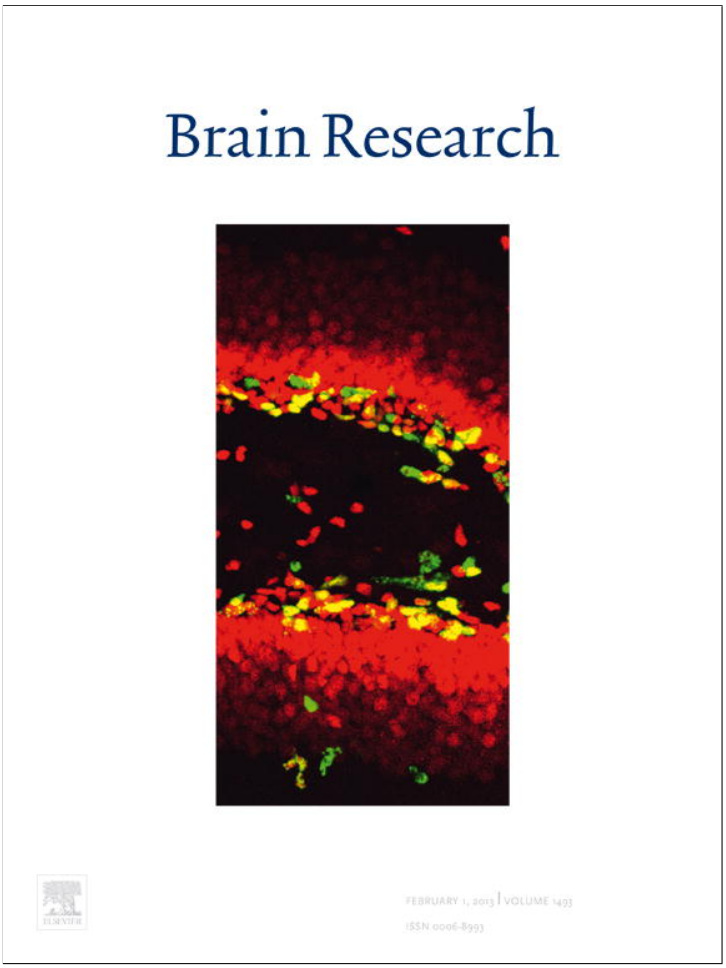

BRAIN RESEARCH 1493 (2013) 27–39

Available online at www.sciencedirect.com

SciVerse ScienceDirect

www.elsevier.com/locate/brainres

Brain Research

Research Report

# Stimulation of the dorsal periaqueductal gray enhances spontaneous recovery of a conditioned taste aversion

G. Andrew Mickley*, Kyle D. Ketchesin, Linnet Ramos, Joseph R. Luchsinger, Morgan M. Rogers, Nathanael R. Wiles, Nita Hoxha

*The Neuroscience Program, Baldwin Wallace University, 275 Eastland Rd., Berea, OH 44017, USA*

ARTICLE INFO

*Article history:*
Accepted 18 November 2012
Available online 23 November 2012

*Keywords:*
Periaqueductal gray
PAG
Central gray
Conditioned taste aversion
CTA
Extinction
Spontaneous recovery
*c-fos*

ABSTRACT

Due to its relevance to clinical practice, extinction of learned fears has been a major focus of recent research. However, less is known about the means by which conditioned fears re-emerge (i.e., spontaneously recover) as time passes or contexts change following extinction. The periaqueductal gray represents the final common pathway mediating defensive reactions to fear and we have reported previously that the dorsolateral PAG (dlPAG) exhibits a small but reliable increase in neural activity (as measured by *c-fos* protein immunoreactivity) when spontaneous recovery (SR) of a conditioned taste aversion (CTA) is reduced. Here we extend these correlational studies to determine if inducing dlPAG *c-fos* expression through electrical brain stimulation could cause a reduction in SR of a CTA. Male Sprague-Dawley rats acquired a strong aversion to saccharin (conditioned stimulus; CS) and then underwent CTA extinction through multiple non-reinforced exposures to the CS. Following a 30-day latency period after asymptotic extinction was achieved; rats either received stimulation of the dorsal PAG (dPAG) or stimulation of closely adjacent structures. Sixty minutes following the stimulation, rats were again presented with the saccharin solution as we tested for SR of the CTA. The brain stimulation evoked *c-fos* expression around the tip of the electrodes. However, stimulation of the dPAG failed to reduce SR of the previously extinguished CTA. In fact, dPAG stimulation caused rats to significantly suppress their saccharin drinking (relative to controls) – indicating an enhanced SR. These data refute a cause-and-effect relationship between enhanced dPAG *c-fos* expression and a reduction in SR. However, they highlight a role for the dPAG in modulating SR of extinguished CTAs.



## 1. Introduction

In order for animals to survive they must be able to perceive and then react to a variety of environmental dangers with effective defensive behaviors. In some cases these defensive behaviors are innate and occur without benefit of experience (e.g., antipredator responses) (Blanchard and Blanchard, 1971). However, initially neutral environmental events may also be associated with noxious stimulation and thereby adaptive fear responses may be acquired

*Corresponding author. Fax: +1 440 826 8549.
E-mail addresses: amickley@bw.edu (G. Andre Mickley), kketches@umich.edu (K.D. Ketchesin), lramos1984@gmail.com (L. Ramos), joeluchsinger@gmail.com (J.R. Luchsinger), mrogers10@mail.bw.edu (M.M. Rogers), nwiles@mail.bw.edu (N. Wiles), nhoxha@mail.bw.edu (N. Hoxha).

0006-8993/$ - see front matter © 2012 Elsevier B.V. All rights reserved.
http://dx.doi.org/10.1016/j.brainres.2012.11.029

(Fanselow, 1991) via classical conditioning processes (Pavlov, 1927).

In the laboratory, conditioned fear is frequently studied by pairing a neutral conditioned stimulus (CS; e.g., a tone) with an intrinsically frightening stimulus [unconditioned stimulus (US); e.g., shock]. Later, the CS is sufficient to produce a variety of physiological and species-specific behavioral reactions (e.g., increases in heart rate, freezing) that have been associated with the subjective feelings of fear and anxiety (Barad, 2005). These conditioned emotional response (CER) paradigms model a variety of human anxiety disorders such as phobias and post-traumatic stress disorder (PTSD; for review, see Herry et al., 2010).

As clinicians seek effective therapies for anxiety disorders, there has been an intense focus on the behavioral and neurological processes that subserve the extinction of conditioned fears (Groblewski and Stafford, 2010; Herry et al., 2010). Extinction is defined as a reduction of the previously acquired conditioned fear and is typically achieved through many non-reinforced presentations of the CS with the aim of breaking, or weakening, the CS+US bond (Norrholm et al., 2011). Unfortunately, fears can re-emerge following the passage of time (i.e., spontaneous recovery; SR) or through other forms of relapse (Bouton, 1993; Rescorla and Heth, 1975). Therefore, alternative methodologies such as explicitly unpairing a CS and US have recently been employed in attempt to reduce the reoccurrence of defensive reactions to conditioned fears (Mickley et al., 2009; Rauhut et al., 2001; Thomas et al., 2005).

The neural pathways that mediate the acquisition and extinction of conditioned fears have been revealed through extensive studies (for reviews see Bush et al., 2009; Pare et al., 2004; Quirk and Mueller, 2008) that have highlighted the importance of a network of structures including the amygdala, hippocampus, prefrontal cortex, and periaqueductal gray (PAG). Different combinations of these neural structures are engaged at different phases of acquisition/extinction (Quirk and Mueller, 2008) and paradigm-specific nuances in the CNS structures that subserve extinction have also been revealed (Mickley et al., 2009). However, the PAG has been referred to as the "… final common pathway…" of affective defensive behavior (Graeff, 1990, p. 324).

As such, the PAG mediates parasympathetic fear responses by regulating vasomotor, cardiovascular, and respiratory responses (Horiuchi et al., 2009). Furthermore, functioning of the PAG is important for the production of panic (Del-Ben and Greaff, 2009; Moers-Hornikx et al., 2011) and subsequent behavioral responses to fear and anxiety (Blanchard et al., 1981; Borelli et al., 2004). Less is known about the role that the PAG plays in the extinction of conditioned fears although McNally et al. (2004, 2005) have established the importance of endorphinergic neurons of the ventrolateral PAG (vlPAG) in the extinction of CERs. It is clear that the neural processes of fear extinction and those processes that allow the reoccurrence of once-extinguished fears must be related (Costanzi et al., 2011) and a recent study indicates that the PAG may be involved in the spontaneous recovery (SR) of a conditioned aversive response (Mickley et al., 2011). However, there remains a substantial gap in our knowledge of the means by which the brain produces SR of conditioned fears and the possible role that the PAG may play in this relapse phenomenon.

The midbrain PAG is an anatomically and functionally heterogeneous structure which is longitudinally organized around the cerebral aqueduct and may be sub-divided into 4 regions: dorsomedial PAG (dmPAG), dorsolateral PAG (dlPAG), lateral PAG (lPAG) and ventrolateral PAG (vlPAG) (Carrive, 1993; Vianna and Brandao, 2003; Bittencourt et al., 2004) – each of which has different afferents/efferents and appear to play different roles in defensive reactions to stressors that are escapable or inescapable (Bernard and Bandler, 1998; Bandler et al., 2000). For example, stimulation of the dlPAG produces active responses to fear, such as initial freezing and then escape behaviors. Whereas, the vlPAG appears to have a functional opposition to the dlPAG and stimulation of this region produces behaviors that may begin with an active coping reaction but end up with passive coping (e.g., freezing; Bandler et al., 2000; De Oca et al., 1998; Vianna and Brandao, 2003).

Several laboratories have examined PAG functional systems by combining 2 of the 4 longitudinal columns into dorsal PAG (dPAG; dmPAG+dlPAG) and ventral PAG (vPAG; lPAG+vlPAG) (Zanoveli et al., 2007; Walker and Davis, 1997; Oliveria et al., 2004) or other columnar combinations. Importantly, both the dorsal and ventral portions of the PAG are not only involved in mediating innate defensive reactions to stressors but also play an important role in the acquisition and extinction of learned fears (Resstel et al., 2008; McNally et al., 2011). For example, Reimer et al. (2012) showed that intracranial administration of α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA)/Kainate and N-methyl-D-Aspartate (NMDA) receptor agonists into the dPAG decreased the fear potentiated startle (FPS) response and increased conditioned freezing. AMPA/Kainate and NMDA receptor antagonists showed no effect on FPS or conditioned freezing, but when the antagonists were paired with the active doses of the agonists, the initial effect was remediated. Thus, glutamatergic signaling has been implicated in the expression of conditioned fear responses in the dPAG.

The midbrain PAG's role in fear conditioning is mediated through its connections to a series of other brain structures that deal with emotional states and conditioned emotional reactions (Carrive, 1993). Plentiful intrinsic PAG connections (Beitz, 1982) are complemented by numerous extrinsic connections with insular cortex, mPFC, amygdala, hypothalamus and the parabrachial nucleus (PBN) (Carrive, 1993; Bittencourt et al., 2004; Chan et al., 2011; Vianna and Brandao, 2003; Bandler et al., 2000; Floyd et al., 2000; Krout et al., 1998; Mantyh, 1982; Bernard and Bandler, 1998; Marchand and Hagino, 1983). Inactivation of the PAG attenuates US-evoked responses in the amygdala and impairs fear acquisition (Johansen et al., 2010) – suggesting a role for the PAG in prediction of negative outcomes (McNally and Westbrook, 2010), risk assessment (Blanchard and Blanchard, 1988), and resetting of expectations (Johansen et al., 2010) that may be relevant to not only fear acquisition but also fear extinction and SR.

Groblewski et al. (2009) have called for studies that go beyond the CER paradigm in order to take advantage of our knowledge about how learning, more generally, could influence development of therapies for fear and anxiety disorders. Therefore, our laboratory has expanded our study

of defensive reactions to conditioned fears and we are examining the conditioned taste aversion (CTA) (Garcia et al., 1955). Rats will avoid a taste that has been previously associated with malaise (Mickley et al., 2004, 2005). CTA is a form of aversive learning that is biologically meaningful in that cessation of eating can be life threatening to an animal. It also has distinct characteristics (e.g., rapid acquisition and resistance to extinction; Nolan et al., 1997) that make it a useful paradigm by which we may study, not only learning, but also the elimination of this defensive reaction to a conditioned fear (Parker, 2003; Mickley et al., 2004, 2005), and its SR (Mickley et al., 2007). CTA extinction employs some of the same neural circuits as does the extinction of CERs. Specifically, we have reported changes in neural activity (as measured through *c-fos* immunoreactivity) in the amygdala, mPFC and PAG that correlate with various stages of extinction and SR of a CTA (Mickley et al., 2004, 2005, 2007, 2011). Several of these changes parallel alterations that are known to occur in these brain areas as a CER is extinguished (Quirk and Mueller, 2008).

Yamamoto et al. (1994) proposed a neural model in which the brain areas important in CTA acquisition include the nucleus of the solitary tract (NST), PBN, amygdala (especially the basolateral nucleus), and the gustatory neocortex (GNC). According to this model, information regarding the taste of the CS and the visceral experience of the US pass through the NST to the PBN (Spector, 1995) where the association between the taste and the visceral experience of malaise occurs (Yamamoto et al., 1994). The PAG is not part of the classic taste aversion pathway summarized by Bures et al. (1998) and Norgren (1995) but there is some evidence that it may be involved in CTA acquisition under certain conditions (Blair and Amit, 1981). Moreover, afferents to the PAG and efferents leaving this structure communicate with a variety of brain areas that are important to CTA acquisition, extinction, or SR. These include the PBN (Bernard and Bandler, 1998; Marchand and Hagino, 1983), amygdala (Carrive, 1993), mPFC (Chan et al., 2011; Bandler et al., 2000; Floyd et al., 2000), and insular cortex (Jasmin et al., 2004). Further, our laboratory has reported significant changes in neural activity (as measured by *c-fos* protein expression) in the amygdala, GNC, mPFC and PAG that accompany either extinction or SR of a CTA (Mickley et al., 2004, 2005, 2007).

Therefore, in the current study we aimed to determine if we could modulate SR of a CTA through stimulation of the PAG. CTAs are typically extinguished by presenting the CS repeatedly without the US (CS-only procedure; CSO). Building on some foundational work by Thomas et al. (2005) and Rauhut et al. (2001), our laboratory has recently employed an explicitly unpaired extinction (EU) methodology in which subjects are presented with the CS and US on alternate days, resulting in an explicit disassociation between the two stimuli. We have reported that asymptotic extinction of a CTA is achieved more rapidly if the EU procedure is employed instead of the CSO procedure. Further, rats that underwent the EU extinction procedure showed significantly less SR of a CTA than did rats that underwent the CSO procedure (Mickley et al., 2009). A subsequent analysis of *c-fos* expression in the PAG indicated that there was a small, but reliable, increase in *c-fos* protein immunoreactivity in the dlPAG in rats that extinguished their CTA using the EU methodology but this effect was not seen in rats that went through CSO extinction (Mickley et al., 2009). Therefore, enhanced *c-fos* protein expression in the dlPAG was correlated with reduced SR.

This was of particular interest since recent use of *c-fos* antisense has allowed investigators to test the hypothesis that the *c-fos* protein is a necessary, and specific, substrate of the associative aspects of CTA formation. In this regard, Lamprecht and Dudai (1996) investigated whether the expression of *c-fos* protein was obligatory for the encoding of a CTA or was merely correlated with CTA training. This group injected phosphorothioate modified oligodeoxynucleotides (ODNs; Wahlestedt, 1994; Chiasson et al., 1994) antisense to *c-fos* into the amygdala several hours before CTA training. The antisense ODNs bind to the target mRNA and hence, specifically block translation (Helene, 1991). As expected, the injection of *c-fos* antisense into the amygdala impaired the formation of CTAs while injections in adjacent brain areas did not. Similar disruptions of CTA acquisition were reported by Swank et al. (1996) who injected *c-fos* antisense into the 4th ventricle (apparently targeting the periventricular structures – NST and PBN). Further, if antisense was given several days after conditioning, extinction of an aversion was blocked. The authors attribute these findings to blockade of associative events since they also demonstrated that *c-fos* antisense does not impair gustatory sensory functioning or the gastrointestinal distress associated with LiCl exposures. Thus, these data suggest that *c-fos* protein immunohistochemistry may be used to identify brain areas involved in not only the sensory experiences of a CS and US but also the associative processes that mediate a CTA. Moreover, the use of *c-fos* antisense has been helpful in determining the extent to which the *c-fos* protein is a *specific* physiological substrate of CTA formation and extinction.

These data lead us to evaluate the extent to which the increased *c-fos* protein expression we observed in the dlPAG (Mickley et al., 2011) caused the reduction of CTA SR. In the current study we used electrical brain stimulation to enhance *c-fos* protein expression in the dPAG. We predicted that, if we could use electrical stimulation to increase *c-fos* protein in the dlPAG of rats that had acquired a potent CTA and then experienced CSO extinction, we would also decrease SR of a CTA. In fact, our data revealed just the opposite finding – dPAG stimulation actually intensified SR of a CTA.

## 2. Results

Rats in the PAG stimulation and stimulation control groups all acquired a strong CTA. Moreover, all the rats extinguished the CTA at about the same rate and achieved similar levels of saccharin (SAC, the CS) reacceptance. However, rats that received electrical stimulation of the dPAG exhibited a more-intense SR of the CTA than did the stimulation controls.

### 2.1. *Histology*

Brain slices were viewed in order to determine the location of the tips of the electrode and also to determine if *c-fos* protein

expression could be observed in this area. Electrode placements were characterized as in ($N=10$) or outside ($N=9$) the dPAG (i.e., dmPAG or dlPAG). See Fig. 1 for illustration of electrode locations. As reported previously (Vianna et al., 2003; Lamprea et al., 2002), electrical brain stimulation can enhance *c-fos* protein expression in the area adjacent to the electrode tip. We observed such expression in our animals (see Fig. 2).

### 2.2. Behavioral results

#### 2.2.1. CTA acquisition

All rats in this study exhibited a strong CTA such that, on the third day of conditioning (following 2 SAC+LiCl exposures), the volume of SAC consumed was less than 1 ml (See Fig. 3). This represented a significant decline from the first day of conditioning before the rats had the initial SAC+LiCl pairing [$t(21)=7.58$, $p<0.001$].

#### 2.2.2. CTA extinction

Our data indicate that the CSO extinction procedure we employed produced reacceptance of the SAC solution. On the first day of extinction training the volume of SAC consumed was very low for rats in our 2 treatment groups [PAG stimulation rats: $0.12\pm0.03$ ml (Mean$\pm$SEM); stimulation control rats: $0.12\pm0.01$ ml (Mean$\pm$SEM)] and similar to that recorded during the final day of conditioning (see Fig. 3). The number of days to achieve asymptotic extinction and the slopes of the extinction curves (derived from each rat's linear regression of daily SAC drinking) were comparable between PAG stimulation rats [$27.90\pm3.59$ days to extinguish; slope$=0.63\pm0.11$ (Mean$\pm$SEM)] and stimulation control rats [$24.08\pm2.40$ days to extinguish; slope$=0.66\pm0.17$ (Mean$\pm$SEM)]. Ultimately, the amount of SAC consumed on the day that rats achieved the criterion for asymptotic extinction was also similar between PAG stimulation rats [$16.21\pm0.48$ mls (Mean$\pm$SEM)] and stimulation control rats [$17.08\pm0.68$ mls (Mean$\pm$SEM)] (see Fig. 4).

#### 2.2.3. Spontaneous recovery test

Rats that experienced electrical stimulation of the dlPAG and/or the dmPAG exhibited a more-potent spontaneous recovery of their CTA (i.e., suppression of SAC consumption) than did rats that were stimulated in closely adjacent brain areas (see Figs. 1 and 4). A 1-way ANOVA (PAG stimulation or stimulation control conditions) with repeated measures (SAC consumed at asymptotic extinction or SR tests) indicated that all rats exhibited a SR of their CTA [$F(1,17)=173.96$, $p<0.001$]. However, rats receiving dorsal PAG Stimulation drank significantly less SAC at the SR test than did the Stimulation Controls [$F(1,17)=8.39$, $p=0.01$] (see Fig. 4).

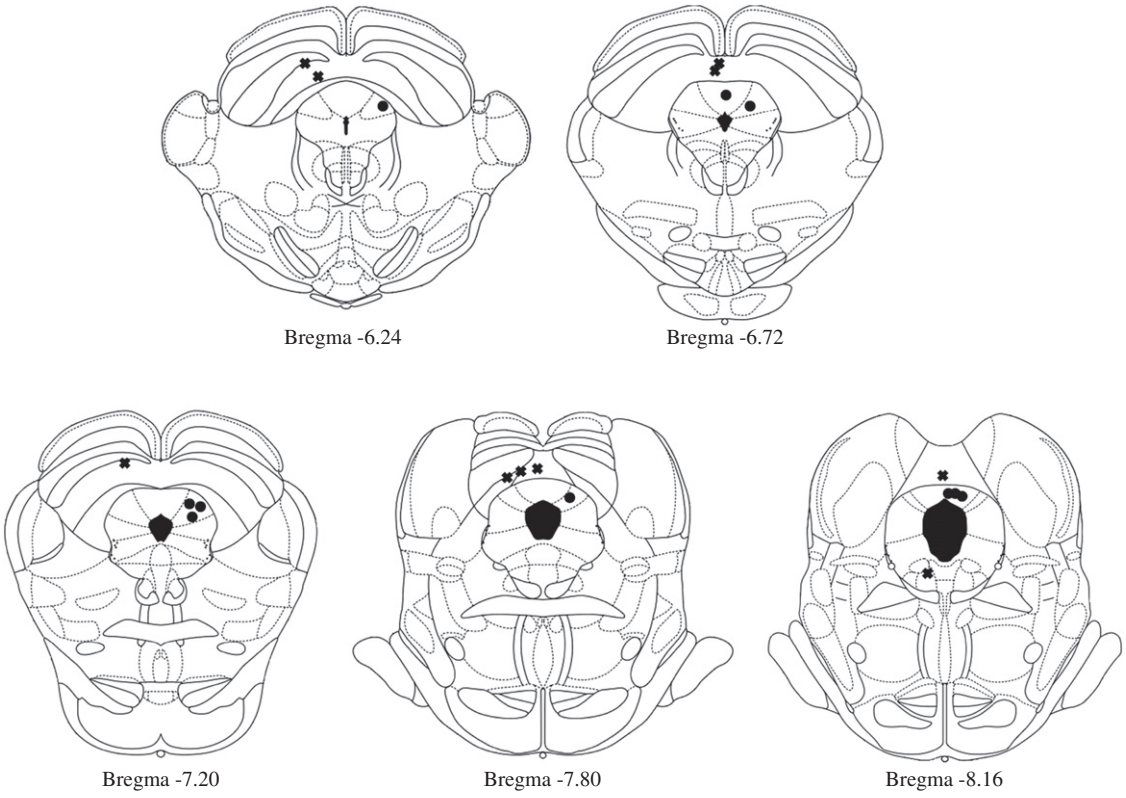


**Fig. 1 – Line drawings of coronal rat brain sections illustrating the electrode tip locations for rats that received electrical stimulation of the dorsomedial PAG (dmPAG) or the dorsolateral PAG (dlPAG) (indicated by a dot on the right side of these illustrations). The electrode placements that *missed* either the dmPAG or dlPAG are indicated by an X on the left side of the drawings. Note: Although stimulation was bilateral, electrode placements are illustrated here in one hemisphere only (left showing target "misses" and right indicating target "hits") for simplicity of presentation. Drawings are modified from the rat stereotaxic atlas of Paxinos and Watson (2008).**

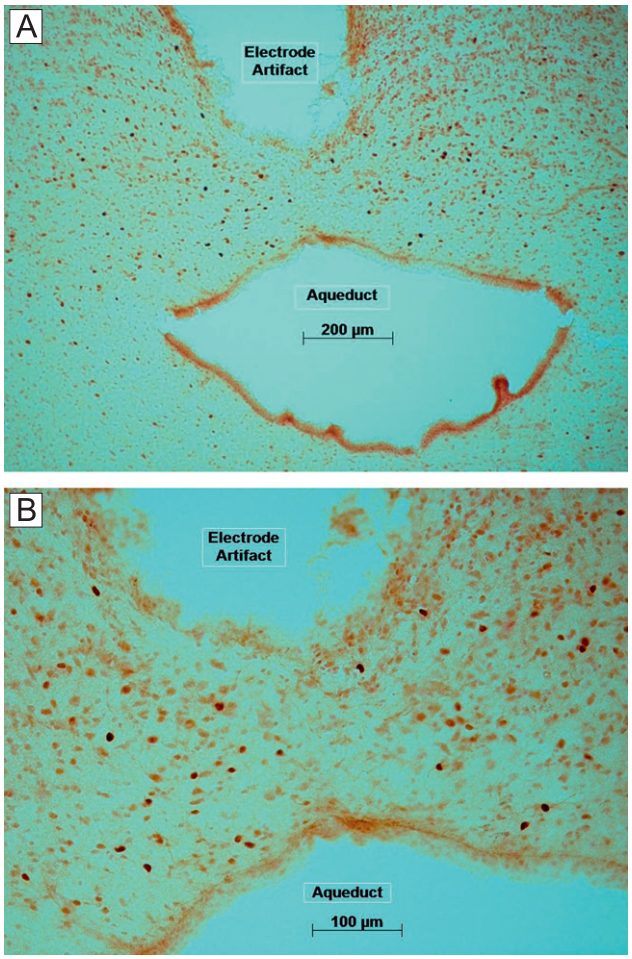


**Fig. 2 – Low (Panel A) and higher (Panel B) magnification of a representative section of the dorsal PAG illustrating *c-fos* protein expression. Electrical stimulation of the PAG induced *c-fos* protein expression concentrated adjacent to the tip of the electrode. This placement was in the dorsomedial PAG at an anterior/posterior plane approximately −8.16 mm posterior to bregma (Paxinos and Watson, 2008).**

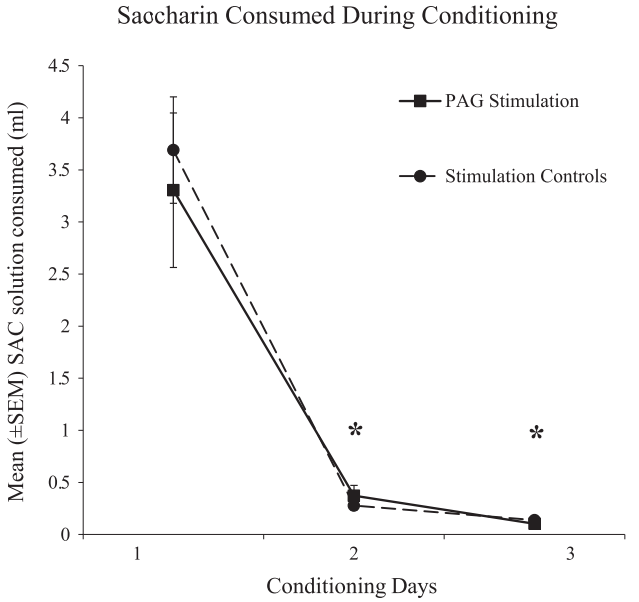


**Fig. 3 – Suppression of 0.3% SAC consumption following multiple pairings with LiCl (81 mg/kg, i.p.). *=significant reduction in SAC consumption ($p<0.001$, see text, Section 2.2.1) compared to the first conditioning day. The CTA of rats in the PAG stimulation group did not differ from the animals that were assigned to the stimulation control group.**

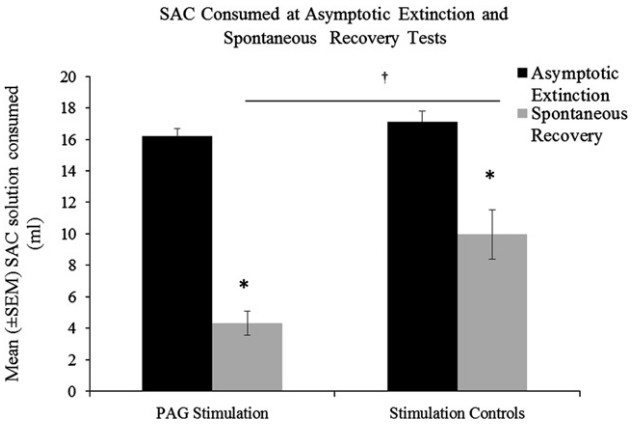


**Fig. 4 – Volume of 0.3% SAC consumed at the end of CTA extinction and during the spontaneous recovery (SR) test 30 days later. All rats achieved asymptotic extinction (90% of baseline – see definition in text) and the animals in each group exhibited a SR of the CTA. *=significant suppression of SAC consumption compared to that consumed at the end of extinction training ($p<0.001$, see text). However, stimulation of the dmPAG/dlPAG potentiated the SR as compared to rats experiencing stimulation outside the dorsal PAG (†=significantly different, $p=0.01$, see text, Sections 2.2.2 and 2.2.3).**

## 3. Discussion

### 3.1. *General summary*

Our previous studies (Mickley et al., 2011) indicated that enhanced *c-fos* expression in the dlPAG was correlated with a reduction in the SR of a previously extinguished CTA. However, we sought evidence that *c-fos* protein is a necessary, and specific, substrate of CTA SR (Lamprecht and Dudai, 1996). Therefore, our original hypothesis for the current study was that electrical stimulation of the dorsal PAG would produce an increase in *c-fos* protein and thereby reduce CTA SR. In fact, just the opposite occurred. Rats receiving dPAG stimulation exhibited a significantly *enhanced* SR of their CTA as compared to control animals that received comparable electrical stimulation outside the dPAG. Thus the correlation we originally observed between enhanced *c-fos* protein expression in dlPAG and reduced SR (Mickley et al., 2011) did not prove to reflect a cause-and-effect relationship. However, our data show that the dPAG is involved, directly or indirectly, in SR of a CTA.

### 3.2. Stimulation controls

We report here that rats receiving electrical stimulation of the dPAG exhibited an enhanced SR of a CTA (i.e., suppressed saccharin consumption) when their drinking was compared to the saccharin consumption of control animals with electrodes that missed their target and were found to be outside the dPAG. However, an alternative explanation might be that the saccharin drinking of our controls was enhanced by the stimulation. Therefore, it is noteworthy that our misplaced stimulation control rats exhibited SRs that are comparable to those observed in non-stimulated rats that went through similar CTA acquisition and EXT procedures (see Mickley et al., 2009). Thus, a comparison of the current data with our previously published results indicates that the brain stimulation experienced by our stimulation controls did not substantially alter their saccharin drinking during the SR test.

### 3.3. Is dPAG stimulation acting as a US?

A possible explanation for our data is that the dPAG brain stimulation produced US-like properties that somehow became associated with the taste of saccharin and helped to partially reinstate the extinguished CR (i.e., avoidance of saccharin). Previous studies have shown that PAG stimulation can be used as a US to establish a CER (Di Scala et al., 1987), conditioned place aversion (CPA) (Zanoveli et al., 2007), or conditioned antinociception (Castilho and Brandao, 2001). However, the conditioning procedures used in studies designed to produce conditioned fears do not closely parallel those employed here on our SR test day. In particular, in the CER and CPA experiments cited above, the PAG stimulation was presented concurrently or immediately after the animal was exposed to the CS. Testing for conditioned effects was conducted in the same context as was the training. However, the timing in our study was quite different in that rats received the dPAG stimulation in a different context and were not offered the saccharin CS until one hour later in the home cage. The use of a testing environment different from that employed for the dPAG stimulation is also a deviation from reinstatement procedures that typically use the same context for presentation of both unsignaled USs and the test (Westbrook et al., 2002). The context specificity of reinstatement has been demonstrated in both animal and human studies (see Bouton, 2004, LaBar and Phelps, 2005). These data make it seem unlikely that the suppression of SAC consumption we observed at our SR test was due to reinstatement or direct conditioning effects. Likewise, the extended time between the end of the brain stimulation and the SR test reduces the possibility that the freezing behavior observed during the stimulation carried over and generally suppressed behavior in the home cage testing environment. Although we made no formal measures during this 1-h interval, rats did exhibit spontaneous locomotion after the dPAG stimulation was switched off – suggesting that the suppression of saccharin drinking we observed was not due to a general inhibition of motor behavior.

### 3.4. Is there a role for PAG in CTA acquisition, extinction and spontaneous recovery?

If our results may not be explained by direct re-acquisition of a CTA or reinstatement, then perhaps the dPAG is involved in the phenomenon of CTA SR more specifically. Are there already known neural circuits involved in extinction and SR that communicate with dPAG? Based on our histological analysis and the similarity of the stimulation-induced behavioral outcomes we observed compared to published data (Jenck et al., 1995; Bandler et al., 2000), we feel confident in concluding that neurons of the dPAG were primarily affected by our stimulation procedures. However, brain stimulation can also cause activation of polysynaptic pathways (Lamprea et al., 2002) that may have influenced the results we report here. As described in the introduction, the PAG has complex connections with multiple brain areas (e.g., mPFC, insular cortex, PBN, amygdala, and hypothalamus) (Carrive, 1993; Bandler et al., 2000; Floyd et al., 2000; Jasmin et al., 2004; Bruchey et al., 2007; Krout et al., 1998; Mantyh, 1982) known to be important in either CTA or CER acquisition/extinction (Bures et al., 1998; Milad and Quirk, 2012; Yamamoto et al., 1994; Mickley et al., 2004, 2005, 2007). In particular, the connections between the dPAG, mPFC, amygdala and GNC provide a likely avenue for modulation of the processes directing CTA extinction and SR reported here (Mickley et al., 2004, 2005, 2007).

There is a substantial body of evidence suggesting a dominant role for enkephalinergic neurons of the vlPAG in the extinction of conditioned fear (McNally et al., 2004; Parsons et al., 2010). Activation of enkephalins in the vlPAG facilitated CER extinction (McNally et al., 2005) whereas the blocking of opioid receptors in the vlPAG prevented CER extinction (Parsons et al., 2010). However, injections of naloxone into the vlPAG did not reinstate the expression of an already-extinguished CER (McNally et al., 2004), suggesting that extinction and SR phenomena may have different neural substrates.

Perhaps this is not surprising since what happens during a test for SR is different from the processes occurring during acquisition or extinction. Rescorla and Wagner (1972) noted that learning occurs when an experience is unexpected. The initial pairing of a CS with a US produces a predictive error between the actual outcome and the expected outcome. For example, during CTA acquisition, an expectation of safety upon tasting saccharin is violated when followed by sensations of malaise. Likewise, once a CTA is formed and then the CS is presented without a US during extinction, expectancy is again violated and learning consistent with inhibition of the original CTA occurs. McNally and colleagues (McNally and Westbrook, 2010; McNally et al., 2011) have emphasized the importance of the vlPAG in determining the nature of US inputs to the amygdala so that unexpected dangerous events are learned about and expected ones are not. However, it is not clear the extent to which presentation of an extinguished CS during an SR test violates any expectations since information about the US (or lack of a US) is not readily available. This may be especially true in the case of CTA when feelings of malaise may not appear immediately and so the perception of any change in the ability of a CS to predict danger is

delayed. As we have reported previously, all 4 longitudinal columns of the PAG reduced their *c-fos* protein expression following the extinction of a CTA. However, only the dlPAG exhibited changes in *c-fos* expression when we compared brains from rats that reached asymptotic extinction with those that experienced the CS during the SR test (Mickley et al., 2011). Thus it seems that the neural mechanisms in the PAG that subserve extinction and SR may be quite different.

It may be important to the interpretation of our SR data to understand the evidence suggesting that the vlPAG and dlPAG are part of intrinsic PAG systems that may function in opposition (Beitz, 1982; Vianna and Brandao, 2003). For example, when the dPAG is electrically stimulated, the vlPAG shows signs of inhibition (Chandler et al., 1993; Behbehani, 1995). Walker and Davis (1997) have also suggested that, under circumstances of high threat, the dlPAG may be excited and thereby inhibit the vlPAG that is primarily engaged in producing low threat responses. If it is the case that the vlPAG plays an important role in fear prediction and learning, it may be hypothesized that stimulation of the dPAG prior to presentation of a CS could interfere with the circuitry involved in registering a predictive error. Parsons et al. (2010) have suggested that the blockade of vlPAG opioid receptors alters the functions of the mPFC and amygdala as they relate to fear extinction. If so, then perhaps dPAG-stimulation-induced inactivity of the vlPAG, leads to inactivity of the mPFC, disinhibition of the amygdala, and subsequent disinhibition of the original behavioral response to our CTA memory. When presented with saccharin during our SR test, the rats may have feared/avoided the conditioned stimulus because inhibition of the original fear was disabled. It must be noted, however, that the current data do not speak directly to the veracity of this proposed neural circuit and our speculations should be tested in future experiments.

### 3.5. PAG and risk assessment

Consistent with other views of the defensive systems managed by the PAG, the sampling of saccharin that occurs during our SR test may be interpreted as an attempt at risk assessment (Blanchard and Blanchard, 1988; Graeff, 1990). Our rats were fluid deprived for 23 h, so perpetuating thirst and risking survival must be weighed against the possibility of re-experiencing the symptoms of poisoning originally felt following LiCl exposure. Here, rats needed to assess the risk of drinking a substance that had, in the past, made them sick, while not disregarding their daily opportunity for liquid consumption. Studies using the CER paradigm have concluded that the dPAG, in particular, seems to be primary in responding to immediate, proximal threats to survival (Oliveria et al., 2004). Although the appetitive task employed here has different parameters and engaged different motivations than do CER paradigms, dehydration and thirst may have been perceived as a significant proximal threat to our rats.

### 3.6. Summary and conclusions

Behaviors indicative of SR reflect a combination of influences ranging from the intensity of the original association, the extent to which the original conditioned response was extinguished and the length of latency period between extinction and the SR test (Mackintosh, 1974). Thus, performance during an SR test represents an integration of the animal's knowledge of the CS at a particular point in time. The measurement of SR or other similar phenomena (relapse; reinstatement) is particularly important to clinicians concerned about the effectiveness and persistence of beneficial therapeutic outcomes (Cammarota et al., 2007; Barad, 2005). Our findings that activation of the dPAG can enhance SR of a CTA may prompt future studies that aim to manipulate this structure through pharmacological or other means to reduce the reoccurrence of conditioned defensive reactions to fears.

## 4. Experimental methods

### 4.1. Subjects

This study used 19 experimentally naïve male Sprague-Dawley rats (274.78±16.97 g; Mean±SEM) purchased from Charles River Laboratories (Wilmington, MA) (see Table 1). The rats were individually housed in plastic cages measuring 45 cm × 21.59 cm × 20.32 cm lined with "Bed o'cobbs" corncob bedding (Andersons Industrial Products, Maumee, OH.). The cages were placed in a temperature/humidity-controlled room maintained between 23–26 °C and 30% humidity (±5%) with a 12-h light/dark cycle (lights on at 0600 h; off at 1800 h). All rats had constant access to LabDiet 5001 (PMI Nutrition International, Brentwood, MO). As the study began, rats were acclimated to a 23-h water deprivation schedule (see details below). The Baldwin Wallace University

**Table 1 – Group nomenclature, number of subjects, and timeline.**

| Group Nomenclature | N | 23-h water deprivation acclimation 3 days | Conditioning | | Extinction | | Latency period 30 days | SR test day 1 day |
|---|---|---|---|---|---|---|---|---|
| | | | Days 1, 3, 5 | Days 2, 4, 6 | Odd days | Even days | | |
| ***PAG Stimulation*** | 10 | Water | SAC[a]+LiCl[b] | Water | SAC | Water | Water | PAG Stimulation+SAC |
| ***Stimulation Controls*** | 9 | Water | SAC+LiCl | Water | SAC | Water | Water | Non-PAG Stimulation+SAC |

[a] 0.3% sodium saccharin salt dissolved in water (SAC).
[b] Lithium Chloride 81 mg/kg, i.p. (LiCl).

Institutional Animal Care and Use Committee approved all methods. Animals were handled according to the instructions found in the *Guide for the Care and Use of Laboratory Animals* (National Research Council, 1996) and the Animal Welfare Act.

### 4.2. Drugs & solutions

All chemicals for the experiment were purchased from Sigma–Aldrich Chemical Company (St. Louis, MO). In this CTA paradigm, the CS was 0.3% saccharin sodium (SAC), %w/v, p.o. The saccharin salt was dissolved in deionized water to create the final concentration. The US was lithium chloride (LiCl) 81 mg/kg, i.p. The LiCl was dissolved in physiological saline to produce a final concentration of 81 mg/ml. All consummatory tests (SAC or water) involved a single bottle with a sipper tube.

A Porter anesthesia machine, Vapor 19.1 (Deerfield, IL) was used to administer Isoflurane during electrode implantation. Atropine (0.52 mg/ml) was administered at a dose of 0.4 mg/kg, i.p., prior to surgery to reduce salivation. One ml of Marcaine (Bupivacaine HCL) 0.5% (Hospira, Inc., Lake Forest, IL) was used to infiltrate the scalp wound and produce post-operative analgesia. *C-fos* protein antibodies were purchased from Calbiochem (Merck, Darmstadt, Germany).

### 4.3. Apparatus

A Kopf stereotaxic apparatus (David Kopf Instruments Model 900, Tujunga, CA), Leica Angle Two™ software (Leica Microsystems, Richmond, IL), and the Paxinos and Watson (2008) stereotaxic atlas guided the placement of the electrode array. An AM Systems (Sequim, WA) Model 2100 brain stimulation unit provided the current. Electrode arrays used for chronic intracranial implantation were fabricated in house and resembled similar ones used by Mickley and Teitelbaum (1978). In brief, they were made of gold Amphenol™ connectors soldered to insulated nichrome #60 wire (approximately 0.1 mm in diameter; 3.40 Ω/31 cm; Pelican Wire Co., Naples, FL) and embedded in a plastic headpiece. The electrode array was fastened to the skull via 1.17 mm diameter steel screws (Item no: 19010-00; Fine Science Tools, Inc. Foster City, CA) and Maxcem Elite™ resin cement (Kerr Corporation, Orange, CA). Stainless steel surgical staples were used for skin closure (size 4/0, Fine Science Tools, Foster City, CA). A cryostat (Leica CM1950, Richmond, IL) was used to slice the brains for immunohistochemical analyses.

### 4.4. Conditioned taste aversion acquisition

Three days before the CTA acquisition began, rats were acclimated to a twenty-three hour/day water deprivation schedule. This water deprivation schedule was maintained throughout the study (see Table 1 for timeline). On experimental days 1, 3 and 5 all animals received the CS, 0.3% saccharin (SAC), for 30 min (starting at 12:00) followed by a 15-min period in which no fluid consumption was allowed and the US was administered (81 mg/kg LiCl, i.p.). This was followed by 30-minute access to water. On experimental days 2, 4 and 6 (rehydration days) all animals received water for one full hour beginning at 12:00. All fluids were made available in a 50 ml drinking bottle placed onto the animal's home cage.

### 4.5. CTA extinction

A series of every-other-day CS-only (CSO) SAC presentations were used to extinguish the CTA. Starting the day after the last post-conditioning rehydration day (experimental day 7), the animals were given a 50 mL bottle of 0.3% saccharin (SAC=CS) for 30 min. Maintaining the drinking schedule initiated at the start of the study, a 15-min period followed the SAC exposure during which no fluids were available to the rat. Subsequently, another 30 min of access to tap water was given. On the alternate days during the extinction phase of the study, the animals were given 60 min of tap water along the same daily schedule described above. This timetable was followed until rats achieved asymptotic extinction of their CTAs.

We wished to estimate levels of baseline/familiar SAC drinking as a means to evaluate the degree to which the rats in this study had extinguished their CTA. However, recording several days of baseline SAC pre-exposure in our animals would have impeded future CTA training, due to latent inhibition effects (Bakner et al., 1991). Baseline SAC consumption was determined in a previous study (Mickley et al., 2004) in which a group ($N=10$) of similarly sized rats were exposed to saccharin for several days. We wished to avoid the bias associated with the rat's initial hesitation to consume novel substances, referred to as neophobia (Gillan and Domjan, 1977). Therefore, on the third day, SAC consumption was averaged (mean consumption $\pm$ SEM $= 17.57 \pm 1.29$ ml) and used as the baseline. Asymptotic extinction was operationally defined as 90% of baseline SAC drinking (15.7 ml).

### 4.6. Latency period and electrode implantation

In order to prepare for an evaluation of the spontaneous recovery of the CTA following extinction, we established a 30-day latency period in which the rats received no further exposures to the taste of SAC. This latency period has been shown to produce a robust SR of the CTA in rats that have experienced the CSO extinction procedure (Mickley et al., 2007, 2011). During this latency period the animals were given 60 min/day access to tap water using the same schedule as described previously.

Rats were chronically implanted with intracranial electrodes aimed at the dlPAG. For most of the rats, this surgery was performed 15 days into the latency period. This surgery schedule ensured that the animal would have ample time to recover prior to the SR test. After surgery, the rats had ad libitum exposure to water for $3 \pm 1$ days before going back on the 23 h water deprivation schedule. A small number of rats were implanted before CTA acquisition but their behavioral data (SAC consumption during extinction and SR testing) did not differ from the rats that received surgery later in the study. Therefore the data were combined for statistical analysis (see below).

### 4.7. Surgical procedures

On the date of surgery, the animal received a 0.4 mg/kg (i.p.) Atropine Sulfate 15 min prior to surgery in order to decrease salivation. Once ready to be anesthetized, the animal received isoflurane (~1.3%) via a Porter anesthesia machine, Vapor 19.1 (Hatfield, PA.). Each rat was placed into a Kopf™ rodent stereotaxic instrument immediately following determination of its unresponsiveness to tail and skin pinch test.

The aseptic surgery for implantation of chronic electrodes involved drilling a trephine hole in the skull over the target coordinates of each brain hemisphere. The *dura mater* at the sites of implantation was removed with a small wire pick. Bilateral bipolar electrodes were centered on the following coordinates to deliver electrical stimulation to the dPAG PAG (Paxinos and Watson, 2008): −7.68 mm, posterior to Bregma, ±0.90 mm lateral to midline, and 5.4 mm ventral (depth from skull surface). The plastic headpiece was made to protrude slightly out of the scalp so the electrodes could be visible post-surgically and a connector could be attached to them. The skin surrounding the implant was stapled and 1 cc of 0.5% Marcaine was infused at the site. The animals' health was monitored until the scalp incision healed completely. During this time, animals received Marcaine infusions twice daily for three days.

### 4.8. Brain stimulation and spontaneous recovery test

Following the end of the latency period (day 31), rats received 30 min of bilateral electrical brain stimulation (15 min/hemisphere). To prepare for brain stimulation, the electrode array was connected to the AM Systems™ brain stimulation generator through a flexible cable attached to a commutator swivel. This allowed free movement of the animal during the stimulation. The rat was placed in a round Plexiglas™ open-topped chamber (~61 cm in diameter) 5 min before the brain stimulation period began. The constant-current brain stimulator delivered biphasic pulses at 100 Hz, with pulse duration of 5 ms and an inter-pulse period of 10 ms. We employed stimulation amplitudes of 56.97±7.47 μA (Mean±SEM). The stimulation amplitudes administered to our experimental rats [51.48±11.34 μA (Mean±SEM)] and stimulation control animals [63.08±9.77 μA (Mean±SEM)] were not significantly different.

Using procedures established by other laboratories (Di Scala et al., 1987), the intensity of the brain stimulation employed was determined by the behavior of the animal. Other laboratories have noted that electrical brain stimulation amplitude is a better predictor of behavioral outcomes than is stimulation frequency (Lim et al., 2008). The literature also suggests that activation of dmPAG and dlPAG produces freezing and flight behaviors (Bittencourt et al., 2004; Dipaulis et al., 1992; Carrive, 1993; Jenck et al., 1995). Our experience paralleled these published findings. Our procedure involved starting at low stimulation amperages and, in a step-wise manner, increasing the intensity until we achieved a frantic running response. We then reduced the amperage by 5–10 μA and this typically produced a stable freezing/immobility response with infrequent sniffing, scanning, or raising/rearing (see descriptions in Bittencourt et al., 2004). Once the stimulation achieved the desired behavioral arrest with elevated muscle tonus (i.e., freezing) the rats typically maintained this for the 15 min session. At the end of this period the procedure was replicated for the other brain hemisphere.

Multiple labs have established the basic axiom that the amount of current passed through an electrode required to directly stimulate a neuron is proportional to the square of the distance between the neuron and the electrode tip (for reviews, see Ranck, 1975; Tehovnik, 1996). However beyond this simple rule, multiple factors [e.g., electrode composition, diameter, shape, and corrosion; electrical pulse shape and duration; and tissue (neuron/glia) composition] work together to determine the spread of current in the brain and the extent to which the current can influence neuronal functioning (Tehovnik, 1996; Buston and McIntyre, 2006; McIntyre et al., 2004; Yousif and Liu, 2007). Empirical estimates of suprathreshold current spread using electrode and stimulation parameters similar to ours, indicate that the average current we employed (i.e., ~50 μA) spread approximately ≤1.0 mm ventral from the electrode tip and ~0.75 mm lateral to each side (Olds, 1958). This was roughly confirmed in our rats by observing the area of *c-fos* protein expression near the electrode tip (see Fig. 2). *C-fos* expression is activated by electrical brain stimulation (Vianna et al., 2003; Sandner et al., 1992; Lamprea et al., 2002; Krukoff et al., 1992). Note, however, that this method does not differentiate between direct vs. indirect (i.e., trans-synaptic "recruited") activation produced by the electrical stimulation (Vianna et al., 2003). A 1 mm spread of current in our PAG Stimulation rats would have largely confined neuronal activation to the dmPAG and dlPAG. Whereas, the electrode placements that missed these structures (see Fig. 1) were predominantly dorsal to the PAG and therefore would have more likely stimulated superior/inferior colliculi and (in rare cases) ventral placements would have activated portions of the tegmental nucleus.

Sixty minutes after the end of the PAG stimulation rats were tested for SR of the CTA. Animals were provided a final opportunity to drink 0.3% SAC from a 50 mL bottle for 30 min. In order to assess *c-fos* protein immunoreactivity, rats were transcardially perfused 90 min after their SR spontaneous recovery test. The *c-fos* protein is part of the cellular signaling mechanism that results in long-term physical changes; thus, it is a useful marker for synaptic stimulation (Sandner et al., 1992). *C-fos* levels have been shown to be highest, 90–120 min after post-synaptic neuronal activity (Herrera and Robertson, 1996).

### 4.9. c-fos immunohistochemistry

We looked at the expression of *c-fos* protein around the electrode tip as an indicator of neural activation (Herrera and Robertson, 1996) during brain stimulation and as a rough reflection of current spread (Krukoff et al., 1992, Lamprea et al., 2002). Prior to sacrifice, the animals were deeply anesthetized with sodium pentobarbital (100 mg/kg, i.p.; 2 ml/kg) and perfused transcardially with heparinized saline followed by 4% paraformaldehyde. The brains were immediately extracted, placed in 4% paraformaldehyde, and stored at ~4 °C. The following day, brains were placed in a 30% sucrose solution consisting of phosphate buffered saline and 0.01%

Thimerosal. Brains were then kept at ~4 °C until slicing. Forty micrometer coronal sections were cut using a cryostat and stored in phosphate buffered saline containing 0.2% sodium azide until they were assayed for *c-fos* protein immunoreactivity (see seminal studies by Hsu et al., 1981).

The *c-fos* protein assay was performed on floating sections using the following procedure. First, the 0.2% sodium azide was removed and sections were washed in PBS. Hydrogen peroxide was then applied for a 30-min period. After another two PBS washes, the sections were incubated in PBS/0.2% Triton/1.5% goat serum for 1 h. The sections were then washed once more in PBS before overnight incubation in the primary antibody, rabbit against anti-*c-fos* (Calbiochem). On the following day, the primary antibody was removed and the sections were washed twice with PBS. Subsequently the sections were incubated in biotinylated goat anti-rabbit antibody (Calbiochem) for 1 h. Next, the secondary antibody was removed and the sections were washed twice in PBS. The sections were then incubated in avidin–biotin complex for 1 h. Finally, the sections were washed twice more with PBS followed by PBS/0.5% Triton before 3, 3′-diaminobenzidine (DAB) was applied. After 2 min on the shaker, the sections were washed twice in DI water and left in PBS at 4 °C (for further details see Mickley et al., 2004). Once assayed, the sections were mounted on gelatin and chrom-alum coated slides, dehydrated, counterstained with neutral red, and cover slipped with Permount™ (Thermo Fisher Scientific, Cleveland, OH).

To ensure that negative *c-fos* expression was not a result of faulty staining procedures, we employed a positive control procedure as described by Rinaman et al. (1997). Adult male rats received an injection of 8% saline solution (2.0 ml/100 g, i.p.) to produce an osmotic thirst, which leads to certain *c-fos* expression in the periventricular nucleus (PVN) of the hypothalamus. Ninety minutes later, the rats were given a supra-lethal dose of sodium pentobarbital (100 mg/kg, i.p.), perfused, and prepared for *c-fos* immunohistochemical procedures. Brain slices including the PVN from these animals were included in all the immunohistochemical assays of the experimental and control groups to ensure that successful staining had occurred. If positive staining was not observed in the positive control sections, the entire assay was discarded. Likewise, in order to ensure that artifacts associated with counter-staining were not counted, negative control brain sections were included along with each assay and treated as described above but were not incubated in the primary antibody.

Slides were viewed using an Olympus™ BX-60 microscope (Olympus, Center Valley, PA.) equipped with an Axiocam MRc5 camera. The images captured were viewed on a computer using Carl Zeiss AxioVision™ v. 4.5 software (Carl Zeiss Microimaging, Germany). Cells with dark, punctate nuclear staining were counted as *c-fos*-positive. Diffusely stained cell bodies were not counted.

### 4.10. *Statistical analysis*

Upon review of the brain histology and behavioral responses during the brain stimulation, criteria were established to separate the rats into two groups. Rats were placed in the "PAG stimulation" group if (1) the histology indicated that the tips of the electrodes were in either the dmPAG or dlPAG, (2) cells labeled for *c-fos* protein were seen at the end of the electrode artifacts, and (3) the rat's behavior during the brain stimulation included a freezing response. Rats in the "stimulation control" group failed to meet one, or more, of these criteria. Unless otherwise specified, we employed one-way ANOVAs or *t*-tests to compare the PAG Stimulation and Stimulation Control groups on various measures of CTA acquisition, extinction and spontaneous recovery (see Results section, below). An $\alpha = 0.05$ was used throughout.

## Acknowledgments

The authors wish to acknowledge the following students and technicians for their contributions to this work: Sean Anderson, Benjamin Brown, Armand DeAsis, Kara Gawelek, Janace Gifford, Sarah Hummel, Aaron McNair, Kim Parkinson, Doug Placko, Ginger Portman, Dave Revta, Marcial Rodriguez, James Romanchik, Hayleigh Sanders, Ashley Sandmann, Taylor Shreve, Logan Sirline, Diana Slawski, Agata Szwaykowska, Stephen Toth, Kate Tylicki, and Rachel Zacharias. *Supported by NIMH Grant no.: 2-R15-MH063720-03.*

## REFERENCES

Bakner, L., Strohen, K., Marvin, N., Riccio, D.C., 1991. Postconditioning recovery from the latent inhibition effect in conditioned taste aversions. Physiol. Behav. 50, 1269–1272.

Barad, M., 2005. Fear extinction in rodents: basic insight to clinical promise. Curr. Opin. Neurobiol. 15, 710–715.

Bandler, R., Keay, K., Floyd, N., Price, J., 2000. Central circuits mediating patterned autonomic activity during active vs. passive emotional coping. Brain Res. Bull. 53, 95–104.

Behbehani, M.M., 1995. Functional characteristics of the midbrain periaqueductal gray. Prog. Neurobiol. 46, 575–605.

Beitz, A.J., 1982. The organization of afferent projections to the midbrain periaqueductal gray of the rat. Neuroscience 7, 133–159.

Bernard, J., Bandler, R., 1998. Parallel circuits for emotional coping behavior: new pieces in the puzzle. J. Comp. Neurol. 401, 429–436.

Bittencourt, A.S., Carobrez, A.P., Zamprogno, L.P., Tufik, S., Schenberg, L.C., 2004. Organization of single components of defensive behaviors within distinct columns of periaqueductal gray matter of the rat: role of N-methyl-D-aspartic acid glutamate receptors. Neuroscience 125, 71–89.

Blair, R., Amit, Z., 1981. Morphine conditioned taste aversion reversed by periaqueductal gray lesions. Physiol. Biochem. Behav. 15, 651–653.

Blanchard, R.J., Blanchard, D.C., 1971. Defensive reactions in the albino rat. Learn. Motiv. 2, 351–362.

Blanchard, D.C., Williams, G., Lee, E.M.C., Blanchard, R.J., 1981. Taming of the wild *Rattus norvegicus* by lesions of the mesencephalic central gray. Physiol. Psychol. 9, 157–163.

Blanchard, D.C., Blanchard, R.J., 1988. Ethoexperimental approaches to the biology of emotion. Ann. Rev. Psychol. 39, 43–68.

Borelli, K.G., Nobre, M.J., Branda, M.L., Coimbra, N.C., 2004. Effects of acute and chronic fluoxetine and diazepam on freezing behavior induced by electrical stimulation of dorsolateral and lateral columns of the periaqueductal gray matter. Pharmacol. Biochem. Behav. 77, 557–566.

Bouton, M.E., 1993. Context, time and memory retrieval in the interference paradigms of Pavlovian learning. Psychol. Bull. 114, 80–99.

Bouton, M.E., 2004. Context and behavioral processes in extinction. Learn. Mem. 11, 485–494.

Bruchey, A.K., Shumake, J., Gonzalez-Lima, F., 2007. Network of fear extinction and renewal functional pathways. Neuroscience 145, 423–437.

Bures, J., Bermudez-Rattoni, F., Yamamoto, T., 1998. Functional morphology of CTA, Conditioned Taste Aversion: Memory of a Special Kind. Oxford University Press, Oxford, pp. 92–107 (Chapter 7).

Bush, D.E.A., Schafe, G.E., LeDoux, J.E., 2009. Neural basis of fear conditioning. In: Berntson, G.G., Cacioppo, J.T. (Eds.), Handbook of Neuroscience for the Behavioral Sciences, vol. 2. John Wiley and Sons, Hoboken, NJ, pp. 762–780 (Chapter 39).

Buston, C.R., McIntyre, C.C., 2006. Role of electrode design on the volume of tissue activated during deep brain stimulation. J. Neural Eng. 3, 1–8.

Cammarota, M., Bevilaqua, L.R., Vianna, M.R., Medina, J.H., Izquierdo, I., 2007. The extinction of conditioned fear: Structural and molecular basis and therapeutic use. Rev. Bras. Psiquiatr. 29, 80–85.

Carrive, P., 1993. The periaqueductal gray and defensive behavior: functional representation and neuronal organization. Behav. Brain Res. 58, 27–47.

Castilho, V.M., Brandao, M.L., 2001. Conditioned anyinociception and freezing using electrical stimulation of the dorsal periaqueductal gray or inferior colliculus as unconditioned stimulus are differentially regulated by 5-HT2A receptors in rats. Psychopharmacology 155, 154–162.

Chan, T., Kyere, K., Davis, B.R., Shemyakin, A., Kabitzke, P.A., Shair, H.N., Barr, G.A., Wiedenmayer, C.P., 2011. The role of the medial prefrontal cortex in innate fear regulation in infants, juveniles, and adolescents. J. Neurosci. 31, 4991–4999.

Chandler, S.C., Liu, H., Murphy, A.Z., Shipley, M.T., Behbehani, M.M., 1993. Columnar organization in PAG: physiological evidence for inter-columnar interactions. Soc. Neurosci. Abstr. 19, 1408.

Chiasson, B.J., Armstrong, J.N., Hooper, M.L., Murphy, P.R., Robertson, H.A., 1994. The application of antisense oligonucleotide technology to the brain: some pitfalls. Cell Mol. Neurobiol. 14, 507–520.

Costanzi, M., Cannas, S., Saraulli, D., Rossi-Arnaud, C., Cestari, V., 2011. Extinction after retrieval: effects on the associative and nonassociative components of remote contextual fear memory. Learn. Mem. 18, 508–518.

Del-Ben, C.M., Greaff, F.G., 2009. Panic disorder: is the PAG involved? Neural Plast. Available from: 〈http://www.ncbi.nlm.nih.gov/pmc/articles/PMC2654309/pdf/NP2009-108135.pdf〉 (accessed 20.09.12).

De Oca, B.M., DeCola, J.P., Maren, S., Fanselow, M.S., 1998. Distinct regions of the periaqueductal gray are involved in the acquisition and expression of defensive responses. J. Neurosci. 18, 3426–3432.

Dipaulis, A., Keay, K.A., Bandler, R., 1992. Longitudional neuronal organization of defensive reactions in the midbrain periaqueductal gray region of the rat. Exp. Brain Res. 90, 307–318.

Di Scala, G., Mana, M.J., Jacobs, W.J., Phillips, A.G., 1987. Evidence of Pavlovian conditioned fear following electrical stimulation of the periaqueductal grey in the rat. Physiol. Behav. 40, 55–63.

Fanselow, M.S., 1991. The midbrain periaqueductal gray as a coordinator of action in response to fear and anxiety. In: Depaulis, A., Bandler, R. (Eds.), The Midbrain Periaqueductal Gray Matter: Functional, Anatomical, and Neurochemical Organization. Plenum Press, New York, pp. 151–173.

Floyd, N.S., Price, J.L., Ferry, A.T., Keay, K.A., Bandler, R., 2000. Orbitomedial prefrontal projections to distinct longitudional columns of the periaqueductal gray in the rat. J. Comp. Neurol. 422, 556–578.

Garcia, J., Kimeldorf, D.J., Knelling, R.A., 1955. Conditioned aversion to saccharin resulting from exposure to gamma radiation. Science 122, 157–158.

Gillan, D.J., Domjan, M., 1977. Taste-aversion conditioning with expected versus unexpected drug treatment. J. Exp. Psychol. Anim. Behav. Process. 3, 297–309.

Graeff, F.G., 1990. Brain defense systems and anxiety. In: Burrows, G.D., Roth, M., Noyes, R. (Eds.), The Neurobiology of Anxiety. Elsevier, Amsterdam, pp. 307–354 (Chapter 11).

Groblewski, P.A., Lattal, M.K., Cunningham, C.L., 2009. Effects of D-cycloserine on extinction and reconditioning of ethanol-seeking behavior in mice. Alcohol. Clin. Exp. Res. 33, 772–782.

Groblewski, P.A., Stafford, J.M., 2010. When the medial frontal cortex fails: implications for extinction and posttraumatic stress disorder treatment. J. Neurosci. 30, 7124–7126.

Helene, C., 1991. Rational design of sequence-specific oncogene inhibitors based on antisense and antigene olgionucleotides. Eur. J. Cancer 11, 1466–1471.

Herrera, D.G., Robertson, H.A., 1996. Activation of *c-fos* in the brain. Prog. Neurobiol. 50, 83–107.

Herry, C., Ferraguti, F., Singewald, N., Letzkus, J.J., Ehrlich, I., Luthi, A., 2010. Neuronal circuits of fear extinction. Eur. J. Neurosci. 31, 599–612.

Horiuchi, J., McDowall, L., Dampney, R., 2009. Vasomotor and respiration responses evoked from the dorsolateral periaqueductal gray are mediated by the dorsomedial hypothalamus. J. Physiol. 587, 5149–5162.

Hsu, S.M., Raine, L., Fangler, H., 1981. Use of avidin-biotin peroxidase complex (ABC) in immunoperoxidase techniques: A comparison between ABC and unlabeled antibody (PAP) procedures. J. Histochem. Cytochem. 29, 577–580.

Jasmin, L., Granato, A., Ohara, P.T., 2004. Rostral agranular insular cortex and pain areas of the central nervous system: a tract-tracing study in the rat. Comp. Neurol. 468, 425–440.

Jenck, F., Moreau, J.-L., Matrin, J.R., 1995. Dorsal periaqueductal gray-induced aversion as a simulation of panic anxiety: elements of face and predictive validity. Psychiat. Res. 57, 181–191.

Johansen, J.P., Tarpley, J.W., LeDoux, J.E., Blair, H.T., 2010. Neural substrates for expectation-modulated fear learning in the amygdala and periaqueductal gray. Nat. Neurosci. 13, 979–986.

Krout, K.E., Jansen, A.S.P., Loewy, A.D., 1998. Periaqueductal gray matter projection to the parabrachial nucleus in the rat. J. Comp. Neurol. 401, 437–454.

Krukoff, T.L., Morton, T.L., Harris, K.H., Jhamandas, J.H., 1992. Expression of *c-fos* protein in rat brain elicited by electrical stimulation of pontine parabrachial nucleus. J. Neurosci. 12, 3582–3590.

LaBar, K.S., Phelps, E.A., 2005. Reinstatement of conditioned fear in humans is context dependent and impaired in amnesia. Behav. Neurosci. 119, 677–686.

Lamprea, M., Cardenas, F.P., Vianna, D.M., Castilho, V.M., Cruz-Morales, S.E., Brandao, M.L., 2002. The distribution of fos immunoreactivity in rat brain following freezing and escape responses elicited by electrical stimulation of the inferior colliculus. Brain Res. 950, 186–194.

Lamprecht, R., Dudai, Y., 1996. Transient expression of *c-fos* in rat amygdala during training is required for encoding conditioned taste aversion memory. Learn. Mem. 3, 31–41.

Lim, L.W., Blokland, A., Visser-Vandewalle, V., Vlamings, R., Sesia, T., Steinbusch, H., Schruers, K., Griez, E., Temel, Y., 2008. High-frequency stimulation of the dorsolateral periaueductal gray and ventromedial hypothalamus fails to inhibit panic-like behavior. Behav. Brain Res. 193, 197–203.

Mackintosh, N.J., 1974. The Psychology of Animal Learning. Academic Press, London, pp. 468–472.

Mantyh, P.W., 1982. Forebrain projections to the periaqueductal gray in the monkey, with observations in the cat and rat. J. Comp. Neurol. 206, 146–158.

Marchand, J.E., Hagino, N., 1983. Afferents to the periaqueductal gray in the rat. A horseradish peroxidase study. Neuroscience 9, 95–106.

McIntyre, C.C., Mori, S., Sherman, D.L., Thakor, N.V., Vitek, J.L., 2004. Electric field and stimulating influence generated by deep brain stimulation of the subthalamic nucleus. Clin. Neurophysiol. 115, 589–596.

McNally, G.P., Pigg, M., Weidemann, G., 2004. Opioid receptors in the midbrain periaqueductal gray regulate extinction of Pavlovian fear conditioning. J. Neurosci. 24, 6912–6919.

McNally, G.P., Lee, B.-W., Chiem, J.Y., Choi, A.A., 2005. The midbrain Periaqueductal gray and fear extinction: opioid receptor subtype and roles of cyclic AMP, protein kinase A, and mitogen-activated protein kinase. Behav. Neurosci. 119, 1023–1033.

McNally, G.P., Westbrook, R.F., 2010. Predicting danger: the nature, consequences, and neural mechanisms of predictive fear learning. Learn. Mem. 13, 245–253.

McNally, G.P., Johansen, J.P., Blair, H.T., 2011. Placing prediction into the fear circuit. Trends Neurosci. 34, 283–292.

Mickley, G.A., Teitelbaum, H., 1978. Movement induced in cataleptic rats: differential effects produced by electrical stimulation of the lateral hypothalamus, substantia nigra, and reticular formation. Psychopharmacology 57, 145–149.

Mickley, G.A., Kenmuir, C.L., McMullen, C.A., Yocom, A.M., Valentine, E.L., Dengler-Crish, C.M., Weber, B., Wellman, J.A., Remmers-Roeber, D.R., 2004. Dynamic processing of taste aversion extinction in the brain. Brain Res. 1061, 79–89.

Mickley, G.A., Kenmuir, C.L., Yocom, A.M., Wellman, J.A., Biada, J.M., 2005. A role for prefrontal cortex in extinction of a conditioned taste aversion. Brain Res. 1051, 176–182.

Mickley, G.A., Hoxha, Z., Bacik, S., Kenmuir, C.L., Wellman, J.A., Biada, J.M., DiSorbo, A., 2007. Spontaneous recovery of a conditioned taste aversion differentially alters extinction-induced changes in *c-fos* protein expression in rat amygdala and neocortex. Brain Res. 1152, 139–157.

Mickley, G.A., Disorbo, A., Wilson, G.N., Huffman, J., Bacik, S., Hoxha, Z., Biada, J.M., Kim, Y.-H., 2009. Explicit disassociation of a conditioned stimulus and unconditioned stimulus during extinction training reduces both time to asymptotic extinction and spontaneous recovery of a conditioned taste aversion. Learn. Motiv. 40, 209–220.

Mickley, G.A., Wilson, G.N., Remus, J.L., Ramos, L., Ketchesin, K.D., Biesan, O.R., Luchsinger, J.R., Prodan, S., 2011. Periaqueductal gray *c-fos* expression varies relative to the method of conditioned taste aversion extinction employed. Brain Res. 1423, 17–29.

Milad, M.R., Quirk, G.J., 2012. Fear extinction as a model for translational neuroscience: ten years of progress. Ann. Rev. Psychol 63, 129–151.

Moers-Hornikx, V.M.P., Vles, J.S.H., Lim, L.W., Ayyildiz, M., 2011. Periaqueductal gray stimulation induced panic-like behavior is accompanied by deactivation of the deep cerebellar nuclei. Cerebellum 10, 61–69.

National Research Council, 1996. Guide For the Care and Use of Laboratory Animals. National Academy Press, Washington, DC.

Nolan, L.J., McCaughey, S.A., Giza, B.K., Rhinehart-Doty, J.A., Smith, J.C., Thomas, R.S., 1997. Extinction of a conditioned taste aversion in rats: I. Behavioral effects. Physiol. Behav. 61, 319–323.

Norgren, R., 1995. Gustatory system. In: Paxinos, R. (Ed.), The Rat Nervous System. Academic Press, Australia, pp. 751–771 Chapter 29.

Norrholm, S.D., Jovanovic, T., Olin, I.W., Sands, L.A., Karapanou, I., Bradley, B., Ressler, K.J., 2011. Fear extinction in traumatized civilians with posttraumatic stress disorder: relation to symptom severity. Biol. Psychiatry. 69, 556–563.

Olds, J., 1958. Self-stimulation of the brain. Science 127, 315–324.

Oliveria, L.C., Nobre, M.J., Brandao, M.L., Landeira-Fernandez, J., 2004. Role of amygdala in conditioned and unconditioned fear generated in the periaqueductal gray. Learn. Mem. 15, 2281–2285.

Pare, D., Quirk, G.J., Ledoux, J.E., 2004. New vistas on amygdala networks in conditioned fear. J. Neurophysiol. 92, 1–9.

Parker, L.A., 2003. Taste avoidance and taste aversion: evidence for two different processes. Learn. Behav. 31, 165–172.

Parsons, R.G., Gafford, G.M., Helmstetter, F.J., 2010. Regulation of extinction-related plasticity by opioid receptors in the ventrolateral periaqueductal gray matter. Front. Behav. Neurosci. 4, 1–11.

Pavlov, I.P., 1927. Conditioned Reflexes. (G.V. Anrep, Trans.). Oxford University Press, London.

Paxinos, G., Watson, C., 2008. In: The Rat Brain in Stereotaxic Coordinates 6th edition Academic Press, London.

Quirk, G.J., Mueller, D., 2008. Neural mechanisms of extinction learning and retrieval. Neuropsychopharmacology 33, 56–72.

Ranck, J.B., 1975. Which elements are excited in electrical stimulation of mammalian central nervous system: a review. Brain Res. 98, 417–440.

Rauhut, A.S., Thomas, B.L., Ayres, J.J., 2001. Treatments that weaken Pavlovian conditioned fear and thwart its renewal in rats: implications for treating human phobias. J. Exp. Psychol. Anim. Behav. Process. 27, 99–114.

Reimer, A.E., De Oliveira, A.R., Brandão, M.L., 2012. Glutamatergic mechanisms of the dorsal periaqueductal gray matter modulate the expression of conditioned freezing and fear-potentiated startle. Neuroscience 219, 72–81.

Rescorla, R.A., Wagner, A.R., 1972. A theory of Pavlovian conditioning: variations in the effectiveness of reinforcement and nonreinforcement. In: Black, A.H., Prokasy, W.F. (Eds.), Classical Conditioning II: Current Research and Theory. Appleton Century Crofts, New York, pp. 64–99.

Rescorla, R.A., Heth, C.D., 1975. Reinstatement of fear to an extinguished conditioned stimulus. J. Exptl. Psychol. 1, 88–96.

Resstel, L.B.M., Lisboa, S.F., Aguiar, D.C., Correa, F.M.A., Guimaraes, F.S., 2008. Activation of CB1 cannabinoid receptors in the dorsolateral periaqueductal gray reduces the expression of contextual fear conditioning in rats. Psychopharmacology 198, 405–411.

Rinaman, L., Stricker, E.M., Hoffman, G.E., Verbalis, J.G., 1997. Central *c-fos* expression in neonatal and adult rats after subcutaneous injection of hypertonic saline. Neuroscience 79, 1165–1175.

Sandner, G., Di Scala, G., Rocha, B., Angst, M.J., 1992. *C-fos* immunoreactivity in the brain following unilateral electrical stimulation of dorsal periaqueductal gray in freely moving rats. Brain Res. 573, 276–283.

Spector, A.C., 1995. Gustatory function in the parabrachial nuclei: implications from lesion studies in rats. Rev. Neurosci. 6, 143–175.

Swank, M.W., Ellis, A.E., Cochran, B.N., 1996. *c-fos* antisense blocks acquisition and extinction of conditioned taste aversion in mice. NeuroReport 7, 1866–1870.

Tehovnik, E.J., 1996. Electrical stimulation of neural tissue to evoke behavioral responses. J. Neurosci. Method 65, 1–17.

Thomas, B.L., Longo, C.L., Ayres, J.J.B., 2005. Thwarting the renewal (relapse) of conditioned fear with the explicitly unpaired procedure: possible interpretations and implications for treating human fears and phobias. Learn. Motiv. 36, 374–407.

Vianna, D.M.L., Brandao, M.L., 2003. Anatomical connections of the periaqueductal gray: specific neural substrates for different kinds of fear. Braz. J. Med. Biol. Res. 36, 557–566.

Vianna, D.M., Borelli, K.G., Ferreira-Netto, C., Macedo, C.E., Brandao, M.L., 2003. Fos-likeimmunoreactive neurons

following electrical stimulation of the dorsal periaqueductal gray
at freezing and escape thresholds. Brain Res. Bull. 62, 179–189.
Walker, D.L., Davis, M., 1997. Involvement of the dorsal periaqueductal gray in the loss of fear-potentiated startle accompanying high footshock training. Behav. Neurosci. 111, 692–702.
Wahlestedt, C., 1994. Antisense oligonucleotide strategies in neuropharmacology. Trends Pharmacol. Sci. 15, 42–46.
Westbrook, R.F., Iordanova, M., McNally, G., Richardson, R., Harris, J.A., 2002. Reinstatement of fear to an extinguished conditioned stimulus: two roles for context. J. Exp. Psychol. Anim. Behav. Process. 28, 97–110.
Yamamoto, T., Shimura, T., Sako, N., Yasoshima, Y., Sakai, N., 1994. Neural substrates for conditioned taste aversion in the rat. Behav. Brain Res. 65, 123–137.
Yousif, N., Liu, X., 2007. Modeling the current distribution across the depth electrode–brain interface in deep brain stimulation. Expert Rev. Med. Devices 4, 623–631.
Zanoveli, J.M., Ferreira-Netto, C., Brandao, M.L., 2007. Conditioned place aversion organized in the dorsal periaqueductal gray recruits the laterodorsal nucleus of the thalamus and the basolateral amygdala. Exp. Neurol. 208, 127–136.