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Prefrontal-limbic Functional Connectivity during Acquisition and Extinction of Conditioned Fear

Douglas W. Barrett [†] **and F. Gonzalez-Lima** [*†]

Department of Psychology and Institute for Neuroscience, University of Texas at Austin, Austin, TX 78712, USA

Abstract—This study is a new analysis to obtain novel metabolic data on the functional connectivity of prefrontal-limbic regions in Pavlovian fear acquisition and extinction of tone-footshock conditioning. Mice were analyzed with the fluorodeoxyglucose (FDG) autoradiographic method to metabolically map regional brain activity. New FDG data were sampled from the nuclei of the habenula and other regions implicated in aversive conditioning, such as infralimbic cortex, amygdala and periaqueductal gray regions. The activity patterns among these regions were inter-correlated during acquisition, extinction or pseudorandom training to develop a functional connectivity model. Two subdivisions of the habenular complex showed increased activity after acquisition relative to extinction, with the pseudorandom group intermediate between the other two groups. Significant acquisition activation effects were also found in centromedial amygdala, dorsomedial and ventrolateral periaqueductal gray. FDG uptake increases during extinction were found only in dorsal and ventral infralimbic cortex. The overall pattern of activity correlations between these regions revealed extensive but differential functional connectivity during acquisition and extinction training, with less functional connectivity found after pseudorandom training. Interestingly, habenula nuclei showed a distinct pattern of inter-correlations with amygdala nuclei during extinction. The functional connectivity model revealed changing interactions among infralimbic cortex, amygdala, habenula and periaqueductal gray regions through the stages of Pavlovian fear acquisition and extinction. This study provided new data on the contributions of the habenula to fear conditioning, and revealed previously unreported infralimbic-amygdala-habenula-periaqueductal gray interactions implicated in acquisition and extinction of conditioned fear.

Key words: aversive conditioning, fear extinction, habenula, infralimbic cortex, amygdala, intercalated cells.

INTRODUCTION

The neural mechanisms that underlie the process of Pavlovian fear extinction are intricate, controversial, and of great interest to those that study learning phenomena (Auchter et al., 2017) as well as its clinical applications (Zoellner et al., 2017). This paradigm is a simple model of how a memory is formed, then modified on the basis of experience. Extinction may be defined as the reduction of a previously learned response that occurs because a conditioned stimulus (CS) is no longer paired with an unconditioned stimulus (US). In Pavlovian fear conditioning of rodents, a tone CS is often paired with an aversive footshock US during acquisition, such that presentations of the CS alone will elicit freezing behavior, a defensive conditioned response (CR). During extinction, repeated presentation of the tone alone (in the absence of footshock) will cause the fear-evoked CR to decrease over time.

In terms of neural mechanisms, increased habenula fluorodeoxyglucose (FDG) uptake has been linked to the prediction of the US by a tone CS in an early Pavlovian conditioning study in rats with aversive electrical stimulation of the brain as US (Gonzalez-Lima and Scheich, 1986). Our subsequent FDG study in the mouse brain (Barrett et al., 2003) examined the metabolic effects of Pavlovian acquisition and extinction of tone-footstock conditioning, but there was no investigation of the habenula. Renewed interest in the habenula in recent years (Velasquez et al., 2014), such as new findings on the negative-reward-predicting properties of habenula neurons (Matsumoto and Hikosaka, 2007) have prompted

*Corresponding author. Address: 108 E. Dean Keeton Stop A8000, University of Texas at Austin, Austin, TX 78712, USA.
E-mail address: gonzalezlima@utexas.edu (F. Gonzalez-Lima).
[†] These authors contributed equally to this work.

Abbreviations: CeL, amygdala, centrolateral nucleus; CeM, amygdala, centromedial nucleus; CR, conditioned response; CS, conditioned stimulus; DMPAG, dorsomedial periaqueductal gray; FDG, fluorodeoxyglucose; HBa, anterior habenula; HBl, posterior habenula, lateral; HBm, posterior habenula, medial; HRP, horseradish peroxidase; ILd, infralimbic cortex, dorsal; ILv, infralimbic cortex, ventral; ITCd, amygdala, intercalated cells, dorsal; ITCv, amygdala, intercalated cells, ventral; PAG, periaqueductal gray; PFC, prefrontal cortex; PTSD, post-traumatic stress disorder; US, unconditioned stimulus; VLPAG, ventrolateral periaqueductal gray.

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us to obtain new data from the habenula from our FDG study of tone-footstock conditioning as well as obtain new data from other limbic regions related to acquisition and extinction. We hypothesized that habenula metabolism may be involved in the prediction of aversive events more generally, i.e., the withdrawal of a reward stimulus or the delivery of an aversive one (Shumake and Gonzalez-Lima, 2013). Therefore, we predicted that habenula metabolism will increase during acquisition and decrease during extinction of tone-footstock conditioning. In addition, the patterns of metabolic activity correlations found between these regions of interest were analyzed for the first time to inform the question of how interactions between the habenula and other brain regions implicated in fear conditioning change from acquisition to extinction training.

Limbic regions such as the amygdala may be specialized for fear acquisition processes in general (Balderston et al., 2014; Schultz et al., 2016), and expression of the freezing CR in particular. For example, many studies have documented the involvement of the central amygdala in CR expression (Helmstetter, 1992; Kim et al., 1993; LeDoux, 1995), in fear-potentiated startle (Davis et al., 1993), and avoidance learning (Poremba and Gabriel, 1997). The central nucleus is the main output zone of the amygdala, and its activity probably reflects the expression of the freezing CR at the time of sampling. After extinction training, the CR is no longer present; thus, the central amygdala may show opposite effects after acquisition vs. extinction. This might not reflect a general "unlearning" process, however; it is also consistent with the involvement of CR inhibition from outside the amygdala.

This CR inhibition is likely provided by the prefrontal cortex (PFC), which has been extensively implicated as playing a crucial role in extinction of conditioned fear (Quirk et al., 2006; Gilmartin et al., 2014). For example, lesions of medial PFC disrupt CR extinction in rats (Morgan et al., 1993), and Milad and Quirk (2002) showed that neurons in infralimbic cortex develop enhanced firing rates during extinction, which are correlated with the decrease in the freezing CR. If infralimbic cortex neurons inhibit the amygdala, metabolic activation of the infralimbic cortex would be present only after extinction, and not after acquisition or pseudorandom training in the Pavlovian fear conditioning paradigm.

Most lesion and stimulation studies of the effects of Pavlovian fear extinction on the rodent brain have focused on the basolateral amygdala and infralimbic cortex as the sites of acquisition and extinction of tone-footstock conditioning, respectively. However, other brain regions such as the habenula may be also involved in these learning processes in the tone-footstock conditioning paradigm. Our lab was the first to use the FDG autoradiographic method to metabolically map brain activity after other learning paradigms, including aversive conditioning (Gonzalez-Lima and Scheich, 1986), operant extinction (Nair and Gonzalez-Lima, 1999; Nair et al., 2001a, 2001b), blocking (Jones and Gonzalez-Lima, 2001a), differential inhibition (Jones and Gonzalez-Lima, 2001b), and habituation (Gonzalez-Lima et al., 1989a, 1989b). One advantage of the FDG autoradiographic technique is the ability to take optical density readings from any brain region. Thus, any region recently implicated in aversive conditioning, such as the intercalated cells of the amygdala (Asede et al., 2015; Strobel et al., 2015) or the nuclei of the habenula (Shumake and Gonzalez-Lima, 2013) can be sampled, and this activity can be correlated with that of other brain systems after fear acquisition or extinction to develop a functional connectivity model.

Therefore, the objective of this study was to obtain new FDG data on the habenula and other limbic regions implicated in aversive conditioning, and to construct a novel functional connectivity model of infralimbic-amygdala-habenula-periaqueductal gray interactions after Pavlovian fear acquisition and extinction.

EXPERIMENTAL PROCEDURES

This manuscript is a new analysis that provides new FDG data from regions not previously investigated in the experiment described in Barrett et al. (2003). Images from the autoradiographic films obtained from those subjects were used to take new optical density readings from new brain regions of interest based on recent findings on the brain metabolic effects of Pavlovian fear extinction. All animal experimentation was carried out in accordance with the National Institute of Health Guide for the Care and Use of Laboratory Animals (NIH Publications No. 80-23) revised 1996 and was approved by the University of Texas Institutional Animal Care and Use Committee. All efforts were made to minimize the number of animals used and their suffering.

A timeline diagram of the fear conditioning protocol and experimental design is provided in Fig. 1. The methods for behavioral training and tissue processing for FDG autoradiography were those described in Barrett et al. (2003). Briefly, 32 male CBA/J mice, 5 weeks of age when delivered from Jackson Laboratory (Bar Harbor, ME), were handled for 7 days prior to training on Pavlovian acquisition (n = 11), extinction (n = 11), or pseudorandom (n = 10) presentations of a tone CS (frequency-modulated tone, 1–2 kHz, 65 dB, 15 s) and footshock US (0.5 mA, 0.75 s). Phase I (acquisition training) occurred in context A. Since our objective was to map neural effects evoked by the tone rather than by context A, all subsequent steps (probe trials, extinction training, and FDG uptake) were conducted in context B to minimize the effects of excitatory conditioning to context A. After training, subjects were given an intraperitoneal injection of 18 μCi/100 gm body weight of ^{14}C(U)-FDG, placed in context B, and exposed to the tone CS in a 5 s on, 1 s off cycle for 45 min. Subjects were then decapitated and brains were rapidly removed and frozen in isopentane at −40 °C. Sections of the brain were cut at 40 μm, picked up on slides, dried, and exposed to autoradiographic film (along with standards of known ^{14}C concentration) for 2 weeks. Films were developed, dried, and stored in protective covers.