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Prefrontal-limbic functional connectivity during acquisition and extinction of conditioned fear

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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.

Keywords

aversive conditioning; fear extinction; habenula; infralimbic cortex; amygdala; intercalated cells

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Conflicts of interest

The authors declare no conflict of interest.

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 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 & 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 & 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 & Gonzalez-Lima, 1999; Nair et al., 2001a, 2001b), blocking (Jones & Gonzalez-Lima, 2001a), differential inhibition (Jones & 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 Figure 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 sec) and footshock US (0.5 mA, 0.75 sec). 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 $\mu\text{Ci}/100\text{ gm}$ body weight of $^{14}\text{C}(\text{U})\text{-FDG}$, placed in context B, and exposed to the tone CS in a 5 sec on, 5 sec 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 $40\text{ }\mu\text{m}$, 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.

Films were placed on a light box and artifact-free images were captured, digitized, and corrected for film background and optical distortions from the camera via background subtraction. Absolute gray levels of the ^{14}C standards were used to create a calibration curve unique to each individual film. Optical density measurements from brain regions of interest were automatically expressed in terms of isotope incorporation per gram of brain tissue (nanocuries per gram). The Paxinos and Franklin (2001) mouse brain atlas was used to locate and delineate the boundaries of each region measured as illustrated in the diagrams shown in Figures 3–6. For the purpose of sampling regional FDG uptake, the infralimbic cortex was subdivided into two equal halves by a line at the center of the dorsal-ventral axis (Fig. 6). Each region was sampled from three adjacent sections, with four non-overlapping readings taken in each section; these measurements were averaged together to give one measurement per region per subject. The brain regions sampled were: Infralimbic cortex, dorsal (ILd); Infralimbic cortex, ventral (ILv); Anterior habenula (HBa); Nucleus reunions; Paraventricular hypothalamus; Amygdala, lateral nucleus (LA); Amygdala, basolateral nucleus (BLA); Amygdala, centrolateral nucleus (CeL); Amygdala, centromedial nucleus (CeM); Amygdala, intercalated cells, dorsal (ITCd); Amygdala, intercalated cells, ventral (ITCv); Posterior habenula, lateral (HBl); Posterior habenula, medial (HBm); Periaqueductal gray, anterior; Posterior thalamus; Posterior intralaminar nucleus; Dorsomedial periaqueductal gray (DMPAG); Ventrolateral periaqueductal gray (VLPAG); Pontine reticular formation. Bregma levels and FDG data for all regions are listed in the appendix table.

Statistical analysis

We found normal distributions and equal variances in our sampled distributions. Mean brain activity was assessed as in Barrett et al. (2003). Briefly, to reduce variability resulting from individual differences in FDG uptake unrelated to the experimental paradigm, white matter readings were used as covariates in an analysis of covariance (ANCOVA). Because there was no statistically significant difference in the white matter readings between groups, the ANCOVA can use the covariate to compensate for small variations in isotope incorporation across individuals. Mean activity readings were then expressed in nanocuries per gram of tissue for each region of interest, adjusted by the covariate white matter readings, with 99% confidence intervals for each group. If the measurements for two groups were both outside the other's 99% confidence interval, the effect was considered significant at uncorrected $p < 0.01$ and at corrected $p < 0.05$ adjusted for multiple comparisons among the groups with the Hochberg procedure, which is a sharper Bonferroni procedure for multiple tests of

significance (Hochberg, 1988). Effect sizes were estimated using Cohen's D [(Mean1-Mean2)/Pooled standard deviation]. A Cohen's D larger than 0.8 is considered a large effect size (Cohen 1977).

Functional connectivity is an analytic approach that has been used for several decades to refer to the correlations of activity between neural elements. It was first introduced using electrophysiological data and glucose metabolic rates (Horwitz et al., 1984; Aertsen et al., 1987). Functional connectivity using autoradiographic FDG data was introduced by McIntosh and Gonzalez-Lima (1991, 1992). This approach was then introduced using fMRI data (Friston, 1994). To state that two neural elements (neurons or brain regions) display functional connectivity is to say that these elements show statistically significant correlated activity without reference to how that correlation is mediated by anatomical connections (McIntosh and Gonzalez-Lima, 1994). Because extinction of conditioned behavior is manifested as neural changes in functional connectivity of FDG data in our previous studies, the functional relationships among the regional brain activity data were analyzed in terms of pair-wise correlations within each group, exactly as done before in our FDG studies of extinction of instrumental behavior (Nair and Gonzalez-Lima, 1999) and Pavlovian fear conditioning (Barrett et al., 2003). Briefly, for the interregional correlation analysis, Pearson product-moment correlations were computed using pair-wise comparisons of each region. To ensure the statistical reliability of correlations, a jackknife procedure was performed to avoid inflated type 1 errors caused by the large number of interregional correlations computed relative to the sample sizes. In the jackknife procedure, each individual subject was dropped from a group, and then correlations were calculated again without that subject's data. This procedure was iterated until each subject had been sequentially dropped and the analysis performed again. Correlations were considered to be reliably corrected as significant only if they remained significantly ($p < 0.05$) different from zero throughout all iterations. This is a conservative correction method sensitive to outliers that avoids inflated type 1 errors in the functional connectivity analysis of metabolic brain data (Sakata et al., 2000).

Results

Representative FDG autoradiographs are shown in Figure 2. Bregma levels with regions showing a significant mean difference in FDG uptake are shown in Figures 3–6. The means, standard errors, and confidence intervals of each region's FDG uptake are listed in a table in the Appendix. Correlation coefficients, which remained significant at $p < 0.05$ after the jackknife procedure, are shown in Figure 7.

Behavior

Table 1 summarizes the percent freezing to the CS for the three groups. After acquisition training, freezing was significantly increased in both the acquisition group [$F(1,19) = 40.4$, $p < 0.01$] and extinction group [$F(1,19) = 41.9$, $p < 0.01$]. After extinction training, the extinction group had a significant decrease in freezing [$F(1,20) = 123.0$, $p < 0.01$].

Mean FDG uptake changes

Table 2 shows the effect sizes (Cohen's D) for each of the significant group differences in FDG uptake. Novel findings during Pavlovian tone-footshock conditioning were found in the habenula (HB, Fig. 2). Habenular regions showed increased activity after acquisition relative to extinction, with the pseudorandom group intermediate between the other two groups. Statistically significant (uncorrected $p < 0.01$ and corrected $p < 0.05$) acquisition effects (increased metabolic activity after acquisition training) were found in two subdivisions of the habenula complex: anterior habenula (HBa, Fig. 3) and medial habenula (HBm, Fig. 4); but the increase in lateral habenula did not reach uncorrected $p < 0.01$.

Another significant acquisition effect was found in centromedial amygdala (CeM, Fig. 4). The acquisition group showed significantly greater metabolic activity relative to the pseudorandom group, with the extinction group showing an intermediate value. However, other amygdala subdivisions such as basal, lateral, centrolateral, or intercalated cells of the amygdala did not show this increase at uncorrected $p < 0.01$.

Significant acquisition effects were also found in dorsomedial and ventrolateral periaqueductal gray (DMPAG, VLPAG, Fig. 5). Both regions showed significantly greater metabolic activity after acquisition training, relative to the control group. The values for the extinction group were intermediate between acquisition and control groups.

Significant mean FDG uptake increases during extinction were found only in dorsal and ventral infralimbic cortex subdivisions (ILd, ILv, Fig. 6), among all the regions sampled. Both infralimbic subdivisions showed increased activity after extinction, but not acquisition or pseudorandom training.

Functional connectivity

Pairwise within-group correlations between FDG uptake of infralimbic cortex, amygdala, habenula and periaqueductal gray regions for each of the three groups showed that all significant correlation coefficients ($p < 0.05$ after jackknife) were positive (Fig. 7). This is indication that for each unit increase or decrease in FDG uptake in one region, the correlated region showed a corresponding increase or decrease in FDG uptake (i.e., positive functional connectivity). The overall pattern of significant correlations between these systems revealed extensive but differential functional connectivity after acquisition and extinction training, with less functional connectivity found after pseudorandom training.

Whereas infralimbic cortex, amygdala, habenula and periaqueductal gray showed various positive correlation coefficients in both pseudorandom and acquisition groups, their pattern of relationships was modified by extinction training. For example, infralimbic regions showed extensive positive functional connectivity with amygdala regions after acquisition training; however, extinction training reversed this trend, resulting in a decrease in the positive correlative activity between infralimbic cortex and amygdala, and an increase in intra-amygdala positive correlations.

Specifically, habenula regions showed a distinct pattern of inter-correlations with amygdala nuclei during extinction. The extinction pattern was characterized by a strong medial

habenula-intercalated cell (HBm-ITCv) correlation; and the ITCv was positively correlated with the lateral amygdala (ITCv-LA). This pattern of habenula-amygdala inter-regional correlations was not found during acquisition or pseudorandom control training. In addition, metabolic activity in the habenula was positively correlated with that of the ventrolateral PAG (HB1-VLPAG) only in the acquisition group. Furthermore, the acquisition group was characterized by a network of infralimbic-amygdala functional connections in which ILd-ILv were strongly inter-correlated with CeL, ITCd and LA. In this network, the strongest intra-amygdala correlations were among CeL, ITCd and LA regions. The extinction group, however, showed a loss of ILd-ILv positive functional connectivity with CeL-ITCd-LA, a pattern more similar to that of the control group.

Therefore, the functional connectivity models revealed changing interactions among infralimbic cortex, amygdala, habenula and periaqueductal gray regions through the stages of Pavlovian acquisition and extinction.

Discussion

Current understanding of neural pathways relevant to auditory fear acquisition and extinction (Amano et al., 2011, Nair 2012, Gilmartin et al., 2014) suggest that: 1) tone and footshock information arrive at the lateral amygdala nucleus (LA) via thalamic and cortical pathways; 2) LA projects to the basolateral amygdala nucleus (BLA) and also to dorsal intercalated cells (ITCd) and centrolateral amygdala (CeL); 3) BLA fear acquisition neurons project to centromedial amygdala (CeM) and BLA fear extinction neurons project to ventral intercalated cells (ITCv); and 4) the central nucleus provides the amygdala output which projects to brainstem regions such as the periaqueductal gray (PAG) and other regions eliciting fear-related behavior. Consistent with the hypothesis that infralimbic cortex (IL) plays an active modulatory role in Pavlovian extinction (Quirk et al., 2006), both dorsal (ILd) and ventral (ILv) infralimbic cortex subdivisions showed significantly greater metabolic activity after extinction training; no other region sampled showed increased activity after extinction. Infralimbic cortex showed the single largest percentage increase in mean FDG uptake after extinction training in our previous study (Barrett et al., 2003). The pairwise correlations between infralimbic cortex and amygdala, which are significantly positive after acquisition but not extinction (Fig. 7), also indicate a shift in the role of infralimbic cortex after Pavlovian conditioning. In particular, the infralimbic-amygdala positive functional connectivity in which ILd-ILv were strongly inter-correlated with CeL, ITCd and LA during acquisition were not found during extinction. The extinction group showed a decrease in the positive correlative activity between infralimbic cortex and amygdala, and an increase in intra-amygdala correlations. The observed intra-amygdala activity correlations among CeL, ITCd and LA are consistent with direct projections from ITCd to both CeL and LA (Asede et al., 2015, Strobel et al., 2015).

The acquisition effect found in CeM supports this region's important role in fear conditioning (Barrett et al., 2003). Other amygdala regions (ventral intercalated cells, lateral nucleus) also showed greater activity after acquisition relative to pseudorandom training, but only at the less stringent uncorrected $p < 0.05$ level. The stronger effect seen in CeM may be consistent with this region's role as the response expression area of the amygdala. If activity

in this region is more coupled to the expression of the conditioned response (freezing behavior), then the higher levels of conditioned freezing in the acquisition group may be driving the increase in metabolic activity in CeM. There are extensive reciprocal anatomical connections between the central amygdala and the periaqueductal gray (PAG) (Rizvi et al., 1991).

This argument, in which regions tied to the expression of conditioned freezing behavior show elevated activity after acquisition training, also applies to the changes seen in PAG. Anterior PAG showed an acquisition effect at uncorrected $p < 0.05$, but this effect was not significant at $p < 0.01$. However, posterior PAG (both dorsomedial and ventrolateral periaqueductal gray) showed significant acquisition effects at $p < 0.01$, in which metabolic activity was elevated by acquisition training, then attenuated by extinction training. In both of these regions, the level of FDG uptake was proportional to the levels of conditioned freezing exhibited by the three experimental groups. These two regions, however, may be differentiated by their specialized roles in the expression of extinguished freezing behavior, as the magnitude of FDG uptake was greater in ventrolateral than in dorsomedial periaqueductal gray in all three groups. This is consistent with the finding that naloxone (an opioid antagonist) injection into ventrolateral (but not dorsal) PAG impaired fear extinction (McNally et al, 2004). Ventrolateral PAG appeared therefore more involved than dorsomedial PAG in the functional connectivity model, as a final output mediating the expression of conditioned freezing behavior. Furthermore, FDG uptake in the ventrolateral PAG was better correlated with that of the habenula than to the amygdala during acquisition. In terms of anatomical circuits that may mediate functional connectivity between these brain regions, there is evidence by the HRP method that neurons in the ventral PAG project to the habenula (Herkenham and Nauta, 1977).

The extinction, acquisition and pseudorandom group received an equal number of tones and footshocks; but only the extinction group received additional tone-alone presentations. If limbic regions like the habenula mediate the prediction of aversive events signaled by the CS, then the acquisition group where the CS triggers a defensive CR would show habenula-PAG functional connectivity, but not the extinction and pseudorandom groups without CRs, as seen in the model. With extinction, the predictive negative value of the tone CS, presented repeatedly without footshock US, was gradually neutralized, decreasing the functional coupling between habenula-PAG pathways. Again, these changes provide multiple routes for the limbic system to adaptively modify behavior, by decreasing conditioned freezing after extinction training. Gonzalez-Lima and Scheich (1986) were the first to link metabolic changes in HBI activity to learning. HBI metabolism was selectively increased by pairings of a tone CS with an aversive US consisting of electrical stimulation to the midbrain reticular formation. Since the HBI was not activated by presentations of the CS alone or the US alone, this implied that the HBI responded to the prediction of the US by the CS. This early FDG finding not only anticipated the discovery of the negative-reward predicting properties of HBI neurons (Matsumoto and Hikosaka, 2007), but it is also consistent with the present FDG findings, suggesting that HBI neurons may be involved in the prediction of aversive events more generally.

The acquisition effect found in the habenula, at both anterior and posterior levels, is consistent with this region's possible role in the prediction of aversive events that may support the persistence of fear memories (Shumake and Gonzalez-Lima, 2013). The congenital helpless rat, which our lab has extensively characterized in terms of baseline brain metabolism using cytochrome oxidase histochemistry (Shumake et al., 2001, 2002, 2003, 2004), shows increased habenula activity, which may play a role in the predisposition to develop affective disorders such as post-traumatic stress disorder (PTSD) and depression (Shumake and Gonzalez-Lima, 2003). The behavioral characteristics of the congenital helpless rat also show remarkable parallels with those of humans predisposed to the development of PTSD (Shumake et al., 2005), including a dramatic deficit in extinction learning, in which presentations of the tone CS during extinction actually result in "paradoxical enhancement" of the original fear memory and increased freezing behavior, instead of inhibition of the CS-evoked freezing response. This role of the habenula in the persistence of fear memories may be reflected in both the significant mean activity differences and in the significant pairwise correlation between lateral habenula and ventrolateral PAG, found only in the acquisition group (Fig. 7). If acquisition training results in increased functional coupling between these two regions, the habenula may be partially driving the increase in CS-evoked freezing behavior after acquisition.

The experience of fear learning and modifying an associative relationship between a CS and US seems to result in increased pairwise correlations between several brain systems (Barrett et al., 2003), as if Pavlovian conditioning drives these systems to become more interactive and more functionally coupled, at least in terms of FDG uptake. However, in terms of inhibitory influences on amygdala from infralimbic cortex, the pairwise correlations during extinction provided evidence for only weak negative correlations between infralimbic and amygdala regions that did not reach statistical significance after the jackknife procedure. The change from strong positive correlations during acquisition to weak correlations during extinction may be considered perhaps as uncorrelated infralimbic-amygdala activity during extinction.

The pattern of habenula-amygdala inter-regional correlations we found in the extinction group (but not in the acquisition or pseudorandom groups) together with our lab's previous data (Barrett et al., 2003) suggests that infralimbic-amygdala interactivity alone may not account for extinction (Amano et al., 2011). Other regions may also contribute to extinction, such as habenular, thalamic and hippocampal regions. For example, the medial dorsal thalamus, which projects reciprocally to medial prefrontal cortex, showed greater activity, as well as a significant negative pairwise correlation with dorsal frontal cortex, after extinction but not after acquisition (Barrett et al., 2003). This thalamic region, when stimulated, can also modify CR extinction (Herry et al., 1999; Herry & Garcia, 2002), indicating that this thalamic region may also be involved in CR inhibition. Hippocampal regions also appear to be involved, and previous metabolic findings on the contextual modulation of extinction (Bruchey et al., 2007) support the importance of the hippocampus in this role.

In conclusion, the present FDG study provided new data on the contributions of the habenula to fear conditioning, and it revealed previously unreported infralimbic-amygdala-habenula-periaqueductal gray functional interactions during acquisition and extinction of tone-

footshock conditioning. Regional metabolic mapping and functional connectivity modeling of FDG data served to reveal functional influences between brain regions that may be ultimately responsible for the adaptive behavioral changes seen after fear extinction learning.

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Appendix

Appendix Table

Means, standard errors, and 99% confidence intervals from analysis of covariance.

Region	Bregma Level	Group	Mean	Std. Error	99% Confidence Interval	
					Lower	Upper
Infralimbic cortex, dorsal (ILd)	1.98 mm	Acquisition	320.1	18.0	270.4	369.8
		Extinction, 1	371.5	18.0	321.7	421.3
		Control	300.8	19.3	247.5	354.1
Infralimbic cortex, ventral (ILv)	1.98 mm	Acquisition	304.8	14.8	263.9	345.7
		Extinction, 1	348.2	14.8	307.2	389.2
		Control	288.5	15.9	244.7	332.4
Anterior habenula (HBa)	−0.82 mm	Acquisition, 2	320.2	20.9	262.4	377.9
		Extinction	248.0	21.0	190.1	305.9
		Control	287.7	22.4	225.8	349.7
Nucleus reunions	−0.82 mm	Acquisition	324.4	17.9	274.9	373.8
		Extinction	300.2	18.0	250.6	349.8
		Control	275.4	19.2	222.3	328.5
Paraventricular hypothalamus	−0.82 mm	Acquisition	257.0	10.8	227.2	286.8
		Extinction	238.3	10.8	208.5	268.2
		Control	230.1	11.6	198.1	262.0
Amygdala, lateral nucleus (LA)	−1.58 mm	Acquisition	295.0	18.3	244.3	345.8
		Extinction	290.1	17.3	242.2	338.0
		Control	252.2	18.8	200.2	304.1
Amygdala, basolateral nucleus (BLA)	−1.58 mm	Acquisition	294.6	15.7	251.1	338.1
		Extinction	302.5	14.8	261.4	343.5
		Control	280.0	16.1	235.5	324.5
Amygdala, centrolateral nucleus (CeL)	−1.58 mm	Acquisition	252.9	18.4	202.1	303.8
		Extinction	252.9	17.3	204.9	300.9
		Control	218.3	18.8	166.3	270.4
Amygdala, centromedial nucleus (CeM)	−1.58 mm	Acquisition, 3	260.5	12.8	225.0	296.1
		Extinction	239.6	12.1	206.0	273.1
		Control	222.8	13.1	186.4	259.2

Region	Bregma Level	Group	Mean	Std. Error	99% Confidence Interval	
					Lower	Upper
Amygdala, intercalated cells, dorsal (ITCd)	−1.58 mm	Acquisition	274.8	18.7	223.0	326.5
		Extinction	273.6	17.6	224.7	322.4
		Control	239.9	19.1	186.9	292.9
Amygdala, intercalated cells, ventral (ITCv)	−1.58 mm	Acquisition	286.6	13.0	250.6	322.6
		Extinction	271.8	12.3	237.8	305.7
		Control	258.5	13.3	221.7	295.4
Posterior habenula, lateral (HBl)	−1.58 mm	Acquisition	435.2	22.2	373.8	496.5
		Extinction	376.0	22.3	314.5	437.6
		Control	401.7	23.8	335.9	467.5
Posterior habenula, medial (HBm)	−1.58 mm	Acquisition, 2	384.0	18.8	332.1	436.0
		Extinction	319.3	18.8	267.2	371.4
		Control	335.9	20.2	280.2	391.7
Periaqueductal gray, anterior	−3.08 mm	Acquisition	353.9	15.9	309.8	398.0
		Extinction	354.6	16.0	310.5	398.8
		Control	312.7	17.1	265.5	360.0
Posterior thalamus	−3.08 mm	Acquisition	375.9	14.5	335.7	416.1
		Extinction	367.0	14.6	326.7	407.3
		Control	353.0	15.6	309.9	396.1
Posterior intralaminar nucleus	−3.08 mm	Acquisition	370.8	15.1	329.1	412.6
		Extinction	358.9	15.1	317.1	400.7
		Control	346.2	16.2	301.5	391.0
Dorsomedial periaqueductal gray (DMPAG)	−4.72 mm	Acquisition, 3	286.4	10.3	258.0	314.8
		Extinction	258.6	10.3	230.1	287.0
		Control	243.7	11.0	213.3	274.2
Ventrolateral periaqueductal gray (VLPAG)	−4.72 mm	Acquisition, 3	359.3	13.4	322.2	396.5
		Extinction	326.5	13.5	289.2	363.7
		Control	315.1	14.4	275.3	355.0
Pontine reticular formation	−4.72 mm	Acquisition	330.9	11.1	300.2	361.6
		Extinction	308.4	11.1	277.6	339.1
		Control	313.9	11.9	281.0	346.8

Regional FDG uptake values are expressed as nanocuries of isotope incorporation per gram tissue wet weight. Indicated group differences are significant at $p < 0.01$ after ANCOVA and at $p < 0.05$ after correction by the Hochberg procedure for multiple group comparisons. **1**, Extinction group significantly greater than both acquisition and control. **2**, Acquisition group significantly greater than extinction. **3**, Acquisition group significantly greater than control.

Abbreviations

CeL amygdala, centrolateral nucleus

CeM amygdala, centromedial nucleus

CS	conditioned stimulus
CR	conditioned response
DMPAG	dorsomedial periaqueductal gray
FDG	fluorodeoxyglucose
HBa	anterior habenula
HBi	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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Highlights

A new analysis of brain effects of Pavlovian fear conditioning revealed increased fluorodeoxyglucose uptake in the habenula.

Metabolic increases were also found in infralimbic cortex in extinction, and amygdala and periaqueductal gray in acquisition.

Functional connectivity models revealed novel interactions between these regions during fear acquisition and extinction.

Fig. 1.

Timeline of the experimental procedures for each of the three groups.

Fig. 2.

Representative FDG autoradiographs showing the habenula (HB), thalamus (TH), and hippocampus (HIPP) in images developed from coronal sections of the mouse brain at the level of HB. The white lines point to HB to illustrate the increase in FDG uptake in A as compared to B. However, the results reported in the paper are not based on visual inspection of autoradiographs; rather, they are based on quantitative densitometric analysis and group statistics that showed that HB had a statistically significant FDG increase in the acquisition group as compared to the extinction group.

Fig. 3. Anterior habenula FDG uptake in the extinction, acquisition and pseudorandom control groups.

Fig. 4. Medial habenula and centromedial amygdala FDG uptake in the extinction, acquisition and pseudorandom control groups.

Fig. 5.
Periaqueductal gray FDG uptake in the extinction, acquisition and pseudorandom control groups.

Fig. 6. Infralimbic cortex FDG uptake in the extinction, acquisition and pseudorandom control groups.

Fig. 7.

Functional connectivity models of infralimbic-amygdala-habenula-periaqueductal gray interactions based on pairwise inter-regional correlations of FDG uptake in the extinction group, acquisition group and pseudorandom control group. The lines with numbers indicate the inter-regional correlation coefficients that remained reliably significant at $p < 0.05$ after the jackknife procedure.

Table 1

Percent freezing behavior in the three groups.

Group	Percent Freezing	
	Probe 1 (post-acquisition)	Probe 2 (post-extinction)
Extinction	83%	21%
Acquisition	82%	83%
Control	30%	27%

Table 2

Summary of effect sizes (Cohen's D) of significant comparisons.

Brain Region	Comparison	Effect Size
Infralimbic cortex, dorsal	Extinction > Control	1.23
Infralimbic cortex, dorsal	Extinction > Acquisition	0.90
Infralimbic cortex, ventral	Extinction > Control	1.26
Infralimbic cortex, ventral	Extinction > Acquisition	0.93
Anterior habenula	Acquisition > Extinction	1.09
Amygdala, centromedial nucleus	Acquisition > Control	0.94
Posterior habenula, medial	Acquisition > Extinction	1.09
Dorsomedial periaqueductal gray	Acquisition > Control	1.30
Ventrolateral periaqueductal gray	Acquisition > Control	1.03