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Research Report

Effects of loud noise on hippocampal and cerebellar-related behaviors. Role of oxidative state

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ABSTRACT

Living organisms are exposed to potentially hazardous noise levels coming from the environment. Besides the direct effect on hearing, extra-auditory noise-associated effects should be considered. Since loud noise has been suggested to induce central nervous system symptoms, the aim of the present work was to investigate the effect of acute (ANE) and chronic noise exposures (CNE) on different behavioral tasks. To understand the mechanisms involved, levels of oxidative status markers were determined in two areas related to memory processes, the hippocampus (Hip) and the cerebellum (CE). 15-day-old male Wistar rats were exposed to loud noise (95–97 dB, 2 h/day), at ANE or CNE. At 30 days, rats were subjected to different CE and Hip-related behavioral tasks. Reactive oxygen species (ROS) levels and antioxidant enzyme activities (CAT and SOD) were also assessed. Results show impairments in spatial and associative memory in noise-exposed animals. Moreover, a decrease in anxiety levels and an increase in habituation memory were observed in CNE animals. While an increase in cerebellar ROS levels was found early after the first noise exposure, a decrease was found in the CE and the Hip at 30 days. The activity of hippocampal CAT was increased early and remained high in ANE rats, while it was unchanged in the CE. Finally, although SOD activity was decreased immediately after the first noise exposure, its levels were increased at 30 days in ANE rats. In summary, the present study shows that an imbalance in oxidative status induced by noise exposure could underlie behavioral changes, some of which would be long-lasting.

1. Introduction

During daily life, people are exposed to potentially hazardous noise levels coming from work environment, urban traffic, household appliances or discotheques (Frenzilli et al., 2004). High levels of occupational noise become a problem in all regions of the world. In the United States of America, for example, more than 30 million workers are exposed to hazardous noise (NIOSH, 1998). In addition, the World Health Organization (WHO, 1999) estimates that approximately 20% of the European population are exposed to noise generated by urban traffic above 65 dB. This level is considered a maximum safety threshold and might be responsible for several disorders that affect not only the auditory organs, but also structures belonging to the nervous, endocrine, and cardiovascular systems (Lenzi et al., 2003). Therefore, although the auditory effects of noise have been long studied, they do not appear to be the only factors responsible for many of the

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effects of noise on living organisms. In fact, noise-associated disorders are mainly extra-auditory (Lenzi et al., 2003; Rabat, 2007) and become evident after exposure to noise level as low as 50 dB. In addition, if the absolute difference between peak and background levels exceeds 30 dB, different types of disturbances might take place (WHO, 1999).

Epidemiological and laboratory studies involving both workers exposed to occupational noise and individuals of the general population – including children – living in areas around airports, industries and noisy streets, indicate that noise may have both temporary and permanent impacts on different body functions. Acute noise exposure can activate the autonomic and endocrine systems and can lead to usually transient changes, such as increased blood pressure and heart rate, together with vasoconstriction and sleep disturbances (Gitanjali and Ananth, 2003). On the contrary, after a prolonged exposure, susceptible individuals may develop permanent effects, such as hypertension, ischemic heart disease, as well as memory deficits (Turner et al., 2005).

The adverse effects of environmental noise on human mental health have been reported by several authors. In particular, it has been suggested that loud noise can induce a variety of symptoms, including changes in anxiety, emotional stress, increase in social conflicts, as well as general psychiatric disorders (Rabat, 2007). Similarly, animal experiments demonstrated that acute and chronic noise exposures can also induce temporary or permanent changes related to the central nervous system (CNS) (Ising and Braun, 2000; Manikandan et al., 2006; Goble et al., 2009).

Certain environmental challenges can increase the production of reactive oxygen species (ROS) in different structures, which may override the cellular antioxidant defenses and can lead to oxidative stress (Vicente et al., 2004; Guelman et al., 2005; Sathyasaikumar et al., 2007; Di Toro et al., 2007; Caceres et al., 2009). Interestingly, the abundance of polyunsaturated fatty acids and the low-level of defensive mechanisms, together with the high oxygen consumption, make the brain more susceptible to oxidative damage than other organs. In particular, after experimental noise exposure in laboratory animals, superoxide anion radicals emerge in the stria vascularis (Yamane et al., 1995), hydroxyl radicals significantly increase in the cochlea (Ohlemiller et al., 1999), hydrogen peroxide-induced cell damage to the inner ear occurs *in vitro* (Dehne et al., 2000), glutathione increases in the lateral wall (Yamasoba et al., 1998) and glutathione peroxidase and malondialdehyde activities increase progressively with noise intensity in hair cells (Yamashita et al., 2004). Although experimental data show that loud noise can increase ROS in the auditory pathway (Yamashita et al., 2004; Pouyatos et al., 2005; Le Prell et al., 2007), reports concerning the influence of noise stress on oxidative status in extra-auditory CNS structures are scarce (Turner et al., 2005; Manikandan et al., 2006; Rabat, 2007). In addition, experimental data in developing animals are lacking. In consequence, since the tonotopic organization of the auditory cortex is much more susceptible to perturbations of acoustic inputs in infancy than in older animals, as suggested by Wang studies (2004), the concept of a 'critical' or sensitive period of development after which plasticity is more restricted could be tested by exposing immature animals.

The hippocampus (Hip) is a structure involved in various memory processes that are essential for creating new memories (Izquierdo and Medina, 1997; Davidson and Jarrard, 2004; Kosten et al., 2007). In addition, the involvement of the cerebellum (CE) in spatial memory has been postulated (Mandolesi et al., 2001, 2007). Since lesions to the Hip and the CE have shown to impair learning and memory in a variety of behavioral paradigms (Jarrard, 1993; Hall et al., 1996; Le Marec et al., 1997; Mandolesi et al., 2001; Davidson and Jarrard, 2004; Kosten et al., 2007; Caceres et al., 2009, 2010), it would be hypothesized that the finding of CE and/or Hip-related behavioral disturbances in noise-exposed animals may be related to a primary damage to the CE and/or the Hip. Further, oxidative stress has been implicated in a number of neurodegenerative diseases (Cassarino and Bennett, 1999) as well as in neurotoxicity models (Goodlett and Horn, 2001). Since results from our laboratory and from others demonstrated that cerebellar and hippocampal oxidative stress and histological disorganization, together with behavioral alterations, are triggered after ionizing radiation exposure (Harman, 1992; Guelman et al., 2003, 2005; Limoli et al., 2004; Mecocci et al., 2004; Di Toro et al., 2007; Caceres et al., 2009), it would be suggested that the balance between oxidants and antioxidants must be maintained to minimize tissue damage and behavioral impairment.

Therefore, the aim of the present work was to investigate the effect of acute and chronic noise exposures (ANE and CNE, respectively) on different behavioral parameters. In consequence, new data about CNS susceptibility to noise damage in developing rats would be obtained through the use of cerebellar and hippocampal-dependent behavioral tasks, such as open field habituation (Vianna et al., 2000), radial maze (Dubreuil et al., 2003), elevated plus maze (Brenes et al., 2009) and inhibitory avoidance (Roozendaal, 2002) tasks. To understand the mechanisms involved, brain ROS levels and free radical scavenging enzyme activities in two areas mainly related to associative, habituation and spatial memory, as well as to anxiety Hip and CE, were determined at different developmental stages: immediately after the first noise exposure, immediately after the last noise exposure in CNE rats, and after 15 days in ANE rats.

2. Results

2.1. *Behavior*

Data show that noise-exposed rats committed more errors than control animals in the full baited radial maze (RAM) ($F_{2,148}=6.03$, $p<0.01$), both after ANE (first block, C vs. ANE, $p<0.05$) and CNE (first block, C vs. CNE, $p<0.05$; second block, C vs. CNE, $p<0.01$, Fig. 1a). In addition, CNE animals made an increased number of choices when compared with control rats ($F_{2,148}=8.88$, $p<0.01$. First block: C vs. ANE, NS; C vs. CNE, $p<0.05$; second block: C vs. ANE, NS; C vs. CNE, $p<0.01$, Fig. 1b). Interestingly, a significant increase in errors or choices made during the entire training (sum of errors made in the 10 sessions) was observed only in CNE animals (total errors: C: 32.86±4.17; ANE: 36.86±4.75; CNE: 54.33±7.21. $F_{2,29}=4.51$, $p<0.05$; C vs. ANE, NS; C vs. CNE, $p<0.05$. Total choices: C: 55.81±1.86; ANE: 56.93±3.15; CNE: 67.28±3.65. $F_{2,29}=5.22$,

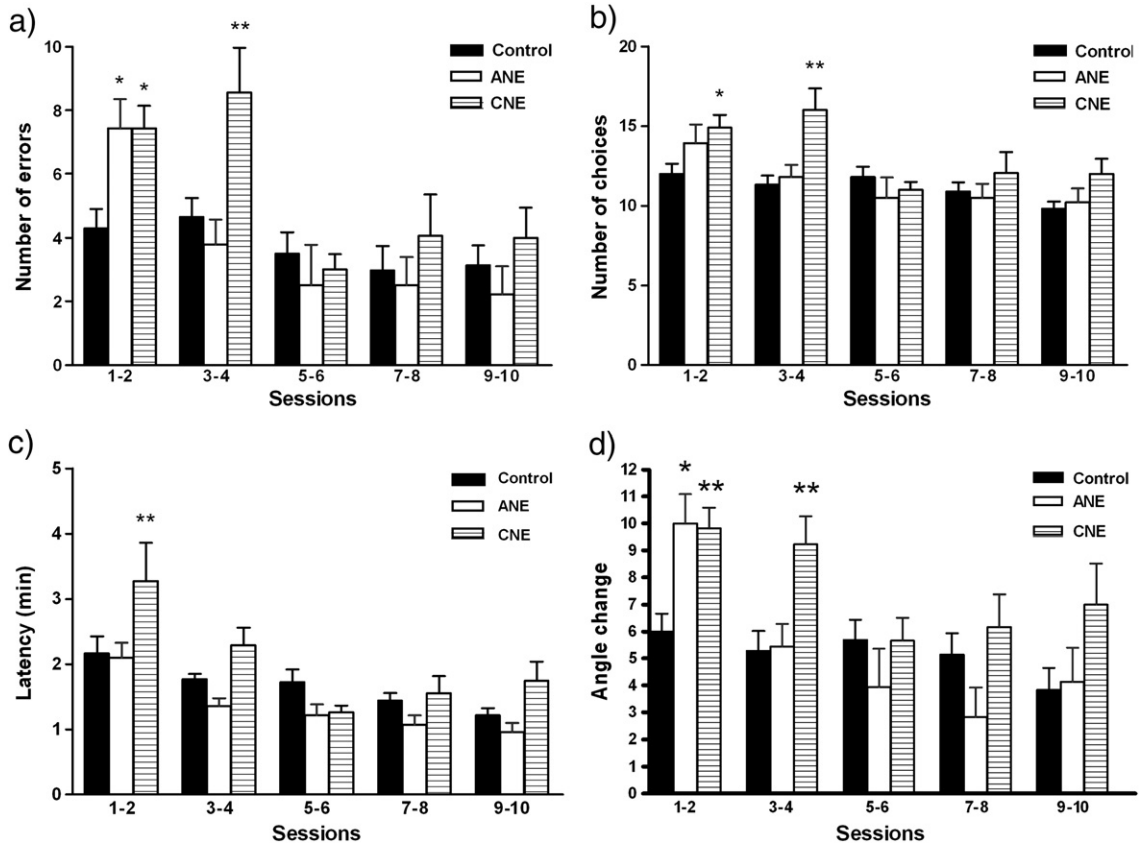

Fig. 1 – Performance of control and noise-exposed rats in full baited RAM memory task. Noise-exposed rats committed more errors (a), made an increased number of choices (b), required more time to complete the maze (c) and made more angle changes (d) in the first blocks than control animals.ANE: acute noise exposure; CNE: chronic noise exposure. Filled bars: control rats; open bars: ANE rats; stripped bars, CNE rats. *, **, $p<0.05$ and $p<0.01$ when compared with control animals. Data are mean ± SEM of the number of errors, choices, time required to complete the maze (in min) or angle changes made by control and noise-exposed animals in the full baited RAM task.

$p<0.05$; C vs. ANE, NS; C vs. CNE, $p<0.05$). Moreover, the time taken to complete the maze (latency) was increased in the first block of sessions in animals exposed for 15 days to noise ($F_{2,148}=8.64$, $p<0.01$. First block: C vs. ANE, NS; C vs. CNE, $p<0.01$, Fig. 1c). When angle change was studied, an increase was observed in noise-exposed animals in the first two blocks of the radial maze ($F_{2,148}=9.56$, $p<0.01$. First block: C vs. ANE, $p<0.05$; C vs. CNE, $p<0.01$. Second block: C vs. ANE, NS; C vs. CNE, $p<0.01$, Fig. 1d). All these parameters returned to control values as the training progressed.

No significant changes were observed either in spatial span of the last session ($F_{2,26}=1.77$, NS, data not shown) or in the percentage of 45° angles made by noise-exposed rats ($F_{2,26}=1.51$, NS, data not shown). However, while a significant correlation between the spatial span and the percentage of 45° angles in the last session was observed in control rats ($r=0.84$, $p<0.01$), no significant correlation was observed either in ANE ($r=0.7$, NS) or CNE rats ($r=0.64$, NS) (Fig. 2).

Interestingly, a significant increase in reference errors in the first three blocks was observed when noise-exposed rats were tested in the win-shift delayed RAM ($F_{2,59}=18.52$, $p<0.01$. First block: C vs. ANE, $p<0.05$; C vs. CNE, $p<0.05$. Second block: C vs. ANE, $p<0.01$; C vs. CNE, $p<0.01$. Third block: C vs. ANE, $p<0.01$; C vs. CNE, NS, Fig. 3).

Fig. 4a shows no differences among the number of lines crossed by control and exposed animals in the open field (OF) ($F_{2,22}=1.63$, NS). When we divided the 6 minute test period in two periods of 3 min to estimate habituation, we observed that all groups significantly decreased the number of lines crossed in the second period ($F_{1,45}=52.63$, $p<0.01$. C, $p<0.01$; ANE, $p<0.01$; CNE, $p<0.01$, Fig. 4b). Importantly, while the percent of decrease of the lines crossed in the second period was similar in control and ANE rats (35.42±2.86 and 29.25±4.6, respectively), a more pronounced decrease was observed in CNE animals (54.4±4.1) when compared with control animals ($F_{2,22}=10.91$, $p<0.01$; C vs. ANE, NS; C vs. CNE, $p<0.05$).

The number of total rearings was increased in CNE animals ($F_{2,22}=3.57$, $p<0.05$; C vs. ANE, NS; C vs. CNE, $p<0.05$, Fig. 5a). However, when we divided the 6 minute test period in two periods of 3 min, we observed in CNE animals an increase in the number of rearings only in the first period when compared with control animals ($F_{2,41}=4.16$, $p<0.05$; C vs. ANE, NS; C vs. CNE, $p<0.05$), while neither group decreased the number of rearings in the second period (Fig. 5b). Lastly, the total time of rearing was increased only in CNE animals ($F_{2,22}=3.71$, $p<0.05$; C vs. ANE, NS; C vs. CNE, $p<0.05$, Fig. 5c) and this increase was due to the increase in the first period ($F_{2,41}=4.158$, $p<0.05$; C vs. ANE, NS; C vs. CNE, $p<0.05$, Fig. 5d).

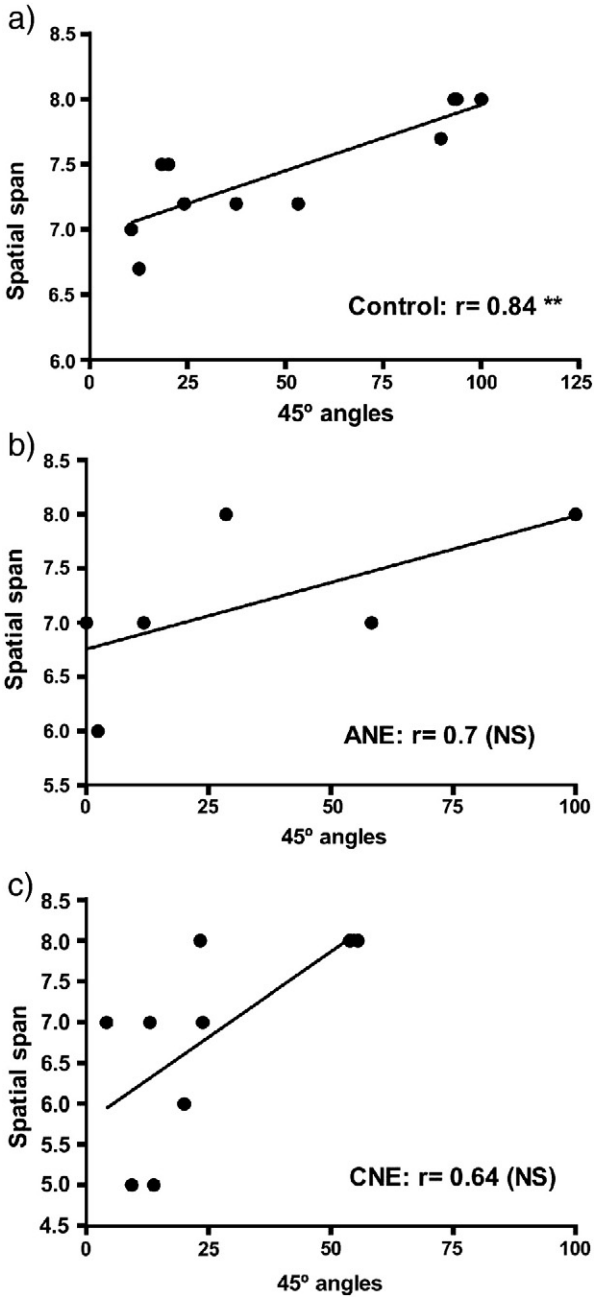

Fig. 2 – Relationship between spatial span and percentage of 45° angles in the last session in the full baited RAM task in control and noise-exposed rats. Although a significant correlation was observed in control rats (a), a lack of a significant correlation could be shown in noise-exposed animals (b and c).ANE: acute noise exposure; CNE: chronic noise exposure. R: correlation coefficient (Pearson). **, p<0.01, signification of correlation.

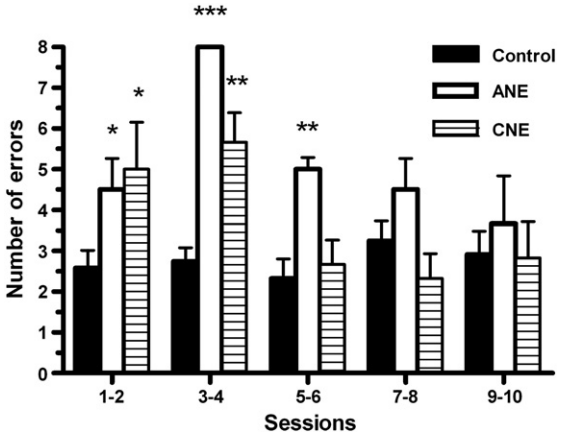

Fig. 3 – Reference errors of control and noise-exposed rats in delayed win-shift RAM memory task. Noise-exposed rats committed more reference errors in the first blocks than control animals.ANE: acute noise exposure; CNE: chronic noise exposure. Filled bars: control rats; open bars: ANE rats; stripped bars, CNE rats. *, **, ***, p<0.05, p<0.01 and p<0.001 when compared with control animals. Data are mean ± SEM of the number of reference errors made by control and noise-exposed animals in the delayed win-shift RAM task.

When total wall climbs were computed, a significant decrease was observed in CNE animals ($F_{2,22}=5.09$, $p<0.05$; C vs. ANE, NS; C vs. CNE, $p<0.05$, Fig. 5e). While no differences in the number of wall climbs were observed in control animals between the first and the second period of 3 min of the OF, the number of wall climbs significantly decreased in CNE and ANE rats among these periods ($F_{1,41}=15.92$, $p<0.01$. C, NS; ANE, $p<0.05$; CNE, $p<0.01$, Fig. 5f). Since the decrease was more pronounced in CNE animals, only this group differed statistically from control animals in the second period ($F_{2,41}=8.076$, $p<0.05$. C vs. ANE, NS; C vs. CNE, $p<0.05$).

Moreover, while total center incursions and time in center remained unaffected in exposed animals (total center incursions, $F_{2,22}=2.1$, NS; time in center, $F_{2,22}=1.1$, NS, data not shown), latency to center was decreased in CNE rats ($F_{2,22}=7.09$, $p<0.01$; C vs. ANE, NS; C vs. CNE, $p<0.01$, Fig. 6a). Finally, when noise-exposed animals were tested in the elevated plus maze (EPM), a significant increase in time spent in open arms, as well as a significant decrease in time spent in the closed arms was found only in CNE rats ($F_{2,22}=4.31$, $p<0.05$; C vs. ANE, NS; C vs. CNE, $p<0.05$, Fig. 6b).

Fig. 7 shows that noise exposure decreased the latency to enter the dark compartment in the inhibitory avoidance (IA) task when compared to control animals ($H_{2,27}=9.23$, $p<0.01$), both in ANE ($p<0.05$) and CNE rats ($p<0.01$).

2.2. *Oxidative status markers*

Table 1 shows that noise exposure induces an increase in cerebellar reactive oxygen species (ROS) levels immediately after the first day of noise exposure ($t_{14}=2.29$, $p<0.01$) and a subsequent significant decrease in 30-day-old rats, not only in ANE, but also in CNE rats ($F_{2,22}=7.56$, $p<0.01$; C vs. ANE, $p<0.01$; C vs. CNE, $p<0.01$). However, cerebellar catalase (CAT) and superoxide dismutase (SOD) levels remained unaffected in exposed rats at the stages tested.

On the other hand, while hippocampal ROS levels were unchanged immediately after the first noise exposure, a decrease in ROS levels was observed in ANE and CNE rats when tested in 30-day-old rats ($F_{2,22}=6.4$, $p<0.01$; C vs. ANE, $p<0.01$; C vs. CNE, $p<0.01$). Moreover, hippocampal CAT activity was increased and SOD activity was decreased

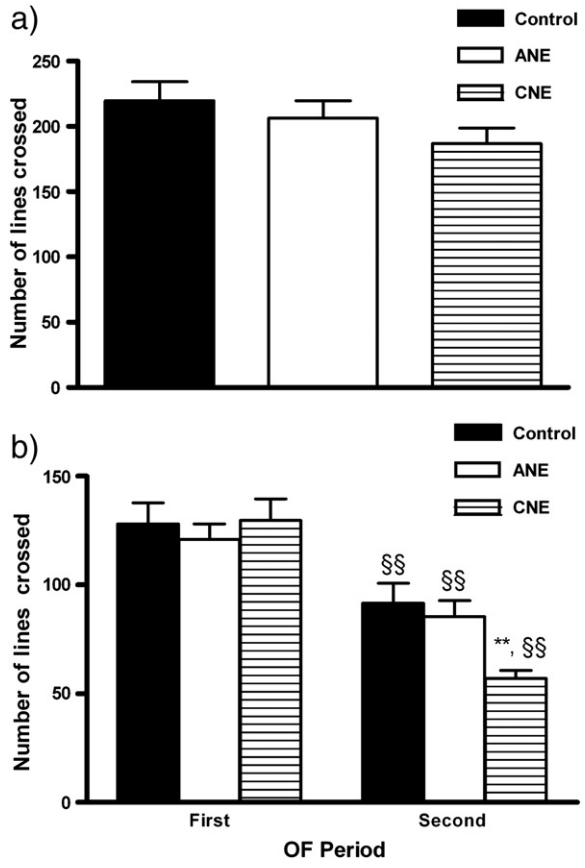

Fig. 4 – Performance of control and noise-exposed rats in the number of lines crossed in the OF task. Although the number of lines crossed through the entire period in the OF remained unchanged in all groups (a), control and noise-exposed animals decreased significantly the number of lines crossed in the second period of the OF (b).ANE: acute noise exposure; CNE: chronic noise exposure. Filled bars: control rats; open bars: ANE rats; stripped bars, CNE rats. **, $p<0.01$ when compared with control animals. §§, $p<0.01$ when compared with the respective first period of the OF. Data are mean ± SEM of lines crossed by control and noise-exposed animals in the OF task.

immediately after the first noise exposure (CAT: $t_8=2.82$, $p<0.05$. SOD: $t_{11}=5.44$, $p<0.01$), whereas their activities were increased at 30 days only after ANE (CAT: $F_{2,22}=16.83$, $p<0.01$; C vs. ANE, $p<0.01$; C vs. CNE, NS. SOD: $F_{2,24}=3.52$, $p<0.05$; C vs. ANE, $p<0.05$; C vs. CNE, NS).

3. Discussion

The extra-auditory impact of noise exposure is generally an overlooked factor in animal research. Since a key function of sensory systems input is to maintain an appropriate level of arousal, noise can affect different organ systems: from immune response to social behavior, the potential impact of environmental noise on different processes should be taken into account in order to either minimize the noise when it is excessive or to use noise experimentally to study its effects (Turner et al., 2005).

At present, the NIOSH recommends a 3-dB exchange rate for the calculation of time-weighted average (TWA) exposures to noise, being the noise levels used in the present work (95–97 dB, 2 h) equivalent to 92–94 dB for 4 h or 89–91 dB for 8 h. Since 94 dB has been reported to be harmful in different models (Ising and Braun, 2000; Mahendra Prashanth and Sridhar, 2008), noise levels reported in the present paper appear to be relevant to human exposure.

The present results show that a transient impairment in spatial memory performance was produced in 30-day-old animals exposed to 95–97 dB of white noise, both in ANE and in CNE rats, together with impaired inhibitory avoidance performance. In addition, an increase in habituation memory was found in CNE rats, together with decreased anxiety levels. On the other hand, an imbalance in oxidative status markers was found in the CE and the Hip of noise-exposed rats, although a differential vulnerability was observed in these structures.

Evidence from different animal models demonstrate the existence of two windows of increased susceptibility, early and late in life (Ohlemiller, 2006), supporting the high vulnerability of developing rodents to noise exposure reported in this paper. Moreover, the period comprising from adolescence until early adulthood seems to be the most sensitive (Henry, 1983).

The increase in spatial (the number of errors), exploratory (choices and the time taken to visit all bitted arms) and procedural (angle change) parameters in the full baited task in the radial maze, observed in noise-exposed rats only in the first two blocks of sessions is confirmed by the finding of increased reference errors in the win-shift delayed maze in the same blocks. These observations suggest that noise might induce a transient spatial memory deficit. Therefore, the increase in total (sum of 10 sessions) errors and choices observed in CNE rats might be attributed to the increase observed in the first 4 sessions.

The normalization observed after removal of the stimulus (ANE rats) as well as even though the acoustic stimulus was still present (CNE rats) supports the hypothesis of adaptability triggered by different types of environmental stressors (Grissom and Bhatnagar, 2009). These observations are supported by the results from Cui et al. (2009), which reported transient alterations in spatial memory after chronic noise exposure.

The spatial span in the last session is an index of spatial working memory abilities and the percentage of 45° angles is an index of the use of procedural or response strategies. Our finding of a significant correlation between spatial span and the percentage of 45° angles made in the last session could indicate the use of working memory and procedural components in the spatial task (Caceres et al., 2009), as found in control animals. However, a lack of correlation between spatial span and the percentage of 45° angles in the last session was found in ANE and CNE animals, demonstrating an enduring disruption of spatial working memory to solve the maze.

The full baited task of the RAM does not discriminate between spatial and response strategy components of spatial learning. In addition, it could be possible that rats adopt response strategies without involving a significant mnemonic

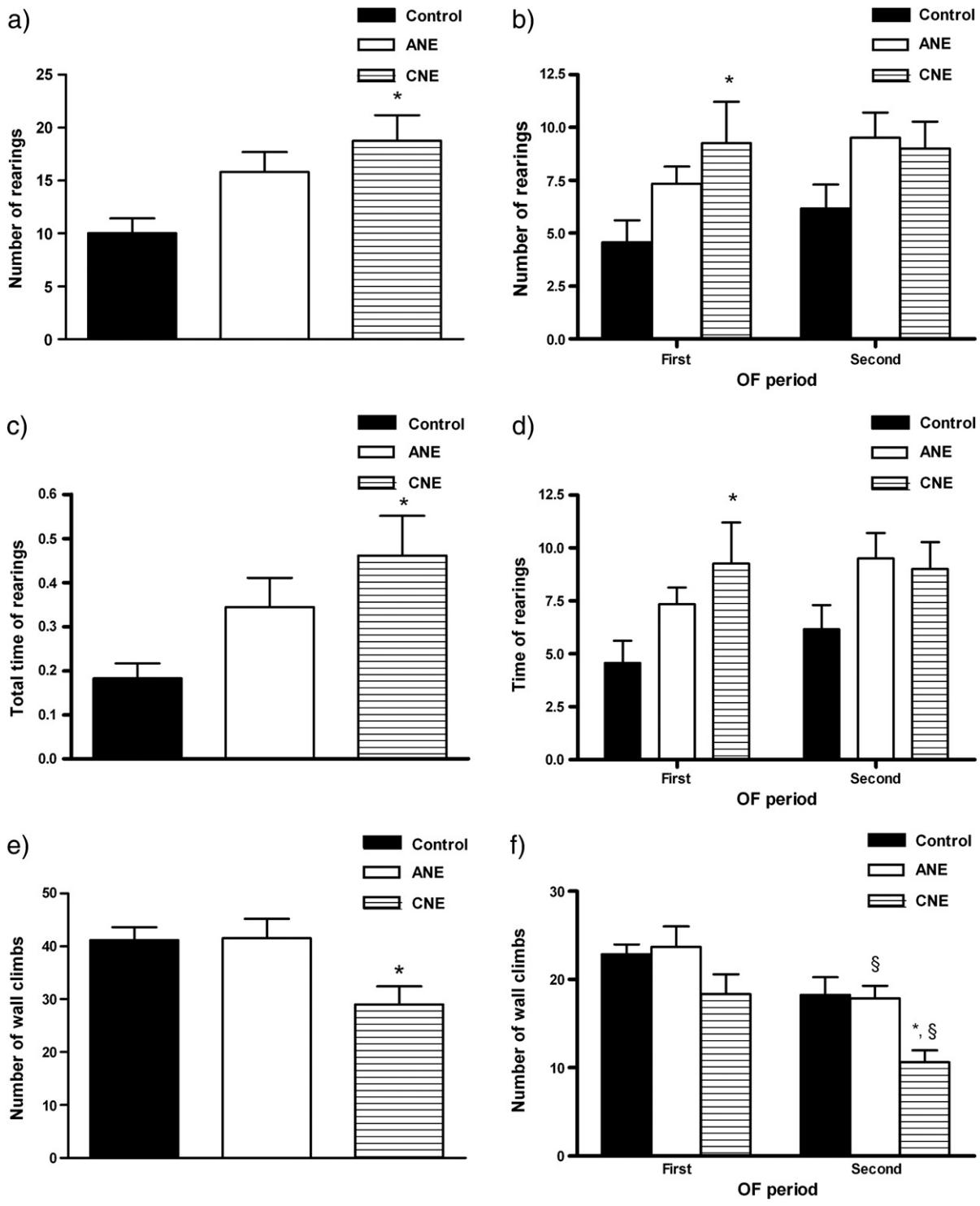

Fig. 5 – Performance of control and noise-exposed rats in the number of rearings, total time of rearings and wall climbs made in the OF task. CNE rats significantly increased total number of rearings when compared with control animals (a), due to the increase observed in the first period of the OF (b). Total time of rearing was increased only in CNE rats (c), owing to the increase observed in the first period (d). Total wall climbs made through the entire period in the OF were decreased in CNE animals when compared with control rats (e), mainly due to the decrease in the second period of the OF (f).ANE: acute noise exposure; CNE: chronic noise exposure. Filled bars: control rats; open bars: ANE rats; stripped bars, CNE rats. *, $p<0.05$ when compared with controls. §, $p<0.05$ when compared with the respective first period of the OF. Data are mean ± SEM of number of rearings, total time of rearing (in min) or wall climbs made by control and noise-exposed animals in the OF task.

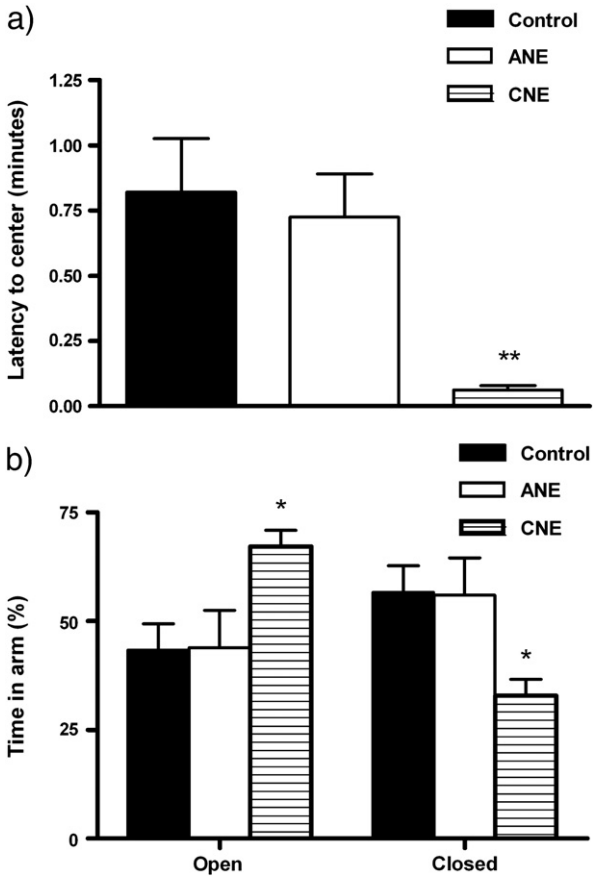

Fig. 6 – Latency to the center in the OF task and percent of time spent in the arms in EPM task, in control and noise-exposed rats. A significant decrease in the latency to center of the OF task was observed in CNE animals when compared with control rats (a). An increase in the % of time spent in open arms and a decrease in the % of time spent in closed arms were observed in CNE rats when tested in EPM task (b). ANE: acute noise exposure; CNE: chronic noise exposure. Filled bars: control rats; open bars: ANE rats; stripped bars, CNE rats. *, **, $p<0.05$ and $p<0.01$ when compared with control animals. Data are mean ± SEM of the latency to center (in min) of control and noise-exposed animals in the OF task or the % of time spent in open or closed arms in the EPM task.

process. For this reason, the win-shift delayed task was used to better isolate spatial working memory component from response strategy component, since in this paradigm the angles used by the animals are non-45° and allow evaluating only the spatial strategy component. Results show that ANE and CNE rats made more reference errors than control animals in the first few blocks, supporting the hypothesis of a transient deficit in the spatial strategy component. Therefore, it would be suggested that noise exposure induces a spatial memory deficit, involving both response and spatial strategies. Moreover, the decreased latency of noise-exposed rats to enter the dark compartment in the IA test suggests a disruption in Hip-dependent associative memory, which seems to be long-lasting, since this alteration was observed even in ANE rats, which were tested 15 days after single exposure. Since the Hip is known to be involved in spatial

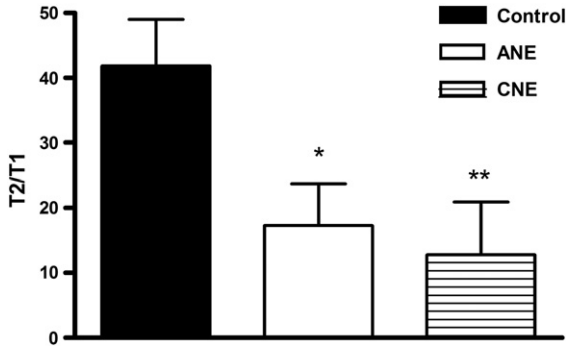

Fig. 7 – Latency to enter the dark compartment of control and noise-exposed animals in the IA task. A significant decrease in the latency to enter the dark compartment was observed in noise-exposed animals when compared with control rats. ANE: acute noise exposure; CNE: chronic noise exposure. Filled bars: control rats; open bars: ANE rats; stripped bars, CNE rats. *, **, $p<0.05$ and $p<0.01$ when compared with control animals. Data are mean ± SEM of the latency to enter the dark compartment (in min) of control and noise-exposed animals in the IA task.

navigation (Roozendaal, 2002; Izaki et al., 2008) as well as in IA task (Izquierdo and Medina, 1997; Moshfegh et al., in press) and the CE would be involved in the spatial system that guides the animal from one place to another (procedural component) (Federico et al., 2006; Mandolesi et al., 2007), it would be suggested that these structures might be potential targets of noise-induced damage. Measurements of oxidative status markers in these structures confirm this hypothesis. Due to the existence of relationships between the CE, the Hip and other CNS regions, it would not be discarded that structures involved in highly emotional tasks might contribute to the behavioral alterations observed in noise-exposed animals (Izquierdo and Medina, 1997; Zinn et al., 2009).

The greatest decrease in the number of lines crossed in the last period of the OF observed in CNE rats suggests that these rats are more able to habituate to the environment than control or ANE rats. Interestingly, considering the OF as a stressful environment, it would be suggested that previous exposure to chronic noise might predispose the animals to better cope with the stressful environment. The increase in habituation memory observed in CNE rats demonstrates that a stressful experience is not uniformly health impairing (McEwen and Gianaros, 2011). Therefore, meeting the demands imposed by stressful experiences can lead to adaptation and beneficial forms of learning that promote resiliency. By contrast, other stressful experiences can foster behavioral changes that increase vulnerability. Results from IA experiments support this statement and suggest that the performance in this task would be impaired by the decreased hippocampal ROS levels, being these species part of the key mediators of associative memory (Jung et al., 2004).

On the other hand, since a decrease in the latency to center was observed in CNE rats in the OF, it would be suggested that a reduced anxiety-like behavior could have been triggered in chronic noise-exposed rats as a way to cope with this stressor. Moreover, EPM results support OF data, confirming that a

Table 1 – Oxidative status in Hip and CE of noise-exposed rats.

Brain region	Parameter	15 days		30 days		
		Control	Noise	Control	Acute noise (ANE)	Chronic noise (CNE)
CE	ROS	0.477 ± 0.07	0.691 ± 0.023**	0.734 ± 0.05	0.145 ± 0.012**	0.112 ± 0.024**
	SOD	0.297 ± 0.008	0.276 ± 0.0063	0.247 ± 0.026	0.211 ± 0.040	0.169 ± 0.028
	CAT	0.025 ± 0.002	0.0318 ± 0.0033	0.170 ± 0.022	0.215 ± 0.021	0.126 ± 0.016
Hip	ROS	0.297 ± 0.028	0.292 ± 0.015	0.522 ± 0.038	0.129 ± 0.012**	0.180 ± 0.022**
	SOD	0.467 ± 0.023	0.198 ± 0.036**	0.343 ± 0.039	0.458 ± 0.029**	0.364 ± 0.031
	CAT	0.0093 ± 0.00043	0.0132 ± 0.001*	0.079 ± 0.013	0.170 ± 0.011**	0.102 ± 0.009

Data are means ± SEM of the levels of ROS (pmol DCF/mg tissue/min) and the activity of the antioxidant enzyme SOD (SOD units/mg tissue) and CAT (CAT units/mg tissue) in the cerebellum (CE) and the hippocampus (Hip) of 15 and 30-day-old rats. Noise: first day of noise exposure (is shared by ANE and CNE rats). ANE: acute noise exposure; CNE: chronic noise exposure. *, **, $p<0.05$ and $p<0.01$ when compared with control animals.

reduced anxiety state was induced by chronic noise exposure, supporting the hypothesis of adaptability to a chronic stressor (Grissom and Bhatnagar, 2009). Interestingly, several authors reported that, in addition to spatial function, it seems that the CE would be involved in emotional behavior, since it has been demonstrated that cerebellar mutants exert reduced anxiety levels, since they spent more time than controls in the open arms (aversive space) of an EPM (Fiez, 1996; Tanaka et al., 2003; Hilber et al., 2004; Mandolesi et al., 2007). Therefore, cerebellar oxidative imbalance could underlie the reduced anxiety levels observed in noise-exposed animals in the present study.

Since milder behavioral changes have been observed after ANE, it would be suggested that noise should be present for longer periods to induce long-lasting behavioral damage.

Several authors reported that some hippocampal functions were altered by noise exposure (O'Keefe, 1999; Goble et al., 2009), strengthening the hypothesis that brain regions that are not part of the classical auditory pathways might be involved in the pathophysiology of some auditory diseases, such as tinnitus, and might be one of the targets for noise exposure, supporting the present data. Therefore, these alternative pathways may begin to explain affective symptoms that many patients with tinnitus have, including depression and phonophobia (Goble et al., 2009).

The negative effects of noise on cell structure and function could be, at least in part, mediated by the increase in ROS (Lenzi et al., 2003). ROS levels in the cochlea were found to be significantly higher 1 h after exposure to 110 dB noise and persisted after the cessation of the exposure (Ohlemiller et al., 1999). The early increase in cerebellar ROS levels observed in noise-exposed animals in the present work supports these results. In contrast, the unchanged levels of hippocampal ROS immediately after first noise exposure might be the result of the observed imbalance in CAT and SOD levels in this tissue. The decrease of ROS levels in 30-day-old ANE and CNE rats, both in the CE and the Hip, which persisted even after the cessation of the stimulus (ANE rats), might be explained based in a report of Owusu-Ansah and Banerjee (2009), which hypothesized that keeping ROS levels slightly elevated puts the cells on alert, sensitized and ready to respond to any threat quickly. Although it would be thought that having low ROS levels would be a good thing, the decrease in ROS levels observed in the CE and the Hip after noise exposure might reflect the *inability* of these rats to cope with environmental changes. Namely, having low ROS levels seems to prevent the cell to adequately respond to the external stimulus. Therefore, it is important to underline that beyond the traditional role of ROS as dangerous by-products of cellular metabolism inducing cellular damage – including cell mutations, cancer, some symptoms of aging, diabetes neuropathy, vascular problems, schizophrenia, Alzheimer's and Parkinson's diseases – ROS can also trigger various physiological reaction cascades. The influence of ROS on physiological processes is based on their ability to modify the activity of key protein molecules, which contain domains sensitive to redox conditions. Examples of physiological processes in which ROS are involved are: cell respiration, phagocyte defense system, thyroid gland, reproductive system, apoptosis and necrosis. Importantly, different signaling pathways require very low concentrations of ROS to act as second messengers (Massaad and Klann, in press).

In consequence, it must be highlighted that considering the physiological role of ROS, either an increase or a decrease in ROS levels might underlie behavioral disruption. All these results support the hypothesis of oxidative imbalance in noise-exposed animals and also support previous reports in which an increase in ROS levels was initially found and different events were triggered thereafter to compensate for the oxidative challenge (Di Toro et al., 2007; Sun et al., 2009; Caceres et al., 2010). Therefore, low ROS concentration, as found in the present paper in CE and Hip of ANE and CNE rats, can also be noxious to cells, since ROS are vital for the organism (Massaad and Klann, in press; Pourova et al., 2010).

It would not be discarded that the persistent low levels of hippocampal ROS found in ANE rats could be due, at least in part, to the increases in hippocampal CAT and SOD activities. If CAT and SOD activities had not been increased, an excess of ROS would have been harmful. Therefore, these changes in the oxidative profile might underlie the abnormal Hip-related behavior found in noise-exposed animals. As the increase in CAT and SOD activities were observed only in ANE rats, it would be suggested that the acute exposure might trigger defensive mechanisms that in the chronic exposure could be counteracted by adaptive mechanisms.

Although cerebellar ROS levels were decreased 15 days after exposure, no changes in cerebellar antioxidant enzyme levels were found, suggesting that CE would be more resistant to antioxidant enzyme changes than Hip, as suggested by Mandavilli and Rao (1996) and Clement et al. (2009), who

reported that regions like the cerebral cortex, the hypothalamus, the Hip and the striatum are more susceptible structures when compared to the CE. Alternatively, it would not be discarded that other components of the antioxidant system could underlie the changes in ROS levels.

In summary, the present study shows that exposure of rats to loud noise (95–97 dB/2 h/day) leads to behavioral changes, in some cases long-lasting, which could be related to the imbalance in oxidative status, underlying the altered ability to respond to the stressor. Consequently, it would be considered that noise-exposed workers are under an increased risk of memory changes. Therefore, further studies in animals are needed to continue understanding the effects of noise on brain structures.

According to these findings, the association between noise exposure, oxidative processes, and behavioral alterations deserves further attention due to the long-lasting consequences.

4. Experimental procedures

4.1. *Animals*

Pregnant albino Wistar female rats were isolated in a cage a few days before delivery. The day of birth (day 0) was known by daily inspection of the cages. 15-day-old males were randomly separated into three treatment groups, control (C), acute noise exposure (ANE) and chronic noise exposure (CNE). Rats were exposed or sham-exposed to noise, according to the scheme, and kept with their dam until 22 days of age. After weaning, they were separated and maintained four per cage until 30 days, with food and water *ad libitum*, on 12 hour light–dark cycles (lights on at 7 am) at 22±2 °C.

For radial maze testing, rats were food-deprived to 85% of their free-feeding weight, beginning the diet approximately 5 days before the start of the test and lasting until the end of the testing period, to ensure adequate motivation.

Animals were handled and sacrificed according to the Institutional Committee for the Use and Care of Laboratory Animal rules (CICUAL, School of Medicine, University of Buenos Aires, Argentina) and the protocol was approved by this Committee under resolution 2079/07. The CICUAL adheres to the rules of the "Guide for the Care and Use of Laboratory Animals" (NIH) and the institution has an Animal Welfare Assurance approved by the Public Health Service (PHS) with the assurance number A5801-01. Adequate measures were taken to minimize animal pain or discomfort.

For all experimental procedures, 7–9 rats of 15 or 30 days were used and were killed by decapitation (total animals used: 124).

4.2. *Noise exposure*

To obtain white noise, a computer software (TrueRTA) was chosen, using a bandwidth from 20 Hz to 20,000 Hz in octave bands. For sound amplification, an active 2 way monitor (SKP, SK150A, 40 W RMS per channel) located at 30 cm from the animal cage in a sound chamber of 1 m×1 m×1 m was used. Noise intensity was measured by an omnidirectional measurement condenser microphone (Behringer ECM 8000) each day prior to animal exposure, by placing the microphone in the acoustic chamber at several locations, and taking an average of the different readings.

Animals were exposed to white noise at 95–97 dB (20–20,000 Hz) 2 h/day, for one day (ANE) or for 15 days (CNE). Control animals were kept in the noise chamber for the same period of time, but without being exposed to noise. Background noise level was 55 dB.

Lighting was provided by a 20 W lamp located in the upper left corner of the sound chamber.

Immediately after the first exposure (at 15 days), Hip and CE were dissected for biochemical determinations. At 30 days, ANE and CNE animals were tested in the different behavioral tasks or were used for biochemical determinations.

4.3. *Behavioral testing*

4.3.1. *Radial arm maze (RAM, spatial memory task)*

Apparatus: A custom-made, automated, eight arm metallic radial maze painted black was used, elevated 50 cm above the floor and situated in a 2.6 m×2.3 m room. It consists of eight identical arms (40 cm×10 cm), radiating from a circular platform (20 cm in diameter), with 35 cm surrounding walls. Each arm is equipped with two infrared diodes located in both lateral walls, at 5 cm from the end of the arm and placed 3 cm from the floor. The sequence of photocell beam interruptions is monitored by a computer.

In order to orient themselves in space, animals must look upward: for these reason, several extra-maze cues were placed around the walls of the behavioral room. The visual cues consist of vertical stripe lines, and black and white squares or circles (70 cm×60 cm). The experimenter was sat always in the same corner of the room.

An opaque cylinder (20 cm diameter×26 cm high) was placed over the central platform and the experimenter must raise it at the beginning of each session.

Chocolate cereals were used as food reward. Each time the animal crossed the infrared diodes, a computer connected to the apparatus received a signal and records that the rat has successfully entered the arm, providing an on-line display of the animal's location.

Pre-training and training procedures were done between 10 and 12 am.

Rats had to learn to visit each arm, only once per session. During the tests, animals would therefore have to remember which food rewards had already been eaten.

Testing procedure:

Pre-training: Rats were allowed to familiarize with the radial-arm maze during 2 days: on the first day, 4–5 rats were placed on the maze with several randomly placed food rewards, for 20 min. On the second day, food pellets were placed only at the ends of the arms and rats were allowed to freely explore the maze for 10 min, in groups of 2 or 3.

a) Full baited task (Dubreuil et al., 2003)

Training sessions: A daily session started with the animal placed in the central platform inside the opaque cylinder for 10 s. Then, the cylinder was removed and the session started. Either when the rat entered all eight arms or if the maze was not completed up to 10 min, the session was

considered finished. This training lasted for 10 consecutive days. The maze was cleaned with 10% ethanol between each rat to minimize olfactory intra-maze cues. Data from 2 sessions were averaged and expressed as blocks.

Data analysis: Rats' performances were evaluated by measuring different spatial, exploratory, and procedural parameters.

Spatial parameters: The number of *errors* (reentry to an already entered arm) and *spatial span* (longest sequence of correctly visited arms), were recorded for each rat at each session. Total *errors* (sum of 10 sessions) were also calculated.
Exploratory parameters: *Choices* (number of arms visited, both right and wrong) and *latency* (total time needed to complete the maze) were recorded for each rat at each session. Total *choices* (sum of 10 sessions) were also calculated.

Procedural parameters: The animals' strategies were evaluated by two different measures: the *percentage of 45° angles* (percentage of adjacent arms visited until completion of the maze) and the *angle change* (considering three successive choices, one angle change is recorded if the animal change the angle between the second and third choices when compared to the angles made between the first and second choices). These parameters were assessed to analyze the flexibility of the strategies used. As spatial span in the last session is an index of working memory abilities, the finding of a correlation between spatial span and the percentage of 45° angles made in the last session could indicate the use of working memory or procedural components in the spatial task, while the lack of correlation could demonstrate a disruption of the procedural strategy to solve the maze.

b) Delayed win-shift task (Nagai et al., 2006)
Training sessions: Rats were given a daily trial per day for 10 consecutive days. Each trial was composed of two phases: the training phase and the test phase, separated by a 15-minute delay. Before the training phase, a set of four arms was chosen randomly and blocked by a removable door. This set was the same for all animals for a given day, but different every day. Food rewards were placed at the end of the four remaining open arms.
Training phase: Rats were placed in the center of the maze and were allowed to explore the maze for a total of 10 min, or until all 4 rewards had been retrieved. The animals were then removed from the maze and returned to their home cages for the delay period.
Test phase: Rats were returned to the center of the platform of the maze and were allowed to explore the maze. During this phase, all the arms were open, but only the arms that were blocked in the training phase contained the food reward. When all 4 rewards had been retrieved or when 10 min had elapsed, animals were removed from the maze. The maze was cleaned between each rat to minimize olfactory intra-maze cues.

Data analysis: A reference error was defined as the entry to the four arms open during the training phase. Data from 2 sessions were averaged and expressed as blocks.

4.3.2. *Open field (OF)*

Apparatus: The experiment was conducted in a standard OF apparatus as described by Prut and Belzung (2003). It is made of white Formica, squared base with a side of 50 cm, enclosed by a surrounding wall of 50 cm high and situated 50 cm above the floor. The base of the OF is divided into 25 equal squares.

Testing procedure: Activity and emotionality measures were taken. After 3 min of habituation to the behavioral room, each rat was placed in the near right-hand corner of the box (with respect to the experimenter position), facing the wall and monitored for 6 min using a digital video camera. The number of lines crossed was measured on the open field over a 6 minute session. This is a classical measure of locomotor (horizontal exploration) activity. Moreover, wall climbs and rearings (vertical exploration) were also recorded. Since habituation to the OF can progressively be seen within a session, a comparison was performed between the behavior of all groups observed during the first 3 min (when the environment would have maximal novelty) and the behavior observed during the last 3 min (when control animals would normally exhibit acclimation to the environment).

Habituation to a novel environment is believed to be one of the most elementary forms of non-associative learning, known to depend on the Hip (Vianna et al., 2000), in which the repeated exposure to the same environment induces a reduction in the exploratory behavior.

Data analysis: Locomotor activity (horizontal exploration) was assessed by the number of crossed lines and vertical exploratory activity by the number of rearings and total time of rearing as well as wall climbs.

The field was divided into two zones: a perimeter (approximately 10 cm in width, immediately adjacent to the wall) and a center zone. Anxiety-like behavior was evaluated by latency to center (time elapsed until rat entered the center of the OF: decreased latency means decreased anxiety-like behavior), incursions to the center (number of entries into center area: increased incursions means decreased anxiety-like behavior) and total time spent in the center (increased time means decreased anxiety-like behavior).

To minimize the olfactory stimulus, the floor of the box was cleaned with a 10% ethanol solution between sessions.

4.3.3. *Elevated plus maze (EPM) (Brenes et al., 2009)*

The wooden-made apparatus consisted of four arms of equal dimensions (50 cm × 10 cm) and raised 50 cm above the floor. Two arms, enclosed by walls 40 cm high, were perpendicular to two opposed open arms. To avoid falls, the open arms were surrounded by a Formica rim of 0.5 cm high. The EPM was dimly illuminated with a lamp located 200 cm above the maze. The rats were placed in the center of the maze, facing one of the closed arms, and were recorded for 5 min using a digital video camera. The percent of time spent on each arm was scored. The maze was cleaned between sessions with a 10% alcohol solution. Some rats fell down when walking in open arms. These animals were excluded from the study.

4.3.4. *Inhibitory avoidance task (IA)*

Apparatus: We used an inhibitory avoidance apparatus as described by Roozendaal (2002). It consists of a box (60 cm × 60 cm × 40 cm), divided into two compartments: one is illuminated, while the other is equipped with a removable cover

to allow it to be in the dark. A removable partition divided the two compartments. The floor of the dark compartment consisted of a stainless steel grid at the bottom, through which a continuous current could be delivered.

Habituation session: The rat was placed into the lit box and allowed to freely explore the apparatus. Either after passing 3 times to the dark side or after 3 min spent in the dark side, the rat was removed from the apparatus. After 10 min, the rat was placed again in the lit side and when it entered the dark, the doors closed and the rat was retained for 10 s in this side.

Training session: Each rat was placed in the lit compartment, facing away from the dark compartment and the latency to move into the dark compartment was recorded. When the rat stepped with all four paws into the dark compartment, a foot shock (1.2 mA, 2 second duration) was delivered. The rat was then removed from the apparatus and returned to its home cage.

Retention session: Retention was tested after 24 h following a procedure similar to the training session, except that no shock was delivered. The ratio between the latency of the rat to move into the dark compartment in the *retention* and the *training* sessions (T2/T1) was taken as a measure of associative memory retention.

To minimize the olfactory stimulus, the box and objects were cleaned with a 10% ethanol solution between sessions.

Inhibitory avoidance task measures the memory of an aversive experience through the simple avoidance of a location in which the unpleasant experience occurred. This task depends heavily on the dorsal Hip (Ennaceur and Delacour, 1988; Izquierdo and Medina, 1997).

4.4. *Biochemical procedures*

4.4.1. *ROS determination*

The levels of hippocampal and cerebellar ROS were determined by a method described by Driver et al. (2000). Briefly, tissues were homogenized in an ice Locke's solution (0.5 mg of tissue/ml). Aliquots of the homogenate were taken and left to warm at room temperature during 5 min. After that, 10 μl of dichloro-fluorescein diacetate (0.97 mg DCFH/ml in methanol) was added (10 μM final concentration) and the mixture was incubated at room temperature during 15 min. Finally, the fluorescence was measured at 485 nm (excitation) and 530 nm (emission). A standard curve was performed using oxidized dichloro-fluorescein (DCF). Results were calculated as pmol DCF/mg tissue/min and expressed as mean values ± SEM.

4.4.2. *Antioxidant enzyme assays*

SOD activity measurement: The activity of hippocampal and cerebellar SOD was determined according to McCord and Fridovich (1969). Briefly, the tissues were homogenized at 10% w/v in a 216 nM, pH 7.8 phosphate buffer solution and were centrifuged at 900 × *g* for 10 min. A supernatant aliquot was mixed with 216 nM, pH 7.8 phosphate buffer, 10.7 mM EDTA, 1.1 mM C cytochrome and 0.108 mM xanthine at 25 °C. Reaction started with the addition of 0.1 ml of xanthine oxidase (XO) enzyme solution (2 U/ml). The increase in the absorbance at 550 nm for 5 min was registered. One unit of SOD is defined as the amount that inhibits the rate of reduction of cytochrome c by 50% in a coupled system, using xanthine and xanthine oxidase at pH 7.8 at 25 °C in a 3.0 ml reaction volume. Results were calculated as SOD units/mg tissue and expressed as mean ± SEM.

CAT activity measurement: The activity of CAT was determined according to Beers and Sizer (1952). Briefly, 10% w/v tissue homogenates were made in 50 mM phosphate buffer and therefore were centrifuged at 42,000 × *g* for 15 min. A supernatant aliquot was incubated with 0.036% (w/w) hydrogen peroxide solution (H_2O_2). The time required to decrease the absorbance at 240 nm from 0.45 to 0.40 absorbance units was registered.

One unit will decompose 1.0 μmol of H_2O_2/min at pH 7.0 at 25 °C. Results were calculated as CAT units/mg tissue and expressed as mean ± SEM.

4.5. *Statistical analysis*

Significant differences between the two groups were determined by the Student test. When more than 2 groups were compared, ANOVA statistical was used and Tukey test was applied for post-hoc comparisons. Since inhibitory avoidance data did not pass the normality test, Kruskal Wallis ANOVA on ranks test was performed with Dunn's method for post-hoc comparisons. Results are expressed as mean values ± SEM. A probability <0.05 was accepted as significant. Relationships between some parameters studied were evaluated by the linear Pearson's correlation coefficient (r).

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