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Behavioral effects in mice of postnatal exposure to low-doses of 137-cesium and bisphenol A

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Abstract

Bisphenol A (BPA) is the most important plasticizer used in many household products such as polycarbonate plastics or epoxy resins. Public and scientific concerns exist regarding the possibility that the neonatal exposure to BPA may contribute to neurobehavioral disorders. On the other hand, there is little information on the effects of low doses of ionizing radiation during critical phases of postnatal brain development, as well as the combination of radiation and environmental chemicals. In this study, C57BL/6J mice were exposed to low doses of internal radiation (^{137}Cs), and/or BPA on postnatal day 10 (PND10). At the age of two months, animals were submitted to several tests to assess anxiety, activity, learning, and memory. Results showed that exposure to ^{137}Cs, alone or in combination with BPA, increased the anxiety-like of the animals without changing the activity levels. Animals exposed to ^{137}Cs showed impaired learning, and spatial memory, an impairment that was not observed in the groups co-exposed to BPA.

***Keywords**:* ionizing radiation, 137-cesium, bisphenol A, postnatal, behavior, mice

1. Introduction

The biological effects of ionizing radiation (IR) from environmental, medical, and man-made sources, as well as from space exploration are of broad health concern (Spitz and Hauer-Jensen 2014). The distribution of artificial radionuclide species in the environment depends on the sources, the release conditions and the subsequent transformation processes (Ashraf et al. 2013). Among radionuclides, 137-Cesium (^{137}Cs) is considered an important indicator of radiative pollution in aquatic environments. The nuclear reactor waste, and accidental releases from Fukushima Daiichi Nuclear Power Plant (FNPP) disaster, and the Chernobyl accident in the Ukraine have been the major sources of ^{137}Cs in the environment. Because ^{137}Cs has a longer half-life (30.07 years) compared with other radiocesium isotopes, it tends to accumulate in bottom sediments, aquatic plants, and fish. Thus, the contamination of drinking water and the consumption of contaminated vegetables and fish are the main pathways for potential human exposure (Ashraf et al. 2013; Heredia et al. 2015). Previous studies showed that neurogenic areas of the brain, including the dentate subgranular of hippocampus, are extremely sensitive to IR (Haridas et al. 2012; Rola et al. 2004). Epidemiological studies conducted after the Hiroshima/Nagasaki atomic bombing showed an increased risk of mental retardation in children who were exposed *in utero* exposure to radiation (Verheyde and Benotmane 2007). Moreover, a reduction in both learning abilities and high school attendance has been observed in children exposed to therapeutic doses of radiation (Hall et al. 2004; Kumar et al. 2013). Epidemiological evidence suggests that low doses of ionizing radiation (≤1.0 Gy) produce persistent alterations in cognition, if the exposure occurs at a young age. It has been long recognized that, cranial irradiation used for the treatment of primary and metastatic brain tumor, often causes neurological side-effects such as intellectual impairment, memory loss and dementia, especially in children (Zhang et al. 2014). It has been suggested that a IR in a critical period of brain development might be sufficient to cause persistently reduced cognitive function (Buratovic et al. 2014).

It is important to note that humans are usually co-exposed to various environmental toxicants. Thus, people are daily exposed to several toxicants such as bisphenol A (BPA). BPA is a compound used in the manufacture of many plastics and

resin products (e.g., bathtubs, countertops, microwaveable food containers and shatterproof beverage containers). In humans, the main pathway of exposure is the intake of food and beverages being, the estimated daily intake <1 μg/kg body weight/day (Kang et al. 2006; Vandenberg et al. 2010). However, pregnant women show higher levels than the general population (Vandenberg et al. 2010). Similarly, urinary BPA levels are significantly higher in occupationally exposed man than in non-exposed workers (Hanaoka et al. 2002). Detectable BPA levels are found in a variety of physiological locations of humans (e.g., saliva, urine, blood, breast milk, placenta) (Rubin 2011). *In vitro* and *in vivo* studies show that BPA exerts estrogenic and anti-androgenic effects by competing with the endogenous hormones at their receptor level (Negishi et al. 2003; Zhang et al. 2014). Recent studies have reported that BPA exposure induces abnormal neurogenesis and hyperplasia in different regions of the mouse embryo (Itoh et al. 2012; Komada et al. 2012). Additionally, it has been observed that BPA induces synaptic remodeling in the nervous system, while it impairs the development of higher cognitive functions (Hajszan and Leranth 2010). Viberg et al. (2011) reported that a single dose of BPA at postnatal day (PND) 10 can induce altered spontaneous behavior and cognitive function in mice. In turn, various studies have described an increased anxiety-like and fear memory of mice after BPA exposure (Luo et al. 2013; Zhang et al. 2014). In a recent study by Jasarevic et al. (2013) pregnant mice were fed during pregnancy and lactation with control diet and different doses of BPA. The offspring showed increased anxiety-like levels and reduced exploratory behavior. Moreover, enhanced depression-like responses were also observed after postnatal exposure (Fujimoto et al. 2013). However, a very consistent finding is that exposure to low doses of BPA early in life disrupts the development of normal sexually dimorphic behaviors (e.g., anxiety and social interaction) (Rubin et al. 2006; Tian et al. 2010; Wolstenholme et al. 2011).

Investigations with rodents have shown that neurodevelopmental exposure to some toxicants affect adversely postnatal orientation, attention, motor activity, and executive function later in life (Schneider et al. 2011). Therefore, co-exposure to ionizing radiation and toxics at very young age might also influence the development of central nervous system (CNS) and could have a negative impact on cognitive development during childhood (Buratovic et al. 2014; Hall et al. 2004). Taking all the

above into account, the main objective of this study was to investigate the effects of postnatal co-exposure to internal radiation (^{137}Cs), and BPA on the neurobehavior of mice.

2. Material and Methods

2.1 Animals

All experiments were performed in pregnant C57BL/6J mice (Charles River, CRIFFA, Barcelona, Spain). Mice were kept in standard animal cages under a 12 h light/dark cycle (light: 8:00-20:00 h), at a temperature of 22±ºC and a relative humidity of 50±10%, with *ad libitum* access to tap water, and food (Panlab rodent chow, Barcelona, Spain). The use of animals and the experimental protocol were approved by the Animal Care and Use Committee of the Universitat Rovira i Virgili (Tarragona, Catalonia, Spain) following the "Principles of laboratory animal care", being carried out in accordance with the European Union Directive 2010/63/EU for animal experiments.

2.2 Groups and treatment

Sixty female mice were randomly assigned to different experimental groups (n=10) and received a single subcutaneous dose of 0.9% saline solution, cesium (^{137}Cs, provided by CIEMAT, Madrid, Spain) and/or bisphenol A (BPA, 239658-50G, provided by Aldrich, Barcelona, Spain) on postnatal day 10 (PND10). There is enough evidence that developing brain is susceptible to permanent impairment during this time window of vulnerability (Stein et al. 2002). Six experimental groups were established: control group (0.9% saline solution used as vehicle), BPA group (25 µg/kgbw of BPA), Cs 4000 group (^{137}Cs with activity of 4000 Bq/kgbw), Cs 8000 group (^{137}Cs with activity of 8000 Bq/kgbw), BPA/Cs 4000 group (25 µg/kgbw of BPA and ^{137}Cs with activity of 4000 Bq/kgbw) and BPA/Cs 8000 groups (25 µg/kgbw of BPA and ^{137}Cs with activity of 8000 Bq/kgbw). The ^{137}Cs, and BPA doses were based on the results of previous investigations (Cao et al. 2013; Lestaevel et al. 2008; Walser-Kuntz et al. 2014). At the

age of two months, animals were submitted to different behavioral tests in the following sequence: elevated plus maze (anxiety), open-field test (anxiety/activity), water maze test (learning and spatial memory), and radial maze test (learning and spatial-working memory). Each test was conducted with an interval of 24 hours of rest for animals. Animals were tested during the same light phase of light/dark cycle.

2.3 Behavioral tests

2.3.1 Elevated Plus Maze test (EPM)

The Plus Maze test is one of the most used mazes to assess anxiety-like levels in mice. It was developed to screen anxioselective effects of drugs (Almatroudi et al. 2015; Lister 1987; Pellow et al. 1985). The apparatus used for the EPM comprises two open arms (25x5x0.5cm) across from each other and perpendicular to two closed arms (25x5x16cm), with a center platform (5x5x0.5cm). The small wall (0.5cm) in the open arms is used to decrease the number of falls. The entire apparatus is 50cm above the floor. In our protocol, mice were transported to the behavioral testing room 30min prior the behavioral testing. Each animal was placed in the central square at the start of 5min session, being allowed to explore freely the environment. After every observation period, and before placing the next animal, the apparatus was cleaned with 70% ethanol in order to remove the olfactory cues left by the previous animal. Performance was recorded by a video camera placed above the maze, being the data analyzed by the video tracking program Ethovision XT© (Noldus Information Technologies, Wageningen, The Netherlands). The following parameters were registered: latency to first entry into the closed arms, time spent in open arms, number of entries into the open arms, and total distance travelled over the maze (Heredia et al. 2015; Walf and Frye 2007) Moreover, an experimenter registered the number of head dips (downward movements of the head towards the floor) (Heredia et al. 2015; Rodgers et al. 1997).

2.3.2 Open-Field test (OF)

To assess the anxiety-like levels and the activity levels of the animals the OF test was used. The OF consisted in a 47cmx47cm wooden square surrounded by a 40cm

high dark wall. The area of the maze that was within 15cm from the wall was considered as peripheral (Franco-Pons et al. 2007; Vicens et al. 2011). The rest of the OF was considered as the central area. At the beginning of the test period, mice were placed in the center of the arena. They were allowed to move freely around the maze and to explore the environment for 15min. After every observation period, and before placing the next animal, the apparatus was cleaned with 70% ethanol in order to remove the olfactory cues left by the previous animal. The video tracking software Ethovision XT© was used to measure the following parameters: distance traveled over the maze and over the central area, and time spent in the central area. Additionally, an experimenter registered the number of rearings (vertical standing of mice on two hindlegs). From these parameters, the distance ratio (distance traveled in central area/total distance traveled in the maze) was calculated. During the behavioral testing, indirect lighting was used, being the lighting levels maintained at ~ 100 lux in the testing room (Lalonde and Strazielle 2012).

2.3.3 Water Maze test (WM)

To evaluate spatial learning and memory, animals were subjected to the Water Maze test (Morris 1984). The WM consisted of a circular tank (diameter 1m; height 60cm), divided into four quadrants. An escape platform (diameter 10cm) was located 1 cm below the surface of the water in the target quadrant. Animals performed 5 trials per day for 3 consecutive days. During each trial, mice were allowed 60s to find the hidden platform and to remain on it for 30s. If the animal failed to find the platform within this period, it was placed on it by the experimenter. The order of the three starting positions was randomized, throughout the day, for each mouse. Extra-maze clues were located around the pool to provide a spatial configuration of the task. To avoid proximal cues and prevent egocentrical learning, an internal mobile wall was added to the maze, being the wall randomly moved between trials. This seems to increase Morris water maze sensitivity (Ribes et al. 2008). At the end of the third acquisition day, retention of the task was assessed by a probe trial, which consisted of a 60s free swim without the escape platform (acquisition Probe). An additional probe trial to evaluate the spatial memory of animals (retention Probe) was performed 48 h after the last training day. Animal performance was recorded using a video camera

placed above the maze. Data were analyzed by the video tracking program Ethovision XT© (Noldus Information Technologies). Latency to escape the platform during the training sessions was measured. During the probe trial, total time spent in the target quadrant, as well as the time spent in other quadrants was also measured in order to compare the time spent searching in the target quadrant between groups (Belles et al. 2010).

2.3.4 Radial Arm Maze test (RAM)

The radial maze was developed by Olton and Samuelson (1976) to evaluate spatial-working memory in rodents. The maze consists of a central square (20 cm diameter) and eight radially attached arms (6cm wide x 35cm long). In this study, animals were trained 3 days (one trial/day) to collect a small food pellet placed at the end of each arm. After every observation period, and before placing the next animal, the apparatus was cleaned with 70% ethanol in order to remove the olfactory cues left by previous animal. This allows facilitating the effectiveness of the visual cues.

Mice had free access to water, but were deprived to food 12h before the initial trial, to increase their motivation for the task (Dale and Roberts 1986). The start of each trial began with the mouse placed in the central platform facing the same arm. The trial finished after 10min, or when the animal had eaten all food rewards (8 food pellets). In this task, animals store information continuously on which arm has already been visited, and which has not, using the extra-maze clues (spatial-working memory) (Suzuki et al. 1980). The optimum strategy implies a minimum number of visits to empty arms. In each session, the time spent in the arms was measured, as well as the number of incorrect arm choices (visitation to the same arm more than once during a single test session), and the number of incorrect arm entries (animal visits an arm and did not eat the reward) (Komatsu et al. 1998).

2.4 Statistics

Data are given as the mean ± standard error mean (SEM). Homogeneity of variances was analyzed using the Levene's test. If variances were homogeneous, ANOVA was used followed by the Tukey post hoc test to evaluate all dose groups simultaneously. If

the variances were not homogeneous, the Kruskal–Wallis test was used. Differences between groups were analyzed using the Mann–Whitney U-test. Moreover, the paired t-test was used to compare the two different point of time tested. The ANOVA test for repeated measures and post hoc analyses adjusted by Bonferroni's correction were used to analyze the progression of parameters recorded by the Ethovision XT© software. The level of statistical significance for all tests was established at $p < 0.05$. All data were analyzed by means of the statistical package SPSS© v.21 (SPSS Sciences, Chicago, USA).

3. Results

3.1 Elevated Plus Maze (EPM)

ANOVA analysis showed significant differences between groups in the time spent in open arms ($p<0.001$) and the number of head dips ($p=0.011$). Post hoc analysis revealed that all treatment groups spent less time in the open arms than the control group. Significant differences were observed between control group and BPA group ($p=0.001$), Cs 4000 group ($p<0.001$), Cs 8000 group ($p<0.001$), BPA/Cs 4000 ($p<0.001$) and BPA/Cs 8000 group ($p<0.001$) (Fig. 1A). However, no differences between treatment groups were observed. On the other hand, the number of head dips were lower in the BPA group ($p=0.043$), Cs 4000 group ($p=0.011$), BPA/Cs 4000 group ($p=0.032$) and BPA/Cs 8000 group ($p=0.032$) compared to control group (Fig. 1B).

3.2 Open-Field test (OP)

We registered the time spent in the central area (anxiety) and the total distance travelled over the maze (activity), as the best indicators of anxiety-like levels and activity levels. ANOVA analysis did not show significant differences between groups in these parameters (Fig. 2A,B). In turn, the number of rearings, and mean and distance ratio (distance center/total distance) were not significantly different between groups. ANOVA for repeated measures, with the variable distance travelled, was performed to analyze the habituation process of animals. A main effect of the time was noted

(p=0.001). However, post hoc analyses did not revealed significant differences between groups.

3.3 Water Maze test (WM)

In the WM acquisition, an ANOVA for repeated measures was performed to analyze the progression of the latency escapes across the training days. Results showed a significant effect of time (p=0.001), an interaction effect Time x Group (p=0.004), and a main effect of the group (p=0.029) in the performance. Post hoc analyses revealed that escape latencies of BPA group were lower than those in the Cs 4000 (p=0.034) and Cs 8000 groups (p=0.024) (Fig. 3). In the probe trial performed the last training day (Acquisition probe), statistical analysis revealed significant differences between groups (p=0.001). Specifically, ^{137}Cs exposed groups (Cs 4000 and Cs 8000), and BPA/Cs 4000 group spent less time searching in the target quadrant than the BPA group (p=0.004, p=0.001 and p=0.037, respectively) (Fig. 4A). No differences between groups were observed in the probe trial (Retention trial) performed 48 h after the last training day (retention trial) (Fig. 4B).

3.4 Radial Arm Maze test (RAM)

We compared in the RAM the total number of errors and the ratio performances (first day errors/second and third day errors) to evaluate the learning of animals. Results showed a main effect of time in the total number of errors (p<0.001) and ratio performances (p=0.021) across the days. An interaction effect Time x Group was detected for the total number of errors (p<0.001). However, no differences between groups were found (Fig. 5A,B). We also compared the number of incorrect arm choices, the number of incorrect arms entries, and the time spent in the arms. No significant differences between groups were noted.

4. Discussion

The present study aims to explore de long-term behavioral alterations induced by perinatal combined exposure to low doses of irradiation and BPA. When the animals were submitted to the EPM, we found that mice in all groups of treatment spent less

time in the open arms than those in the control group. These results indicate that mice in all treatment groups showed higher anxiety-like than those in the control group. A reduction in this variable is commonly explained as an increase in the anxiety-like levels (Walf and Frye 2007). Also, this is supported by the reduction in the number of head dips of treatment groups. These results are in agreement with previous studies that showed behavioral anomalies induced by radiation. In relation with the IR effects, Ganesan et al. (2014) assessed the behavioral effects of radiation from whole-body gamma irradiation (6.7 Gy) in rats. In this case and following the same line of our results, an increase of anxiety-like levels of animals in the EPM was registered. Recently, Kokosova et al. (2015) showed that the exploratory behaviour and locomotor activity were significantly lower and the level of anxiety was higher measured in EPM test in prenatal irradiated (1 Gy) rats aged 2 months. The increase of anxiety was expressed by significantly shortening the time spent in the open arms of the maze and low level of exploration. Another study conducted by Kumar et al. (2013) exposed mice to whole-body doses γ-radiation (2-8 Gy) and cognitive functions were tested using the EPM. The results indicated that radiation caused short-term memory dysfunctions at lower doses, but the long-term memory processing was disrupted at higher doses. Regarding to BPA exposure, several prior studies have reported evidence of heightened anxiety following developmental exposure to BPA (Mileva et al. 2014; Mustieles et al. 2015; Rebuli et al. 2015). Our results also demonstrated an increase in the anxiety-like levels of animals treated with BPA alone or in combination with IR. Similar results were also registered by Gioiosa et al. (2013). The authors showed evidence of increased anxiety in pre- and postnatally exposed offspring mice with a low dose of BPA (10 μg/kg bw/day). Also, Diaz et al. (2013) tested the effects low-dose BPA exposure (40 μg/kg body weight) in rats and the results showed an increase on anxiety.

In the present study, no significant effects of IR and BPA were observed in the activity-like levels of OF. The total distance travelled over this maze is considered as an indicator of activity levels (Carter et al. 2013). Our results showed no significant differences between groups in this parameter. However, previous studies reported an increase in the activity-like of animals after BPA exposure (Komada et al. 2014; Viberg et al. 2011). The early life BPA exposure has been related with conduct problems and

hyperactivity in children (Harley et al. 2013). In the OF maze, the time spent in the central area is considered as anxiety-like parameter (Lipkind et al. 2004). Previous studies did not report changes in the activity levels of animals exposed to ^{137}Cs and/or enriched uranium (Houpert et al. 2007a; Houpert et al. 2007b). In contrast, Soares et al. (2014) reported a reduction in the activity levels and impaired habituation of animals after whole body irradiation. In the present study, results of OF test are not in agreement with the results obtained in the EPM. However, differences between sensitivity of various tests depends on both strain and maze used (Mathiasen et al. 2008). Recently, the sensitivity to changes in anxiety-like levels of OF test using C57BL/6J strain has been reported (Heredia et al. 2014). Moreover, we found an increase in the time spent in the central area in BPA-treated mice. This parameter is interpreted as anxiety-like or activity-like, depending on the authors (Heredia et al. 2012; Naghibi and Rayatnia 2011; Weiss et al. 1998). Consequently, this result could indicate an increase in the activity-like levels of animals exposed to BPA. With similar results, Wang et al. (2014) reported that perinatal BPA exposure (0.05-50 mg/kgbw/day) showed no adverse effect on locomotor activity in the offspring.

In the MWM test, we assessed learning and spatial memory. Regarding to spatial learning, the current results showed that groups exposed to IR did not reduce their scape latencies across the training days, indicating impaired learning in these groups. Moreover, significant differences between BPA and irradiated groups were observed, indicating a differential effect of these toxicants. In fact, mice co-exposed to IR and BPA showed similar performance levels than those in the control group. These results may suggest a possible protective effect of BPA on spatial learning capabilities against IR exposure. A Previous study did not find impaired learning after a perinatal irradiation (8 Gy) in mice (Kalm et al. 2013). In contrast, Kokosova et al. (2015), demonstrated that the prenatal irradiation (1 Gy) negatively influenced the short-term spatial memory in rats in MWM. Our results agree with those of previous studies showing that BPA exposure does not impair spatial learning assessed in the MWM test (Ferguson et al. 2012; Viberg et al. 2011). However, impaired spatial learning was observed in mice by perinatal exposure to BPA (50 mg/kgbw/day). In this case, BPA extended the escape latency time to locate the hidden platform (Kumar et al. 2013) . In the current investigation, as in previous studies, the last training day we assessed the

learning levels through a probe trial (Puzzo et al. 2014). In the acquisition probe we found significant differences between BPA and irradiated groups. Nevertheless, no significant differences between control and treated groups were observed. It would indicate a general tendency of BPA to improve the learning process against Cs exposure. In the probe trial performed 48 h after the last training session (spatial memory), no differences between groups were noted.

We assessed spatial-working memory in the RAM test, a test which animals should reduce their number of errors across the training days (Komatsu et al. 1998). We observed a main time effect of time for all groups. Animals reached the same performance level without significant differences between groups. Therefore, no effects on spatial-working memory after exposure to IR and/or BPA were found. So far few studies have evaluated the effects of radiation using the radial arm maze test. Using the RAM test, Hossain and Uma Devi (2001) studied the long-term effect of fetal irradiation (0.25-1.5 Gy) on the learning and memory in the adult mouse. The results showed a significant decrease in the learning ability and memory retention of 6-month-old mice at of 1 Gy. On the other hand, Wang et al. (2014) observed that perinatal exposure to BPA (0.05-50 mg kg bw/day) resulted in significantly more working and reference memory errors in the offspring.

In summary, the results of this study show that exposure and co-exposure to ^{137}Cs and BPA increases the anxiety-like levels of mice. ^{137}Cs impairs the spatial memory at the current doses. Unexpectedly, this impairment was not observed in the mice co-exposed to BPA and ^{137}Cs. Exposure (or co-exposure with BPA) to ^{137}Cs did not modify the activity levels of animals and their performance in the spatial-working memory task. On the other hand, a number of studies have shown a positive relationship between hippocampal function and behavioral performance (Kokosova et al., 2015; Yu et al., 2014). It has been suggested that a possible mechanism involved in IR effects is neuro-inflammation. This inflammatory response can be characterized by expressions of inflammatory molecules including cytokines (Moravan et al., 2011). Recently, Son et al. (2014) showed that IR-induced decrease in hippocampal neurogenesis and hippocampus-related behavioral disability. In relation to BPA exposure, Tiwari et al. (2015) suggested that BPA exposure, both during prenatal and postnatal periods,

altered myelination in the hippocampus of the rat brain, leading to cognitive deficits. Perinatal exposure to BPA might affect neuronal plasticity in the hippocampus, thereby potentially modulating neuronal development and leading to impaired cognitive and memory functions (Masuo and Ishido, 2011). Taking the above into account, we conclude that the current results may contribute to understand the potential risk of cognitive dysfunctions in children environmentally co-exposed to ionizing radiation and environmental chemicals. However, the identification of the possible molecular mechanisms involved in hippocampal dysfunction, after early co-exposure to IR and environmental toxicants such as BPA, needs still to be clarified. Consequently, further investigations are required in order to define the clinical relevance and to elucidate the mechanisms involved.

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FIGURE LEGENDS

Figure 1. Differences between time spent in open arms (Panel A) and number of head dips (Panel B) in the plus maze test. *: indicates significant differences between treatment and control group at $p<0.05$. **: indicates significant differences between treatment and control group at $p<0.01$. ***: indicates significant differences between treatment and control group at $p<0.001$. Data are expressed as mean ± SEM.

Figure 2. Means of time spent in central area (Panel A) and total distance travelled (Panel B) in the open field test. Data are expressed as mean ± SEM.

Figure 3. Escape latencies in the water maze test during training days. An asterisk indicates significant differences between groups. *: indicates significant differences between treatment and control group at $p<0.05$. Data are expressed as mean ± SEM.

Figure 4. Differences between the time searching in target quadrant in the acquisition probe performed the last day of training (Panel A) and the retention probe performed 48 h after the last training session (Panel B) in water maze test. Different letters indicates significant differences between groups at $p<0.05$. Data are expressed as mean ± SEM.

Figure 5. Means of total number of errors (Panel A) and performance ratios (Panel B) during the training days in the radial arm maze. Data are expressed as mean ± SEM.

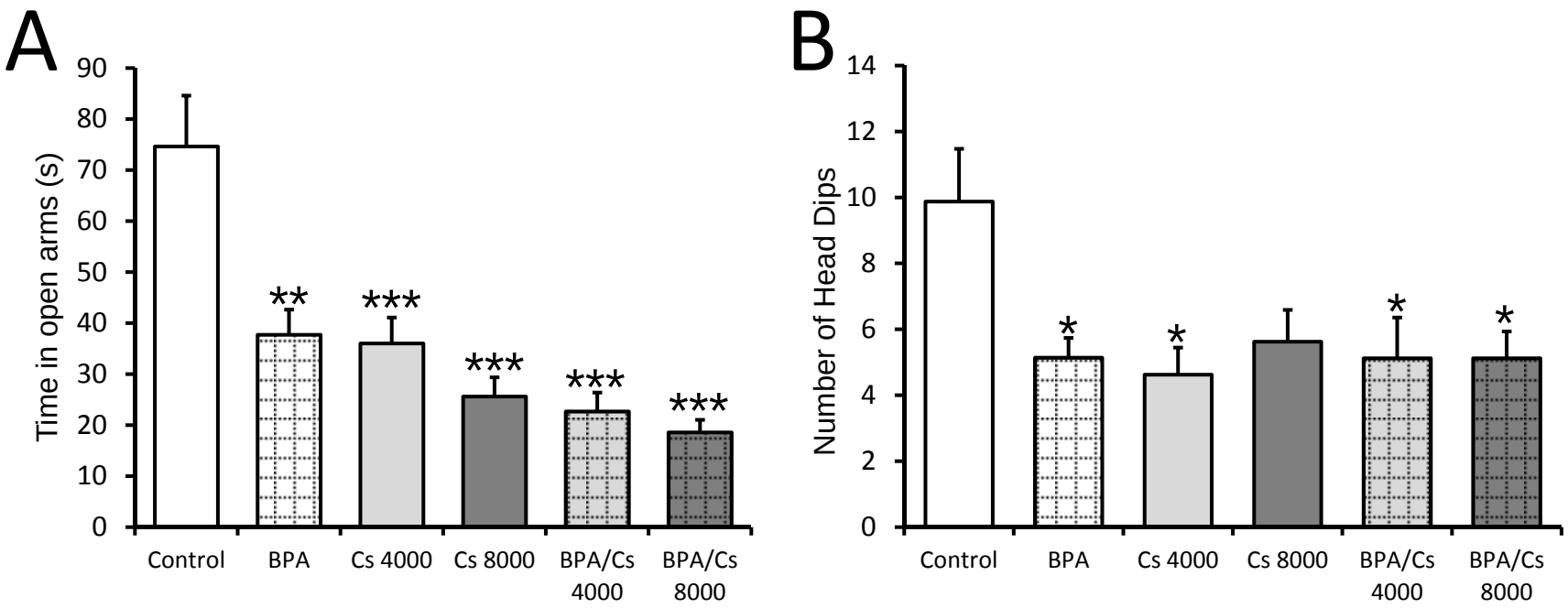
A
Time in open arms (s)
90
80
70
60
50
40
30
20
10
0
**
***
***
***
***
Control
BPA
Cs 4000
Cs 8000
BPA/Cs 4000
BPA/Cs 8000
B
Number of Head Dips
14
12
10
8
6
4
2
0
*
*
*
*
Control
BPA
Cs 4000
Cs 8000
BPA/Cs 4000
BPA/Cs 8000

Figure 1

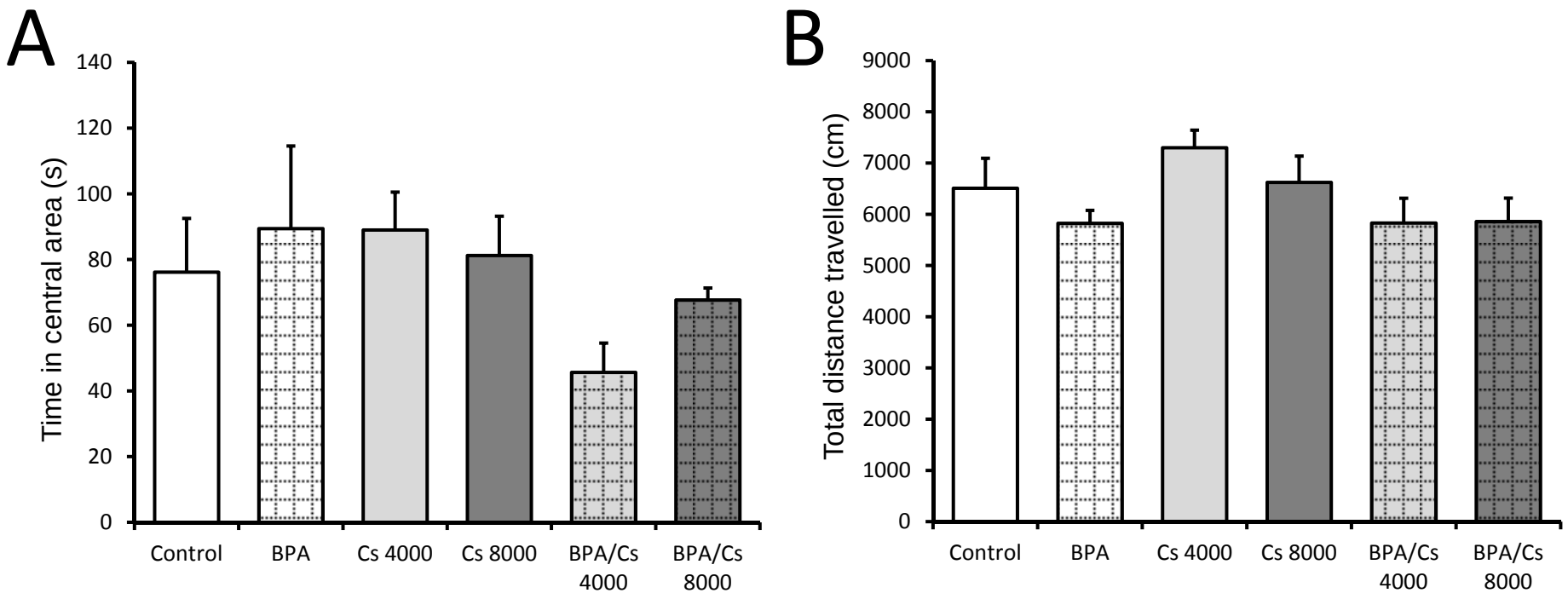
A
Time in central area (s)
140
120
100
80
60
40
20
0
Control
BPA
Cs 4000
Cs 8000
BPA/Cs 4000
BPA/Cs 8000
B
Total distance travelled (cm)
9000
8000
7000
6000
5000
4000
3000
2000
1000
0
Control
BPA
Cs 4000
Cs 8000
BPA/Cs 4000
BPA/Cs 8000

Figure 2

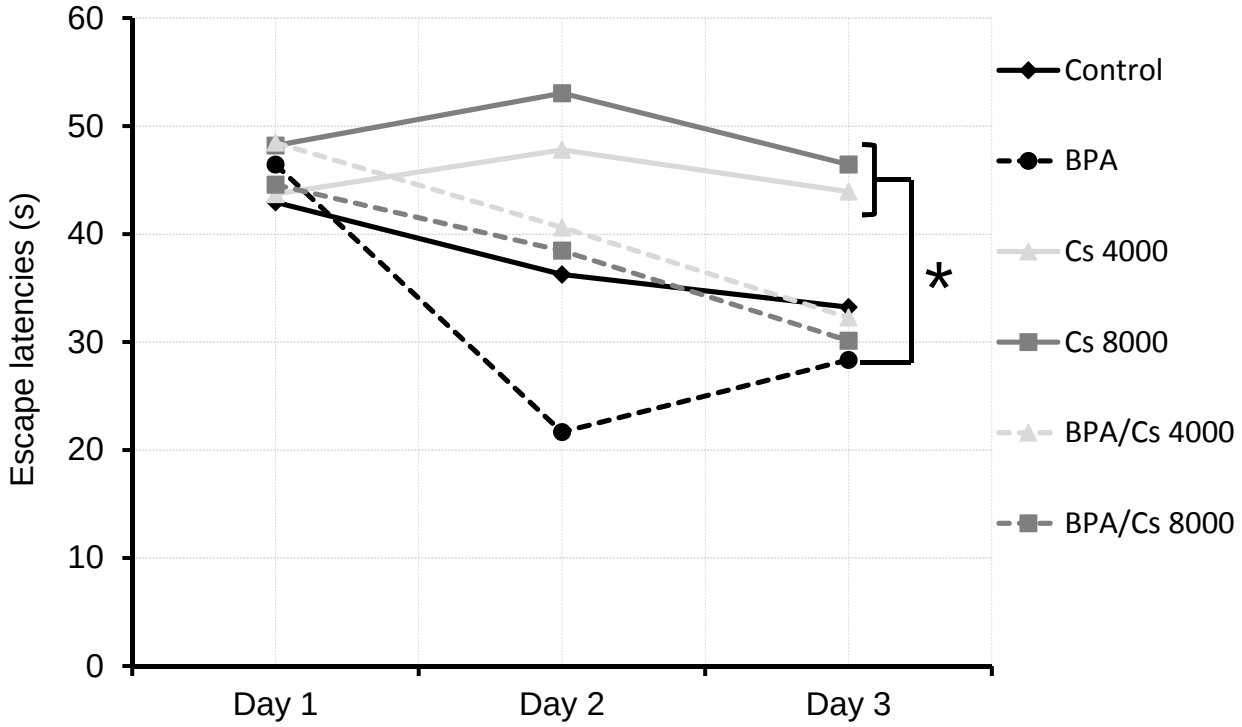
60
50
40
30
20
10
0
Escape latencies (s)
Day 1
Day 2
Day 3
*
Control
BPA
Cs 4000
Cs 8000
BPA/Cs 4000
BPA/Cs 8000

Figure 3

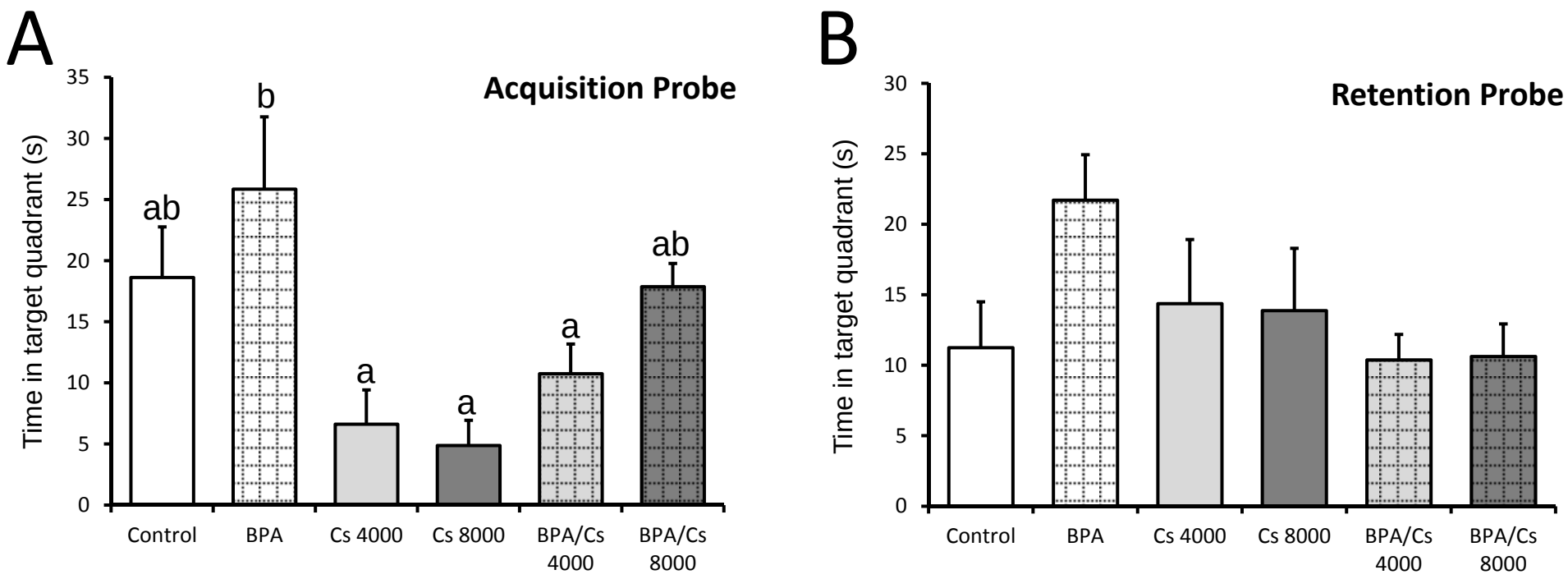
A
Acquisition Probe
Time in target quadrant (s)
35
30
25
20
15
10
5
0
ab
b
a
a
a
ab
Control
BPA
Cs 4000
Cs 8000
BPA/Cs 4000
BPA/Cs 8000
B
Retention Probe
Time in target quadrant (s)
30
25
20
15
10
5
0
Control
BPA
Cs 4000
Cs 8000
BPA/Cs 4000
BPA/Cs 8000

Figure 4

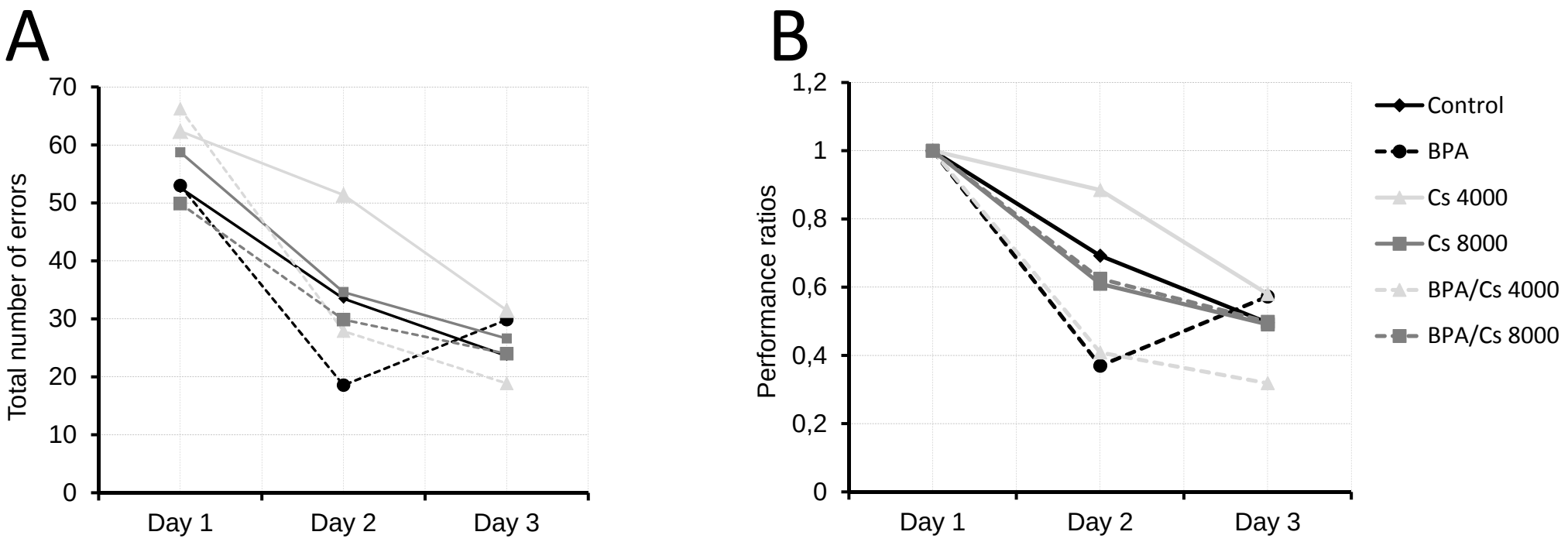
A
Total number of errors
70
60
50
40
30
20
10
0
Day 1
Day 2
Day 3
B
Performance ratios
1,2
1
0,8
0,6
0,4
0,2
0
Day 1
Day 2
Day 3
Control
BPA
Cs 4000
Cs 8000
BPA/Cs 4000
BPA/Cs 8000

Figure 5