Psicothema 2005. Vol. 17, nº 4, pp. 563-568
www.psicothema.com

ISSN 0214 - 9915 CODEN PSOTEG

Brain metabolism after extended training in a fear conditioning task

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Pavlovian fear conditioning (PFC) is one of the most used behavioural paradigms for studying the neurobiology of learning and memory. In this study, rats were trained in a conditioned suppression task receiving either three (limited training) or eight conditioning sessions (extended training). Rats in both training conditions came to suppress lever press behaviour as conditioning proceeded. After training, the neuronal metabolism of brain limbic regions was analysed using cytochrome oxidase histochemistry. A significant decrease in the metabolism of particular brain regions including the medial septum, anteroventral thalamus and medial mammillary nucleus, was observed only after extended training. However, no significant differences were detected after extended training in the basolateral amygdala, brain region traditionally involved in the formation and consolidation of aversive memories formed during PFC. Our results provide support for a differential involvement of the studied limbic regions on fear conditioning and anxiety.

Metabolismo cerebral tras el entrenamiento prolongado en una tarea de condicionamiento del miedo. El condicionamiento Pavloviano del miedo (CPM) es uno de los paradigmas conductuales más usados para estudiar la neurobiología del aprendizaje y la memoria. Se entrenaron ratas en una tarea de supresión condicionada, que recibieron tres (entrenamiento limitado) u ocho sesiones de condicionamiento (entrenamiento prolongado). Ambos grupos llegaron a suprimir la conducta de presión de una palanca cuando se estableció el condicionamiento. Posteriormente, se analizó el metabolismo neuronal de regiones límbicas empleando la histoquímica de la citocromo oxidasa. Se observó una disminución significativa del metabolismo cerebral en septum medial, tálamo anteroventral y núcleo mamilar medial tras un entrenamiento prolongado. Sin embargo, no se detectaron diferencias significativas con el entrenamiento prologando en la amígdala basolateral, región cerebral implicada tradicionalmente en la formación y consolidación de recuerdos aversivos establecidos durante el CPM. Nuestros resultados apoyan la implicación diferencial de las regiones límbicas seleccionadas en el condicionamiento del miedo y la ansiedad.

Pavlovian fear conditioning (PFC) is a ubiquitous form of learning that involves the association of stimuli and their aversive consequences. In this paradigm, the animal receives pairings of an innocuous conditioned stimulus (CS), such as a tone or the context of the conditioning chamber, and a noxious unconditioned stimulus (US), such as a footshock. After a few such pairings, the CS comes to elicit a constellation of conditioned responses (CRs) that are characteristic of fear, including «freezing» or immobility (the species-typical behavioural response to a threatening stimulus), autonomic and endocrine responses (such as changes in heart rate and blood pressure, defecation, and increased levels of circulating stress hormones), and other changes including the potentiation of reflexes such as the acoustic startle response. Associated with the autonomic symptoms of the fear response there are, in humans, cognitive effects such as feelings of dread and despair (de Vicente Pérez and Díaz-Berciano, 2005; Gutiérrez Maldonado and Arbej Sánchez, 2005). Disorders of the storage or expression of fear responses are thought to underlie such mental disorders as panic attacks, anxiety and post traumatic stress disorder. Because fear conditioning is rapidly acquired and persistent, involves well-defined stimuli and responses, occurs in every species that has been examined from flies to humans, and implicates similar neural circuits in different vertebrate species, it has emerged as an especially useful paradigm for understanding the neurobiological mechanisms of this form of behavioural plasticity (e.g. LeDoux, 2000; Maren, 2001; Schafe and LeDoux, 2002; Fanselow and Poulos, 2005).

In recent years, the neural circuitry that underlies fear conditioning, particularly auditory fear conditioning, has been characterized in great detail (Fendt and Fanselow, 1999; LeDoux, 2000; Maren, 2001; Fanselow and Poulos, 2005). One of the most studied brain regions is the amygdala, which seems to be critically involved in fear conditioning. However, there is no general agreement about the role of this structure on PFC. Several authors suggest that the amygdala is not required for the formation of the

Fecha recepción: 11/1/05 • Fecha aceptación: 22/4/05
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aversive memories formed during fear conditioning, but it is essential in the performance of unconditioned fear responses (Vazdarjanova and McGaugh, 1998; Vazdarjanova, Cahill and McGaugh, 2001). Conversely, other authors propose a time-limited role of the amygdala and the hippocampus on fear conditioning, mainly necessary for the acquisition but not the maintenance of associative learning (Poremba and Gabriel, 1997; LeDoux, 2003; Maren, 2003; Sanders, Wiltgen and Fanselow, 2003). In addition, the involvement of other limbic regions anatomically and functionally coupled to the above-mentioned structure, like the mammillary bodies, anterior thalamus and septal area has been less characterized. The exact role of this circuitry in the acquisition and expression of conditioned fear responses to aversive stimuli is not yet fully understood (see Blair, Schafe, Bauer, Rodrigues and LeDoux, 2001; Sah, Faber, López de Armentia and Power, 2003).

The present study examines the changes in brain activity during auditory fear conditioning in rats. Here, we assessed the effect of different levels of training (i.e., the influence of the number of tone-shock pairings) on the metabolic dynamics of several brain regions during fear conditioning. Specifically, we compared rats that received limited training (9 pairings of the tone with the footshock) and animals that had extended training (24 paired presentations). We hypothesize that the pattern of brain activity shifts as classical conditioned fear develops, being brain activity dependent on the learning rate. Behavioural training involved a conditioned fear paradigm (Estes and Skinner, 1941). In this protocol, training establishes a stimulus as predictive of a noxious event and the degree of conditioned fear is assessed by measuring disruption of ongoing behaviour such as leverpressing (conditioned suppression). Cytochrome oxidase (CO) histochemistry was used to evaluate the metabolic capacity of several limbic regions after classical fear conditioning. The histochemical determination of CO activity can be used as a marker of neuronal oxidative metabolism (Wong-Riley, 1989). As compared to other functional methods, CO histochemistry reflects sustained demands of neuronal activity that take place over hours or days (González-Lima and Cada, 1994; Liu and Wong-Riley, 1995; Zhang and Wong-Riley, 2000). This method has been previously applied to study changes in brain metabolism induced by associative learning in several species like rats (Poremba, Jones and González-Lima, 1997, 1998a; Conejo et al., 2005), cephalopods (Agin, Chichery and Chichery, 2001) and insects (Deglise, Dacher, Dion, Gauthier and Armengaud, 2003). In our study, the main brain regions involved in fear and anxiety were selected to assess the effects of repeated exposition to a fear-inducing environment on brain activity.

Methods

Animals

Thirty-eight male Wistar rats (364-573 g) were used in our experiment. They were housed singly in standard plastic cages (27 × 27 × 15 cm) with water freely available in a temperature (23 ºC) and light (12/12 h light-dark cycle) controlled room. Throughout the experiment they were maintained on a 22.5-h schedule of food deprivation. All experimental procedures were performed in accordance with guidelines of the European Council Directive (86/609/EEC).

Apparatus

Four standard operant chambers (Letica Instruments, Barcelona, Spain) housed in sound-and-light resistant shells were used for the behavioural procedure. Each chamber was equipped with a response lever located near a recessed tray, where 45 mg food pellets could be delivered. The floor of the chamber was composed of 0.5-cm diameter stainless-steel rods, spaced 1.5 cm centre to centre, which could be electrified through a scrambler from a shock source. The conditioned stimulus (CS) was a 30 s discrete auditory stimulus (120-Hz tone) delivered through a speaker mounted on the front wall of the chambers. The unconditioned stimulus (US) was a mild footshock (0.2 mA for 0.5 s) delivered through the grid floor of the chambers. An IBM microcomputer controlled the equipment and recorded the leverpresses during instrumental training and testing.

Behavioural procedure

The rats were initially trained to collect food rewards (45 mg food pellets) during two, 30 min magazine training sessions. The rewards were delivered on a random time (RT) 60 s schedule with the levers retracted. On the next day, with the levers replaced, all animals were trained to respond on the lever with a continuous reinforcement (CRF) schedule (i.e., each lever press was rewarded with food pellets) until 30 reinforcers had been earned. On the next four days, the rats received lever training sessions in which lever press was reinforced on a variable interval (VI) schedule (i.e., on average the first response after each interval is reinforced) whose parameter was increased from 5 to 15 s and 30 to 60 s across successive sessions. Each session started with the insertion of the lever and ended with its retraction 40 min later. The variable interval 60-s schedule remained in effect across the experiment. Upon the completion of instrumental training, subjects were assigned either to the limited training condition (18 rats) and the remainder to the extended training condition (20 rats). Specifically, for each training condition the animals were divided in two groups, those that had tone-shock pairings (groups L-P and E-P), and those that received explicitly unpaired presentations of these stimuli (groups L-U and E-U). Thus, the first term in the group designations refers to the training regime (Limited or Extended), while the second term refers to the conditioning treatment (Paired or Unpaired). The animals were randomly assigned to the following groups: L-P (n= 10), E-P (n= 11), L-U (n= 8), and E-U (n= 9).

Upon the completion of the instrumental training, all animals received preexposure to the 120-Hz tone. Preexposure comprised a single 40 min session during which there were two 30 s presentations of the tone. The first tone presentation was accomplished 10 min after the start of the session and the second tone presentation 20 min later. No shock was delivered during this session. This session served to remove the initial disruption of lever pressing observed following presentation of any novel stimulus.

On the day following the preeexposure session, the fear conditioning phase began. There were eight conditioning sessions for the animals assigned to the extended training condition (groups E-P and E-U). For those in the group E-P, they consisted of three pairings a day of the tone, again of 30 s duration, with the footshock for a total of 24 paired presentations. The tone terminated with the footshock. Each session was 40 min long, with 10 min between each conditioning trial. Eight minutes elapsed

from the start of the session to the onset of the first tone-shock pairing. During these sessions, daily training for the subjects in the group E-U consisted of alternating presentations of the tone and the footshock over 40 min, with an interval of 5 min between each presentation. The total numbers of tone and footshock stimuli were the same in both groups. During this phase, the animals in the limited training condition (groups L-P and L-U) were treated in the same way that the corresponding rats in the extended training condition, except that they were given three conditioning sessions. Rats in group L-P received three tone-shock pairing a day for a total of 9 paired presentations. For the group L-U, daily random training consisted of alternating presentations of three tones and three shocks over the course of 3 days.

The degree of conditioning was assessed by determining if the normally ongoing lever press response is disrupted by the tone presentation. Suppression to the tone was measured by a ratio $A/(A+B)$, where A represents the number of lever presses made during the 30 s presentation of the conditioned stimulus (i.e, tone) and B the number of lever presses made during 30 s prior to the onset of the stimulus (pre-CS scores). Hence, a ratio of 0.5 represents no suppression during the stimulus, and a ratio of 0.0 represents maximal conditioned suppression.

Cytochrome oxidase activity

One hour following the onset of last aversive conditioning session, the animals were sacrificed by overdose of sodium pentobarbital (100 mg/kg) and changes in the metabolic activity of selected brain regions were analysed using quantitative cytochrome oxidase (CO) histochemistry, following the method originally described by Wong-Riley (1979). Briefly, 20 µm-thick brain sections were obtained using a cryostat microtome (Microm Heidelberg, Germany) and incubated during 2 hours at 37 ºC in a staining bath containing cytochrome c (Sigma, USA), sucrose, and diaminobenzidine tetrahydrochloride (Sigma) in 0.1 M phosphate buffer (pH 7.4). Finally, the sections are rinsed in phosphate buffer, dehydrated in alcohol and coverslipped with Entellan (Merck, Germany). A series of sections of rat liver cut at different thicknesses (10, 20, 40, and 80 µm) were included together with brain tissue in each bath. These sections were used as standards to control for staining variability across different incubation baths, as previously described (Gonzalez-Lima and Cada, 1994).Using an image processing system (Leica Q-550, Germany), relative optical density (OD) readings were obtained from the septal area, anteroventral and anterodorsal thalamic nuclei, medial and lateral mammillary nuclei, and medial geniculate nucleus in each subject (six readings taken bilaterally in each brain region). Optical density readings between baths were corrected by comparing the measures taken from each liver standard.

Statistical analysis

Evidence of classical fear conditioning was evaluated by comparing the conditioned suppression ratios to tone using a two-way analysis of variance (ANOVA), with training regime (Limited or Extended) and conditioning treatment (Paired or Unpaired) as the factors. The performance during the lever press training and the unconditioned suppression to the tone during the preexposure day were analyzed by a one-way ANOVA. Post hoc tests were done by Tukey-Kramer tests where appropriate.

Differences in CO staining (OD) of each brain region between paired and unpaired groups (three and eight trials) were analysed by one way ANOVA. Values are expressed as means ± S.E.M.

Results

Behavioural effects

Initially, the total number of lever presses during the last session of instrumental training was evaluated by a one-way ANOVA, with the factor groups distinguishing between performances in each of the four groups employed in the experiment. This analysis revealed no effect of groups. The mean number of lever presses during this session for the various groups were: group L-P, 758; group L-U, 707; group E-P, 765; group E-U, 693. An analysis of the unconditioned suppression rates to the tone on the pre-exposure session showed that the differences among the groups were not significant. The mean unconditioned suppression ratio for each group during this session was: group L-P, 0.48; group L-U, 0.45; group E-P, 0.43; and group E-U, 0.46.

The mean suppression ratios to tone for the four groups during classical conditioning phase are displayed in Figure 1. Rats in groups L-P and E-P learnt to suppress lever press behaviour during tone presentations (i.e., tone evoked conditioned fear responses) whereas those in groups L-U and E-U did not suppress. It is important to emphasize that this effect did not depend on the amount of training. The performance during the last session of fear conditioning did not differ between the limited and the extended conditions. A two-way ANOVA conducted on the suppression ratios to tone in this session revealed that the effect of conditioning treatment (Paired or Unpaired) was significant [F (1,34)= 111.6; p<0.001]. By contrast, there was no significant effect of training regime (Limited or Extended), nor was the interaction between the training regime and the conditioning treatment. The mean suppression ratios during this session in the four groups were: group L-P, 0.13; group L-U, 0.56; group E-P, 0.10; group E-U, 0.51.

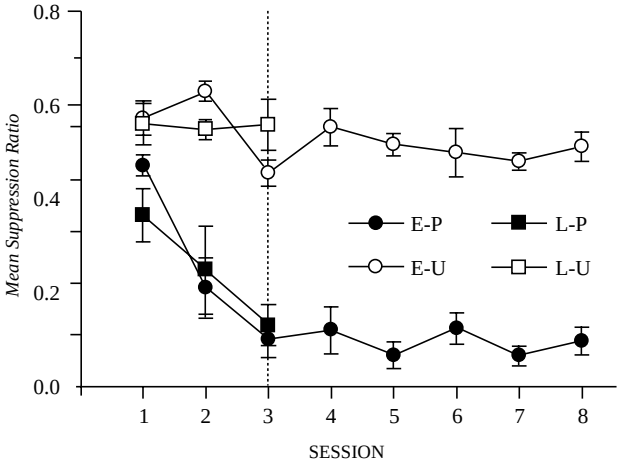

Figure 1. *Mean suppression ratios to tone during fear conditioning sessions. A ratio of 0.5 represents no conditioned fear during the tone, and a ratio of 0.0 represents maximal fear conditioning*

Quantification of CO activity

In a few subjects, the quality of some brain regions was not adequate for quantification. Therefore, data from these subjects were removed, and the statistical analyses were corrected where appropriate taking into account the missing data. A significant decrease in CO activity was found in the septal area [F (3,33)= 3.2; p<0.05], anteroventral thalamic nucleus [F(3,33)= 3.0; p<0.05], medial mammillary nucleus [F(3,34)= 4.8; p<0.01] and medial geniculate nucleus [F(3,33)= 3.1; p<0.05] in the group E-P as compared with the group L-P (see Table 1). Conversely, no significant differences were detected between groups E-P and L-P in the basolateral amygdala, but there was less metabolic activity in basolateral amygdala in the group E-U as compared with the group L-U [F(3,34)= 4.1; p<0.05].

Discussion

Our data represent a look at the dynamic nature of fear conditioning over time and are consistent with the conclusion that the neural substrates of phenomena are not uniform (see Rokers et al., 2002). Certainly, several nuclei in the limbic system become involved during the conditioning of fear responses. However, the temporal pattern of brain activation shifts as fear learning develops. In general, CO activity tends to decrease in both the paired and unpaired groups with extended training in all the limbic regions studied. This result could be interpreted as a habituation effect on neuronal activity after repeated exposition to the experimental setting and discrete stimuli involved in training. However, significant decreases in CO activity were observed only in particular brain regions of the extended group. Therefore, these changes may indicate a differential involvement of these brain regions in fear conditioning. The medial geniculate nucleus is clearly involved, since it is the auditory relay nucleus for CS processing in the amygdala during auditory fear conditioning (LeDoux, 2000). Changes observed in neuronal activity with extended training, could be associated with plastic changes in the synaptic inputs to the amygdala induced by associative learning, as reported previously using CO histochemistry (Poremba et al., 1998b). The medial septum has been involved in Pavlovian conditioning (Berger and Thompson, 1978; Berry and Thompson, 1979; Allen, Padilla and Gluck, 2002), and particularly in contextual fear conditioning due to its close relationship with the hippocampus (Conejo et al., 2005). It is known that the septohippocampal system mediates the effects of novelty (or familiarity) on conditioning. Accordingly, neurons in the medial septum and the dorsal hippocampus respond strongly when novel, but not familiar, stimuli are presented (Rokers et al., 2002). Since the medial septum is the basal forebrain origin of the septohippocampal cholinergic projection, changes in acetylcholine levels in the hippocampus are mainly caused by medial septal activity. Other authors (Orsetti, Casamenti and Pepeu, 1996) reported an increase in hippocampal cholinergic levels during the acquisition of an instrumental conditioning task, followed by a decrease as training progresses. A similar decrease was reported in the activity of medial septum neurons in a Pavlovian conditioning task as a function of training (Berger and Thompson, 1978). The significant decrease in CO activity observed in the medial septum can be attributed to a habituation effect to both the contextual and conditioned stimuli, related with the modulation of hippocampal activity by the medial septum.

Fear conditioning seems to be also mediated by the anterior thalamus, because lesions of this limbic region impair not only contextual PFC, but also auditory PFC (Celerier, Ognard, Decorte and Beracochea, 2000). In particular, the electrical activity of neurons from the anteroventral thalamic nucleus and the mammillary body changes in response to active avoidance learning (Poremba and Gabriel, 1997). This kind of associative learning involves fear conditioning and instrumental learning of the motor avoidance response. Similarly, the conditioned suppression task used in our experiment involves both fear conditioning and instrumental learning of lever press response to obtain food. We obtained a significant decrease in the metabolic capacity of the anteroventral thalamic nucleus in the conditioned group after extended training, which suggests an involvement of this nucleus in both PFC and instrumental conditioning. The anteroventral thalamic nuclei and the cingulate cortex are reciprocally interconnected, playing an important role not only on learning and memory, but also on attention (Aggleton and Brown, 1999; Smith, Freeman, Nicholson and Gabriel, 2002). In this regard, it seems more plausible that the cingulothalamic circuit relevant for attentional processes decreases its activity after extended or prolonged training, as shown by our CO activity data. A similar result was found in the medial mammillary nucleus, which sends a direct projection to the anteroventral thalamus via the mammillothalamic tract. However, recently has been demonstrated that mammillary body lesions do not impair auditory PFC, but induce severe deficits in contextual fear conditioning (Celerier, Pierard and Beracochea, 2004). Although contextual fear conditioning may be important to explain our results, it certainly difficult to interpret why a significant decrease of CO activity was found only in the

Table 1

Effects of training in a Pavlovian fear conditioning on CO activity of selected brain regions. Data represent mean relative optical density of CO histochemical staining. The optical density values are directly related with CO activity. * Significant differences between E-P vs L-P in Group Paired (Tukey-Kramer post hoc tests, * p<0.05 and ** p<0.01); † Significant differences between E-U vs L-U in Group Unpaired (Tukey-Kramer post hoc tests, p<0.05)

Limbic structures	Paired group		Unpaired group	
	Extended (E-P)	Limited (L-P)	Extended (E-U)	Limited (L-U)
Medial septum	63.1 ± 2.4*	82.4 ± 7.6	72.7 ± 4.1	81.4 ± 6.1
Anterodorsal thalamus nucleus	77.5 ± 2.7	95.5 ± 7.1	82.3 ± 4.1	89.7 ± 9.1
Anteroventral thalamus nucleus	54.2 ± 2.4*	66.8 ±5.9	58.8 ± 2.3	69.5 ± 5.3
Lateral mammillary nucleus	80.2 ± 4.4	89.7 ± 7.2	87.4 ± 1.9	88.5 ± 11.9
Medial mammillary nucleus	78.1 ± 2.6**	96.8 ± 2.7	75.5 ± 2.6	91.4 ± 9.6
Basolateral amygdala	71.9 ± 3.7	82.2 ± 4.4	70.4 ± 1.9†	87.3 ± 4.6
Medial geniculate nucleus	52.2 ± 1.3*	70.1 ± 4.4	62.3 ± 3.2	65.6 ± 7.7

conditioned group after extended training. It should be taken into consideration that the mammillary bodies are a main site of action of anxiolytic drugs (Kataoka, Shibata, Gomita and Ueki, 1982; Yamashita et al., 1989), and that lesions of the mammillary bodies decrease anxiety (Beracochea and Krazem, 1991). The decrease in CO activity of the conditioned group can be linked to the predictability of the US, and the familiarization to the fear-inducing environment, that probably decreases fear and anxiety in this group as compared to the group unpaired. Unexpectedly, we did not find significant changes in the basolateral amygdala of the conditioned group with training, a region classically involved in PFC. This result agrees with other data supporting a time-limited involvement of the amygdala in fear conditioning, participating only in the acquisition but not in the consolidation of fear memories (Wilensky, Schafe and LeDoux, 2000).

In conclusion, our study demonstrates that extensive training in a PFC task induces selective decreases in the neuronal metabolism of limbic regions, related with different functional aspects of fear conditioning. Our findings may also account for previous research on the neural substrate for PFC during prolonged exposition to fear- or anxiety-provoking environments, as a cause of anxiety and mood disorders.

Acknowledgements

We wish to thank the laboratory technicians Piedad Burgos and Begoña Valdés for their help. This work was supported by grants MCYT BSO-2002-03404 (Ministerio de Ciencia y Tecnología, Spain) to M.L., and MEC-04-SEJ-07445 (Ministerio de Educación y Ciencia, Spain), SU-04-FMM-02 (Fundación de Investigación Médica) to J.L.A., with the colaboration of FEDER.

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