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Changes in brain oxidative metabolism induced by inhibitory avoidance learning and acute administration of amitriptyline

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Abstract

The effects of antidepressant drugs on memory have been somewhat ignored, having been considered a mere side effect of these compounds. However, the memory impairment caused by several antidepressants could be considered to form part of their therapeutic effects. Amitriptyline is currently one of the most prescribed tricyclic antidepressants, and exerts marked anticholinergic and antihistaminergic effects. In this study, we evaluated the effects of inhibitory avoidance (IA) learning and acute administration of amitriptyline on brain oxidative metabolism. Brain oxidative metabolism was measured in several limbic regions using cytochrome oxidase (CO) quantitative histochemistry. Amitriptyline produced a clear impairment in the IA task. In animals exposed only to the apparatus, amitriptyline decreased CO activity in nine brain regions, without affecting the remaining regions. In animals that underwent the IA training phase, amitriptyline reduced CO activity in only three of these nine regions. In animals treated with saline, IA acquisition increased CO activity in the medial prefrontal cortex, the prelimbic cortex, and the medial mammillary body, and diminished it in the medial septum and the nucleus basalis of Meynert with respect to animals exposed only to the IA apparatus. In animals treated with amitriptyline, IA acquisition did not modify CO activity in any of these regions, but increased it in the anteromedial nucleus of the thalamus, the diagonal band of Broca, and the dentate gyrus. The results reveal a pattern of changes in brain oxidative metabolism induced by IA training in saline-treated animals that was clearly absent in animals submitted to the same behavioural training but treated with amitriptyline.
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Tricylic antidepressants (TCAs) are often prescribed for the pharmacological management of severe major depression, particularly in cases with physical symptoms or pain (Mann, 2005). Amitriptyline is one of the most prescribed of the available TCAs (Barbui and Hotopf, 2001). Although the mechanisms underlying the therapeutic action of antidepressant drugs remain largely unknown, the reuptake inhibition of norepinephrine and serotonin is believed to be related with the antidepressant effects of TCAs. Compared to other TCAs, amitriptyline has a higher anticholinergic potency and a considerably high antihistaminergic effect (Cusack et al., 1994; Richelson, 1994). These properties have been associated with effects in the CNS, where they cause memory impairment and sedation after acute administration. Paradoxically, memory impairment is a common symptom among patients suffering from major depression, and is believed to be restored after chronic treatment with antidepressants (Burt et al., 1995). We have hypothesized that memory impairment is related to the therapeutic action of antidepressants (Parra, 2003). Several studies have examined the effect of antidepressants on memory in rodents (Yau et al., 1995, 2001; Monleón et al., 2002; Parra et al., 2002; Everss et al., 2005; Naudon et al., 2007), however,

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the effects of antidepressants on brain function related with memory processes have yet to be directly addressed. We believe that evaluating the effects of antidepressants on brain function not only at a basal level, but also with respect to the performance of behavioural tasks, is of interest to the study of the mechanisms of action of antidepressants.

One of the most common procedures used to evaluate memory in animals is the inhibitory avoidance (IA) task, in which the animal is punished when it enters a dark compartment, thus learning to inhibit its behavioural response. Several brain regions have been proposed as parts of the neural system of this learning task, including the nucleus basalis, hippocampus, amygdala, nucleus accumbens, septum, and prefrontal cortex (Ambrogi Lorenzini et al., 1999; Roozendaal et al., 2001; Izquierdo et al., 2002; Maren and Quirk, 2004). The involvement of specific neural structures and the neurochemical changes that occur in the formation of memory in inhibitory avoidance are time-dependent (Izquierdo and Medina, 1997). We have previously demonstrated that acute pre- or posttraining administration of amitriptyline causes a memory deficit in IA that is unrelated to its anxiolytic and locomotor effects (Everss et al., 2005; Arenas et al., 2006).

The aim of the present study was to evaluate short-term changes in the brain metabolism induced, separately and in combination, by training in an IA task and acute administration of amitriptyline. Cytochrome oxidase (CO) activity was applied as a functional metabolic marker for neuronal oxidative metabolism. CO quantitative histochemistry has previously been used to study brain function associated with memory processes (Poremba et al., 1998; Agin et al., 2001; Deglise et al., 2003; Hu et al., 2006; Conejo et al., 2004, 2007), and to evaluate the effects of anxiolytic and antipsychotic drugs on brain function in several species (Prince et al., 1997; Prince and Oreland, 1998; González-Pardo et al., 2006). Compared to other functional metabolic mapping techniques, CO provides a measurement of the sustained metabolic demands (known as metabolic capacity) of neurons, which can take place over hours or days (Wong-Riley, 1989; González-Lima and Jones, 1994; González-Lima and Cada, 1998).

1. Materials and methods

1.1. Animals

Male CD1 mice of 42 days of age, obtained from CRIFFA (Lyon, France), were used as experimental subjects. Animals were housed in groups of 5, in standard translucent plastic cages of 27×27×15 cm (Panlab S.L., Barcelona, Spain), in a temperature-controlled room (21±2 °C), under a reversed light/dark cycle (lights off: 07:30–19:30, local time), with food and water available ad libitum. All experimental procedures were carried out during the dark phase of the cycle, after 11 days of acclimatization to the housing conditions. The experimental protocol and use of the animals were in compliance with the European Community's Council Directive of 24 November 1986 (86/609/EEC) and Spanish legislation (R.D. 1201/2005) related to the care and use of experimental animals.

1.2. Drugs and treatments

Animals were randomly assigned into six groups. Three groups received a single i.p. injection of amitriptyline hydrochloride (AMI; Sigma-Aldrich, Madrid, Spain), which was dissolved in a saline solution (0.9% NaCl) in order to provide a dose of 20 mg/kg. In the habituation group (H), subjects were exposed solely to the inhibitory avoidance apparatus. Animals in the acquisition group (A) were subjected to the training phase of the task. Mice in the retention group (R) were also subjected to the test phase of the task (see below for more procedure details). Three additional groups were injected i.p. with saline (SAL). Groups were identified as H-AMI, A-AMI, R-AMI, H-SAL, A-SAL, and R-SAL (H and A groups, $n=6$; R groups, $n=9$). All injections were administered at a volume of 0.01 ml/g body weight, 30 min prior to the behavioural procedure. Animals in the R groups were injected before the training trial but not before the test trial.

1.3. Inhibitory avoidance procedure

1.3.1. Apparatus

A step-through inhibitory avoidance box for mice (Ugo Basile, Comerio-Varese, Italy) was employed. The cage, made of Perspex plates, consisted of two compartments (each one measuring 15×9×16.5 cm), which were separated by an automatic sliding door. One compartment was coloured white and illuminated with a white fluorescent tube (10W), which provided a light intensity of 290 lx, as measured with a Panlux Electronic2 photometer (Gossen, Nürnberg, Germany) placed at floor level in the box (light compartment). The other compartment had black walls and was not illuminated (dark compartment). The floor consisted of a metal grid with 48 stainless steel rods of a diameter of 0.7 mm and separated by 8 mm. The apparatus was placed in a sound attenuation box.

1.3.2. Behavioural procedure

Mice in A and R groups were subjected to the training phase of inhibitory avoidance, which began with a 90 s period of adaptation to the light compartment. The door between the compartments remained closed during this period. The door was then opened for a maximum of 300 s, and if the animal entered the dark compartment it received an inescapable footshock of 0.4 mA that was delivered for 5 s. The time taken to enter the dark compartment, defined as latency, was automatically measured in tenths of a second, and manually recorded after each trial. Animals in the H groups did not receive any shock during training. Mice in the R groups were submitted to the test phase 24 h later. This phase was identical to the previous training phase, with the exception that no shock was delivered.

1.4. Cytochrome oxidase histochemistry

Ninety minutes after the behavioural tests had concluded, all mice, except for those in the R groups, were decapitated and their brains frozen in isopentane and stored at −40 °C. This time period, chosen to detect possible changes in CO activity, was

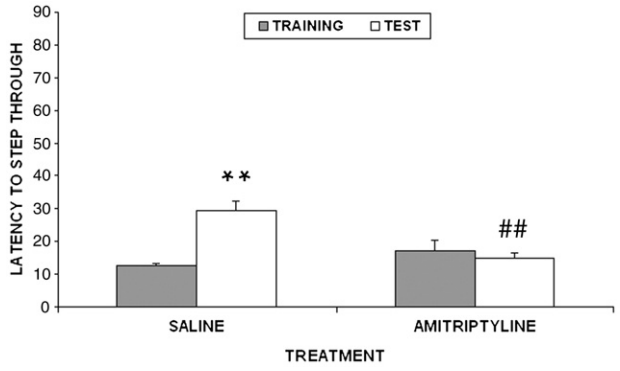

Fig. 1. Effect of pretraining administration of saline or amitriptyline on step-through latencies of an inhibitory avoidance task in the training and test phases. Values are expressed as means (+SEM) of square root of proportions ($p=x/300$) transformed to arcsin. **$p<0.001$ vs. saline in training, ##$p<0.001$ vs. saline in test.

based on experience gained through previous studies performed by our research group (López et al., 1997; Conejo et al., 2005). Tissue from mice in the R groups was not stained in order to avoid confusion among the effects of memory acquisition, consolidation, retrieval, and amitriptyline administration on brain CO activity. Coronal brain sections (30 μm thick) were obtained using a cryostat microtome (Microm HM-505E, Heidelberg, Germany) and processed for CO histochemistry according to the method described by González-Lima and Jones (1994). Additional brains of untreated mice were homogenized on ice and their mean CO activity measured spectrophotometrically in an aliquot of the homogenate, using a commercial kit (Sigma-Aldrich, Madrid, Spain). Cryostat slides containing sections of different thicknesses (10, 30, 50 and 70 μm) were obtained from the homogenate and mounted on a slide. The sets of sections from mouse brain homogenate were used as CO activity standards.

The brain sections and a complete set of standards underwent CO histochemistry. In short, slides were lightly fixed for 5 min with a 1.5% glutaraldehyde, rinsed three times in phosphate buffer and preincubated in a solution containing cobalt chloride and dimethylsulfoxide dissolved in Tris buffer. Once the sections had been rinsed in phosphate buffer (pH 7.6; 0.1 M), they were incubated in darkness for 1 h at 37 °C in a solution containing diaminobenzidine, sucrose, cytochrome *c* and catalase (Sigma-Aldrich, Spain) dissolved in phosphate buffer (pH 7.6; 0.1 M), which was continuously stirred. The slides were rinsed three times with cold phosphate buffer, and then dehydrated and coverslipped with Entellan (Merck, Darmstadt, Germany). CO histochemical staining intensity was measured by means of densitometric analysis, using a computer-assisted image analysis workstation (MCID, InterFocus Imaging Ltd., Linton, England) composed of a high precision illuminator, a digital camera and a computer with specific image analysis software. Relative optical density readings (ROD) were obtained from limbic regions known to be involved in the behavioural effects of anxiolytic drugs, of which the following were selected: the prefrontal and prelimbic cortex, anterior thalamic nuclei (anterodorsal, anteroventral and anteromedial nuclei), nucleus accumbens (core and shell regions), diagonal band of Broca, nucleus basalis, bed nucleus of stria terminalis, dorsal hippocampus (dentate gyrus, CA3 and CA1 subfields), mammillary body (medial and supramammillary nuclei) and amygdala (basolateral and central nuclei). In order to establish comparisons and make allowances for possible variations in the staining of brain sections from different staining baths, measurements were taken from CO-stained brain homogenate standards. Regression curves between section thickness and CO activity for each set of standards were calculated for each incubation bath. Finally, ROD values measured in the selected brain regions were converted into CO activity units using the regression curves calculated.

1.5. Data analysis

The inhibitory avoidance data were transformed into proportion ($p=x/300$) values and then to arcsin ($\arcsin\sqrt{p}$) values according to Snedecor and Cochran (1980). An ANOVA of these data was performed with Drug Treatment (Saline and Amitriptyline) as a between factor, and Phase (Training and Test) as a within factor. Student's *t*-tests were used to compare training vs. test latencies in saline and amitriptyline-treated groups, and performances of saline vs. amitriptyline groups in the training and the test phases. Statistical significance of group differences in CO activity for each brain region was analyzed using one-way ANOVA followed by Newman–Keuls post hoc tests where appropriate. A probability level of less than 0.05 ($p<0.05$) was considered to be statistically significant.

2. Results

Behavioural data analysis showed the main factors and their interaction to be statistically significant: Drug Treatment [$F(1,16)=5.40$, $p<0.05$], Phase [$F(1,16)=8.26$, $p<0.05$], and Drug Treatment × Phase [$F(1,16)=14.31$, $p<0.01$]. The post hoc test for this interaction was not computed, as there is no

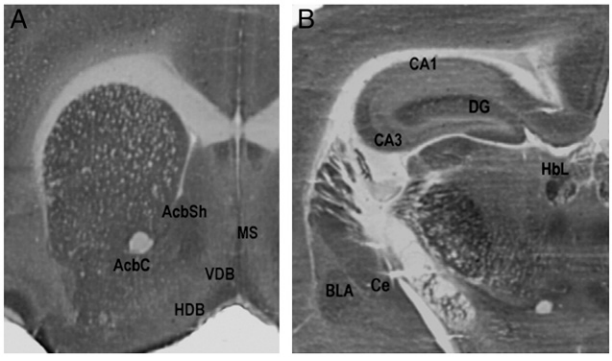

Fig. 2. Microphotographs of CO histochemical staining in coronal sections of mouse brain at different levels (group H-SAL). Note the dark staining of forebrain cholinergic regions in A, also present in limbic regions including the amygdala and hippocampus in B. Abbreviations: AcbC: nucleus accumbens, core. AcbSh: nucleus accumbens, shell. BLA: amygdala, basolateral nucleus. Ce: amygdala, central nucleus. CA1, CA3: Cornu Ammonis of hippocampus. DG: dentate gyrus of hippocampus. HbL: habenula. HDB: horizontal diagonal band of Broca. MS: medial septum. VDB: vertical diagonal band of Broca.

Table 1
Mean CO activity (micromol cytochrome c oxidized/min/g wet tissue at 23 °C) as measured in selected brain regions of animals in different experimental groups

	H-SAL	H-AMI	A-SAL	A-AMI
Medial prefrontal cortex	27.39±0.95	30.22±2.27	35.80±1.48$^{\#}$	29.51±1.89^{+}
Prelimbic cortex	28.69±0.42	27.12±0.92	32.43±0.11$^{\#\#}$	26.12±0.97^{+}
Thalamus, anterodorsal nucleus	53.21±2.05	52.70±1.35	51.01±1.30	52.68±0.89
Thalamus, anteroventral nucleus	41.81±1.59	37.79±1.10	37.53±1.71	37.51±0.94
Thalamus, anteromedial nucleus	36.39±1.19	31.91±0.76**	34.33±1.47	37.65±1.04^{a}
Medial septum	30.42±0.59	26.90±0.68**	26.83±0.55**	24.05±0.54**$^{,+}$
Nucleus accumbens core	38.37±2.34	26.86±1.91**	35.73±1.64	30.82±1.17
Nucleus accumbens shell	39.29±1.18	28.74±1.83**	36.29±1.79	29.87±0.94^{+}
Nucleus basalis of Meynert	34.31±1.59	28.19±1.88**	24.84±1.78$^{\Delta}$	30.39±1.34
Bed nucleus of the stria terminalis	44.32±2.00	38.25±1.39**	44.92±1.66	35.03±0.67^{+}
Diagonal band of Broca	38.76±1.66	30.47±1.28**	38.23±1.40	39.68±1.91^{a}
Basolateral amygdala	36.85±1.21	34.08±1.08	34.40±1.68	35.49±1.39
Central nucleus of amygdala	33.10±2.33	33.46±1.40	34.12±1.50	29.68±1.45
Hippocampus, CA1 subfield	22.66±0.94	23.81±1.01	23.90±1.02	23.08±0.54
Hippocampus, CA3 subfield	37.94±1.27	31.07±1.34**	34.75±0.95	33.98±0.98
Hippocampus, dentate gyrus	37.32±1.79	30.27±1.18**	40.05±2.21	37.51±0.94^{a}
Supramammillary nucleus	23.93±1.34	23.76±0.65	25.38±0.86	22.74±1.01
Medial mammillary nucleus	30.64±1.20	32.19±1.44	37.72±2.29$^{\#}$	30.07±1.11^{+}
Lateral mammillary nucleus	39.34±2.01	36.92±1.83	37.22±1.41	35.85±1.22

Data represent mean CO activity±SEM **$p \leq 0.01$ significantly lower than that in H-SAL group. $^{\Delta}p \leq 0.05$ significantly lower than that in H-SAL, H-AMI and A-AMI groups. $^{\#}p \leq 0.05$, $^{\#\#}p \leq 0.01$ significantly higher than that in H-SAL, H-AMI and A-AMI groups. $^{a}p \leq 0.05$ significantly higher than that in H-AMI group. $^{+}p \leq 0.05$ significantly lower than that in A-SAL group. Newman–Keuls post hoc tests.

unambiguous choice of an appropriate error term for post hoc comparisons involving between-group by within-subject interaction (Winer, 1991). Student's *t*-tests showed the presence of inhibitory avoidance in the saline group [$t(8)=5.56$, $p<0.001$], absence of inhibitory avoidance in the amitriptyline group [$t(8)=0.57$, $p>0.05$], lack of significant differences between the performances of animals in the saline and amitriptyline groups in the training phase [$t(16)=1.49$, $p>0.05$], and the presence of statistically significant differences in the test phase [$t(16)=4.16$, $p<0.001$] (see Fig. 1).

There was less CO staining in animals that had received amitriptyline (H-AMI, A-AMI) than in saline-treated animals (H-SAL, A-SAL) in the basal forebrain and most of the limbic regions studied (see Fig. 2; Table 1). Specifically, the factor Drug Treatment was statistically significant in the following brain regions: medial prefrontal cortex [$F(3,20)=4.33$, $p<0.05$], prelimbic cortex [$F(3,20)=9.5$, $p<0.001$], anteromedial thalamic nucleus [$F(3,20)=4.78$, $p<0.05$], medial septum [$F(3,20)=19.12$, $p<0.001$], nucleus accumbens core [$F(3,20)=8.14$, $p<0.001$] and shell [$F(3,20)=12.69$, $p<0.001$], nucleus basalis of Meynert [$F(3,20)=5.71$, $p<0.01$], bed nucleus of stria terminalis [$F(3,20)=10.02$, $p<0.001$], diagonal band of Broca [$F(3,20)=7.19$, $p<0.01$], CA3 hippocampal subfield [$F(3,20)=6$, $p<0.01$], dentate gyrus of hippocampus [$F(3,20)=6.72$, $p<0.01$], and medial mammillary nucleus [$F(3,20)=4.85$, $p<0.05$].

Post hoc comparisons of these ANOVA analyses revealed the following (see Table 1): among the habituation groups (H), CO activity was significantly lower in the AMI group than in the corresponding SAL group (Newman–Keuls test; $p<0.01$) in the following brain regions: anteromedial thalamic nucleus, medial septum, nucleus accumbens (core and shell), nucleus basalis, bed nucleus of the stria terminalis, diagonal band of Broca, and hippocampal formation (CA3 subfield and dentate gyrus). Regarding the acquisition groups (A), statistically significant decreases in CO activity were detected in the AMI group (Newman–Keuls test; $p<0.01$) in only some of the previously-mentioned brain regions: medial septum, nucleus accumbens shell, and bed nucleus of the stria terminalis. However, an increase in the CO activity of the AMI with respect to that of its SAL group was observed in the nucleus basalis of Meynert (Newman–Keuls test; $p<0.05$). In the A groups, additional brain regions that showed a decrease in CO activity caused by amitriptyline treatment were the medial prefrontal cortex, prelimbic cortex, and medial mammillary nucleus (Newman–Keuls test; $p<0.05$). When A and H saline groups were compared, CO activity was higher in the former in the medial prefrontal cortex, prelimbic cortex, and medial mammillary nucleus, and higher in the latter in the medial septum and nucleus basalis of Meynert. Finally, when A and H groups treated with amitriptyline were compared, CO activity was higher in the former in the anteromedial nucleus of the thalamus, the diagonal band of Broca, and the dentate gyrus.

3. Discussion

Acute administration of amitriptyline caused a general pattern of decrease in the oxidative metabolism when measured in numerous limbic regions of animals exposed to the apparatus. A generalized pattern of decrease in cerebral metabolism has previously been reported following acute administration of the SSRI antidepressant fluoxetine in rats (Freo, 1996; Porrino et al., 1997; Freo et al., 2000). These authors reported a maximum decrease in regional cerebral 2-[^{14}C]deoxyglucose (2-DG) uptake in subcortical regions with a high density of 5-HT reuptake sites or 5-HT innervation, such as the raphe nuclei, amygdala, hypothalamus (paraventricular and mammillary nuclei), hippocampus and frontal cortex (Biegon and Mathis,

1993; Freo et al., 2000). Acute administration of the TCA clomipramine, a non-selective 5-HT reuptake inhibitor, was also reported to cause a similar decrease in regional 2-DG uptake in 5-HT-rich brain regions (Freo et al., 1995; Freo, 1996). However, after amitriptyline administration, we found no significant differences in the CO activity of some limbic regions, including the prefrontal and prelimbic cortices, anterodorsal and anteroventral thalamic nuclei, basolateral and central amygdala nuclei, a part of the hippocampus (CA1 subfield), and mammillary bodies. Other authors report similar results, finding no differences or even increases in 2-DG uptake in several brain regions after acute administration of the TCA desmethylimipramine (Gerber et al., 1983).

In animals submitted to the IA training phase, the decrease in CO activity observed after amitriptyline administration was present in fewer brain regions than in those affected by exposure to the apparatus (see Table 1). In what was an exception to this, treated subjects presented an even higher CO activity in the nucleus basalis of Meynert than did controls. Nevertheless, it seems that these differences were largely due to the low activity in the A-SAL group rather than a high CO activity in the A-AMI group.

Comparison of CO activity in animals treated with saline and subjected to the IA acquisition session with those exposed only to the apparatus proved complex. CO activity increased in the medial prefrontal and prelimbic cortices, and in the medial mammillary nucleus, while CO activity diminished in the medial septum and nucleus basalis of Meynert. No statistically significant changes were detected in the remaining regions. The increases in CO activity in some brain regions and the drop in others seem to be characteristic of changes in brain energy metabolism induced by learning (e.g., Puga et al., 2007). The mammillary body is known to be involved in both anxiety and IA memory (Pratt et al., 1988; Shibata and Furukawa, 1988; González-Pardo et al., 2006). In addition, the prefrontal and prelimbic cortex have been implicated in behavioural inhibition (Jinks and McGregor, 1997; Mello e Souza et al., 2000). However, no differences in CO activity were observed in the basolateral and central amygdaloid nuclei or the hippocampus, which are regions to which IA learning has been attributed to (Maren and Quirk, 2004). In accordance with our results, other authors have reported that only the striatum, and not the hippocampus or amygdala, shows changes in 2-DG uptake 20–30 min after IA training (Doyle et al., 1990). Furthermore, pretraining or posttraining lesions of the amygdala attenuate, but do not block IA learning (Tinsley et al., 2004). On the other hand, CA1 and CA3 hippocampal subfields seem to be necessary for the consolidation of IA memory but not for its acquisition (Martinez et al., 2002). Despite the lack of significant differences in the latter regions, we cannot discard time-limited involvement of the amygdala or the hippocampus at later stages of IA memory consolidation. At the time at which the brains were extracted, a diminution of CO activity, as a trend ($p=0.06$), was observed in the CA3 subfield of the hippocampus.

If the pattern of differences in CO activity observed in the saline groups (i.e. A-SAL vs. H-SAL) represents changes that are necessary for the learning of IA, this pattern should not be observed in animals that do not learn; namely those treated with amitriptyline. Effectively, our results showed no changes in CO activity in any of the regions shown to be involved in learning by our results with saline-treated animals (medial prefrontal cortex, prelimbic cortex, medial septum, nucleus basalis of Meynert and medial mammillary nucleus). In fact, the significant changes observed in subjects administered with amitriptyline (i.e. A-AMI vs. H-AMI) consisted of increases in CO activity in different regions (anteromedial nucleus of the thalamus, diagonal band of Broca, and dentate gyrus) to those mentioned above.

Behavioural results obtained in R groups served as a double control that allowed us to confirm that the behavioural procedure which the A groups underwent produced IA learning in their R counterparts, and, subsequently, that amitriptyline interfered with such learning. Performance in the test phase was impaired in animals that had received a single pretraining administration of amitriptyline. These results support those of our previous studies that demonstrate that amitriptyline also causes a deterioration of memory when administered chronically before IA training or acutely after IA training (Everss et al., 2005; Parra et al., 2006). It also supports research in humans indicating deficits in attention and memory following acute amitriptyline administration (Kerr et al., 1996; van Laar et al., 2002; Veldhuijzen et al., 2006). It can be argued that the decreased ability to learn in amitriptyline-treated animals is due to sensory-motor or arousal impairments rather than associative or memory deficits. Although the data of the present study do not exclude this possibility, findings of a previous report (Arenas et al., 2006) lead us to believe that the results observed can be attributed to memorization deficit. Arenas et al. (2006) administered amitriptyline prior to training as part of a 2×2 experimental design traditionally used to test the existence of state-dependent learning in drug-administered subjects (Overton, 1974). The authors ruled out the presence of state-dependent learning and other effects of the drug, including performance deficit, performance facilitation, and suppressed exploration. They found the memorization deficit pattern proposed by Overton (1974) to be the most appropriate for explaining the effect of amitriptyline on IA.

In conclusion, the present study proves that the technique employed here for measuring regional brain cytochrome oxidase activity is sensitive to one trial learning and acute administration of amitriptyline.

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