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Effects of social stress and clomipramine on emotional memory in mice

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We have previously observed impairing effects of social defeat stress (CSDS) on inhibitory avoidance (IA) in mice. Given the similarity between changes produced by social stress in animals and symptoms of certain human psychopathologies such as depression and anxiety, the effects of the antidepressant clomipramine on IA impairment produced by CSDS were evaluated in the present study. Male CD1 mice were randomly assigned to the groups: non-stressed+saline, non-stressed+clomipramine, stressed+saline and stressed+clomipramine. Stressed animals were subjected to daily agonistic encounters (10 min) in the home cage of the aggressor over a 20-day period. Just before each encounter, non-stressed and stressed mice were injected i.p. with saline or clomipramine (10 mg/kg) according to their experimental condition. 24 hours after the last CSDS session, all the mice were tested in a step-through IA task. In the IA training phase, animals were punished by a shock to the paw when they entered the dark compartment of the apparatus. In the IA test phase (one week later) the same procedure took place, but without shock. Complementary measures were obtained by evaluating all the animals in an elevated plus maze (locomotor activity and emotionality) and on a hot plate (analgesia). IA learning was confirmed in all groups except the stressed+saline group, which was the only one that exhibited higher anxiety levels. No variations were observed in either locomotor activity or analgesia. In conclusion, CSDS induces anxiety and impairs emotional memory in mice; the negative effects of CSDS on memory appear to be attenuated by clomipramine, and these detrimental effects do not seem to be secondary to the effects of CSDS on locomotor activity, emotionality or pain sensitivity.

Key words: chronic social defeat stress, inhibitory avoidance, elevated plus maze, hot plate, locomotor activity, anxiety, analgesia

INTRODUCTION

Social stressors are the main source of stress in humans and contribute to the development and expression of diverse pathologies, representing a major risk factor for depression (e.g. Campeau et al. 2011). In fact, heightened stress reactivity and dysregulation of the HPA (hypothalamus-pituitary-adrenal) axis are some of the most consistent features in patients suffering from major depression (Holsboer 2000, Gallagher et al. 2007, Holsboer and Ising 2010), while remission of this pathology is associated with normalization of the HPA axis (Holsboer 2000, Ising et al. 2007). Experimental studies that address the long-term effects of social stress, due to ethical and time constraints, often require the use of animal models (Tanaś et al. 2015). Social defeat stress in animals, defined as being defeated in confrontations with conspecific animals (Jin et al. 2015), is frequently induced by the resident/intruder paradigm (Björkqvist 2001). This kind of stress has been reported to cause a variety of behavioral, neuroendocrinological, physiological, neurochemical, neurological and immunological changes (Blanchard et al. 2001, Buwalda et al. 2005, Niebylski et al. 2012) that resemble certain symptoms of human psychopathologies, such as depression and anxiety (Bartolomucci and Leopardi 2009). Therefore, animal models based on social stress are becoming increasingly popular for studying the relation between stress and the aforementioned psychopathologies (Sgoifo and Meerlo 2002, Wood et al. 2010). CSDS (chronic social defeat stress) is an animal model widely used nowadays to emulate human diseases related to stress, such as anxiety (e.g. Watt et al. 2009, Toth and Neumann 2013) and depression (e.g. Venzala, et al. 2012, Iñiguez et al. 2014), in order to determine the underlying mechanisms and identify potential pharmacological treatments.

Memory impairment can be also an important negative consequence produced by stress (e.g. Trofimiuk et al. 2006), and has previously been shown to be induced by CSDS in several animal paradigms (Ohl and Fuchs 1998, 1999, Touyarot et al. 2004, Wang et al. 2011, Patki et al. 2013,

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Monleón et al. 2015, 2016). For example, Ohl and Fuchs (1998, 1999) observed memory deficit in a modified hole-board task in male tree shrews exposed to CSDS. Touyarot and others (2004) reported a deficit in spatial learning in the Morris water maze in highly-reactive-to-novelty rats undergoing a 21-day CSDS schedule. Wang and colleagues (2011) also reported impairments in the spatial memory of chronically stressed mice (21 daily encounters of CSDS) in novel object recognition and the Y-maze, while Patki and others (2013) observed worse long-term memory in socially defeated rats tested in a radial arm water maze task.

Inhibitory avoidance (IA), or passive avoidance, is a common procedure used to evaluate memory in animals (Gold 1986), which mainly involves emotional memory. In the step-through version, which was employed in the present and previous studies carried out in our laboratory (e.g. Parra et al. 2000, 2010, 2013, Monleón et al. 2002, 2009, 2015, 2016, Everss et al. 2005, Arenas et al. 2006), the animal is punished by a shock to the paw when it enters the dark compartment, which leads to an inhibition of its behavioral response in order to evade receiving a future shock (Bureš et al. 1983). In previous studies using the CD1 strain as stressed subjects, in place of the C57BL/6J strain used in the standard protocol (Golden et al. 2011), we found that CSDS (a 3-week period of 10-min daily sessions) prevented the formation of IA memory in post-pubertal mice. Furthermore, these effects of CSDS on memory were not secondary to motor or emotional effects of stress (Monleón et al. 2015, 2016).

As previously mentioned, stress has long been linked to neuropsychiatric diseases, such as depression (Barden 2004). Memory impairment is also a symptom of depression (e.g. Ramponi et al. 2010), and, along with other symptoms, is reduced by antidepressant therapy (Antikainen et al. 2001). Thus, the aim of the present work was to investigate whether chronic antidepressant treatment reverses the memory impairment produced by CSDS. In rodents repeatedly exposed to social defeat, there is a decrease in volume and cell proliferation in the hippocampus that can be reversed by chronic antidepressant treatment (Czéh et al. 2007, Becker et al. 2008, Van Bokhoven et al. 2011). Clomipramine, a tricyclic antidepressant widely used to treat depression and obsessive compulsive disorder, has been shown to reverse the behavioral deficits and inhibition of cell proliferation in the adult hippocampus induced by chronic unpredictable stress (Liu et al. 2008, 2012).

We have evaluated the effect of chronic clomipramine on IA impairment produced by CSDS in post-pubertal male CD1 mice, hypothesizing that clomipramine would reverse the negative effects of CSDS on memory. In addition to the IA task, our animals were assessed in an elevated plus maze and a hot plate in order to obtain complementary measures of locomotor activity, emotionality and analgesia, as they could be confounding factors in the animals' performance of the main task.

METHODS

Subjects

Post-pubertal (42 days) male CD1 mice (Charles River, Lyon, France) were used as experimental subjects. The animals arrived at the laboratory weighing 30–43 g and were housed in groups of 4 or 5 in translucent plastic cages (height 14.5 cm, width 27 cm, length 27 cm) with roofs of stainless steel bars (Panlab S.L., Barcelona, Spain). Male CD1 retired breeder mice of over 3 months of age (Janvier, France) were housed individually in similar cages in preparation for their use as aggressors. All the animals were maintained in a temperature-controlled room (21±2°C) under a reversed light-dark cycle (lights off: 7:30 a.m.–7:30 p.m., local time) with food and water available *ad libitum*. Group-housed mice were marked for recognition by painting their fur with purple coloring. The animals were subjected to a stress treatment and to several behavioral tests during the dark phase of the cycle. Adequate measures were taken to minimize any pain or discomfort caused to the animals and they were evaluated daily by veterinary personnel. The experimental protocol and use of animals were in compliance with the European Community's Council Directive of 22 September 2010 (2010/63/EU) and the Spanish Real Decreto 53/2013.

Drugs

Clomipramine hydrochloride (Sigma-Aldrich Química, S.A., Madrid, Spain) was dissolved in physiological saline (0.9% NaCl) and administered i.p. in a volume of 0.01ml/g body weight. The control groups received the same volume of physiological saline.

Behavioral paradigms and apparatus

A step-through inhibitory avoidance apparatus for mice (Ugo Basile, Comerio-Varese, Italy), contained within an isolation box, was employed to evaluate memory. This cage is made of Perspex sheets and is divided into two compartments (both with a height of 15 cm, width of 9.5 cm, and length of 16.5 cm) separated by a partition with an automatically-operated sliding door. The floor is made of 48 stainless steel bars with a diameter of 0.7 mm and situated 8 mm apart. The safe compartment is white and continuously illuminated by a light fixture fastened to the cage lid (24 V, 10 W, light intensity of 290 lux at floor level, measured with the Panlux Electronic2 photometer manufactured by GOSSEN, Nürnberg, Germany), whereas the "shock" compartment is made of black Perspex panels and is maintained in darkness at all times.

An elevated plus maze for mice (Cibertec, Madrid, Spain) was applied to measure unconditioned anxiety-like behavior and locomotor activity. This apparatus consists of two open arms (30×5 cm^2 each) and two enclosed arms with walls (30×5×15 cm^3 each) that extend outwards from a common central square (5×5 cm^2). The maze is made of Plexiglas (black floor and walls) and is elevated to a height of 40 cm above floor level.

To assess nociceptive perception, we employed a hot plate (Mod. Socrel DS37, Ugo Basile, Varese, Italy) consisting of a metal plate (25×25 cm^2) located above a thermoregulator and a plastic cylinder (height 18 cm and diameter 19 cm) made of Plexiglas.

Procedure

After 10 days of acclimatization to the animal facility, mice were randomly distributed into four groups (n=12–14): NS+SAL (non-stressed+saline), NS+CLO (non-stressed+clomipramine), S+SAL (stressed+saline) and S+CLO (stressed+clomipramine).

Following a modified version of the guidelines proposed by Golden and colleagues (2011), a Chronic Social Defeat Stress (CSDS) paradigm was used as an animal model of social stress. In this paradigm, experimental male mice (stressed animals) are repeatedly subjected to bouts of social defeat by a larger CD1 mouse that has screened positive for aggressive behavior. In a pre-stress phase, CD1 retired breeders were selected as aggressors based on their attack latencies and the number of attacks they had launched during a 3-day screening procedure (attack latencies shorter than 60 s and 3 or more attacks in 3 min were the aggressor inclusion criteria). A total number of 28 aggressors were finally selected in this pre-stress phase. In the CSDS phase, the stressed groups were submitted to a daily 10-min social defeat experience by a larger and aggressive mouse on 20 consecutive days. In the agonistic encounters, each stressed mouse (also called the intruder) was placed in the home cage of an unfamiliar male (the aggressor, also called the resident). All the residents rapidly recognized and launched a first attack against the intruder within the 2 first minutes. Once the experimental mouse had been physically stressed by defeat during a 10-min period, both animals (intruder and resident) were maintained in sensory contact for 1 h by means of a clear perforated Plexiglas divider that divided the resident home cage into two halves. Subsequently, the intruder was returned to its home cage. In each subsequent defeat, experimental mice were exposed to a novel resident mouse. Just before each agonistic encounter, all stressed animals were injected daily i.p. with saline or clomipramine (10 mg/kg) according to their experimental condition. Non-stressed mice (NS+SAL and NS+CLO groups) were not submitted to any social exposure, and the only manipulation they underwent was the daily pharmacological treatment. The body weight of all animals was monitored before and after the CSDS procedure. Taking into account that social defeat involves physical aggression and wounding, defeated mice were evaluated daily by veterinary personnel. All the encounters were supervised by a blind experimenter and none needed to be terminated due to excessive aggression. Nevertheless, after successive encounters, and in accordance with criteria of the veterinary personnel in our laboratory, two intruders were removed from the experiment and immediately euthanized because of excessive wounding, as indicated in the protocol of Golden and others (2011).

Twenty-four hours after the last CSDS session, all mice were submitted to a one-trial step-through inhibitory avoidance task, which was the main behavioral test employed in the study. This task consisted of two phases: training and test. The training phase began with a 90-s adaptation period in the light compartment of the apparatus. Following this, the door between the compartments was opened and the time taken to enter the dark compartment – defined as latency – was automatically measured in tenths of a second and manually recorded. The mouse was allowed to remain in the light compartment for a maximum of 300 s after the door had opened. As soon as the animal entered the dark compartment, the sliding door was closed and a foot-shock (0.3 mA for 5 s) was delivered through the grid floor. The test phase took place one week later, following the same procedure as in the training phase, with the exception that no shock was delivered.

Twenty-four hours after the test phase of the inhibitory avoidance task, control and stressed mice were evaluated in an elevated plus maze in a complementary behavioral test, as in previous studies carried out in our laboratory (e.g. Vinader-Caerols et al. 2006, Monleón et al. 2015, 2016). This task consisted of a 5-min session that began by placing the mouse in the central square (facing one of the open arms). All sessions were recorded with a video camera (Sony DCR-SR35) for subsequent analysis. The number of entries into the open and closed arms (entry is defined as all four paws being placed on an arm) was scored by a trained observer who was unaware of the treatment applied. Based on former studies (Lister 1987, Rodgers and Johnson 1995, Rodgers and Dalvi 1997, File 2001, Campos et al. 2013, Sestakova et al. 2013), these scores provide an uncontaminated measurement of locomotor activity through the number of closed arm entries, and one primary anxiety index through the percentage of open arm entries (the lower the score, the higher the anxiety).

Twenty-four hours after the elevated plus maze test, all mice were evaluated on a hot plate as a second complementary behavioral test. The metal plate was heated through a thermoregulator to a fixed temperature of 55°C (the surface temperature was continuously monitored). Each mouse was

placed on the hot plate inside a plastic cylinder to confine it to the heated surface. The latency to the lifting of one or both hind paws was recorded in seconds (s) and provided a nociceptive measurement (the lower the score, the higher the nociception). The animals that failed to lift their paws within 45 s were removed from the plate (to avoid thermal injury) and were assigned a response latency value of 45 s.

Statistical analyses

Inhibitory avoidance data were transformed into proportion (p=x/300) values and then to arc sin (arc sin √p) values according to Snedecor and Cochran (1980). This transformation is appropriate when a cut-off time is applied, and crossing latencies that exceed this limit are interpreted as the maximum trial length. Therefore, all latencies are transformed into a percentage or proportion values, and these percentages (p) are then transformed to arc sin (degree) values (according to the formula: arc sin √p) prior to statistical analysis and graphical constructions. A repeated measures analysis of variance (rANOVA) was then performed, with "Stress" (non-stressed and stressed) and "Drug" (saline and clomipramine) as between-subjects factors and "Phase" (training and test) as a within-subject factor (the repeated-measure factor). Two-way ANOVAs for training and test phases were also performed separately. Training and test sessions were compared within the same group using the Student's t test for dependent samples.

After checking that data fulfilled the criteria for normality and homogeneity, two-way ANOVAs were also carried out for the anxiety, locomotor activity and nociception data obtained in the elevated plus-maze and hot plate tests. The *p* value for statistical significance was set at $p<0.05$.

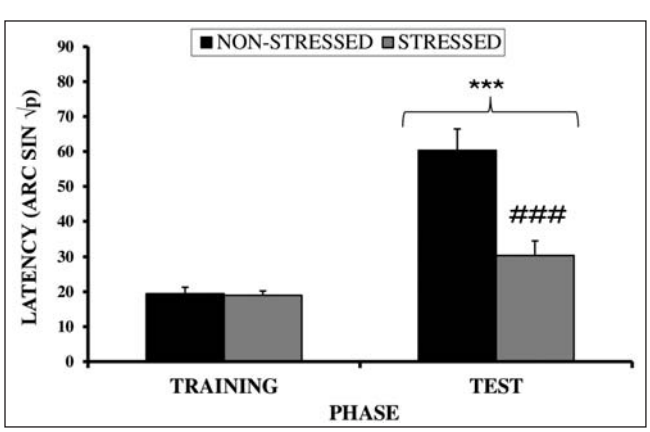

Fig. 1. Effects of CSDS on training and test latencies of post-pubertal male CD1 mice in an inhibitory avoidance task (non-stressed group: n=26; stressed group: n=25). Values are expressed as means (+SEM) of square root of proportions (p=x/300) transformed to arc sin. ***$p<0.001$ *vs.* TRAINING; ###$p<0.001$ *vs.* NON-STRESSED.

All analyses were performed using the "SPSS" software package, version 22 for windows by IBM Corp (2010).

RESULTS

Inhibitory avoidance

The rANOVA revealed that the main factor Phase was statistically significant ($F_{1,47}$=53.25, $P<0.001$), with test latencies being longer than training latencies. The main factor Stress ($F_{1,47}$=12.11, $P<0.001$) was also statistically significant, with stressed animals showing significantly lower escape latencies than non-stressed subjects. The interaction Phase X Stress was statistically significant ($F_{1,47}$=16.53, $P<0.001$), showing that there were differences in escape latencies between non-stressed and stressed mice in the test phase but not in the training phase (see Fig. 1). Neither the main factor Drug ($F_{1,47}$=0.43, n.s.) nor any interaction involving this factor was statistically significant (Phase X Drug: $F_{1,47}$=0.52, n.s.; Stress X Drug: $F_{1,47}$=0.03, n.s.; Phase X Stress X Drug: $F_{1,47}$=0.06, n.s.).

Training

The two-way ANOVA for the training phase showed that the factors Stress and Drug were not statistically significant ($F_{1,47}$=0.04, n.s.; $F_{1,47}$=0.01, n.s.; respectively), and neither was the interaction Stress X Drug ($F_{1,47}$=0.51, n.s.).

Test

The two-way ANOVA for the test phase revealed that the main factor Stress was statistically significant ($F_{1,47}$=18.62, $P<0.001$), with stressed animals showing significantly

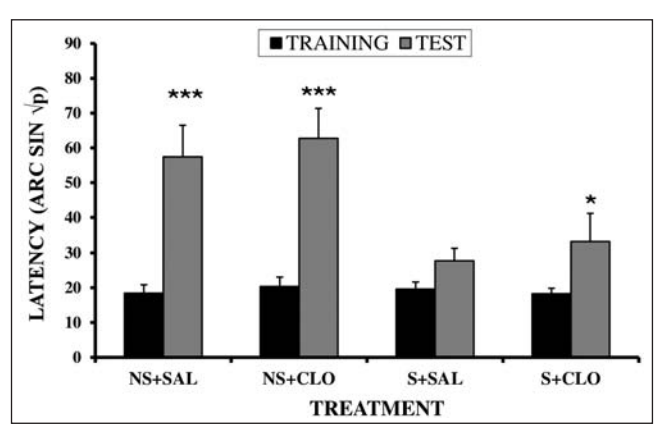

Fig. 2. Effects of CSDS and clomipramine on training and test latencies of post-pubertal male CD1 mice in an inhibitory avoidance task. NS+SAL=non--stressed+saline group (n=12); NS+CLO=non-stressed+clomipramine group (n=14); S+SAL=stressed+saline group (n=13); S+CLO=stressed+clomipramine group (n=12). Values are expressed as means (+SEM) of square root of proportions (p=x/300) transformed to arc sin. *$p<0.05$, ***$p<0.001$ *vs.* TRAINING.

lower test latencies than non-stressed subjects (see Fig. 1). Neither the factor Drug nor the interaction Stress X Drug was statistically significant ($F_{1,47}$=0.39, n.s.; $F_{1,47}$=0.01, n.s.; respectively).

Training vs. test

Inhibitory avoidance learning (significantly longer test latencies than training latencies) was observed in NS+SAL (P<0.0001), NS+CLO (P<0.0001) and S+CLO (P<0.05). However, inhibitory avoidance was absent in the S+SAL group (P>0.05) (see Fig. 2).

Elevated Plus Maze

Locomotor activity (number of entries in enclosed arms)

Stress and Drug were not statistically significant ($F_{1,47}$=1.02, n.s.; $F_{1,47}$=2.75, n.s.; respectively), and neither was the interaction Stress X Drug ($F_{1,47}$=0.05, n.s.) (see Fig. 3).

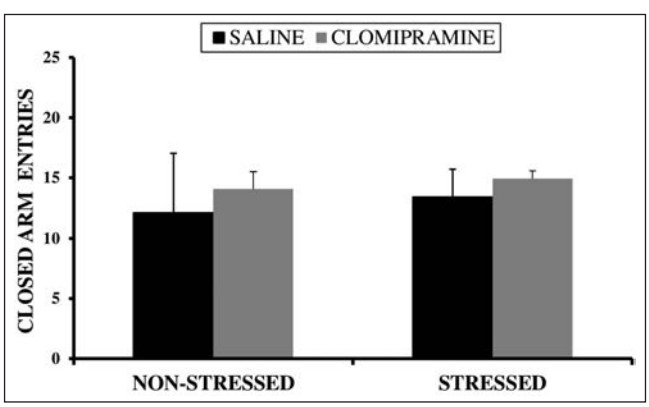

Fig. 3. Effects of CSDS and clomipramine on locomotor activity (number of closed arm entries) in post-pubertal male CD1 mice in an elevated plus maze task (n=12–14 per group). Values are expressed as means (+SEM).

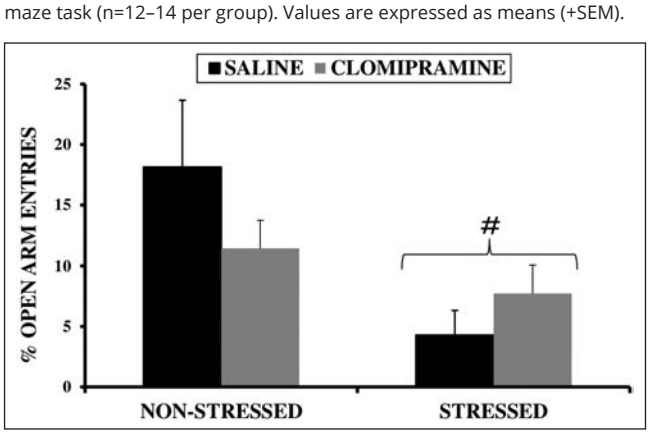

Fig. 4. Effects of CSDS and clomipramine on anxiety (percentage of open arm entries) in post-pubertal male CD1 mice in an elevated plus maze task (n=12–14 per group). Values are expressed as means (+SEM). #p<0.05 *vs.* control (NON-STRESSED groups).

Anxiety (percentage of open arm entries)

The two-way ANOVA showed significant differences in the measurement of anxiety, with the main factor – stress – proving to be statistically significant ($F_{1,47}$=7.3, P<0.05) and significantly higher anxiety (lower scores) being detected in stressed *vs.* non-stressed subjects (see Fig. 4). Neither the factor Drug nor the interaction Stress X Drug was statistically significant ($F_{1,47}$=0.25, n.s.; and $F_{1,47}$=2.41, n.s.; respectively).

Hot plate

No statistically significant differences were observed for either of the main factors – Stress or Drug ($F_{1,47}$=0.14, n.s.; $F_{1,47}$=0.01, n.s.; respectively) – or their interaction ($F_{1,47}$=0.41, n.s.) (see Fig. 5).

DISCUSSION

The aim of the present study was to investigate the effects of the tricyclic antidepressant clomipramine on IA impairment produced by CSDS in post-pubertal male CD1 mice. Overall, stressed subjects performed the IA task more poorly than non-stressed animals, regardless of whether they received saline or clomipramine treatment; and all the groups, with the exception of the non-drugged stressed animals (S+SAL group), showed IA learning.

In accordance with reports of the behavioral deficits, especially on memory, produced by CSDS in other paradigms (Ohl and Fuchs 1998, 1999, Touyarot et al. 2004, Wang et al. 2011, Patki et al. 2013), we have observed that CSDS impaired IA conditioning in post-pubertal mice: stressed subjects exhibited lower test latencies than non-stressed animals, which means that being submitted to CSDS had detrimental effects on their memory. Moreover, only

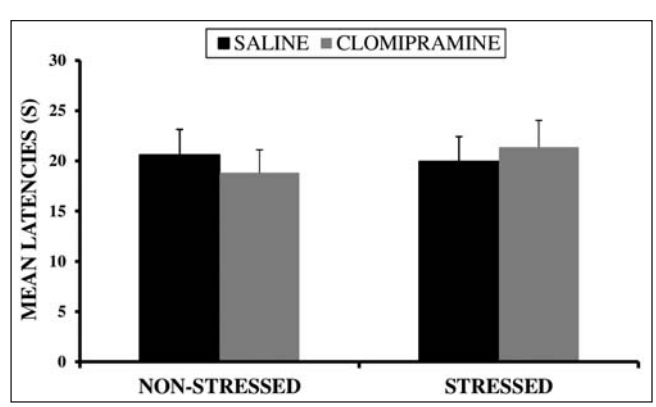

Fig. 5. Effects of CSDS and clomipramine on latencies to lift hind paws in post-pubertal male CD1 mice in a hot plate task (n=12–14 per group). Values are expressed as means (+SEM).

non-drugged stressed animals failed to exhibit IA learning, unlike animals in the other groups, in which IA conditioning was confirmed. In relation to the memory processes most affected by CSDS, and taking into account that social stress was implemented before the memory task, we suspect that CSDS impaired mainly the memory acquisition process. Consolidation and retrieval memory processes were somehow less or not affected by CSDS, as social stress was no longer present (i.e. no agonistic encounters took place between training and test phases of the memory task). In this way, it could be said that CSDS impaired the learning rather than the memory of this task. The present results are also consistent with previous findings in our laboratory using the same degree of CSDS and the same behavioral task (but without pharmacological treatment), in which CSDS produced impairing effects on IA (Monleón et al. 2015, 2016). Several explanations in the neurobiology of these effects have been proposed, such as neuritic atrophy, reduced neurogenesis and decreased neurotrophin levels in the hippocampus, as well as changes in the levels of brain-derived neurotrophic factor within the basolateral amygdala (Krishnan 2014). Actually, this structure is activated during emotional experiences that the individual perceives as anxiety, fear, stress and rage (Rogan and LeDoux 1996). The amygdala, an important brain region involved in the control of affective stimuli (Lehner et al. 2010), including those related to the emotional memory of IA, is possibly also the most important structure affected by the effects of CSDS on IA learning. It is well known that some emotional experiences mediated by the activation of amygdala are better remembered (e.g. McGaugh 2002, 2004, Wolf 2008). Nevertheless, it is also possible that this activation by CSDS is focused on fear-inhibiting pathways (Ehrlich et al. 2009), which prevented IA learning in the S+SAL group in our study.

The antidepressant clomipramine has been widely used to treat psychiatric disorders. Besides its use in depression, it is a reference treatment for anxiety disorders such as obsessive-compulsive disorder and panic disorder (Calegari et al. 2007). Unlike non-drugged stressed animals (S+SAL group), our stressed mice receiving clomipramine (S+CLO group) learned the IA task, which proves that chronic clomipramine treatment is effective in reversing the memory impairment produced by CSDS.

There are reports in the literature that pre-training administration of acute high doses (40 mg/kg) of clomipramine have detrimental effects on IA learning in mice, whilst chronic administration of lower doses (15 mg/kg) improves this kind of learning in rats (see Monleón et al. 2008). In the present study, chronic administration of clomipramine (10 mg/kg) did not have a significant effect when administered to non-stressed animals, but it counteracted the adverse effects of CSDS on memory when administered to defeated animals. It has also been repeatedly observed that infusions of norepinephrine into the amygdala enhance memory consolidation of a wide variety of training experiences (e.g. Hermans et al. 2014). Taking into account that the amygdala is one of the main brain structures involved in the IA task, and that clomipramine, though more selective for serotonin reuptake inhibition, is involved in norepinephrine reuptake inhibition, it is reasonable to think that this drug is capable of reversing the negative effects produced by CSDS in the IA task. Indeed, given that stress is thought to induce a broad spectrum of behavioral changes considered as important analogues of depressive symptoms in both humans (e.g. Lavergne and Jay 2010) and animals (e.g. Gardier 2009, Lee and Kim 2010), it is not surprising that the antidepressant clomipramine counteracts memory impairment produced by CSDS.

Spontaneous locomotor activity in our stressed mice was not affected by CSDS. Several studies have found that defeated animals commonly show a decrease in motor activity, with this hypo-activity being reported in several species, including rats (e.g. Watt et al. 2009), tree shrews (e.g. Van Kampen et al. 2000) and mice (e.g. Iñiguez et al. 2014). In contrast, other studies have reported no significant decrease in activity (Monleón et al. 2015, 2016) or hyperactivity (Venzala et al. 2013). The lack of motor effects observed in the present study was evident not only in the elevated plus-maze task (in which no differences were observed in the number of entries into the closed arms), but also in the IA task, as no statistically significant differences were observed between stressed and control mice in terms of their training latencies. Therefore, it can be concluded that the effects of CSDS on memory are not secondary to the motor effects of stress.

In the present study, CSDS led to changes in the anxiety measure between stressed and non-stressed animals, with socially defeated mice showing anxiety-like behavior (i.e., lower percentage of open arm entries). Some studies have found that social stress enhances anxiety-like behaviors of rats (Haller and Halasz 2000) and mice (Bahi 2013, Monleón et al. 2015, 2016) in the elevated plus-maze test, although others have failed to detect such an enhancement (Martinez et al. 1998). Anxiety could act as a confounding factor in the effects of CSDS on memory, which means that the higher level of anxiety/fear of stressed animals observed in the present study would have resulted in these subjects taking longer to enter the dangerous compartment; as a consequence, their IA performance would have improved. However, our stressed mice did not display IA learning. Thus, the effects of CSDS on memory would appear not to be secondary to its effects on anxiety, as occurs in the case of its motor effects. We observed no effects of chronic clomipramine treatment in either stressed or non-stressed animals in the elevated plus maze in the present study. It has been reported that the effects of clomipramine on heart rate, a physiological

anxiety measure, depend on the dose administrated (Frank et al. 2006). Therefore, it is reasonable to suspect that the lack of effects of clomipramine in reducing the anxiety symptoms of our stressed subjects was due to the dose of clomipramine administered.

It is important to confirm that CSDS does not increase animals' innate fear of light environments, as this could also act as a confounding factor in the effects of social stress on IA performance (an increased fear of light would lead to shorter escape latencies). This can be checked by a Light/Dark transition test (without shock). Although such a Light/Dark test was not included in the present study, it can be argued that, if CSDS had increased the animals' innate fear of light, stressed subjects would have displayed shorter latencies than non-stressed mice in the training phase of the IA task (not only in the test phase). However, no significant differences in training latencies were observed between groups. Therefore, we believe that this potential confounding factor can be ruled out from our study.

A reduced pain perception is a classic effect produced by a variety of acute stressors in humans and animal models (Miguez et al. 2014). However, stress is reported to inhibit or exacerbate pain perception depending on the nature and/or parameters of the stressor (Butler and Finn 2009). This dual action of stress on pain modulation depends to a great extent on pre-existing conditions, in particular previous pain experience associated with methodological factors, along with previous adverse life events (Larauche et al. 2012). In our study, pain sensitivity in chronically defeated mice was indistinguishable from that in non-defeated controls. Therefore, similarly to the rationale applied to motor and emotional effects, we consider that the effects of CSDS on memory are not secondary to its effects on pain sensitivity.

As with any research, our study has limitations and strengths. From our point of view, the main limitation of the present experiment is the use of a single dose of clomipramine, as testing a wider range of doses would have been ideal. We selected a single dose of clomipramine due to limitations in our laboratory in terms of the facilities and resources necessary for submitting such a number of animals to CSDS in an experimental design administrating several doses of drug. The specific dose of clomipramine we have employed was based on previous studies in which pre-training administration of a rather low dose of clomipramine improved IA, whilst high doses had detrimental effects, as mentioned above. With respect to the strengths, the present work represents a step forward in the study of pharmacological treatment of the effects of CSDS on memory. Our results show that treatment with clomipramine is effective in attenuating CSDS-induced memory impairment. Importantly, we have controlled several potential confounding factors (locomotor activity, emotionality and pain sensitivity) through complementary tasks.

CONCLUSIONS

The present study shows that: (i) CSDS impairs emotional memory involved in inhibitory avoidance in post-pubertal mice; (ii) the memory impairment produced by CSDS is slightly attenuated by a low dose (10 mg/kg) of the tricyclic antidepressant clomipramine; (iii) the detrimental effects of CSDS on emotional memory do not seem to be secondary to the effects of stress on locomotor activity, emotionality or pain sensitivity; and (iv) this work provides further evidence that CD1 is a valid strain of mice for use as stressed subjects in the CSDS protocol.

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