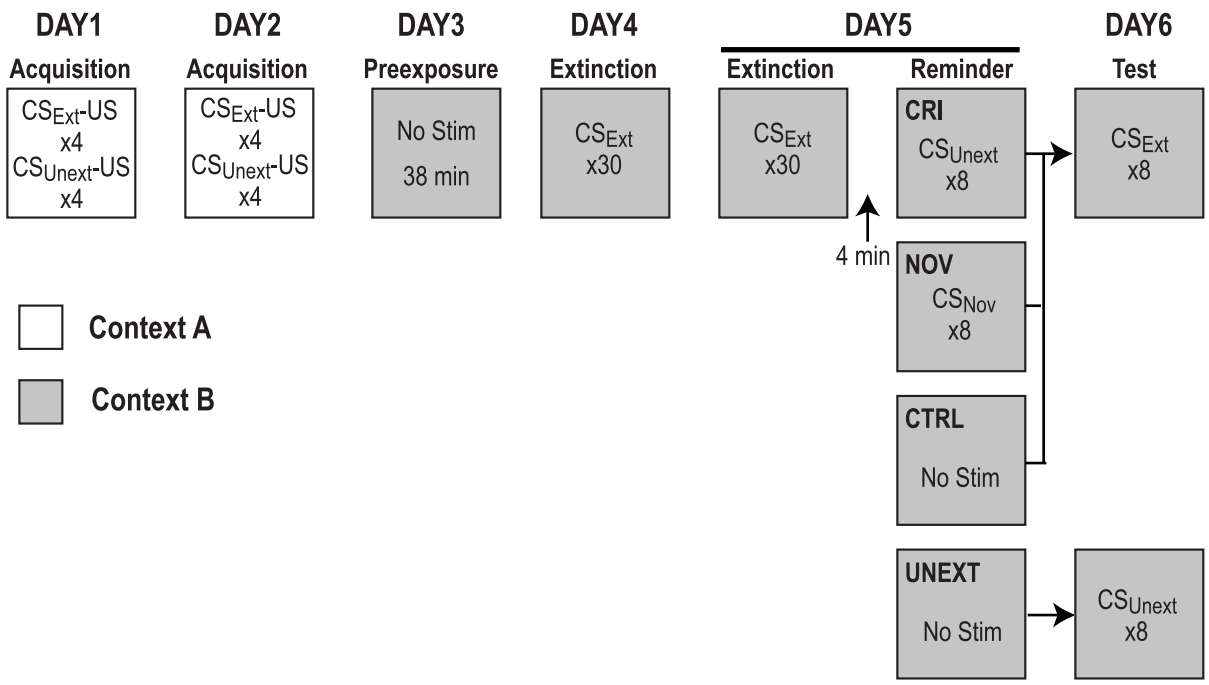

Figure 5.1. *Experimental design.* See main text for explanation.

Figure 5.2

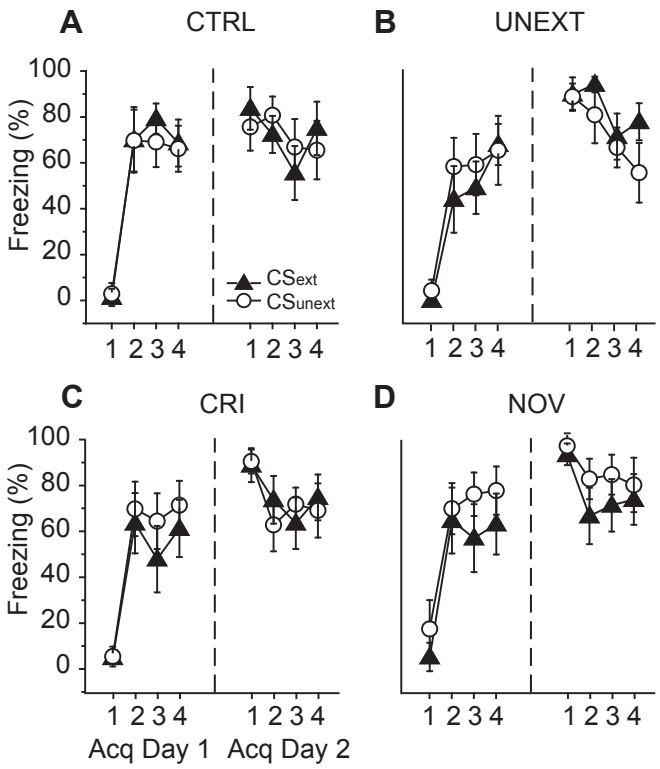

Figure 5.2. *Freezing during training sessions.* Animals were trained to fear a tone and a light CS during two acquisition days consisting of 4 pairings of each CS with the US on each day (for a total of 8 CS-US pairings per day). The Y-axis of each graph indicates percentage of time spent freezing (freezing (%)) to CS_{Ext} and CS_{Unext} (light or tone, counterbalanced) during each conditioning trial. **(A)** CTRL group, **(B)** UNEXT group, **(C)** CRI group, **(D)** NOV group.

Figure 5.3

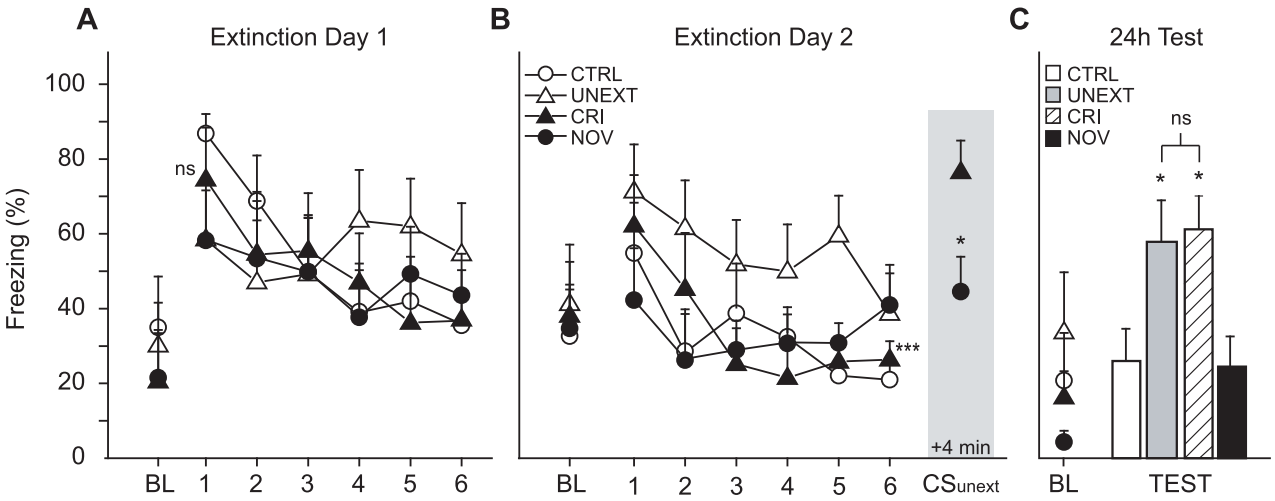

Figure 5.3. *Freezing during extinction and test sessions.* **(A,B)** Line graphs depict percentage of time spent freezing (freezing (%)), plotted as 12 extinction trial blocks (5 trials per block, 6 blocks per day) during Extinction Day 1 and 2. Shaded area in B shows freezing during the "reminder" trials (average of the 8 unpaired presentations of CS_{Unext} or CS_{Nov}), which began 4 min after the last CS_{Ext} trial on Day 2. Asterisks denote comparisons to CRI rats during reminder trials (shaded area); *= p<.05, ***=p<.001; ns=not significantly different. **(C)** Bar graphs depict percentage of time spent freezing in response to the CS during the test session (average of 8 test trials), which occurred 24h after the end of Extinction Day 2. Asterisks denote comparisons to CTRL on test day.

Chapter 6

General Discussion

6.1 STANDARD MODEL FOR ROLE OF mPFC IN FEAR CONDITIONING

Results from past studies have suggested opposing roles for the PL and IL subdivisions of mPFC in the expression of fear (see Chapter 1, Figure 1.2). Burgos-Robles et al. (2009) reported that PL neurons exhibit sustained firing during the presentation of a CS that is correlated with CS duration and freezing expression. Sotres-Bayon et al. (2012) also recently reported that AMG inactivation, which impairs conditional freezing (Goosens and Maren, 2001; Blair et al., 2005; Tarpley et al., 2010; Sierra-Mercado et al., 2011), reduces spontaneous and CS-evoked activity in some PL cells. In contrast, it has been suggested that IL mediates the inhibition of fear expression (Morgan and LeDoux, 2005; Quirk et al., 2006; Vidal-Gonzalez et al., 2006; Santini, et al, 2008) because activity in IL is necessary for extinction learning (Sierra-Mercado et al., 2011) and electrical stimulation of IL reduces the expression of CS-evoked freezing (Vidal-Gonzalez et al., 2006).

While this evidence supports the idea that PL and IL exert opposing influences on the expression of conditioned fear, the findings are rather limited in that they rely upon one specific behavioral defense as a measure for fear: freezing. Studies using other fear measures, such as startle potentiation, have yielded conflicting evidence for the role of mPFC in fear conditioning. Gewirtz et al. (1997) provided evidence that mPFC may not be necessary for the expression or inhibition of fear based on their findings that lesions of mPFC did not impair conditioned inhibition or extinction of the fear potentiated startle reflex, nor did they seem to affect the expression of auditory CS-evoked freezing. But their lesions were not restricted to PL or IL specifically, so a direct comparison to studies

targeting only PL or IL cannot be made. However, results presented in Chapter 3 are in agreement with the findings of Gewirtz et al. (1997) since we found that inactivating mPFC did not impair the expression of freezing.

Burgos Robles and colleagues (2009) consider mPFC cells to be "tone responsive" if they exhibit a change in firing rate during the presentation of a fear-conditioned CS. However, it is difficult to determine whether this "tone response" specifically corresponds with the emotion of fear itself, or a specific mode of behavioral fear expression, or simply inhibition of motor movement that occurs during freezing. Our ability to measure both freezing and flight in the same animals has allowed us to propose a more detailed classification of mPFC cells that purportedly regulate "fear". Like Burgos-Robles and colleagues (2009), we also classify cells based upon firing rate changes during the CS versus CX period, but we have the ability to compare CS-evoked neural activity in freezing versus flight trials. Fear is presumably elicited by the CS in either case, but the behavioral expression of fear differs. This offers us the advantage of distinguishing between cells that respond to the CS itself, versus those that fire selectively during specific defensive behaviors, versus those correlated with motor movement in general.

To directly compare our present results against prior findings, Figure 6.1 illustrates how the neurons we recorded in mPFC would have been classified based on an analysis of freezing trials alone (similar to the analyses performed by Milad and Quirk, 2002; Burgos-Robles et al., 2009; Sotres-Bayon et al., 2012). Without the ability to compare freezing versus flight trials, many of the cell types we found in mPFC would not be distinguishable from one another. For example, MOV- and CSR+ cells would be indistinguishable from FRZ+

cells, since all of these cell types increase their firing rates during CS presentations of freezing trials. Moreover, MOV+ cells and CSR- cells cannot be distinguished from FRZ- cells, because they all decrease firing rates during the CS in freezing trials. If we were to classify the cells we recorded in mPFC using only data from freezing trials, our cell classifications and proportions would very closely resemble those reported by Burgos-Robles et al. (2009) (see Figure 6.1). But because our fear conditioning paradigm provides a way to distinguish between cells that respond differently depending upon what type of defensive behavior is being expressed, we can begin to theorize that mPFC may have a more complex role in fear circuitry than current models suggest.

6.2 REVISING THE FEAR CIRCUIT

Past studies suggest that PL and IL have opposing roles within the fear circuit (Vidal-Gonzalez et al., 2006; Sotres-Bayon and Quirk, 2010; Sierra-Mercado et al., 2011), but results from our recording and infusions studies do not fully corroborate the view that PL and IL oppose one another by facilitating and inhibiting (respectively) fear expression. Results described in Chapter 4 showed that neurons that were responsive to the CS only in freezing trials (FRZ cells) and those that were responsive to the CS only during flight trials (FLT cells) were fairly evenly distributed across the ACC (12% FRZ, 19.7% FLT), PL (20.6% FRZ, 17.2% FLT), and IL (27.3% FRZ, 27.3% FLT) subregions of mPFC (see Figure 4.6B). Although these activity measures are purely correlation, they suggest the possibility that all three subregions may play some role in mediating conditional freezing and flight. Upon examination of cells that were excited by the CS in freezing trials (FRZ+) versus those that

were inhibited (FRZ-), ACC and PL contained an equal number of FRZ+ and FRZ- cells, while IL contained twice as many FRZ- cells as FRZ+ cells, but the low number of total FRZ cells recorded in mPFC (n=13) makes it difficult to draw any real conclusions about whether different subregions contain more or fewer FLT versus FRZ cells. Furthermore, results described in Chapter 3 showed that inactivation and hyperactivation of mPFC (which most likely affected both PL and IL) bidirectionally modulated fear expression; inactivating mPFC did not impair freezing but did impair flight, and hyperactivating mPFC impaired freezing but enhanced flight. Future studies that more specifically target only PL or IL may help elucidate whether the effects we observed were due to actions in PL or IL specifically.

Since our findings do not clearly delineate the roles of PL versus IL in defensive responding, we shall propose a modified model of the fear circuit that distinguishes populations of mPFC neurons by their functions, rather than by their anatomical locations. Figure 6.2 shows a modified version of the 'standard' fear circuit previously depicted in Figure 1.2. We propose that FRZ+ cells in mPFC facilitate freezing behavior via reciprocal excitatory projections to BLA (similar to the proposed role of PL neurons in the standard model), and FLT+ cells inhibit freezing by way of excitatory projections to ITC and Cel_{off} cells (similar to the proposed role for IL neurons in the standard model). FLT+ cells may not only inhibit freezing, but also facilitate flight via direct projections to excite FLT+ neurons in dPAG. Presumably, during test trials when rats have not recently been shocked, the CS evokes freezing behavior by the classical fear circuit (Figure 6.2A), which incorporates direct projections from AMG to vPAG, and may also involve the reciprocal connections between mPFC FRZ+ cells and BLA, which disinhibit CeM output neurons to facilitate freezing

behavior (LedDoux et al, 1988, 1990, 2000). However, after recent encounters with shock (Figure 6.2B), FLT+ cells become 'switched on' so that they begin responding during the CS period, thereby allowing flight behavior to compete with freezing behavior as a defensive response to the CS. The mechanisms by which the shock stimulus can 'switch on' the FLT+ cells requires further investigation, but there are several possibilities to consider.

One possibility is that shock-responsive neurons in mPFC exert direct local influences upon other mPFC neurons, thereby altering their responsiveness to the CS. It is thought that regions of mPFC, especially ACC, integrate negative affect, pain, and cognitive control (Shackman et al., 2011), so it is possible that local input from shock-responsive cells in mPFC might increase CS-responsiveness of FLT cells, and perhaps decrease responsiveness of FRZ cells as well, after the onset of shocks in training trials. The idea that CS responsiveness is modulated locally is consistent with results from the infusions studies in Chapter 4, as discussed further below in section 6.3.

Shock has been shown to induce the release of neuromodulators in mPFC, including dopamine and norepinephrine (Speciale et al., 1986; Sorg and Kalivas, 1993; Gresch et al., 1994; Dazzi et al., 1995, 2001, 2003a,b; Wedzony et al., 1996; Jedema, 1999), acetylcholine (Mark et al., 1996), and serotonin (Inoue et al., 1994). It is possible that US-induced neuromodulator release activates FLT+ cells in mPFC to mediate conditional flight. This idea is consistent with numerous studies showing that fear expression and extinction are modulated by dopamine (Pezze et al., 2003; Shah et al., 2004; Pfeiffer and Fendt, 2006; Mueller et al., 2010; Babaei et al., 2011), norepinephrine (Mueller et al., 2008), and acetylcholine (Raybuck and Gould, 2010; Santini et al., 2012).

Additionally, FLT+ cells in mPFC may become ‘switched on’ by shock-induced activation of afferents originating from structures outside mPFC, such as the AMG. , Consistent with this possibility, Gozzi et al. (2012) have proposed that a subset of neurons in CeL may act as a switch between passive and active coping behaviors. Their model incorporates 'on'- and 'off'-type cells much like Ciocchi et al. (2010)'s CeL_{on} and CeL_{off} cells (described in Chapter 1). According to Gozzi and colleagues (2012), when 'type 1' CeL cells (similar to CeL_{on} cells) are switched on, CeM-mediated freezing behavior is induced and cortical arousal is decreased via CeL projections to the substantia innomniata (SI) of the basal forebrain. Conversely, 'switching off' or inhibiting type one CeL cells removes feedforward inhibition to other inhibitory CeL cells, and as a result, CeM-mediated freezing is inhibited, thus leading to increased 'active coping' (digging, exploration, and rearing), and cortical arousal is increased (via SI disinhibition). If we assume that this CeL-mediated switch is triggered by US input to AMG, then the increased cortical activation observed when the CeL is switched off (when freezing shifts to active behavior) could support our model's assumption that mPFC FLT+ cells become activated following shock.

Shock-responsive cells in mPFC may also increase the excitatory tone of AMG (Bissière et al., 2008), and thereby activate FLT+ cells by enhancing direct inputs from BLA to mPFC FLT+ cells (see Figure 6.2B). This seems plausible because activation of metabotropic glutamate receptors in ACC can be used to fear-condition an auditory tone in the place of a shock US (Tang et al., 2005), and NMDA receptors in AMG are required for this fear conditioning to take place. Importantly, ACC's ability to act as a substitute for shock seems to be a property not shared with other cortical areas like primary somatosensory cortex,

providing evidence in support of human studies suggesting that ACC's role may be to encode information about the aversiveness of shock (Shackman et al., 2011).

6.3 EFFECTS OF mPFC INACTIVATION AND HYPERACTIVATION

In Chapter 3, we showed that inactivating mPFC did not impair conditional movement suppression. This does not rule out the possibility that FRZ+ cells in mPFC might contribute to freezing expression in drug free rats, but pharmacologically inactivating them did not seem to affect the ability of the CS to produce movement suppression. This conflicts with prior data showing that freezing expression is impaired after selective inactivation of PL (Sierra-Mercado et al., 2006; Corcoran and Quirk, 2007; Sotres-Bayon and Quirk, 2010; Sotres-Bayon et al., 2011), but is consistent with some other studies that have shown intact fear responses after disruptions of mPFC (Gewirtz et al., 1997; Garcia et al., 2006; Chang and Maren, 2010). One possible explanation for this discrepancy could be that our infusions affected a larger area of mPFC than the studies showing that PL inactivation impairs freezing. Inactivating a large area of mPFC might not only remove the reciprocal excitation between LA and mPFC FRZ+ cells that normally facilitates freezing (see Figure 6.3A), but also remove input from mPFC FLT+ cells that normally inhibit CeA output and thereby suppress freezing. Thus, removal of FRZ+ cells' facilitatory influence on freezing expression would be compensated by also removing FLT+ cells' inhibitory influence, resulting in net zero change in the drive upon freezing behavior. Hence, if both the excitatory and inhibitory influences of mPFC upon freezing are removed, then CS activation of LA may still be able to produce freezing behavior via the AMG to PAG pathway.

Consistent with this interpretation, we found that inactivating mPFC impaired CS-evoked flight responses, which may have been caused by two factors. First, mPFC inactivation may have blocked the inhibition of freezing normally exerted by FLT+ cells projecting to ITC and CeL (since freezing behavior was exhibited in the place of flight). Second, mPFC inactivation may have disrupted direct projections from FLT+ cells in mPFC to FLT+ cells in dPAG. This interpretation is further supported by results from hyperactivating mPFC, which impaired CS-evoked freezing during pre-shock trials, possibly by increasing tonic activity in the pathway from mPFC to ITC and CeL that inhibits the expression of freezing behavior (Figure 6.3B). Of course, hyperactivating mPFC may have also elevated the activity of FRZ+ cells, and enhanced their output to BLA (which might be expected to lead to greater freezing). But given the architecture of the circuit, that pathway may have been overridden by the competing inhibitory pathway (equated here with FLT+ cells in mPFC), which exerts its inhibitory effects downstream from the excitatory effects of FRZ+ cells. However, hyperactivation of mPFC did not seem to by itself 'switch on' FLT+ cells or make them start responding to the CS, because hyperactivation alone did not cause the CS to evoke flight responses prior to shock delivery. But after shock delivery, mPFC hyperactivation appeared to enhance the transition to CS-evoked flight responses, since post-shock flight responses were greater than the drug-free condition. Enhanced activation of FLT+ cells (and their outputs to AMG and dPAG) after the shock might account for this enhancement in conditional flight behavior that we observed.

6.4 FUTURE STUDIES FOR VALIDATING AND EXPANDING OUR FEAR CIRCUIT MODEL

Our findings raise several questions about the brain's fear circuitry that could be addressed in future research studies. Several of these questions, and possible research strategies for addressing them, are discussed below.

6.4.1 Do FRZ+ and FLT+ cells in mPFC project to PAG?

Our proposed fear circuit suggests that FRZ+ and FLT+ cells may send efferent projections to specific target neurons in AMG and PAG. We posit that FLT+ cells in mPFC project to FLT+ cells in dPAG to facilitate conditional flight behavior, and may also project to ITC neurons in AMG to inhibit freezing. In addition, FRZ+ cells may project to vPAG to drive freezing, and also to principle neurons in BLA that drive freezing responses. These assumptions could be tested in future studies by electrically or optically stimulating neurons in PAG or AMG, to determine whether there is antidromic activation of FRZ+ and/or FLT+ cells in mPFC. The prediction would be that FLT+ cells might respond antidromically to stimulation of dPAG and ITC cells, but not to stimulation of vPAG or BLA cells. Of course, it might be found that FLT+ cells project to both vPAG and dPAG, and in this case it would remain possible that FLT+ cells exert opposing influences on vPAG versus dPAG. Similarly, FRZ+ cells might respond antidromically to stimulation of vPAG and BLA cells, but not to stimulation of dPAG or BLA cells. Determining the patterns of projections from specific subgroups of mPFC neurons to various targets would help to test and refine our hypothesis that mPFC may mediate fear-associated actions, rather than emotions. And more specifically, identifying projections from mPFC FRZ+ cells to vPAG and/or FLT+ cells to dPAG

would support the idea that mPFC modulates defensive response strategies by exerting influences upon neural activity in PAG. Conversely, identifying projections from mPFC FRZ+ cells to dPAG and/or FLT+ cells to vPAG would imply an inhibitory role for mPFC in defensive responding such that FLT+ cells may inhibit vPAG-mediated freezing and FRZ+ cells may inhibit dPAG-mediated flight.

6.4.2. Are FRZ+ and FLT+ cells in mPFC akin to fear "expression" and "extinction" cells?

Projections from our model's FRZ+ and FLT+ cells to areas of AMG are the same as those proposed to mediate fear expression and extinction, respectively (Peters et al., 2009; Sotres-Bayon and Quirk, 2010). As stated above, our ability to distinguish between populations of cells in mPFC mediating freezing and flight behaviors challenges the idea that mPFC contains "extinction" and "expression" cells that mediate fear. Our mPFC FRZ+ cells increase their firing rates during freezing, just as putative "expression" cells do (but not all "expression" cells are FRZ+ cells; see above). But while others have suggested that "extinction" cells inhibit *fear*, we are suggesting that the cells in mPFC that inhibit amygdalar output to vPAG may actually inhibit freezing behavior and not fear, thus allowing for the transition from freezing to various other behaviors such as conditional flight (in the case of an animal that has recently been shocked) or free exploration (in the case of an animal that has been extinguished). If this were true, then our FLT+ cells should be correlated with CS-presentations following extinction training. To test this, after identification of FLT+ cells in mPFC, rats can undergo extinction training, during which we can observe whether FLT+ cells lose their CS responsiveness, indicating that they specifically

mediate fear-evoked flight, or whether they become responsive to the CS after freezing has been extinguished, suggesting a more general role in the inhibition of freezing regardless of the animal's emotional state and behavioral response when freezing is absent.

6.4.3. Is the AMG necessary for CS-induced FRZ+ and FLT+ cell responses?

Past data from our lab has shown that the AMG is necessary for the expression of both freezing and flight responses to a fear conditioned CS (Tarpley et al., 2010). It has also been suggested that brief spiking in AMG evoked by a trained CS triggers sustained responses in mPFC that mediate fear expression (Burgos-Robles et al., 2009). To verify that FRZ+ and FLT+ cells in mPFC are activated by input from AMG, some rats used in our recording studies received bilateral AMG inactivations prior to a fear conditioning session. Preliminary evidence suggests that the AMG is necessary not only for the expression of conditional fear responses, but also for CS-evoked activity in many of the cells in mPFC and PAG. Example cells are shown in Figure 6.4. Figure 6.4A shows behavior and recording data from a FLT+ cell in dPAG during post-shock training trials prior to (15 trials) and following (16 trials) bilateral AMG inactivation. Spike rasters are shown for each trial individually, with colors representing the rat's movement speed during each trial. Blue indicates low movement speed (<5 cm/s), yellow indicates intermediate movement speed (5-10 cm/s), and pink indicates fast movement speed (>10 cm/s). Rate histograms show the FLT+ cell's firing rate averaged over trials (1 sec bins; black=pre-inactivation, orange=post-inactivation). During drug free post-shock trials (top), movement speed during the CS was greater than that during CX because the rat exhibited CS-evoked flight behavior. The FLT+

cell increased its firing rate during the CS of flight trials, but not freezing trials (not shown). Inactivating AMG blocked CS-evoked flight (indicated by the lack of yellow/pink movement speed behind the bottom spike rasters), replicating our lab's past findings (Tarpley et al., 2010). Additionally, CS-evoked activity was also blocked, providing evidence that FLT+ cells only increase their firing rate when conditional flight is expressed. However, it is not clear whether the reduction in CS-evoked firing was due to a lack of input from AMG regarding the salience of the CS, or simply because movement itself decreased. However, this cell displayed a brief increase in spiking immediately after CS onset that did not seem to be correlated with flight. This firing evoked by CS onset may have been a result of input from AMG since it has been shown that cells in AMG exhibit brief spiking at CS-onset (Quirk et al., 1995; Maren, 2001). Supporting this, after AMG inactivation, the FLT+ cell no longer exhibited this CS onset response.

Figure 6B shows a MOV+ cell recorded from ACC on the same inactivation day as the FLT+ cell described above. Prior to AMG inactivation this ACC MOV+ cell increased its firing rate during the CS because compared to CX, the rat exhibited an increase in movement speed (because he was turning in response to the CS). This cell also showed a decrease in firing rate during freezing trials (not shown). After AMG inactivation, a decrease in the MOV+ cell's activity during the CS reflected the rat's decrease in movement speed. Further studies combining mPFC and dPAG recording with AMG inactivation will be necessary to more clearly determine how AMG input affects each cell type within mPFC and dPAG.

Further study of how different types of neurons in mPFC and dPAG respond following amygdala inactivation may help to elucidate the roles of these cells in mediating

defensive behaviors and emotional states, and would also help to discern what sources of afferent input to mPFC are responsible for driving the activity of the specific cell subtypes we have identified there.

6.5 CLINICAL IMPLICATIONS

6.5.1 Etiology of anxiety disorders

Anxiety disorders are most commonly classified according to symptomatology, but classification according to etiology may be more appropriate for the understanding and treatment of these disorders (Damsa et al., 2009). Results from numerous imaging studies have shown a substantial overlap between specific anxiety disorders and their alleged underlying dysfunctional circuitry. Abnormalities of the AMG are thought to contribute to the pathophysiology associated with social anxiety disorder (Evans et al., 2008) and specific phobias (Straube et al., 2007). Dysfunction and decreases in volume of mPFC are often associated with posttraumatic stress disorder (PTSD) (Gueze et al., 2008; Kasai et al., 2008), generalized anxiety disorder (GAD) (Krain et al., 2008), and panic disorder (Asami et al., 2008). PAG abnormalities have also been linked to panic disorder (Graeff, 2004). While human fMRI studies have provided insight that dysfunction in "fear circuitry" can accompany a variety of anxiety-related symptoms, knowledge regarding the pathophysiology of anxiety on a microcircuit level is crucial to understand specifically how these disorders manifest. Elucidating the specific circuits involved in the expression of distinct defenses (e.g., freezing and flight), as well as the mechanism mediating the switch between those defenses, may provide important insight to the distinction between stimuli-

specific fear and anxiety in general (Davis, 2006). Differentiating the etiology of these two types of fear may help develop more effective pharmacological treatments for those suffering from anxiety disorders.

6.5.2 mPFC and Psychopathology

Experiments presented here have focused upon mPFC's role in defensive action selection, but mPFC participates in many other cognitive, emotional, and behavioral processes as well.

Evidence has shown that mPFC is essential for cognitive control (Miller, 2000; Miller and Cohen, 2001) and behavioral flexibility (Lanser et al., 2001; Winter et al., 2009; Kehagia et al., 2010), which are often impaired in individuals with substance dependence and mood disorders (Groman and Jentsch, 2012). Defensive action selection may depend upon behavioral flexibility and/or cognitive control since a prepotent response to a fear conditioned CS (like freezing) might have to be inhibited in order to perform some alternative response (like flight after recent shock, or free exploration after extinction).

Dopaminergic abnormalities in mPFC can lead to various cognitive impairments like working memory deficits (Goldman et al, 1971; Brozoski et al., 1979; Goldman-Rakic, 1991, 1994; Crofts et al., 2001). Since rats in our studies respond to a fear conditioned CS in a way that depends upon recent events, mPFC might maintain a 'working memory' for recent shock that mediates the expression conditional flight. It could be postulated that the bidirectional effect on conditional flight observed after inactivating and hyperactivating mPFC was due to attenuating and enhancing (respectively) mPFC's working memory for

fear. As discussed above, shock can increase dopamine in mPFC (Dazzi et al., 1995, 2001, 2003a,b), so it is possible that similar to other working memory tasks (Sawaguchi and Goldman-Rakic, 1991, 1994), dopamine in mPFC may mediate a working memory for fear. This suggests that the inappropriate defensive responding (excessive fear) inherent to anxiety disorders might be due in part by dysfunctional dopaminergic mechanisms in mPFC that disrupt working memory. This postulation conforms to current theories proposing that working memory impairments associated with disorders like schizophrenia are due to abnormal dopaminergic mechanisms in mPFC (Sawaguchi and Goldman-Rakic, 1994; Goldman-Rakic and Selemon, 1997; Winterer, 2006; and see Eyles et al., 2012).

Figure 6.1

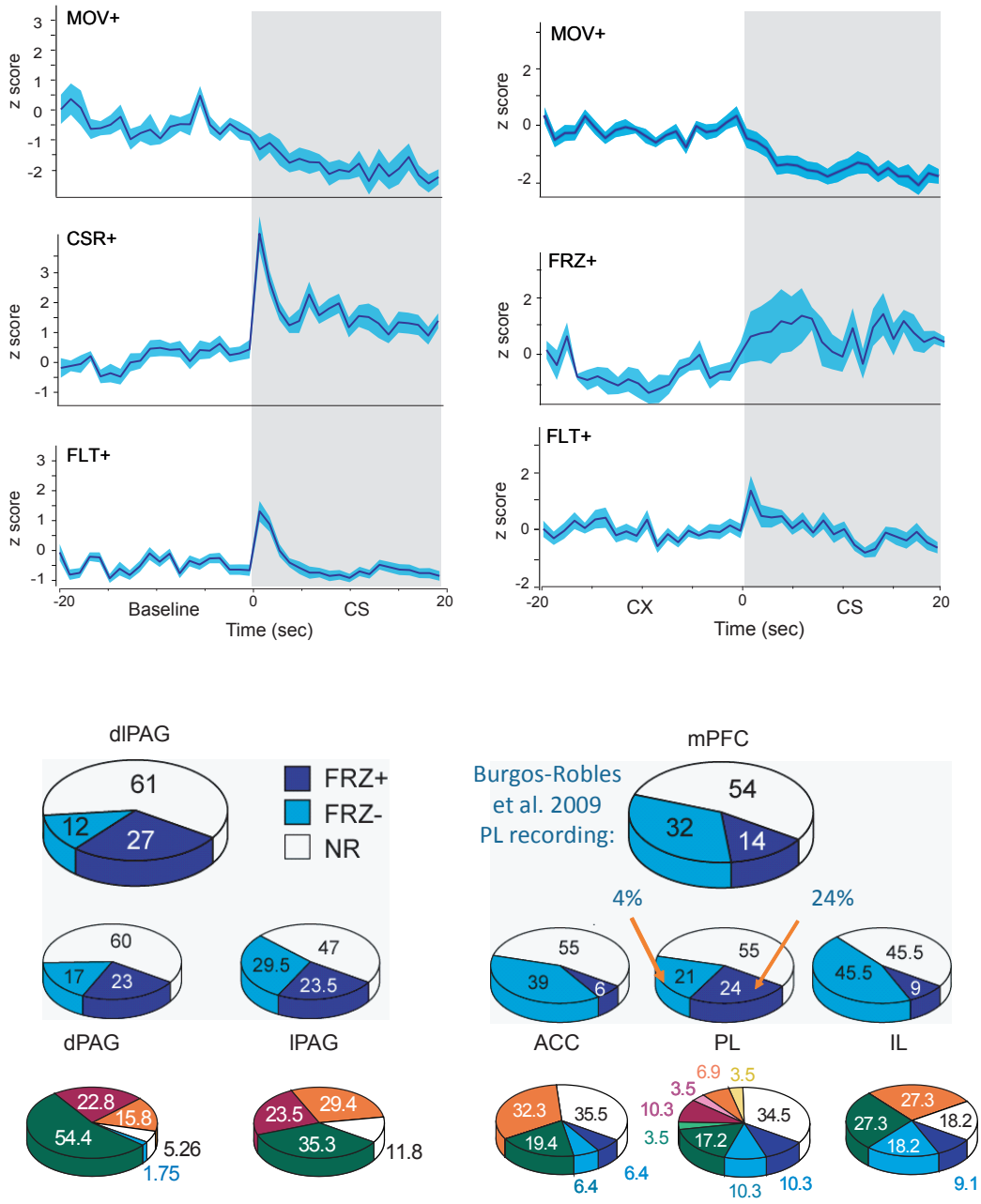

Figure 6.1. *"Reclassification" of dlPAG and mPFC cells using only freezing trials.* Freezing data only from Figures 4.3 and 4.7 are shown (top) to illustrate that without the capability to observe flight behavior, cells from MOV- and CSR+ populations cannot be discriminated from FRZ+ cells. FLT+ cells would not be distinguishable from no response (NR) cells. Bottom panel shows pie graphs of FRZ+/- and NR cells, as classified using only freezing trials. For comparison, pie graphs from Figures 4.2 and 4.6 are depicted below.

Figure 6.2

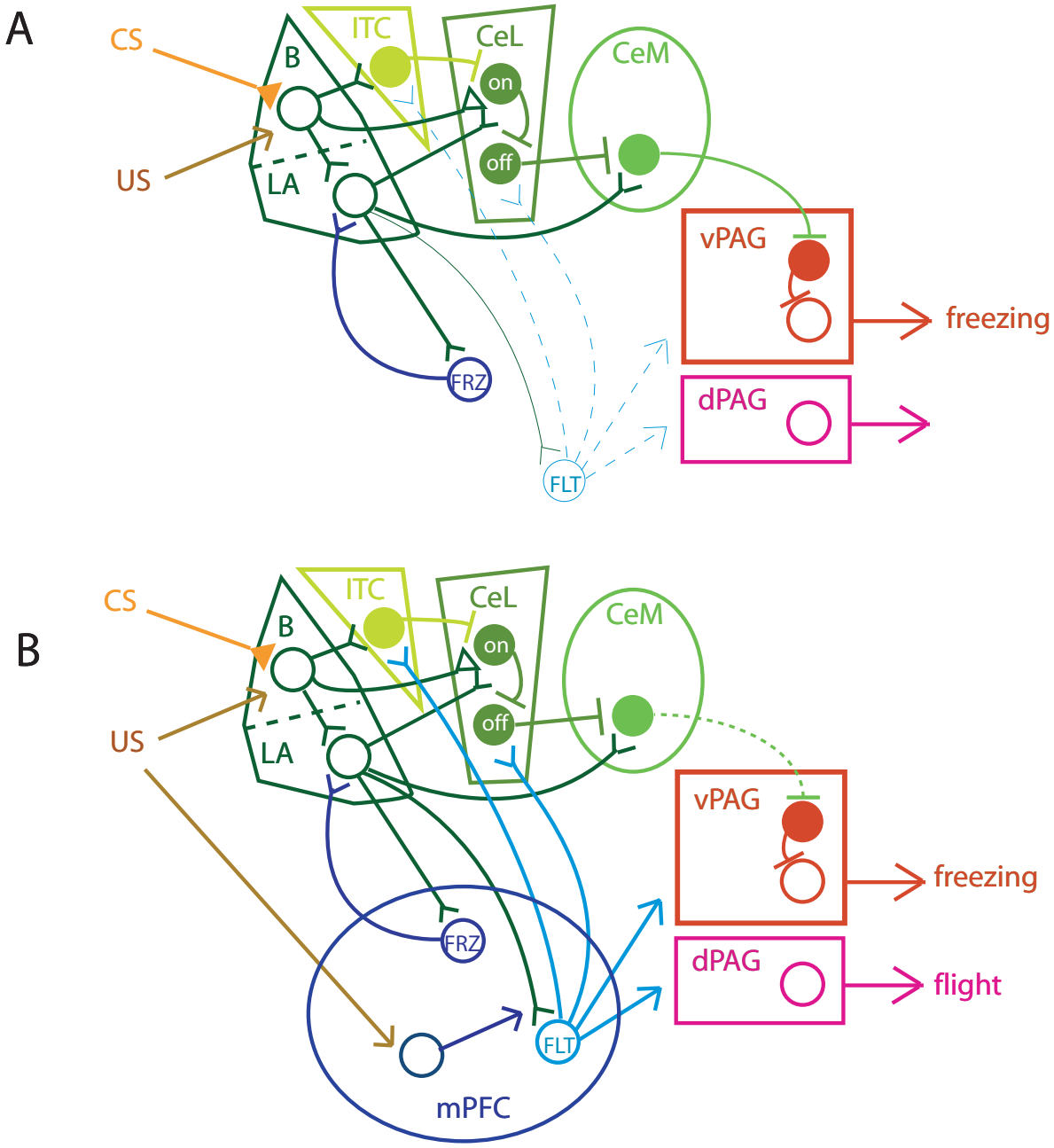

Figure 6.2. *Proposed fear circuit model.* **A**, during pre-shock trials, reciprocal excitation between mPFC FRZ+ cells and LA facilitate freezing expression. **B**, After the onset of shock in training trials, FLT+ cells in mPFC become activated so that they begin responding during the CS period, thereby allowing flight behavior to compete with freezing behavior as a defensive response to the CS.

Figure 6.3

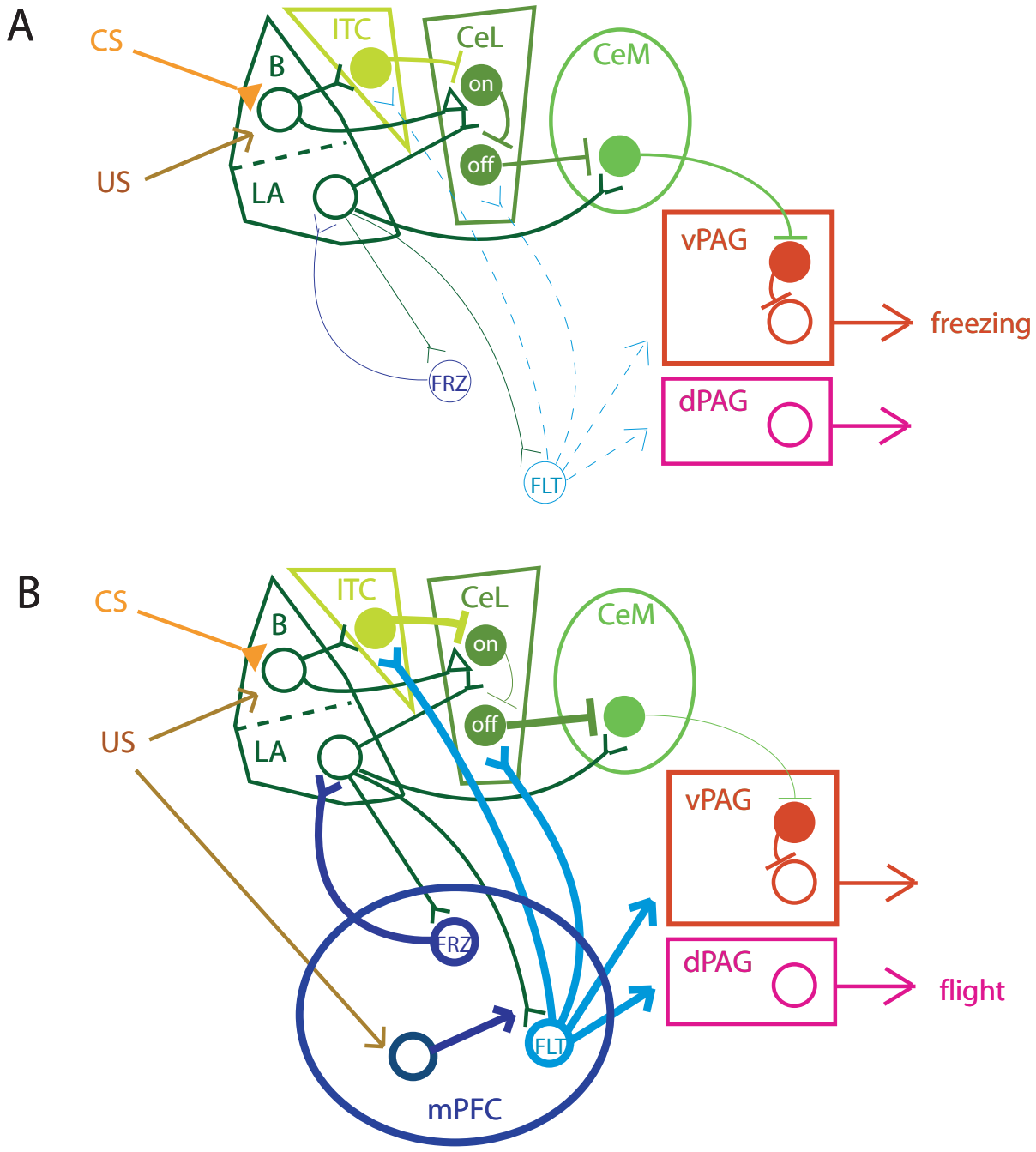

Figure 6.3. *Inactivation and hyperactivation of mPFC.* **A**, after mPFC inactivation, freezing can still be evoked by direct AMG – PAG connections, but lack of FLT+ cell input blocks conditional flight. **B**, After hyperactivation of mPFC, although both FRZ+ and FLT+ cells may increase their output to AMG, FLT+ cells seem to override FRZ+ cells since rats exhibited impaired freezing and enhanced flight.

Figure 6.4

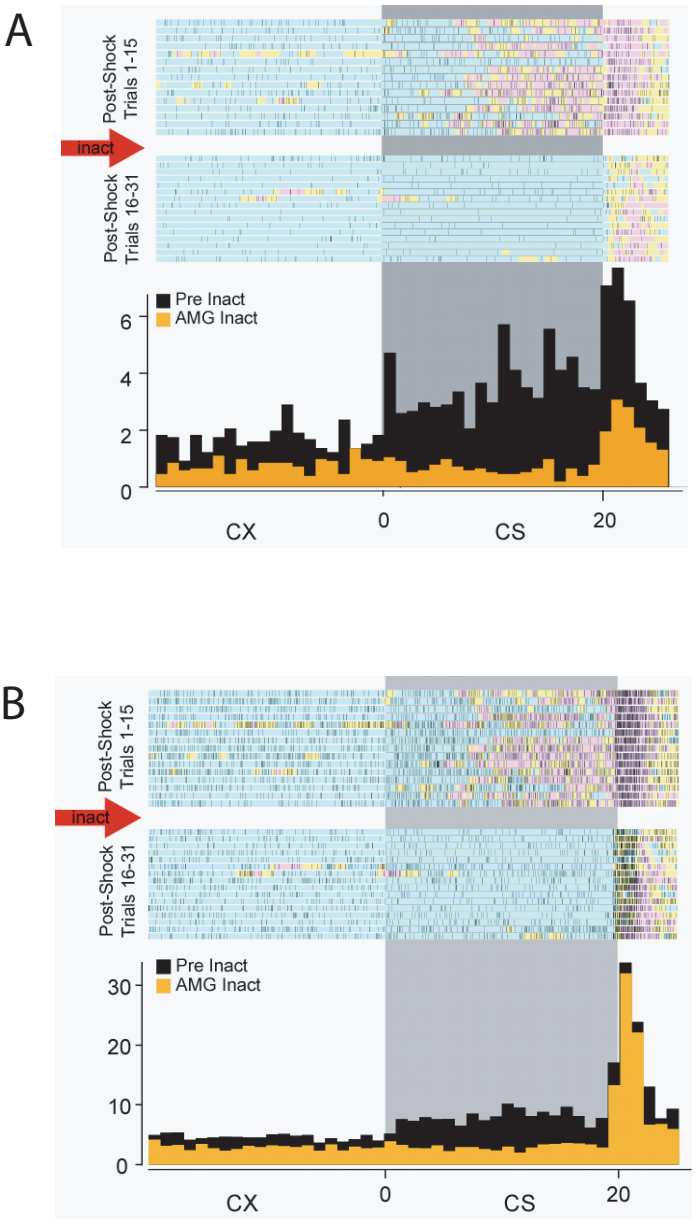

Figure 6.4. *Example cells recorded during AMG inactivation.* Description of spike rasters and rate histograms in main text). **A**, A FLT+ cell in dPAG is no longer responsive to the CS following bilateral AMG inactivation, which corresponds with the elimination of CS-evoked flight. **B**, Similarly, an example MOV+ cell in mPFC loses its CS-responsiveness following AMG inactivation.

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