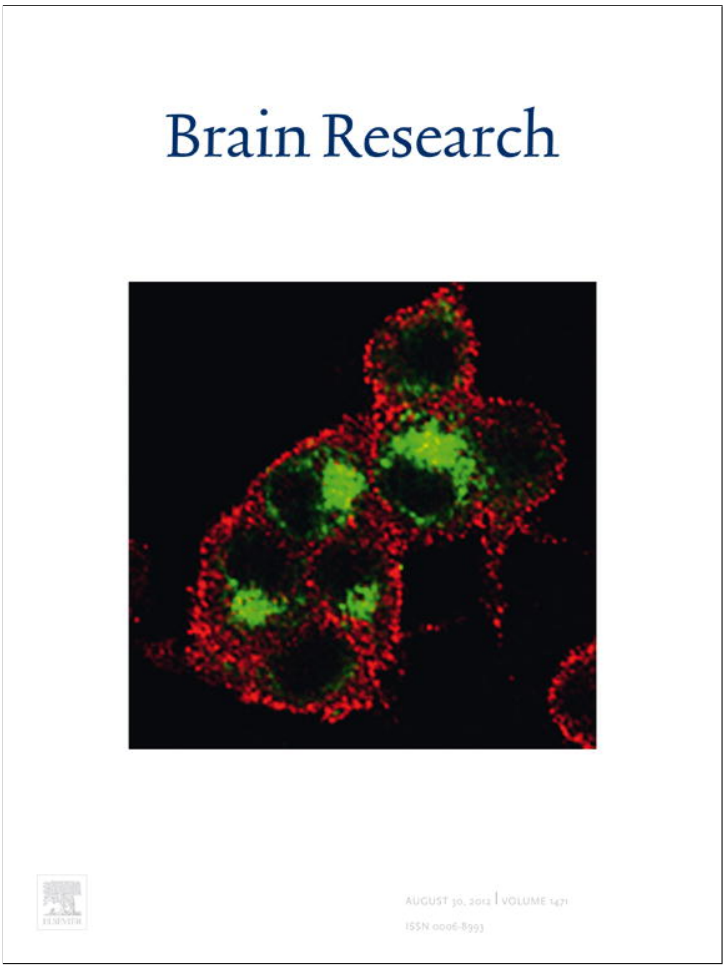

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Research Report

Rat hippocampal alterations could underlie behavioral abnormalities induced by exposure to moderate noise levels

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ABSTRACT

Noise exposure is known to affect auditory structures in living organisms. However, it should not be ignored that many of the effects of noise are extra-auditory. Previous findings of our laboratory demonstrated that noise was able to induce behavioral alterations that are mainly related to the cerebellum (CE) and the hippocampus (HC). Therefore, the aim of this work was to reveal new data about the vulnerability of developing rat HC to moderate noise levels through the assessment of potential histological changes and hippocampal-related behavioral alterations. Male Wistar rats were exposed to noise (95–97 dB SPL, 2 h daily) either for 1 day (acute noise exposure, ANE) or between postnatal days 15 and 30 (sub-acute noise exposure, SANE). Hippocampal histological evaluation as well as short (ST) and long term (LT) habituation and recognition memory assessments were performed. Results showed a mild disruption in the different hippocampal regions after ANE and SANE schemes, along with significant behavioral abnormalities. These data suggest that exposure of developing rats to noise levels of moderate intensity is able to trigger changes in the HC, an extra-auditory structure of the Central Nervous System (CNS), that could underlie the observed behavioral effects.

1. Introduction

During daily life, people are exposed to potentially harmful noise levels coming from work environment, urban traffic, household appliances and/or discotheques (Frenzilli et al., 2004). For these reasons, exposure to loud noise levels represents a problem in all regions of the world. In the United States of America, for example, more than 30 million workers are daily exposed to hazardous noise (NIOSH, 1998). It is estimated that 16% of disabling hearing loss observed in adults worldwide is due to occupational noise (Kopke et al., 2007).

Although it is known that auditory structures of living organisms can be affected by noise exposure (Cappaert et al., 2000; Hu and Zheng, 2008), it should not be ignored that many of the effects are extra-auditory (Lenzi et al., 2003; Rabat, 2007;

Abbreviations: HC, hippocampus; ANE, acute noise exposure; SANE, sub-acute noise exposure; Ct, Control; CNS, Central Nervous System; PND, postnatal day; OF, open field; OR, object recognition; ST, short term; LT, long term

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Uran et al., 2010; Chengzhi et al., 2011). It has been reported that noise can produce severe behavioral disruptions in eating (Krebs et al., 1996) and sleep (Rabat et al., 2004). In addition, CNS-related signs such as emotional stress, enhancement of social conflicts and multiple psychiatric disorders (Rabat, 2007) were observed after noise exposure, along with increases in aggressive behavior and anxiety (Stansfeld and Matheson, 2003). Moreover, animal experiments demonstrated that different schemes of acute and chronic noise exposures can induce temporary or permanent changes in learning and memory processes, both in developing and mature specimens (Ising and Braun, 2000; Prior, 2002; Manikandan et al., 2006; Goble et al., 2009; Uran et al., 2010; Chengzhi et al., 2011). It is to be noted that the level of environmental noise generally experienced by humans is of moderate intensity and its effects on brain function are largely unknown.

It is important to highlight that developing animals might be more vulnerable to noise than adults, given that mammalian CNS undergoes a progressive structural and functional growth during early life stages (Cheng et al., 2011). Consequently, it seems that plastic changes induced by noise are not limited to the auditory pathway (Kaltenbach, 2000; Kaltenbach and Zhang, 2007; Kujawa and Liberman, 2009) but would extend to other parts of the CNS, such as the HC. It has been suggested that, in addition to its involvement in learning and memory processes (Eichenbaum, 2004; Goble et al., 2009), HC might also act in response to auditory stimuli (Sakurai, 2002), as noise can be transmitted through the lemniscal ascending path via the inferior colliculus, then to the auditory cortex and finally to the CA3 region of the HC (Moller and Rollins, 2001; Kim et al., 2008; Gao et al., 2009). Therefore, it could be hypothesized that hippocampal functioning may be affected by noise exposure through an indirect mechanism. Previous findings of our laboratory demonstrated that noise was able to induce behavioral alterations, mainly related to the CE and the HC (Uran et al., 2010). Hippocampal-dependent behavioral alterations included decreased anxiety levels as well as impairments in associative memory and habituation “within session”. Therefore, to confirm if HC is one of the targets of noise-induced damage, it is essential to investigate whether exposure to moderate noise levels can affect other hippocampal-related tasks and to establish a relationship with possible hippocampal histological alterations.

It is to be emphasized that the growing number of adolescents attending discotheques, in addition to the popular use of portable devices at loud intensities among young people, makes the present paper clinically relevant. Importantly, at 15 days of age (the age at which rats were exposed to noise in this work), rat brain development can be comparable to that of a human toddler. Moreover, at 30 days (the age at which noise effects were evaluated in the present work), it would be comparable to an adolescent brain (Chengzhi et al., 2011).

Therefore, the aim of this work was to reveal new data about the vulnerability of developing rat HC to moderate noise levels through the assessment of potential histological changes and hippocampal-related behavioral alterations.

These data were obtained by testing rats’ memory in different hippocampal-dependent tasks, such as habituation memory retention in an open field (OF) device (Vianna et al., 2000) and recognition memory in an object recognition (OR) device (Bevins and Besheer, 2006), both examined at ST (1 h intertrial interval) and at LT (24 h intertrial interval). Animals were evaluated at different post-exposure intervals. Finally, potential hippocampal histological alterations were assessed to test the hypothesis that a plethora of behavioral changes could be underlain by histological changes.

2. Results

2.1. *Behavioral findings at ST*

Results showed a significant decrease in the number of lines crossed in the second session of the OF task at ST in all groups of rats tested at PND 30 (two way ANOVA: between sessions factor: $F_{1,41}=28.17$, $p<0.001$), although a more significant decrease was observed in Ct rats when compared with noise-exposed animals (first session vs. second session: Ct, $p<0.001$; ANE, $p<0.05$; SANE, $p<0.05$, Fig. 2a).

Since OF assessments were performed at 30 days, regardless of the scheme of noise-exposure used, the time elapsed between the completion of noise exposure and the beginning of the behavioral assessment was different in each scheme (it was 15 days in ANE rats and 0 day in SANE rats). Since these discrepancies may produce dissimilar results, SANE rats were also evaluated at PND 45, e.g., 15 days after the end of noise exposure (identical to the length of the interval set for ANE rats), to avoid a potential effect of time on OF performance. Statistical results of lines crossed in the OF task obtained from SANE rats evaluated at PND 45 resulted similar to those observed in SANE animals tested at PND 30 (two way ANOVA: between sessions factor, $F_{1,25}=49.25$, $p<0.001$; first session vs. second session: Ct, $p<0.001$; noise, $p<0.05$, Fig. 2b).

When animals were tested at ST in the OR task at PND 30, Ct and ANE animals showed an increase in exploration time of the novel (*N*) when compared with the familiar (*F*) object, whereas exploration time of each object was similar in SANE

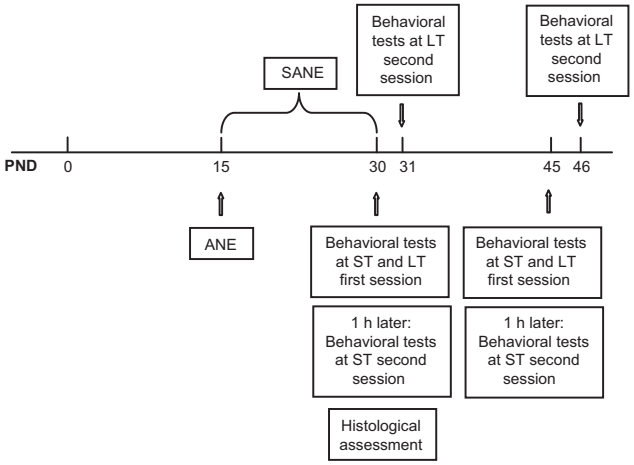

Fig. 1 – Ct: control rats; ANE: acute noise exposed rats; SANE: sub-acute noise exposed rats.ST: short term (1 h intertrial interval); LT: long term (24 h intertrial interval). PND: postnatal day.

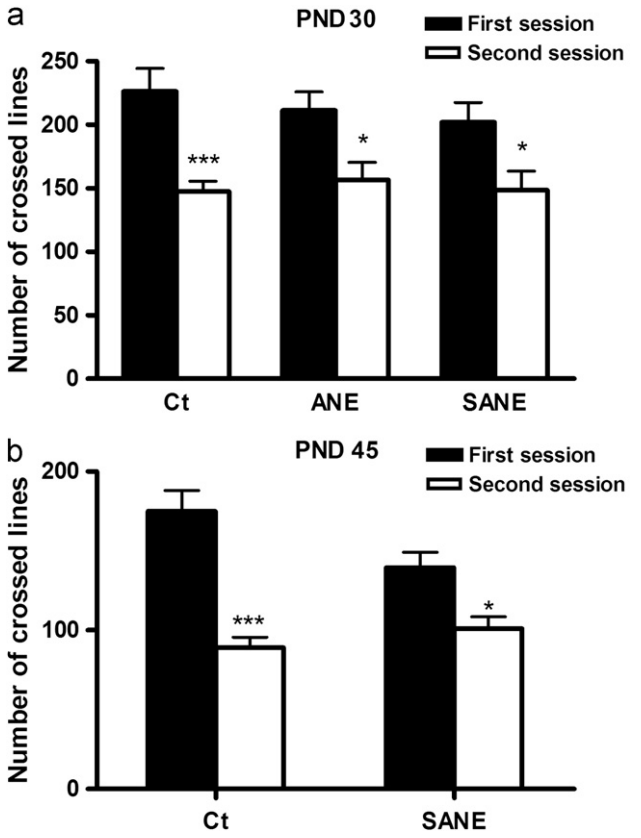

Fig. 2 – Number of lines crossed in the open field task at ST. Filled bars: first session; open bars: second session. Ct: Control rats; ANE: acute noise exposed rats; SANE: sub-acute noise exposed rats; ST: short term (1 h intertrial interval). (a) A decrease in the number of lines crossed was observed at postnatal day (PND) 30 in all groups. (b) The same decrease in the number of lines crossed was observed at PND 45 in all groups., $^{***}p<0.05$ and $^{*}p<0.001$ when compared with the first session, respectively. Data are mean of the number of lines crossed ± SEM. $n=7$ for each group.

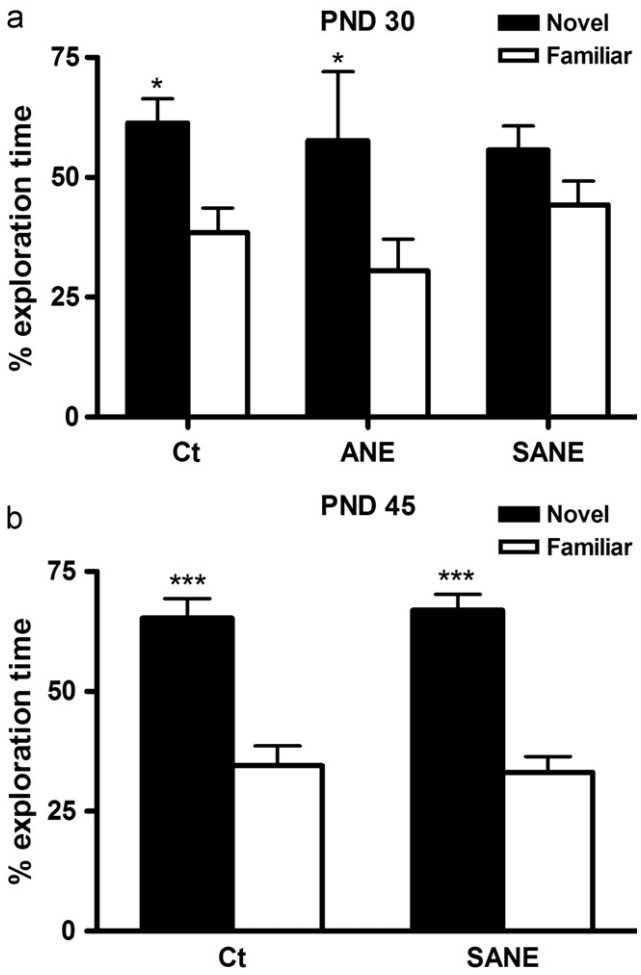

Fig. 3 – Percent of exploration time in the object recognition task at ST. Filled bars: Novel object; open bars: familiar object. Ct: Control rats; ANE: acute noise exposed rats; SANE: sub-acute noise exposed rats; ST: short term (1 h intertrial interval). (a) Ct and ANE groups explored more time the novel than the familiar object at PND 30, whereas SANE rats explored the same time both objects. (b) Ct and noise-exposed animals explored more the novel than the familiar object at PND 45, $^{***}p<0.001$ when compared with the familiar object. Data are mean of the percent of time exploring objects ± SEM. $n=7$ for each group.

animals (two way ANOVA: between objects factor, $F_{1,39}=13.89$, $p<0.001$; F vs. N: Ct, $p<0.01$; ANE, $p<0.001$; SANE, NS, Fig. 3a).

In contrast, results showed that at PND 45 Ct and noise-exposed groups explored more time the N object than the F (two way ANOVA: between objects factor, $F_{1,23}=103.3$, $p<0.001$; F vs. N: Ct, $p<0.001$; Noise, $p<0.001$ Fig. 3b).

2.2. *Behavioral findings at LT*

Fig. 4a showed that whereas the number of lines crossed by Ct rats evaluated at PND 30 decreased significantly in the second session of the OF task at LT, the same number of lines were crossed by noise-exposed animals in both sessions (two way ANOVA: between sessions factor, $F_{1,39}=3.78$ $p<0.05$; first session vs. second session: Ct, $p<0.05$; ANE, NS; SANE, NS). Moreover, similar statistical results were obtained in the OF task performed at LT when animals were evaluated at PND 45 (two way ANOVA: between sessions factor, $F_{1,25}=40.44$, $p<0.001$; first session vs. second session: Ct, $p<0.001$; noise, NS, Fig. 4b).

Likewise, whereas Ct animals at PND 30 explored more the N than the F object when tested in the OR task at LT, exploration time on both objects was similar in noise-exposed animals (two way ANOVA: between objects factor, $F_{1,43}=6.52$, $p<0.05$; F vs. N: Ct, $p<0.001$; ANE, NS; SANE, NS, Fig. 5a). Further, whereas Ct rats tested at PND 45 spent more time exploring the N than the F object, exploration time of both objects was similar in noise-exposed animals (two way ANOVA: between objects factor, $F_{1,23}=14,95$, $p<0.001$; F vs. N: Ct, $p<0.001$; Noise, NS, Fig. 5b).

2.3. *Histological findings*

Results showed that the different hippocampal regions were affected by noise exposure (Figs. 6a–8a). Total cell number was significantly increased in CA1 region of ANE rats when

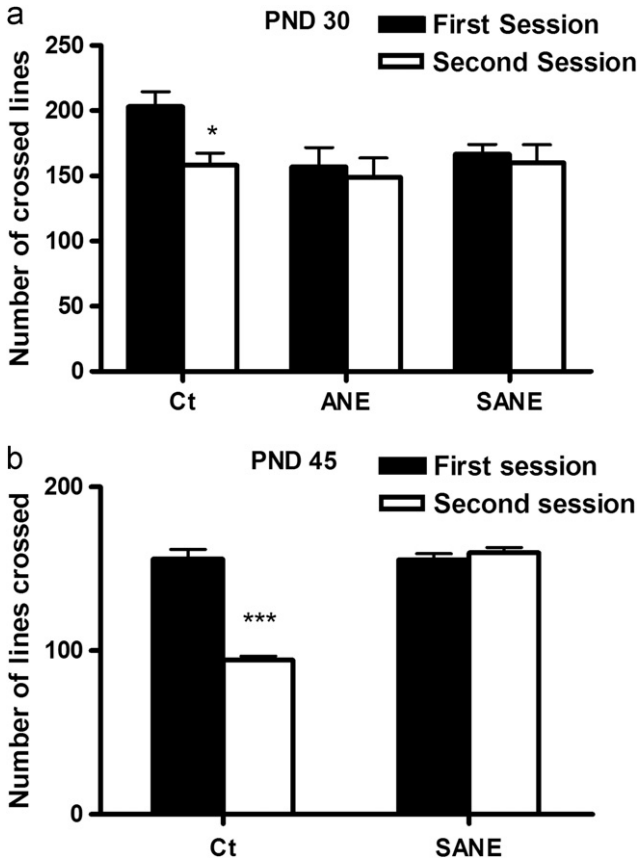

Fig. 4 – Number of lines crossed in the open field task at LT. Filled bars: first session; open bars: second session. Ct: Control rats; ANE: acute noise exposed rats; SANE: sub-acute noise exposed rats; LT: long term (24 h intertrial interval). (a) A decrease in the number of lines crossed was observed at PND 30 in Ct group, while no differences were observed in noise-exposed groups. (b) The same results were observed at PND 45 in all groups., $^{*}p<0.05$ when compared with the first session. Data are mean of the number of lines crossed ± SEM. $n=7$ for each group.

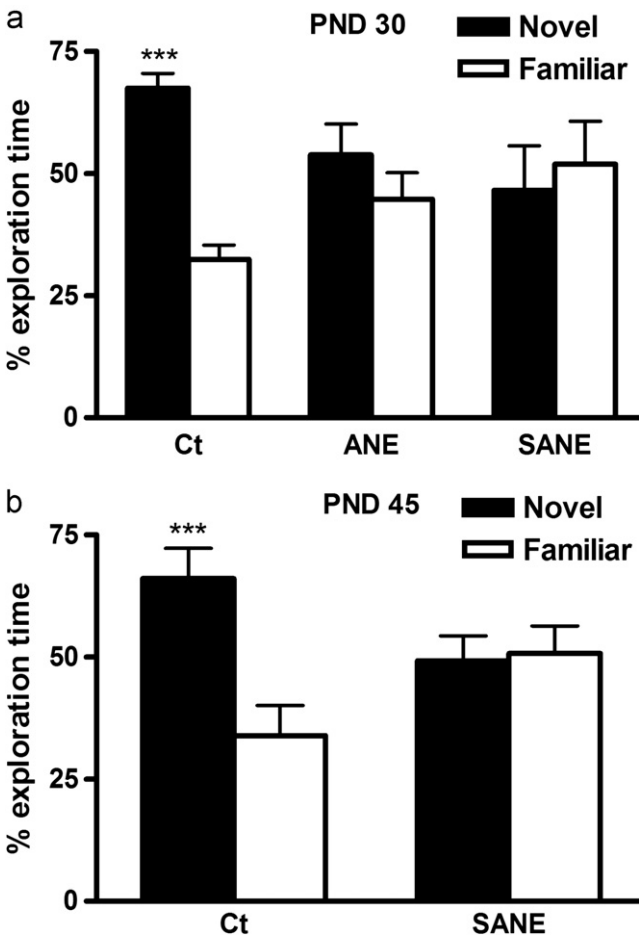

Fig. 5 – Percent of exploration time in the object recognition task at LT. Filled bars: Novel object; open bars: familiar object. Ct: Control rats; ANE: acute noise exposed rats; SANE: sub-acute noise exposed rats. LT: long term (24 h intertrial interval). (a) Ct group explored more time the novel than the familiar object at PND 30, whereas ANE and SANE rats explored both objects the same time. (b) Ct group explored more time the novel than the familiar object, whereas noise-exposed rats explored both objects the same time at PND 45, $^{***}p<0.001$ when compared with the familiar object. Data are mean of the percent of time exploring objects ± SEM. $n=7$ for each group.

compared with Ct, whereas no changes were observed in SANE rats (one way ANOVA: $F_{2,93}=9.647$, $p<0.001$; ANE vs. Ct, $p<0.001$; SANE, NS, Fig. 6b). In CA3 region, total cell number was significantly increased in both ANE and SANE rats (one way ANOVA: $F_{2,71}=5.822$, $p<0.01$; ANE vs. Ct, $p<0.05$; SANE vs. Ct, $p<0.01$, Fig. 7b). Finally, total cell number was significantly increased in DG region of ANE and SANE rats (one way ANOVA: $F_{2,\ 118}=8.941$, $p<001$; ANE vs. Ct, $p<0.01$; SANE vs. Ct, $p<0.001$, Fig. 8b).

A higher number of pyknotic cells was found in ANE animals when compared with Ct animals in CA1 region, while no changes were observed in SANE rats (one way ANOVA: $F_{2,94}=14.54$, $p<0.001$; ANE vs. Ct, $p<0.001$; SANE vs. Ct, NS, Fig. 6c). In addition, pyknotic cells were significantly increased in hippocampal CA3 region, not only in ANE but also in SANE rats (one way ANOVA: $F_{2,71}=9.357$, $p<0.001$; ANE vs. Ct, $p<0.001$; SANE vs. Ct, $p<0.05$, Fig. 7c). Finally, the number of pyknotic cells in DG remained unaffected in noise-exposed rats (one way ANOVA: $F_{2,118}=0.7391$, NS, Fig. 8c).

3. Discussion

Results showed that moderate levels of white noise (95–97 dB SPL, 2h daily), either through acute or sub-acute noise schemes, can induce an impairment in habituation and recognition memory that is observed mainly at long term and seems to be long-lasting. In addition, some hippocampal histological disruption can be observed in exposed animals.

Exposure to loud noise has usually been associated with hearing loss, often related to the injury to auditory pathway (Cappaert et al., 2000). However, it has been reported that noise damage could also be induced to extra-auditory structures. In fact, cardiovascular, endocrine, and nervous systems are among some of the targets of noise injury (Oliveira et al.,

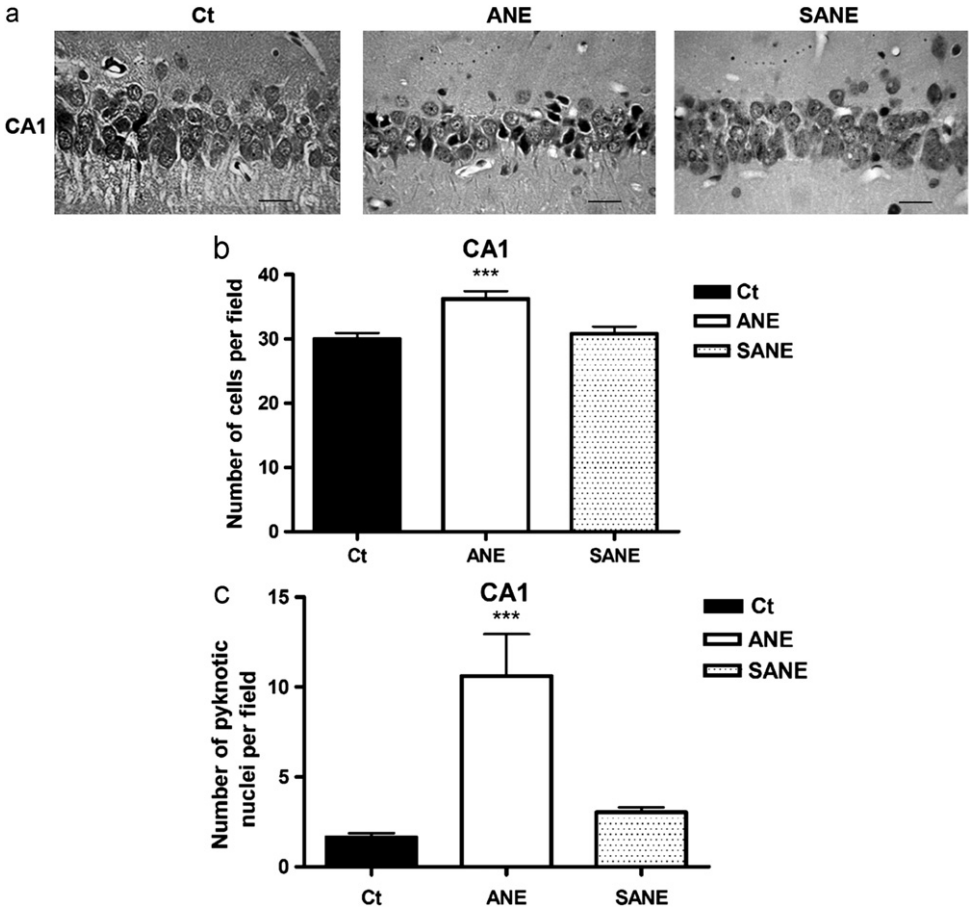

Fig. 6 – Effect of noise exposure on CA1 hippocampal area. Significant changes were induced in hippocampal coronal hematoxilin-eosin CA1 sections of ANE rats when compared with controls. (a) Noise exposure induced a significant increase in the number of pyknotic cells (b) together with an increase in the number of total cells after ANE, without changes after SANE. (c) Data are expressed as the mean number of cells per field ± SEM. Scale bar: 25 μm. Ct: Control; ANE: Acute noise exposure; SANE: Sub-acute noise exposure, $^{***}p < 0.001$ when compared with control animals. $n = 4$ for each group.

2001; Alves-Pereira and Castelo Branco, 2007). Several authors reported different effects of noise in CNS structures. For instance, a series of neurological changes were identified in people exposed to occupational noise, including brain lesions, increased latencies in nerve conduction and cognitive deterioration (Gomes et al., 1999; Pimenta et al., 1999). Therefore, it might be suggested that exposure to noise may have persisting effects on brain function and behavior, even if the peripheral hearing system remains intact. Moreover, according to Alves-Pereira and Castelo Branco (2007), it might be postulated that acoustical phenomena, even if it were not perceived by the auditory system, may cause damage to biological tissues.

Developing CNS, like other immature tissues, seems to be more vulnerable than adult tissue to different environmental factors. In particular, human exposure to loud noise (e.g., aircraft noise) was found to impair cognitive development in children, affecting predominantly reading comprehension (Stansfeld et al., 2005; Chengzhi et al., 2011). However, few reports are available in which the effects of noise are assessed in specific structures of immature CNS (Kim et al., 2006). As a result, the scarcity of reports devoted to study the impact of noise on developing CNS, makes the study of this topic relevant.

Although the effects of noise on CNS could be indirectly mediated by damage to auditory structures (Kim et al., 2008; Gao et al., 2009), it could be postulated that other mechanisms might be involved. For instance, it is known that every organ and tissue have their own acoustical properties such as resonance frequency and acoustical impedance (Alves-Pereira and Castelo Branco, 2007). Therefore, it has been proposed that low-frequency noise transmission extends into objects and living organisms that allowed it to set up resonant vibration in different organs (Berglund et al., 1996; Mahendra Prashanth and Sridhar, 2008). In addition, it must be highlighted that not only the loudness but also the vibration induced by noise exposure could contribute to the overall effects (Alves-Pereira, 1999, Castelo Branco and Alves-Pereira, 2004).

In this study, PND 15 rats were used at the beginning of the exposure to noise. We decided to use specimens of this age because rats are deaf to air-borne sounds until PND 11–12. At this developmental stage, the rat neural circuit that supports hearing becomes functional and extends to PND 30 (Chang and Merzenich, 2003; Chang, 2003; Gao et al., 2009). Since it has been suggested that the extra-auditory effects would take place only with an intact auditory pathway

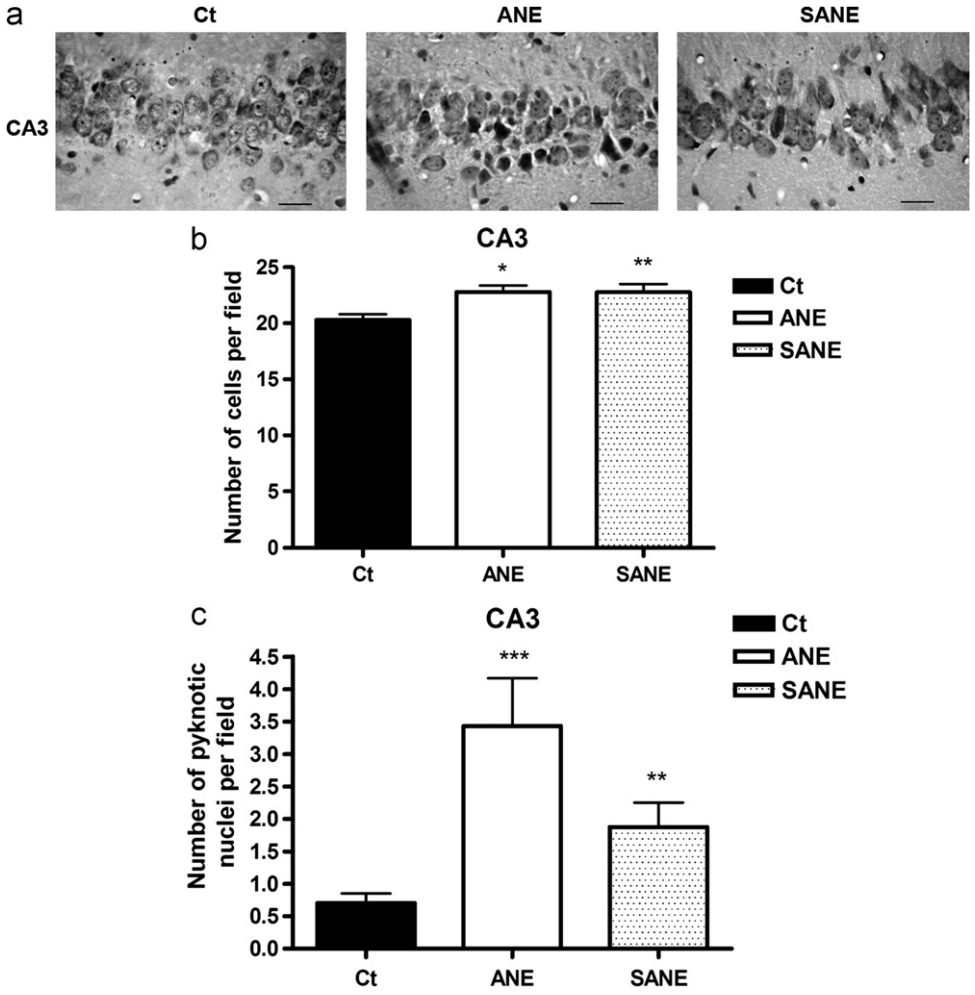

Fig. 7 – Effect of noise exposure on CA3 hippocampal area. Significant changes were induced in hippocampal coronal hematoxilin–eosin CA3 sections of ANE and SANE rats when compared with controls. (a) Noise exposure induced a significant increase in the number of pyknotic cells. (b) together with an increase in the number of total cells after ANE and SANE schemes. (c) Data are expressed as the mean number of cells per field ± SEM. Scale bar: 25 μm. C: control; ANE: acute noise exposure; SANE: Sub-acute noise exposure, $^{*}p<0.05$, $^{**}p<0.01$ and $^{***}p<0.001$, when compared with control animals, respectively. $n=4$ for each group.

(Irvine, 2007; Gao et al., 2009), it was crucial to assure that the rat perceived sound at the beginning of noise exposure. Thus, the choice of PND 15 rats for procedures of noise exposure relied on the rats' hearing competence. Moreover, due to the relationship between HC and auditory cortex, we chose to expose animals in a highly plastic developmental period for both structures (Zhang et al., 2008). Preliminary experiments carried out in our laboratory (not shown) demonstrated that no alterations in auditory function and cochlear histology were induced both in rats exposed to noise either in acute or sub-acute schemes, suggesting that the observed noise-induced damage to HC did not seem to be mediated by auditory pathway injury. Even so, further experiments should be performed to confirm this hypothesis.

It is noteworthy that the level of noise used in the present work is considered to be of moderate intensity. This statement is supported by data of different authors such as Zhang et al. (2008) or Freeman et al. (1999) who demonstrated that exposure of rats to noise levels of 90 dB SPL during PND 15–30 caused minimal long-term changes in the ear function, as shown by the auditory brainstem response, distortion product otoacoustic emissions and transient evoked otoacoustic emissions. Moreover, Gao et al. (2009) reported that rats exhibited non-significant impairment in electrocochleography and distortion product otoacoustic emissions after 5 days exposure to 90 dB SPL, whereas the hearing of the rats was impaired after exposure to 102 dB SPL. In addition, Burow et al. (2005) reported that 98 dB SPL could be considered noise of intermediate intensity, strong enough to allow endocrine axis habituation, but not so robust that adaptation would be prevented. These data were also confirmed by Sasse et al. (2008), Samson et al. (2007) and Turner et al. (2005). In contrast, noise of intensities of 110 to 130 dB demonstrated to be more harmful on auditory pathway, while noise of lower intensity such as the used in the present paper did not seem to have the ability to induce histological or behavioral changes in auditory structures (Cappaert et al., 2000; Kim et al., 2008). Indeed, noise levels used in the present work are

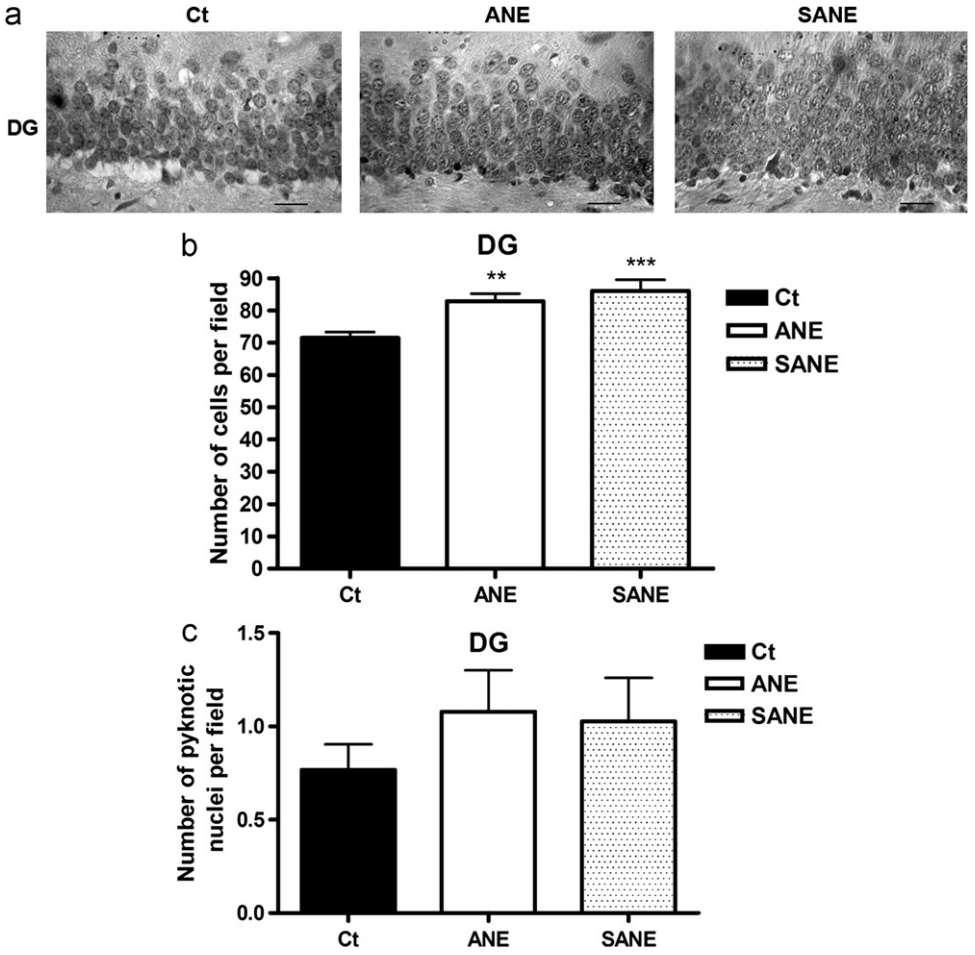

Fig. 8 – Effect of noise exposure on DG hippocampal area.Significant changes were induced in hippocampal coronal hematoxilin-eosin DG sections of ANE and SANE rats when compared with controls. (a) Noise exposure did not induce any change in the number of pyknotic cells. (b) whereas the total number of cells was increased after ANE and SANE. (c) Data are expressed as the mean number of cells per field ± SEM. Scale bar: 25 μm. Ct: Control; ANE: Acute noise exposure; SANE: Subacute noise exposure. $^{**}p<0.01$ and $^{***}p<0.001$, when compared with control animals, respectively. $n=4$ for each group.

comparable to those detected in some industrial workplaces and streets of different cities of the world as reported by Chengzhi et al. (2011). In particular, these levels of noise are actually experienced in the downtown of our city (Buenos Aires, Argentina).

Only slight differences were found between the effects of ANE and SANE schemes on the different endpoints measured, suggesting that the number of exposures to noise could not be a determining factor in noise-induced damage. Considering that noise-exposed rats' behavior was assessed 15 days after the completion of noise exposure both in ANE and SANE animals, and that the only behavioral difference between ANE and SANE schemes disappeared after 15 days from the end of noise exposure (see Fig. 3a and b), it could be suggested that the interval between the last exposure to noise and the beginning of the behavioral testing is critical to consolidate noise-induced changes. In summary, only one exposure to noise at an early developmental age is necessary and sufficient to trigger long-lasting behavioral impairments and histological abnormalities in extra-auditory structures. Moreover, since most SANE changes persisted at least until PND 45, it might be postulated that, despite the time elapsed since last exposure, noise-induced damage cannot be reversed.

Behavioral data suggested that all PND 30 rats habituated successfully to a new environment at short term, as noted by the decrease in the number of lines crossed in the second session of the OF. Moreover, this decrease was maintained for at least 15 days. These results support previous work in which the habituation "within session" was similar in all groups, suggesting that the short term memory would be unaffected in exposed animals (Degroot et al., 2005; Uran et al., 2010; Pereira et al., 2011). In contrast, when rats were tested at long term, no decrease in the number of lines crossed in the second session of the OF was observed in exposed rats, neither at PND 30 nor at PND 45. These differences suggested that, whereas Ct rats were able to habituate to a new environment at either intertrial interval, noise-exposed rats failed to adapt to it when tested at long term. Taken together, it could be suggested that whereas short term processes remained unaffected after exposure to noise, a significant impairment was observed after long term testing in either noise scheme, supporting the hypothesis

that short term and long term processing mechanisms would involve different mechanisms (Izquierdo et al., 2000; Caceres et al., 2010).

The observation of HC histological abnormalities without changes in the performance in the OR task at the shortest delays tested (1 h) indicates that hippocampal damage did not affect the ability to appreciate novelty per se. On the contrary, the impaired novelty preference observed at longer delays (24 h) could be attributed to a disturbance in recognition memory. This delay-dependent memory impairment observed in the OR task of noise-exposed rats, together with the HC disruption, supported the OF results and also results of Clark et al. (2000) which found that rats with hippocampal lesions showed impaired recognition memory. Therefore, it could be suggested that histological changes found in noise-exposed animals might underlie behavioral abnormalities.

Habituation to a new environment was proposed to be related to HC (Thiel et al., 1998; Vianna et al., 2000; Giovanini et al., 2001; Berti et al., 2012). Moreover, object recognition, a type of non-spatial memory, has also been associated to this nervous system structure (Ennaceur and Delacour, 1988; Schröder et al., 2003; de Lima et al., 2006; Bevins and Besheer, 2006). Since an impairment in OF and OR performance at LT was observed in noise-exposed rats, it might be suggested that HC could be a major candidate for being a target of noise injury (Pereira et al., 2011). It should be noted that the increase in the number of pyknotic cells in CA1 and CA3 regions of noise-exposed animals, together with the increase in the number of total cells in such regions, could suggest that cell death could have taken place after exposure to noise (Pawluski et al., 2010). Moreover, it could indicate that the observed deficits in habituation and recognition memory could be underlain by HC damage. These findings are supported by results of Park et al. (2001) who found habituation deficits in a model of stress, suggesting that the reduction in the efficiency of hippocampal-dependent behavioral processing produced a correlated hippocampal lesion. Likewise, a failure to habituate to the environment was also found in a mutant mice model of Alzheimer's disease characterized by hippocampal dysfunction (Deacon et al., 2009, Cui et al., 2009). Therefore, a relationship between hippocampal behavioral and histological damage could be postulated. However, it should not be discarded that other areas of the brain might also be involved in the behavioral changes found in noise-exposed animals (Daenen et al., 2001). For instance, basal amygdala is known to receive input from association cortex areas and from regions processing memory and cognition such as the HC (Kraus and Canlon, 2012). In addition, prefrontal cortex was reported to be involved in behavioral deficits after hippocampal lesions (Daenen et al., 2001). Further neuroanatomical and biochemical studies are needed to elucidate which structures are implicated.

Finally, it could be hypothesized that the previously reported oxidative imbalance found in HC of noise-exposed animals, induced as a consequence of a decrease in hippocampal ROS levels and an increase in antioxidant enzymes activities (Uran et al., 2010, Massaad and Klann 2011; Pourova et al., 2010), might be the triggering factor for the histological impairment found in noise-exposed animals which could underlie the abnormal HC-related behavior.

4. Conclusions

These data suggest that exposure of rats to moderate noise levels at early developmental stages is able to trigger changes in the hippocampus, an extra-auditory structure of the Central Nervous System, that could underlie the behavioral hippocampal-related impairments observed in exposed rats that seem to be long-lasting.

5. Experimental procedures

5.1. *Animals*

Healthy male and female albino Wistar rats were obtained from the Animal Facilities of the Biochemistry and Pharmacy School of the University of Buenos Aires, Argentina.

A total of 10 cages with one male and two females each (total females $n=20$; total males $n=10$) were used in this study for mating procedures. When pregnancy of each female became evident (e.g., few days before delivery), pregnant rats were isolated one per cage and left undisturbed until delivery.

The day of birth was designated as postnatal day (PND) 0 and was determined by the inspection of the cages three times per day. Only male rats, coming from 20 litters, were used for the different experimental procedures. At PND 15, animals were randomly assigned to each experimental group ($n=28$ for each group): control (Ct), acute noise exposed (ANE) and sub-acute noise exposed (SANE). Then, the 28 animals of each group were randomly assigned to each of the following groups: (a) PND 30: OF and OR at short term (ST, 1 h intertrial interval); (b) PND 30: OF and OR at long term (LT, 24 h intertrial interval); (c) PND 45: OF and OR at ST; (d) PND 45: OF and OR at LT. Histological assessment was performed in a subset of each group. Therefore, the number of animals in each experimental group was $n=7$ (total animals used: 84).

Therefore, a subset of rats ($n=56$) was exposed to noise, according to each scheme and another subset of animals-the Ct, sham-exposed rats ($n=28$)-, was placed in the same box than noise-exposed rats, but without being exposed. All groups were kept with their dams until 21 day of age.

After weaning, rats were separated and maintained 3–4 per cage until PND 30 or 45, with food and water *ad libitum*, on 12 h light–dark cycles (lights on at 7 A.M.) at $22\pm2\,^{\circ}C$ and wood shavings for bedding.

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 503/10. The CICUAL adheres to the rules of the "Guide for the Care and Use of Laboratory Animals" (NIH) (2011 revision) and to the EC Directive 86/609/EEC (revision 2010) for animal experiments. Adequate measures were taken to reduce the number of animals used and to minimize their pain or discomfort.

To avoid circadian rhythm alterations, noise exposures were performed in the intermediate phase of the light cycle during 2 h, within the range of 10 A.M. to 2 P.M. Moreover, behavioral tests were performed at the same time interval in

each session. For each experimental condition, seven PND 15 rats were used. At PND 30 or 45, behavioral tests were performed. In a subset of PND 30 animals, HC was dissected to assess possible histological damage. Experimental design is depicted in Fig. 1.

5.2. Exposure to noise

Animals were kept in their home wire-mesh cages (40 cm × 25 cm × 16 cm), so that they were not handled throughout noise exposure period. The cages were introduced into an "ad hoc" ventilated wooden sound chamber of 1 m × 1 m × 1 m, as reported by Cui et al. (2009). Due to the different number of males born in each litter, a different number of animals were exposed each time. Usually, one to four rats per cage were exposed simultaneously.

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

PND 15 animals were exposed to white noise at 95–97 dB SPL (20–20,000 Hz) 2 h per day, either in a single (ANE) or in multiple (15 consecutive days) exposures (SANE). Ct animals were placed in the same box of noise-exposed animals for the same period of time, but without being exposed to noise (sham-exposed). The range of background noise was between 50 and 55 dB SPL, being within the interval suggested by the WHO (World Health Organization) guidelines (NIOSH, 1998) and by other authors (Campeau et al., 2002; Sasse et al., 2008). Lighting was provided by a 20 W lamp located in the upper left corner of the sound chamber. In addition, sound chamber was provided with a sound attenuation system made with Celotex™.

To maintain comparable experimental conditions, SANE pre-weaning rats were separated from their mothers and post-weaning rats were deprived of food and water during noise exposure period.

5.3. Histological assessment

5.3.1. Tissue preparation

Animals were anesthetized with 28% chloral hydrate (0.1 ml per 100 mg of body weight) and perfused intracardially with Ringer's media at 35 °C, followed by 4% paraformaldehyde at pH 7.2. After perfusion, animals were decapitated and each brain was dissected out and maintained for two additional hours in the same fixative solution. The tissues were dehydrated and included in paraffin. After that, coronal brain sections containing the hippocampal area were obtained with a Micron microtome (4–6 μm of thickness) and recovered for light microscopy analysis. Slides were stained with hematoxylin and eosin (HE).

5.3.2. Histological morphometry

The number of neurons and pyknotic nuclei was assessed in the hippocampi of exposed animals and compared with their respective controls using the software NIH ImageJ 1.40 g (Roy et al., 2005). The slides were coded and the examiner was blinded to treatment groups. To guarantee uniform sampling, we maintained the septotemporal and mediolateral orientations and used the positions of blood vessels as landmarks. We took the middle of the ectal limb of the dentate gyrus (DG), as well as the pyramidal cell layer of the CA1 and CA3 regions and counted the number of neurons in a fixed field size (8200 μm^2 for CA1 and CA3 and 5800 μm^2 for DG).

Pyknotic cells were counted on these sections as a morphological measure of cell death. Cells were considered pyknotic if they lacked a nuclear membrane, had pale or absent cytoplasm compared to non-pyknotic cells and had darkly stained spherical chromatin (Pawluski et al., 2010). Using pyknosis as a measure of cell death does not allow distinguishing between the type and age of the dying cells. Therefore, it could not be possible to determine whether a differential cell death between young vs. older neurons has been induced (Pawluski et al., 2010).

5.4. Behavioral assessment

5.4.1. Open field task (OF)

Repeated sessions in an open field device were used to analyze habituation memory. Habituation to a novel environment is believed to be one the most elementary forms of non-associative learning, known to depend on HC (Vianna et al., 2000, Giovanini et al., 2001), in which the repeated exposure to the same environment induces a reduction in the exploratory behavior. A decrease in the number of squares crossed in the second session is taken as a measure of habituation memory retention (Vianna et al., 2000, Pereira et al., 2011). Open field consisted in a 50 × 50 × 50 cm dimly illuminated wooden box with a floor divided into 25 equal squares by black lines. Prior to exposure to the OF task, rats were allowed to habituate to the behavioral room for 3 min in their home cages. After that, rats were taken out of the cage, gently placed on the left rear quadrant of the OF box and allowed to freely explore the arena for 6 min (first session). Then, the number of lines crossed, indicative of locomotor activity, was recorded over the session. In a second session, either at ST (1 h intertrial interval) or at LT (24 h intertrial interval), animals were left to explore the apparatus for another 6 min and the number of lines crossed were recorded again to evaluate habituation to the task (Barros et al., 2006). Comparison between the numbers of lines crossed by each group during the first session (when the environment would have maximal novelty) with the number of lines crossed during the second session (when the environment became familiar) was performed.

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

5.4.2. Object recognition task (OR)

The object recognition task was performed according to the protocol described by Bevins and Besheer (2006). Object recognition task evaluates visual hippocampal functions

(Ennaceur and Delacour, 1988; Bevins and Besheer, 2006) and is used to assess the non-spatial recognition memory performance of rodents (Heldt et al., 2007; Clark et al., 2000).

The task was performed in the same wooden box than that used in the OF task ($50 \times 50 \times 50$ cm). In the habituation session, the rat was placed in the box and allowed to freely explore the apparatus for 5 min. During the training session (first session), two identical objects were placed in the box. The session started when the rat was placed in the apparatus facing the wall at the middle of the front segment and allowed to explore the box and the objects. At the end of session, the rat was immediately put back in its home cage. In the testing session (second session), one object was replaced by a novel, non-familiar, object. Exploration time of each object was assessed in training and testing sessions. Exploration was defined as directing the nose to the object at a distance of no more than 2 cm and/or touching the object with the nose. Sitting on the object was not considered exploration.

Testing session was performed either at ST or at LT after training. Total exploration time was set to 5 min for each session.

Animals' activity was recorded using a camcorder. As a convention, rats were not allowed to displace the objects. Experiments made in a separate cohort of animals demonstrated that rats had no preference for either object or location in the box.

To minimize the olfactory stimulus, the box and objects were cleaned with a 10% ethanol solution between sessions. Different groups of rats were used for each intertrial interval.

5.5. *Statistical analysis*

Significant differences between groups were analyzed through one or two way ANOVA statistical. For post-hoc comparisons, Tukey test was used. Levene's test of equal variances was applied for all tests (SigmaStat, v. 3.5). Results are expressed as mean values $\pm$ SEM. A probability < 0.05 was accepted as significant.

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