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Enhanced metabolic capacity of the frontal cerebral cortex after Pavlovian conditioning

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Abstract

While Pavlovian conditioning alters stimulus-evoked metabolic activity in the cerebral cortex, less is known about the effects of Pavlovian conditioning on neuronal metabolic capacity. Pavlovian conditioning may increase prefrontal cortical metabolic capacity, as suggested by evidence of changes in cortical synaptic strengths, and evidence for a shift in memory initially processed in subcortical regions to more distributed prefrontal cortical circuits. Quantitative cytochrome oxidase histochemistry was used to measure cumulative changes in brain metabolic capacity associated with both cued and contextual Pavlovian conditioning in rats. The cued conditioned group received tone-footshock pairings to elicit a conditioned freezing response to the tone conditioned stimulus, while the contextually conditioned group received pseudorandom tone-footshock pairings in an excitatory context. Untrained control group was handled daily, but did not receive any tone presentations or footshocks. The cued conditioned group had higher cytochrome oxidase activity in the infralimbic and anterior cingulate cortex, and lower cytochrome oxidase activity in dorsal hippocampus than the other two groups. A significant increase in cytochrome oxidase activity was found in anterior cortical areas (medial, dorsal and lateral frontal cortex; agranular insular cortex; lateral and medial orbital cortex and prelimbic cortex) in both conditioned groups, as compared to the untrained control group. In addition, no differences in cytochrome oxidase activity in the somatosensory regions and the amygdala were detected among all groups. The findings indicate that cued and contextual Pavlovian conditioning induces sustained increases in frontal cortical neuronal metabolic demand resulting in regional enhancement in the metabolic capacity of anterior cortical regions. Enhanced metabolic capacity of these anterior cortical areas after Pavlovian conditioning suggests that the frontal cortex may play a role in the retention and regulation of learned associations.

Keywords

Cytochrome oxidase; hippocampus; amygdala; context

Cytochrome oxidase (CO) metabolic brain mapping provides an alternative approach for investigating neural circuits mediating Pavlovian conditioning, and it is a well-suited method for quantifying more stable neuronal metabolic capacity changes that reflect prolonged training (Poremba et al., 1997, Poremba et al., 1998b, Poremba et al., 1998a, Sakata et al., 2000, Villarreal et al., 2002, Conejo et al., 2005, Sakata et al., 2005). CO changes in the brain after prolonged training reach a more stabilized state of oxidative metabolism, and techniques which measure evoked brain metabolic activity, such as fluorodeoxyglucose (FDG) autoradiography,

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positron emission tomography (PET) or functional magnetic resonance imaging (fMRI) may not reflect sustained changes in baseline metabolic capacity (Gonzalez-Lima and Cada, 1998). Thus, CO may be a better metabolic marker of the cumulative effects of experience on brain function, since it is not dependent on stimulus-evoked or event-related activations like FDG, PET or fMRI. CO activity may reflect cumulative functional demands on brain systems across days of conditioning as opposed to techniques using immediate early genes, glucose uptake or hemodynamic responses that reflect on-line responses to a stimulus or condition in a testing period.

This study used quantitative CO histochemistry to examine cumulative differences in regional brain metabolic capacity related to Pavlovian cued and contextual conditioning in rats. Utilization of the CO methodology allowed examination of neural circuits implicated in across-days learning of Pavlovian conditioning, as opposed to memory retrieval. As the neuronal energy demand to process and consolidate learned associations changes, so does the production of the CO enzyme (Wong-Riley, 1989, Gonzalez-Lima, 1992). Earlier findings from our laboratory have demonstrated that auditory fear conditioning in rats results in metabolic capacity changes in the auditory system, confirming that mapping CO metabolic activity using quantitative histochemistry can be used to examine sustained metabolic effects of behavioral training on the brain (Poremba et al., 1997, Poremba et al., 1998a, Poremba et al., 1998b). Another recent study found that several days of the excitatory tone-footshock conditioning in rats changed CO activity in septohippocampal areas (Conejo *et al.*, 2005). However, CO activity changes associated with Pavlovian conditioning in the prefrontal cortex have not been examined, but were likely to occur given the involvement of this cortical brain area in both cued and contextual Pavlovian conditioning (Garcia et al., 1999, Morgan and LeDoux, 1999, Baeg et al., 2001, Holschneider et al., 2006). We hypothesized that CO activity may change as a consequence of Pavlovian conditioning, and that prefrontal cortex may show increased CO metabolic capacity following the cued and contextual Pavlovian conditioning in rats. Conditioned animals may have increased metabolic capacity in prefrontal cortical areas since learned associations may be expressed at a cortical level as changes in synaptic connection strengths (Cai, 1990). There is evidence in both humans and rodents that as learned information becomes reorganized, there is a time-dependent establishment of long-term memory storage in frontal cortical areas (Bontempi et al., 1999, Wiltgen et al., 2004, Takashima et al., 2006). Thus, frontal cortical neurons may have a more sustained energy demand and increased metabolic capacity following Pavlovian conditioning. Aversive cued delay conditioning and contextual Pavlovian conditioning were used to compare neuronal metabolic capacity in prefrontal cortical areas with a handled control group. Since the capacity of a neuron to communicate with others in a network is coupled to its CO activity, quantitative CO histochemistry was used to examine tissue alterations in oxidative metabolic capacity associated with these common associative learning paradigms.

In addition to frontal cortical regions, several other brain areas were included in the analysis based on evidence suggesting their involvement in Pavlovian conditioning. For example, amygdala has been shown to play an important role in the acquisition of the cued Pavlovian conditioning in rats (Maren, 2001, McGaugh, 2004, Pare et al., 2004), while the dorsal hippocampus contributes to contextual conditioning (Holland and Bouton, 1999, Sacchetti et al., 1999, Fanselow, 2000). Since there is evidence for the time-limited involvement of amygdala in conditioning (Poremba and Gabriel, 1999), we hypothesized that CO activity in amygdala may return to baseline by the time of decapitation (three days following the conditioning procedure). In addition, we anticipated that dorsal hippocampus of the rats trained with a contextual conditioning procedure may show altered metabolic capacity as compared to cued-conditioned or handled animals. Finally, we also analyzed CO activity in one cortical (somatosensory cortex) and two subcortical (external cuneate nucleus and lateral ventral basal thalamic nucleus) somatosensory regions, since CO activity in these regions was not likely to

change due to associative learning paradigm, but could potentially show alterations in CO activity as a consequence of somatosensory experience of the footshock unconditioned stimulus (US).

EXPERIMENTAL PROCEDURES

Materials and Methods

An initial pilot study with 16 ($n = 8$ per group) one-month old male Long-Evans rats purchased from Harlan, Inc. (Indianapolis, IN) served to confirm that the behavioral regime resulted in robust conditioned freezing to the conditioning context (11.62 ± 2.2 seconds out of maximum 15) and the conditioned tone (11.25 ± 2.4 seconds out of maximum 15). Following this pilot study, 30 additional one-month old male Long-Evans rats from the same supplier were divided into three groups (cued conditioned, pseudorandom contextually conditioned and handled groups; $n = 10$ per group) and were used to evaluate effects of the same conditioning regime on both behavior and brain cytochrome oxidase activity. All subjects were housed under standard laboratory conditions (12 hour light/dark cycle), 3 per cage, with food and water available *ad libitum*. Upon their arrival to the colony, rats were handled daily for a week to habituate them to the experimenters. At the time of decapitation subjects were approximately 45 days old and weighed 172 g on average (range 157–187 g). The protocol used was approved by the University of Texas Institutional Animal Care and Use Committee and complied with all applicable federal and NIH guidelines.

Apparatus

The experiment took place in MED Associates (St. Albans, VT) sound-attenuated operant chambers illuminated by a bright red light. The chambers had a speaker mounted at the top, through which Wavetek Sweep/Modulation generators (Wavetek, San Diego, CA) produced 65 dB frequency-modulated tones of 1–2 kHz, and 15 seconds in duration. The chambers were also equipped with a grid floor through which an electric shock could be delivered as programmed into a MED-PC protocol supplied by MED Associates. Before each session the chambers were cleaned with diluted detergent which also served as an olfactory cue for this context.

Behavioral training

Animals in conditioned groups were habituated to the conditioning chambers for two days, one hour each day. After habituation, rats in the cued group received two days of four tone-shock pairings (Table 1). US consisted of a footshock of 0.5 mA, 0.75 sec in duration and it co-terminated with a tone conditioned stimulus (CS). Each CS lasted 15 seconds and subjects' behavior was scored by an observer unaware of the subject's group assignment. Pre-CS behavior was recorded for the 15 sec preceding each CS presentation. The conditioned response (CR) that was measured was freezing behavior, defined as lack of any movement except for rapid, shallow breathing, with all feet on the floor.

Subjects in the contextually conditioned group were presented with the same number of tones and shocks, but these stimuli were pseudorandomly paired only once out of eight total stimulus presentations. A single tone-shock paired presentation was given to prevent the sound from becoming a conditioned inhibitor, a stimulus that acquires a "safety" signal value due to reliable absence of shock during sound presentation. The average inter-trial interval was three minutes. Presentation of stimuli was controlled by computer programs created using MED-PC behavioral programming language (MED Associates).

Twenty four hours following the acquisition training, one ten-minute probe trial consisting of three CS presentations was conducted out of the acquisition context to test CR independently

of any possible contextual influence. Freezing behavior was measured in three-second bins, over the entire period of CS presentation (15 seconds), as well as 15 seconds prior to the onset of the CS (pre-CS freezing). The pre-CS freezing measure was used to account for any non-specific freezing due to context generalization or sensitization effects of the US. Forty eight hours after the initial probe trial, another probe trial identical to the first one was administered. This second probe trial assessed long-term effects of conditioning, as well as the extent of possible extinction that may have occurred due to the presentation of 3 unreinforced tone CSs during the first probe trial. Following the second probe trial, subjects were decapitated; their brains were quickly harvested and stored in a -40°C freezer.

Quantitative cytochrome oxidase histochemistry

The brains from twelve additional control rats were used for the preparation of brain homogenate CO standards (Gonzalez-Lima and Cada, 1998). These rats were allowed food and water *ad libitum* and were housed under the standard laboratory rearing conditions (12h light/dark cycle). Following decapitation, brains were removed and stored at 4°C in sodium phosphate buffer (pH 7.6) until all brains were collected. The unfixed brains were then homogenized in a large glass homogenizer kept at 4°C . Aliquots of brain homogenate were placed in chilled plastic tubes and briefly centrifuged at low speed (2000 rpm) in a cold (4°C) centrifuge to remove air bubbles. These aliquots were frozen in the same manner as the experimental brains and stored at -40°C . Immediately prior to the CO histochemistry procedure, the brain homogenate was sectioned at 5 different thicknesses (10, 20, 40, 60 and $80\text{ }\mu\text{M}$) and these CO standards were included with each batch of incubation medium. This is a necessary procedure performed to generate a single regression equation between CO activity and optical density of the sections for the comparison of all tissue in the experiment. The CO enzymatic activity of the brain homogenates was determined using spectrophotometry.

Spectrophotometry of cytochrome oxidase activity

For the spectrophotometric assay, the following reagents (all materials were purchased from Sigma, St. Louis, MO) were prepared: 1) Isolation buffer (pH 7.0): 21.7 g sucrose, 0.076 g EDTA, 0.2644 g Trizma HCl, 0.039 g Trizma base, distilled water to 0.2 liters; 2) Dialysis buffer (pH 7.0): 1.405 g potassium phosphate monobasic, 2.082 g sodium phosphate dibasic, distilled water to 0.5 liters; 3) 10% sodium deoxycholate in distilled water and 4) 1% solution of cytochrome c from the horse heart in the dialysis buffer. Procedure for determining CO activity spectrophotometrically was performed as previously described (Callaway *et al.*, 2004).

The cytochrome oxidase staining procedure

Frozen brain slides were carefully picked to assure best possible section quality. Because of the large number of slides processed, it was necessary to run CO histochemistry in several batches. Each batch contained the slides from the same Bregma level and a CO standard slide. This way, the between-batch differences could be accounted for, and analysis of the between-region differences was done on the slides that were processed at the same time. Frozen slides were first immersed for 5 minutes in 10% phosphate-buffered sucrose (0.1 M, pH 7.6) containing 0.5% glutaraldehyde to aid adherence of the sections to the slides. Following this, three five-minute washes in 10% phosphate-buffered sucrose were done to remove red blood cells and to gradually warm tissue to room temperature. Slides were then preincubated in Tris buffer (0.05 M, pH 7.6) containing 275 mg/l cobalt chloride, 10% sucrose and 0.5% dimethylsulfoxide. This 10 min preincubation step involving metal intensification through cobalt binding has been shown to enhance CO staining (Poremba *et al.*, 1998a). Slides were then rinsed in phosphate buffer and incubated in the 37°C incubator for one hour in an oxygen-saturated phosphate buffer containing diaminobenzidine tetrachloride and dimethylsulfoxide.

Following this incubation step, tissue was fixed by a thirty minute immersion in room-temperature 4% formalin in 10% sucrose phosphate buffer. Next, tissue was dehydrated through a series of five-minute incubations in ethanol dilutions, ending with two submersions in 100% ethanol. Finally, slides were cleared by immersions in xylene and then coverslipped using Permount (Fisher Scientific, Pittsburgh, PA).

Image analysis

Using a stereotaxic atlas of the rat brain (Paxinos and Watson, 1997), as well as a CO atlas of the rat brain (Gonzalez-Lima and Cada, 1998), representative sections for each brain region were chosen for densitometric analysis. The regions of interest (ROI) examined are illustrated by Bregma level in Fig. 1.

The optical density (OD) in the CO stained sections from both hemispheres was analyzed using an image-processing system consisting of high-gain video camera, Targa-M8 image capture board, Everex computer, Sony color monitor, DC-powered illuminator, and JAVA software (Jandel Scientific, San Rafael, CA). The system was calibrated using an OD step tablet (Kodak, Rochester, NY) and the OD of the brain sections was used as a chromatic indicator of CO activity. Possible optical artifacts from the camera were corrected through background subtraction. A calibration curve was created with the standards using a single regression equation (Jones and Gonzalez-Lima, 2001) in order to account for between-batch staining differences. These standards were linear in the 10 to 80 micron range of thickness used.

Multiple readings were taken from left and right sides of each section to avoid artifacts. Thus, the reported CO activity is an average of the readings taken randomly from each hemisphere across three different brain sections. For each brain region analyzed, 12 readings per region per brain were taken (four readings per section from three adjacent sections per brain). For each region measured, the size of the densitometer window was set to approximate one quarter of the size of the whole region. Adjacent measurements were taken and averaged to sample the entire region. The averaged measures resulted in one mean value per region for each subject. The size of the window was held identical across subjects, as was the number of readings for each ROI. Thus the area measured was constant across all animals and was limited to the anatomical region of interest.

Statistical analysis

In addition to the individual regions of interest, an estimate of global brain activity was taken by averaging the CO activity units from all ROIs examined for any given subject. Although this average only reflects CO activity in the grey matter, it has been demonstrated that the whole brain CO activity estimate obtained in this manner is very closely correlated to the global brain average calculated from the entire set of sections in the series of a given subject (grey and white matter, $r = 0.98$, Bruchey, unpublished data). This global measure of activity was used as a reference for group differences in overall metabolic activity, as well as for normalization of the CO activity data (ROI/brain activity) for each subject to minimize inter-subject staining differences unrelated to treatment group effects.

Analysis of variance (ANOVA) was used to examine between-group differences in freezing behavior and regional brain CO activity. Since there were many regions of interest that were examined, Hochberg's Bonferroni corrected post-hoc tests were used to test the statistical significance of the results at $p < 0.05$. Using this approach reduced the risk of inflated Type I error rates associated with multiple tests while not discarding true differences as a result of being overly conservative.

Brain-behavior correlations between the regions which showed statistically significant differences in CO activity and behavioral measures of freezing during probe trials were calculated for the two conditioned groups. Negative brain-behavior correlations reflect a linear relationship between increased regional brain metabolic capacity and reduction of the freezing CR.

RESULTS

Behavioral results

The cued conditioned group rats showed a strong CR (average CS - preCS freezing duration = 12 ± 0.45 seconds out of maximum 15) to the tone CS when tested outside the context of acquisition twenty four hours following the training, indicating that this group had successfully associated the CS with the US. Seventy two hours after the completion of associative conditioning, animals were retested in another probe trial, and displayed a strong CR to the CS (average CS - preCS freezing duration = 14.8 ± 0.27 seconds), suggesting a robust memory for the previous training episodes. As expected, the contextually conditioned group animals did not display a strong CR to the tone CS (average CS - preCS freezing duration = 1.5 ± 0.6 seconds) when tested outside of the acquisition context in either probe trial. The control, handled rats were never exposed to the CS or the US, and thus were not included in the probe trials.

Brain cytochrome oxidase activity

Significant training-dependent changes in CO activity were found in both anterior and posterior infralimbic cortex, as well as the anterior cingulate cortex, with the cued conditioned group having 20% or higher CO activity in these brain areas than the other two groups (Figure 2). Granular insular cortex showed a similar pattern of CO activity increases, although the conditioned groups' CO activity did not statistically differ from the handled group. CO activity in the somatosensory cortex also did not differ among groups.

Both the cued and the contextually conditioned group animals had significantly ($F_{(26,2)} > 6$, $p < 0.01$) elevated CO activity in the prefrontal cortical regions, as compared to the control group. Figure 3 shows means and standard errors of the normalized CO activity in the regions of interest examined.

As shown in Fig. 3, frontal cortical regions such as medial, dorsal and lateral frontal cortex, agranular insular cortex, lateral and medial orbital cortex and prelimbic cortex had significantly ($p < 0.01$) elevated CO activity as compared to the handled group. While there was a range of CO activity readings (0.62 to 1.92) within these regions, all of them showed a 52% or higher average increase in CO metabolic capacity associated with Pavlovian cued or contextual conditioning, suggesting that functional demands of conditioning modify tissue metabolic responses in these cortical areas.

On the other hand, dorsal hippocampus of the cued conditioned rats showed a relative decrease in CO activity (Figure 4). The CA1 subfield in the cued conditioned group showed a decrease in CO activity as compared to the other two groups, while CO activity measured in the CA3 subfield and dentate gyrus in the cued conditioned group was significantly lower in the cued group, as compared to the contextually conditioned group.

The CA1 subfield of the ventral hippocampus (measured at -5.8 Bregma level) showed elevated CO activity (1.44 ± 0.07) in the handled group, as compared to cued (0.76 ± 0.03) and contextual (0.82 ± 0.04) groups. Similarly, ventral hippocampal CA2 subfield also showed relative increase in CO activity in the handled group ($1.26 \pm .13$), as compared to cued (0.99 ± 0.03) and contextual (0.99 ± 0.05) groups. Finally, CA3 subfield of the ventral hippocampus

in the handled group also had elevated CO activity (1.4 ± 0.14), as compared to cued (0.89 ± 0.31) and contextual (1 ± 0.05) groups.

Finally, external cuneate nucleus, lateral ventral basal thalamic nucleus, as well as central, medial, basal and lateral nuclei of amygdala showed no significant differences ($p > 0.05$) in CO labeling among all groups. Mean normalized CO activity in the lateral amygdaloid nucleus was 1.14 ± 0.09 in the cued group, 1.24 ± 0.07 in the contextual group and 1.12 ± 0.07 in the handled group. For the basal amygdaloid nucleus mean normalized CO activity was 1.11 ± 0.09 in the cued group, 1.19 ± 0.07 in the contextual group and 1.14 ± 0.07 in the handled group. Since basal and lateral nuclei of the amygdala had similar CO activity, the readings from these nuclei were combined and presented as the basolateral amygdala (BLA; Figure 4).

Brain-behavior correlations

Correlations between CS-evoked freezing behavior and regional CO activity were examined to further discriminate cued vs. contextual conditioning effects in the brain. While no statistically significant correlations between CS-evoked freezing and CO activity were found for the contextually conditioned group, the cued group's freezing behavior was positively correlated ($r = 0.68$, $p = 0.03$) with the CO activity in the anterior cingulate cortex (CG2). Therefore, cue-conditioned rats with more increased CO activity in the CG2 also displayed stronger CRs during the final probe trial.

DISCUSSION

Quantitative CO histochemistry was used to evaluate cumulative changes in prefrontal cortical, amygdaloid and hippocampal metabolic capacity associated with Pavlovian conditioning. Neuronal metabolic capacity is largely determined by the ability to use ATP for the resetting of membrane potentials involved in synaptic communication (Wong-Riley, 1989). ATP production itself is limited by mitochondrial enzymatic complexes which create a proton gradient that is used by ATP synthase. CO is the rate-limiting respiratory chain enzyme for oxygen consumption of neurons that is coupled to ATP production (Capaldi, 1990). Thus, quantitative CO histochemistry can be used to determine alterations in neuronal metabolic capacity resulting from the entire training period. This technique provides a more stable representation of the metabolic state than FDG or fMRI techniques, which measure shorter-term stimulus-evoked neuronal energy demand (Gonzalez-Lima and Cada, 1998).

The main general finding of this study was that cued and contextual associative conditioning resulted in over 50% increases in CO metabolic activity in anterior cortical regions, supporting Pavlov's original hypothesis that the cerebral cortex plays an important physiological role in conditioning (Pavlov, 1927). Because CO activity may be used as a marker of sustained energy demand in a brain region, then the findings of increased CO activity in the prefrontal cortex of conditioned animals suggest that Pavlovian conditioning may elevate metabolic demand in frontal cortical neurons for prolonged periods, such as across days of training.

Increased research into the neural substrates of Pavlovian conditioning and extinction has suggested that medial prefrontal cortex (mPFC) may influence behavioral performance through processes occurring during acquisition (Morgan *et al.*, 2003), and increased mPFC activity has also been proposed to correlate with the maintenance of anxiety during a repeated confrontation with phobia-related stimuli (Straube *et al.*, 2006). Neurons in the infralimbic (IL) region of mPFC have been shown to respond to conditioned stimuli following the tone-footshock and context-footshock Pavlovian conditioning (Baeg *et al.*, 2001), and cerebral blood flow is increased in IL following auditory cued fear conditioning in rats (Holschneider *et al.*, 2006). However, lesion literature regarding involvement of mPFC in rodent tone-footshock conditioning is equivocal. There are some reports that mPFC lesions do not disrupt conditioned

fear acquisition (Gewirtz et al., 1997, Garcia et al., 2006), and others that show that mPFC plays an important, if not essential role in this process (Morrow et al., 1999, Laviolette et al., 2005). CO histochemistry of cortical regions associated with Pavlovian conditioning may improve our understanding of the role of prefrontal cerebral cortex in retention and recall of associative learning.

The contextual group underwent pseudo-random exposure to the tones and footshocks, thus serving as a comparison group where the tones were not paired with the footshocks. Although there are many similarities between cued and contextual fear conditioning, such as experience of the footshock, exposure to the tone and to the conditioning context, there are also differences in associations learned and brain regions involved. For example, entorhinal lesions or lesions of the bed nucleus of the stria terminalis have little effect on conditioned responding to discrete cues, but diminish responding to contextual cues (Sullivan et al., 2004, Majchrzak et al., 2006), while voluntary exercise improves conditioned responding to contextual, but not discrete conditioned stimuli (Baruch *et al.*, 2004). Conversely, lesions of the nucleus accumbens core have no effect on responding to the contextual cues, but disrupt conditioned responding to the tone CS (Cassaday *et al.*, 2005). In the present study, contextually conditioned and the cue-conditioned group showed increased CO activity in the prefrontal cortex, suggesting that both cued and contextual Pavlovian conditioning increase metabolic capacity in the anterior regions of the cortex. On the other hand, infralimbic cortex and anterior cingulate cortex showed CO activity increase associated with cued conditioning.

Anterior cingulate cortex not only showed metabolic capacity increase following cued conditioning, but CO activity in this brain region was also significantly correlated with behavioral response to the tone CS. In addition to CO histochemistry, various other markers of brain metabolism point to an increase in CG2 activity following Pavlovian aversive conditioning. For example, fear conditioning increases regional cerebral blood flow (Holschneider *et al.*, 2006), c-fos expression (Beck and Fibiger, 1995) and stimulus-related functional activation in the anterior cingulate cortex (Knight et al., 1999, 2004). Because feature extraction from the environment and sensorimotor integration are associated with CG2 (Vogt and Miller, 1983), then the CO activity increase in this area following aversive Pavlovian conditioning may reflect CG2 involvement in the assemblage of the conditioned freezing response.

However, CG2 role exceeds that of a purely motor-response integrating region. Medial PFC areas such as CG2 may be part of a core anxiety system relevant for sustained evaluation of potential danger and the subjective experience of fear (Rauch *et al.*, 1997). Furthermore, CG2 has been implicated in the elicitation and control of sympathetic autonomic arousal (Critchley, 2003), which is one of the main physiological features present during the processing of fear-related stimuli.

Cognitive-behavioral therapy strongly reduces stimulus-evoked phobic symptoms and hyperactivity of CG2, suggesting that increased activation in CG2 is associated with specific phobias, while an attenuation of this brain response correlates with successful therapeutic intervention (Straube *et al.*, 2006). We found that both CG2 and IL cortical regions had increased CO activity in the cued conditioned group, as compared to the other groups. This finding is consistent with the CG2's role in multimodal processing and sensorimotor integration which could facilitate a modality-specific involvement in the expression of fear, as in the case of specific phobias, or our tone CS-evoked freezing.

Several lines of evidence also suggest a role of the prelimbic cortex in associative learning. For example, multiple unit recordings from prelimbic cortex in rabbits show systematic increases in response to tone CSs paired with paraorbital electric shock (Maxwell *et al.*,

1994), and lesions of this area retard Pavlovian eye-blink trace conditioning (McLaughlin *et al.*, 2002). In addition, Fos-like immunoreactivity is increased in prelimbic cortex following tone-footshock conditioning in rats (Morrow *et al.*, 1999). Hence, the increased CO activity in prelimbic cortex following fear conditioning reported here supports its role in Pavlovian conditioning.

The agranular insular cortex (AI) has been depicted as a visceral-sensory region of the prefrontal cortex (Cechetto and Saper, 1987). Lesions of the AI have been found to impair aversive cued and contextual conditioning (Hankins *et al.*, 1974, Morgan and LeDoux, 1999), and these findings are in line with our results of increased CO metabolic activity in the AI following aversive Pavlovian conditioning. AI afferents to prelimbic and infralimbic cortical areas have also been described (Conde *et al.*, 1995), suggesting that AI may influence the activity of these areas associated with Pavlovian conditioning.

Lateral and medial orbital cortical regions of the rat brain have also been implicated in cued and contextual conditioning by both lesion and brain mapping studies (Morgan and LeDoux, 1999, Nordquist *et al.*, 2003). Neurons in this area may be part of the limbic circuit that participates in the processing of emotion and memory consolidation (Oyoshi *et al.*, 1996). Thus, it is not surprising that fear conditioning was associated with elevated CO activity in the orbital cortex in the present study.

While it is possible that the observed increases in CO activity in the prefrontal cortical regions may be induced by the exposure to the footshock US rather than by the associative learning, this is not the case for any of the other brain regions examined in this study. As shown in Figure 2, anterior cingulate, anterior infralimbic and posterior infralimbic cortex of contextually conditioned rats did not have increased CO activity, although these animals received the same number of footshocks as the cued conditioned group. Similarly, no sensitization effects were seen in somatosensory, amygdala or hippocampal areas, suggesting that the observed alterations in CO activity were more likely the product of associative conditioning than a non-specific generalized metabolic capacity increase in the cortical neurons, or sensitization due to the exposure to the footshock. Rather, only certain frontal and limbic regions are specifically involved in the processing of cued and contextual Pavlovian conditioning.

Another alternative interpretation of the findings is that the observed changes in CO activity may reflect differences in experience during the probe trials. Since CO enzymatic activity reflects cumulative energy demand on any particular brain region, it is possible that some of the effects measured may have resulted from both training (learning) and probe trials (memory retrieval). This is a limitation of the CO technique; however, when brain CO activity patterns associated with Pavlovian fear conditioning are compared to FDG uptake, a metabolic brain mapping technique which measures memory retrieval in probe trial-evoked responses, there is no overlap in the regions of interest which show statistically significant differences between the cue-conditioned and contextually conditioned groups and the memory retrieval of conditioned freezing (Barrett *et al.*, 2003). It seems also unlikely that the CO effects were due to extinction effects since we observed no decrement in the conditioned freezing response during the second probe trial, and there was no evidence of extinction, as the cue-conditioned rats displayed strong freezing during the second probe trial.

Another region that was included in the analysis of CO activity changes in the brain associated with Pavlovian conditioning was the amygdala. This region was chosen based on its proposed involvement in aversive Pavlovian conditioning (Ledoux *et al.*, 1984, Miserendino *et al.*, 1990, Maren, 2001), as well as its purported interactions with the cortical systems described above (Maren, 2005, Quirk *et al.*, 2006). Interestingly, we found no differences in CO activity among groups in this brain area. Because CO activity is a marker of sustained metabolic

demand in a brain region, one possible interpretation is that amygdala's time-limited involvement in aversive conditioning may not be readily observed with CO histochemistry. In other words, amygdala could be activated during the initial formation of the CS-US association, but then return to baseline activity. This is in agreement with another study which examined CO activity changes in septohippocampal metabolism in rats following classical fear conditioning, and found no significant differences in CO activity in the BLA between the conditioned and control groups (Conejo *et al.*, 2005). Both of these CO studies suggest an alternative interpretation to the notion that fear memories are stored in the amygdala rather than the cerebral cortex (Gale *et al.*, 2004). Lesions of amygdala may impair conditioned freezing by interfering with amygdala-cortical interactions rather than interfering with amygdaloid memory storage. Additionally, human brain mapping studies using fMRI have shown that amygdala activity may specifically reflect the expression of learned fear responses (Cheng *et al.*, 2003, Cheng *et al.*, 2006), which in this study occurred briefly during the probe trials, and may not have produced enough sustained energy demand in the amygdala to result in the CO activity increase.

As hypothesized, contextual conditioning altered CO activity in the dorsal hippocampus. Cued conditioned group showed a relative CO activity decrease in the hippocampal regions examined, suggesting that changes in neural metabolism in this brain area associated with aversive auditory cued conditioning depend on the learned value of the tone CS. Our data is supported by other studies which also demonstrated a decrease in hippocampal CO activity following Pavlovian fear conditioning (Conejo *et al.*, 2005), and conditioned response inhibition (Jones and Gonzalez-Lima, 2001). Additionally, local cerebral perfusion is decreased in response to a fear conditioned tone CS (Holschneider *et al.*, 2006), and neurotoxic lesions of the dorsal hippocampus have been found to impair cued Pavlovian conditioning in rats (Maren *et al.*, 1997), further indicating that dorsal hippocampus plays an important role in the Pavlovian fear conditioning in rats.

Taken together, the present findings support recent theories about memory consolidation in the prefrontal cortex (Frankland and Bontempi, 2006). There is evidence for a shift in the organization of memories which are initially consolidated in subcortical regions such as the amygdala or the hippocampus to more distributed prefrontal cortical circuits in both animals and humans (Bontempi *et al.*, 1999, Pasupathy and Miller, 2005, Takashima *et al.*, 2006). Amygdala and hippocampus are thought to "participate in a larger limbic circuit whose contribution to conditioning and learning is time-limited. This circuit may actively mediate consolidation processes, or it may simply support behavioral acquisition and performance for an interim period until more permanent memory coding is established in nonlimbic regions" (Poremba and Gabriel, 1999). Our findings of increased metabolic capacity in prefrontal cortical areas following associative conditioning in rats support this view of distributed memory integration within the prefrontal cortex following the initial memory consolidation.

In summary, quantitative CO histochemistry successfully revealed cumulative changes in frontal cortical metabolic capacity associated with both cued and contextual Pavlovian conditioning in rats. Relatively large increases in metabolic capacity were found in frontal and limbic cortex, especially in the mPFC, suggesting that Pavlovian conditioning induces distributed increases in frontal cortical neuronal metabolic demand resulting in enhanced metabolic capacity of anterior cortical regions.

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Glossary

LIST OF ABBREVIATIONS

CO	CYTOCHROME OXIDASE
FDG	FLUORODEOXYGLUCOSE
PET	POSITRON EMISSION TOMOGRAPHY
fMRI	FUNCTIONAL MAGNETIC RESONANCE IMAGING
US	UNCONDITIONED STIMULUS
CS	CONDITIONED STIMULUS
CR	CONDITIONED RESPONSE
ROI	REGION OF INTEREST
OD	OPTICAL DENSITY
ANOVA	ANALYSIS OF VARIANCE
CG2	ANTERIOR CINGULATE CORTEX
mPFC	MEDIAL PREFRONTAL CORTEX
BLA	BASOLATERAL AMYGDALA
IL	INFRALIMBIC CORTEX
AI	AGRANULAR INSULAR CORTEX

Figure 1. Localization of regions of interest by Bregma level

The location of the regions examined is shown in each diagram on the right side of each level. The corresponding section stained with cytochrome oxidase is shown on the left side of each level. List of abbreviations: MFC- medial frontal cortex; DFC- dorsal frontal cortex; LFC- lateral frontal cortex; AI- agranular insular cortex; LO- lateral orbital cortex; MO- medial orbital cortex; PRL- prelimbic cortex; ILA- infralimbic cortex, anterior; ILP- infralimbic cortex, posterior; CG2- anterior cingulate cortex; GI- granular insular cortex; S1- primary sensorimotor cortex; LA- lateral nucleus of amygdala; BA- basal nucleus of amygdala; CEA- central nucleus of amygdala; ME- medial amygdala; VBL- lateral ventral basal thalamic

nucleus; CA1- field CA1 of hippocampus; CA3- field CA3 of hippocampus; DG- dentate gyrus; ECU- external cuneate nucleus.

Figure 2. Regional differences in cytochrome oxidase metabolic activity following Pavlovian conditioning

List of abbreviations: ILA- infralimbic cortex, anterior; ILP- infralimbic cortex, posterior; CG2- anterior cingulate cortex; GI- granular insular cortex; S1- primary sensorimotor cortex.

* = $p < 0.05$ significantly different than handled control; # = $p < 0.05$ significantly different than the contextual group.

Figure 3. Cytochrome oxidase metabolic activity in prefrontal cortex following Pavlovian conditioning

List of abbreviations: MFC- medial frontal cortex; DFC- dorsal frontal cortex; LFC- lateral frontal cortex; AI- agranular insular cortex; LO- lateral orbital cortex; MO- medial orbital cortex; PRL- prelimbic cortex. * = $p < 0.05$ significantly different than handled control; # = $p < 0.05$ significantly different than the contextual group.

Figure 4. Cytochrome oxidase metabolic activity in subcortical regions following Pavlovian conditioning

List of abbreviations: CA1- field CA1 of hippocampus; CA3- field CA3 of hippocampus; DG- dentate gyrus; BLA- basolateral nucleus of amygdala; CEA- central nucleus of amygdala; ME- medial amygdala; ECU- external cuneate nucleus; VBL- lateral ventral basal thalamic nucleus. * = $p < 0.05$ significantly different than handled control; # = $p < 0.05$ significantly different than the contextual group.

Table 1

Behavioral Conditioning Protocol

	Cued conditioned group	Contextually conditioned group
Day 1,2	1h Habituation to conditioning context.	1h Habituation to conditioning context.
Day 3,4	15 min session of paired presentations of T (4) and S (4) in conditioning context.	15 min session of pseudorandom presentations of T (4) and S (4) in conditioning context.
Day 5,7	10 min Probe session out of the conditioning context.	10 min Probe session out of the conditioning context.

T = Tone CS; S = shock US