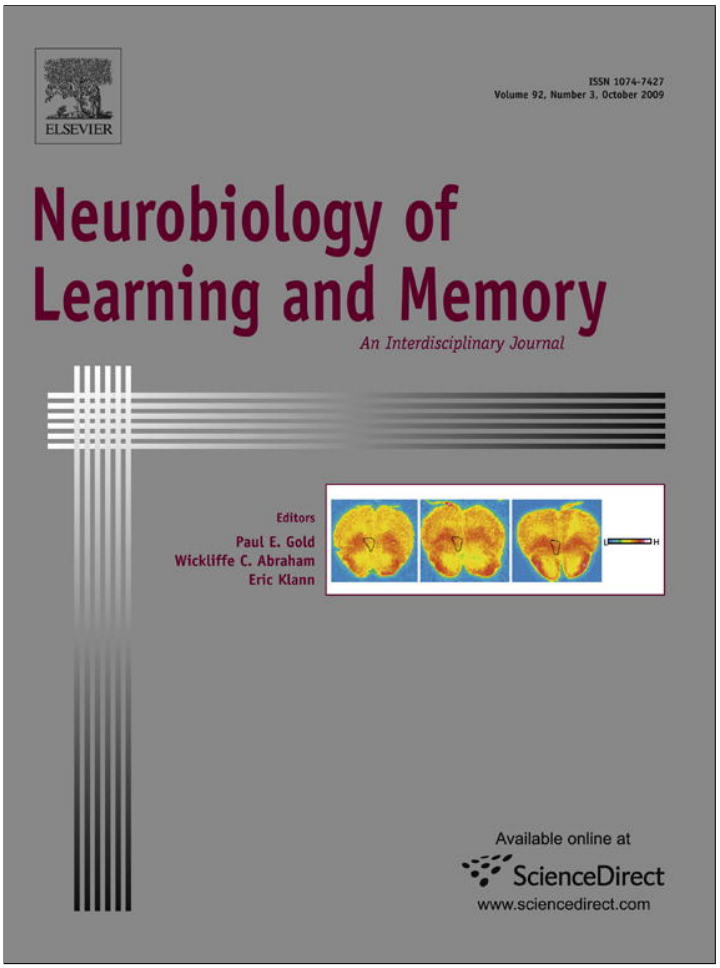

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Effects of prolonged iron overload and low frequency electromagnetic exposure on spatial learning and memory in the young rat

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ABSTRACT

Low-frequency electromagnetic fields (EMF) have been suggested to affect the brain via alterations of blood–brain barrier permeability to iron. Because of an immature blood–brain barrier, the young brain may be particularly vulnerable to EMF exposure. It is therefore possible that behavioral and neurotoxic effects resulting from EMF-induced iron excess in the brain would be greater in young adults. The objective of the present study was to investigate the interaction between low-frequency EMF and iron overload in young rats. In Experiment 1, we tested the effects of iron overload on spatial learning and memory. Iron treatment did not affect performance in a reference (Morris water maze) and a working memory task (8-arm radial maze). In contrast, detection of a spatial change in an object exploration task was impaired. These effects correlated with modifications of the serotoninergic metabolism. In Experiment 2, the combination of EMF exposure and iron overload was tested. As in Experiment 1, rats were not impaired in reference and working memory tasks but were mildly impaired in the detection of the spatial change. Overall, the results showed an effect of iron overload on spontaneous spatial memory processes. However, low-frequency EMF exposure did not potentiate the effects of iron overload in young rats.

1. Introduction

During the last decade, considerable research has focused on the biological effects of electromagnetic fields (EMF, ranging from low frequencies 0 Hz–10 MHz to radiofrequencies 10 MHz–300 GHz) generated by electrical and communication technologies. A substantial number of studies have indicated that EMF has an impact on biological functions thus raising concerns about health risks. Whether EMF exposure results in adverse health outcomes is abundantly debated and controversial. As repeatedly claimed, potential risks may include childhood leukemia, breast cancer, DNA damage, neurological effects, neurodegenerative diseases, immunological changes (Repacholi, 1998; Ahlbom et al., 2001; Hardell & Sage, 2008) but this remains to be clarified (Habash, Brodsky, Leiss, Krewski, & Repacholi, 2003a, 2003b).

Particular interest has concentrated on the impact of EMF on brain functions. Several neurobiological and neurophysiological effects have been reported. Exposure to EMF induces modifications of excitatory and inhibitory neurotransmitter systems (ACh: Testylier, Tonduli, Malabiau, & Debouzy, 2002; NMDA: Mausset-Bonnefont et al., 2004; GABA: Mausset, De Seze, Montpeyroux, & Privat, 2001), and affects electrophysiological activity in human and animals (e.g. Hardell & Sage, 2008; Huber et al., 2002; Krause, Pesonen, Haarala Bjornberg, & Hamalainen, 2007; Maby, Jeannes, Faucon, Liegeois-Chauvel, & De Seze, 2005; Vecchio et al., 2007). A number of reports show effects of EMF exposure on cognitive functions and performance in various behavioral tasks in human (e.g., Keetley, Wood, Spong, & Stough, 2006; Koivisto et al., 2000; Lee, Lam, Yee, & Chan, 2003) but other failed to find any effect (Haarala et al., 2007; Russo et al., 2006; Terao, Okano, Furubayashi, & Ugawa, 2006). Animal studies also yielded inconsistent results. Performance in a spatial working memory task using the 8-arm radial maze has been found to be altered in a study (Lai, Horita, & Guy, 1994) but unaffected in several others (Cobb, Jauchem, & Adair, 2004; Cosquer, Kuster, & Cassel, 2005; Dubreuil, Jay, & Edeline, 2002, 2003; Sienkiewicz, Blackwell, Haylock, Saunders, & Cobb, 2000). Object recognition was also non-altered (Dubreuil, Jay, & Edeline, 2003).

It is nevertheless necessary to distinguish between high frequency EMF generated by mobile communication and low frequency EMF generated by power lines, household electrical wiring and medical devices. Although exposure to low frequency EMF has been shown to induce a variety of biological effects (Cec-

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coni et al., 2000; Fear & Stuchly, 1998; Hendee et al., 1996), and to interfere with brain activity (Bawin, Satmary, Jones, Adey, & Zimmerman, 1996; Carruba & Marino, 2008; Gona et al., 1993), their impact on cognitive processes is so far poorly known (Gerth, Schlykowa, Thoss, & Drischel, 1983; Rudolph, Krauchi, Wirz-Justice, & Feer, 1985).

There is evidence that high and low frequency EMF alters blood–brain barrier permeability (Oscar & Hawkins, 1977; D'Andrea, Chou, Johnston, & Adair, 2003; Hossmann & Hermann, 2003; Nittby et al., 2008). It has been suggested that changes in the integrity of the blood–brain barrier may result in excess accumulation of heavy metals and in particular of iron in the brain (Castelnau et al., 1998; Thompson, Shoham, & Connor, 2001). Iron is an ubiquitous component that plays an essential role in many metabolic functions including brain functions. It is carried by transferrin – a Fe-binding glycoprotein – and transported across the blood–brain barrier mainly by transferrin receptor (Moos & Morgan, 2000). Thus, damage to the blood–brain barrier that would induce iron excess or deficiency may have neurotoxic effects (Zheng, Aschner, & Ghersi-Agea, 2003). Iron accumulation in the brain has been shown to generate cytotoxic effects associated with behavioral disruptions (Sobotka et al., 1996). For example, unilateral injection of iron in the substantia nigra in rats produces a drop of striatal dopamine that is associated with motor impairments (Ben-Shachar & Youdim, 1991; Sengstock, Olanow, Menzies, Dunn, & Arendash, 1993; Sengstock, Olanow, Dunn, Barone, & Arendash, 1994). Thus, oxidative damage due to iron may contribute to the development of human neurodegenerative disorders including Parkinson's and Alzheimer's disease (Bressler et al., 2007; Thomas & Jankovic, 2004; Thompson, et al., 2001; Zecca, Youdim, Riederer, Connor, & Crichton, 2004). In animals, dietary or administrated iron overload results in deficits in motor activity, habituation, startle reflex, active avoidance, inhibitory avoidance, working memory in the radial maze, and object recognition (Fredriksson, Schröder, Eriksson, Izquierdo, & Archer, 1999, 2000; Schröder et al., 2001; Sobotka et al., 1996). Interestingly, these studies promote the idea that the young brain is particularly sensitive to iron excess, possibly due to an immature iron permeability of the blood–brain barrier. It is therefore possible that behavioral and neurotoxic effects resulting from EMF-induced iron excess in the brain would be greater in young rats.

The objective of the present study was therefore to investigate the possible interaction between low frequency EMF and iron supplementation in young rats. First, we examined the impact of iron overload on spatial learning and memory using various tasks including a reference memory task in the Morris water maze, a working memory task in the radial 8-arm maze, and a non-associative memory task in an open field containing several objects. Second, we examined whether combining repeated exposure to low-frequency EMF and iron overload would produce greater memory deficits. Neurotoxic effect of iron supplementation in the brain was evaluated by measuring monoamine levels.

2. Material and methods

2.1. Subjects

One month-old male Wistar rats, purchased from a commercial supplier (Janvier, Le Genest-St-Isle, France) and weighting 130–150 g (4 weeks old) served as subjects. Upon arrival, they were housed by groups of two with food and water ad libitum and kept in a temperature-controlled room (20 ± 2 °C) with a 12/12 light/dark cycle. In Experiment 1, two groups were tested: (1) Iron overload (IO, $n = 8$), and (2) Saline (SAL, $n = 8$). In Experiment 2, three groups were tested: (1) EMF exposed (EMF, $n = 6$), (2) EMF exposed and iron overload (EMF-IO, $n = 6$), and (3) Sham exposed and non-treated with iron, (SHAM-EMF, $n = 6$). Iron overload and MF exposure treatment started one week after arrival.

Experiments were performed in accordance with the NIH guide for the care and use of laboratory animals (NIH publication no. 86–23, revised 1978), European guidelines (European Community Council Directive, november 2004, 1986, 86/609/EEC) and National guidelines (Council directive no. 87848 of the Direction des Services Vétérinaires de la Santé et de la Protection Animale permission no. 13.24 from the Ministère de l'Agriculture et de la Pêche to E.S.).

2.2. Iron overload (Experiments 1 and 2)

Rats received daily one intraperitoneal injection of ferrous sulfate (Fe SO4 7H2O, Sigma Aldrich, France) dissolved in sodium chloride 0.9% or vehicle (i.p.; 3 mg of FeSO4 per kg of body) during 21 consecutive days. It has been shown in a previous study that a daily 3 mg/kg dose of iron administrated in adult rats during 5 days results, 16 days after treatment, in significant iron accumulation in the hippocampus and basal ganglia (Maaroufi et al., 2009). This accumulation was correlated to behavioral deficits. Based on this result, longer treatment using the same dose is expected to ensure iron accumulation in the brain.

2.3. EMF exposure system (Experiment 2)

To generate a magnetic field, we used a coil of Helmholtz with two parallel spires (Fig. 1), which generated an EMF (intensity: 5 V/m). EMF was measured and standardized in the total floor area of the Plexiglas cage at 150 kHz. Radiated and conducted electromagnetic emissions (150kHz–1 GHz range) is generated by numerous electric/electronic devices, household appliances, and industrial equipments. Rats were exposed to low frequency 150 kHz EMF, 1 h/day (between 9 and 12 h) during 21 consecutive days. The cage contained six rats for each assay. The sham-exposed control rats were placed under the same conditions without applying EMF. Our aim in Experiment 2 was to examine the effects of combining EMF exposure with iron overload in rats that had similar level of systemic iron as in Experiment 1. We therefore used similar iron administration procedure.

2.4. Behavioral testing (Experiments 1 and 2)

Rats were tested after completion of iron overload and/or EMF exposure treatment. They were first trained in a reference memory

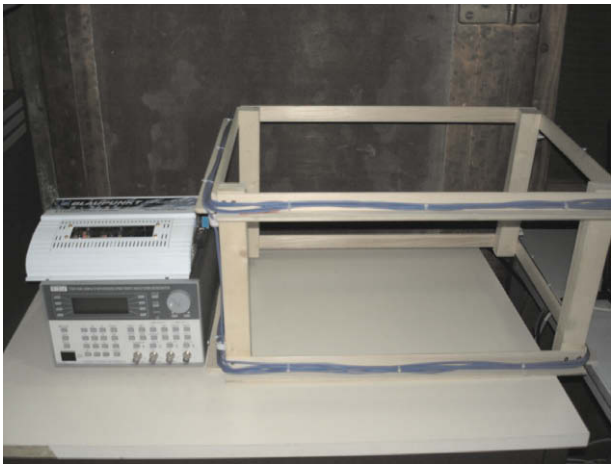

Fig. 1. 150 kHz EMF irradiation apparatus (Experiment 2).

task in the Morris water maze (post-treatment days 1–8), second in a working memory task in the eight-arm radial maze (post-treatment days 9–15) and third in a non-associative object exploration task (day 16).

2.4.1. Morris water maze

The water maze was an elevated circular pool (diameter 1.80 m) located in the middle of a room containing a large variety of cues. The pool was filled with 20 °C water that was made opaque by addition of 2 kg of white chalk powder. A white-painted circular platform (diameter 8 cm) was placed inside the pool, 30 cm away from the wall. Its top surface was 1 cm below the surface of the water and was therefore invisible to the animals. A camera positioned above the apparatus and connected to a DVD recorder and to a monitor allowed tracking the trajectory of the animal. The animals received four trials per day for 8 days. A typical trial consisted of releasing gently a rat in the water, its head facing the wall, from one of four possible starting places (N, E, S, and W) around the perimeter of the pool. The four starting positions were used in a pseudo random order within a four trial block. Once in the water, the rats swam until they eventually came across and climbed on the escape platform that was always located in the middle of the NW quadrant (reference memory task). When a rat had not reached the platform after 60 s swimming, it was gently guided by hand towards the platform. After the last daily trial, rats were dried in a towel and put back in their home cage. After all trials had been run on day 8, each rat was given a probe trial. The platform was removed and the rats were allowed to swim until 60 s have elapsed. All trials were processed on line by a Videotrack tracking system (ViewPoint, Champagne-au-Mont-d'Or, France). Raw data were processed using customer-made computer programs to calculate navigation parameters. Escape latency was used to measure learning and memory in rats. For the probe trial, we calculated the amount of time spent in the quadrant where was located the platform (goal quadrant) and in the three other quadrants of the pool.

2.4.2. Eight-arm radial maze

The apparatus was a black-painted elevated (50 cm above the floor) stainless steel radial maze with eight arms (60 cm long, 10 cm wide) radiating from an octagonal shaped central platform (30 cm large). The maze was located in a small experimental room (3×2 m) that provided numerous environmental cues. A food cup was placed 0.5 cm from the distal part of each arm. All eight cups were baited with one 45 mg sucrose pellet. A preliminary phase consisted of depriving the animals to reduce their body weight to 85% of their initial weight. The animals were then placed by groups of two on the maze and were trained to eat 45 mg sucrose pellets scattered over the central platform and arms during 15 min daily sessions (familiarization phase). Training consisted of two daily trials with a 60 min inter-trial interval for 8 days. For each trial, an animal was placed on the central platform and was allowed to visit all 8 baited arms to eat the pellets. After the animal found all pellets or after 15 min have expired, it was removed from the maze. An arm entry was registered if the rat placed its four paws within the alley. Re-entries in already visited arms were counted as working memory errors. The mean number of errors in each day was used for analysis.

2.4.3. Object exploration task

The apparatus was a square open field (80 cm on a side) with a painted flat white wooden floor. It contained four objects (A, B, C, and D). Object A was a plastic house-shaped object ($11 \times 8 \times 20$ cm), object B a dark gray painted bottle (8 cm diameter, 20 cm high), object C a Rubik's cube (5.5 cm on each side) placed on a cylindrical metal base (total height, 22.5 cm), and object D a white plastic cylinder (8 cm diameter, 12 cm high). In the last session, a novel object was used to replace a familiar object. The novel object (E) was a cylindrical spray-paint container (6.5 cm diameter, 20 cm high). The initial arrangement of the four objects (A, B, C, and D) was a triangle (Fig. 2). The distance between objects A, B, and D was approximately 30 cm, and object C was located approximately 20 cm from the three other objects. The open field was surrounded by a white curtain so that the environment was visually uniform. Each rat was submitted to eight successive 4-min sessions separated by 4-min intervals during which the animal was returned to its home cage. From sessions 2 to 5, the rats were exposed to the initial configuration of objects. In session 6 (spatial change), object D had been displaced toward the peripheral wall therefore yielding a new spatial configuration (see Fig. 2). Note that all objects were at the same distance from the wall (approximately 17 cm). Session 7 was similar to session 6. In session 8 (non-spatial change), one of the familiar objects (A) was replaced by a novel object (E). This protocol allowed to measure the ability of rats to habituate (session 2–5), detect a spatial change (session 6) and detect a non-spatial change (session 8). All sessions were recorded on DVD for off line analysis. As in previous studies (Parron, Poucet, & Save, 2006; Parron & Save, 2004; Save, Poucet, Foreman, & Buhot, 1992), object exploration, i.e. the contact duration of the rat's snout with the object was measured with a stopwatch by a person who was blind to the different groups. The amount of habituation, reaction to spatial change and non-spatial change was quantified by calculating three indexes (Lee, Hunsaker, & Kesner, 2005). An habituation score was calculated by subtracting the contact duration for each object in session 5 from the contact duration for the same objects during session 1. The habituation index was then defined as the average of the scores calculated for all four objects between sessions 1 and 5. For spatial change, we considered separately the displaced object (D) and the non-displaced objects (A, B, and C). The spatial mismatch index was the contact duration for object D during sessions 4 and 5 averaged subtracted from the contact duration for the same object during ses-

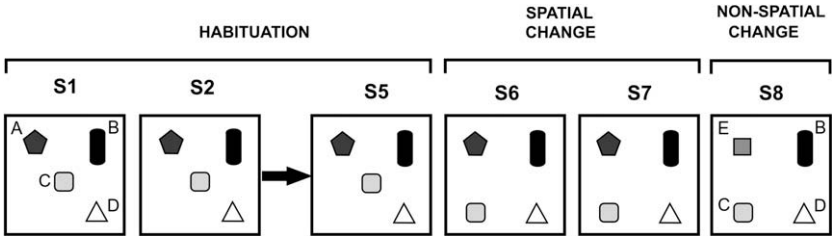

Fig. 2. Protocol of the object configuration memory task. Each rat was submitted to eight successive 4-min sessions separated by 4-min intervals during which the animal was returned to its home cage. From sessions 2 to 5, the rats were exposed to the initial configuration of objects (habituation). In session 6 (spatial change), object D had been displaced toward the peripheral wall therefore yielding a new spatial configuration. The same configuration was maintained in session 7. In session 8, a familiar object (A) was replaced by a novel object (E).

sions 6 and 7 averaged. A similar spatial mismatch index was calculated for the non-displaced objects. The difference of contact duration between sessions 4 and 5 averaged and sessions 6 and 7 averaged was calculated for each object and averaged for each rat. The amount of reaction to non-spatial change was quantified by calculating an object mismatch index defined as the average contact duration for the familiar objects (B, C, and D) subtracted from the novel object (E) contact duration in session 8.

2.5. Data analyses

Behavioral data were analyzed using two-way ANOVAs with repeated measures, factorial ANOVAs, and Newman–Keuls post hoc tests. Biochemical data were analyzed using student *t* tests.

2.6. Biochemical measurements (Experiments 1 and 2)

At the end of the experimental period, the rats were given a lethal dose of sodium pentobarbital (80 mg/kg) and then decapitated. Brain was removed, rapidly washed with cold phosphate buffered saline (PBS), then immediately frozen on dry ice and finally stored at −80 °C. Thick coronal slices (2–3 mm) of various brain regions (cortex 5.16 mm to 2.16 mm; striatum 2.16 mm to −0.48 mm; hippocampus −2.52 mm to −4.56 mm and cerebellum −9.36 mm to −14.04 mm relative to bregma; (Paxinos and Watson, 2004) were made at −20 °C using a cryostat (Leica CM3050). Micropunches were taken from these different brain regions.

Samples were weighed and homogenized (1/10, w/v) in cold 20 mM HEPES buffer, pH 7.2, containing 1 mM EDTA, (HEPES/EDTA) using a Potter–Elvehjem homogenizer fitted with a pestle for microtubes. Part of homogenate was immediately prepared for biochemical measurements of monoamines while the remaining part was stored on ice. Sample contents in monoamines (5HT and DA) and their respective catabolites, 5-hydroxyindolacetic acid (5HIAA) and 3, 4-dihydroxyphenylacetic acid (DOPAC) were measured as described previously (Dusticier & Nieoullon, 1987). Briefly, homogenate was sonicated in 1 volume of 0.1 M perchloric acid, centrifuged at 10,000 g for 15 min at 4 °C, and supernatants were kept at −80 °C until being assayed for monoamines and catabolites by high performance liquid chromatography with electrochemical detection. The mobile phase consisted of 0.1 M sodium acetate, 0.17 mM octyl sulfate, 8% methanol, 0.7 mM EDTA, pH 4.5, and was delivered through an LC-10ADvp Shimadzu pump (Kyoto, Japan) into a C-18 (ODS2, 4.6 × 150 mm) Spherisorb column (Waters, Milford, MA, USA). Twenty microliter samples were injected and analyzed using a Coulochem II, ESA detector (Chelmsford, MA, USA). The limit of detection was 20 fmol/sample.

3. Results

3.1. Experiment 1: effects of iron overload

3.1.1. Morris water maze: reference memory task

As shown in Fig. 3A, escape latencies decreased over days during acquisition of the Morris water maze task in the two groups. This was confirmed by a two-way ANOVA with repeated measures showing no effect of group ($F(1, 14) = 0.39$, $P > 0.05$); a significant effect of session ($F(7, 98) = 29.99$, $P < 0.001$) and no group × session interaction ($F(7, 98) = 1.72$, $P > 0.05$). Fig. 3B shows the time spent in each quadrant of the pool during the probe trial. A factorial ANOVA indicated a significant effect of quadrant ($F(3, 56) = 107.45$; $P < 0.001$) and no effect of group ($F(1, 56) = 0.12$; $P > 0.05$) and group × quadrant interaction ($F(3, 56) = 1.22$; $P > 0.05$). Newman–Keuls post-hoc tests showed that in the two groups, rats spent more time searching in the goal quadrant than in the other quadrants (SAL: all *P*s < 0.001; IO: all *P*s < 0.001). In addition, there was not difference in the time spent in the goal quadrant between SAL and IO groups ($P > 0.05$).

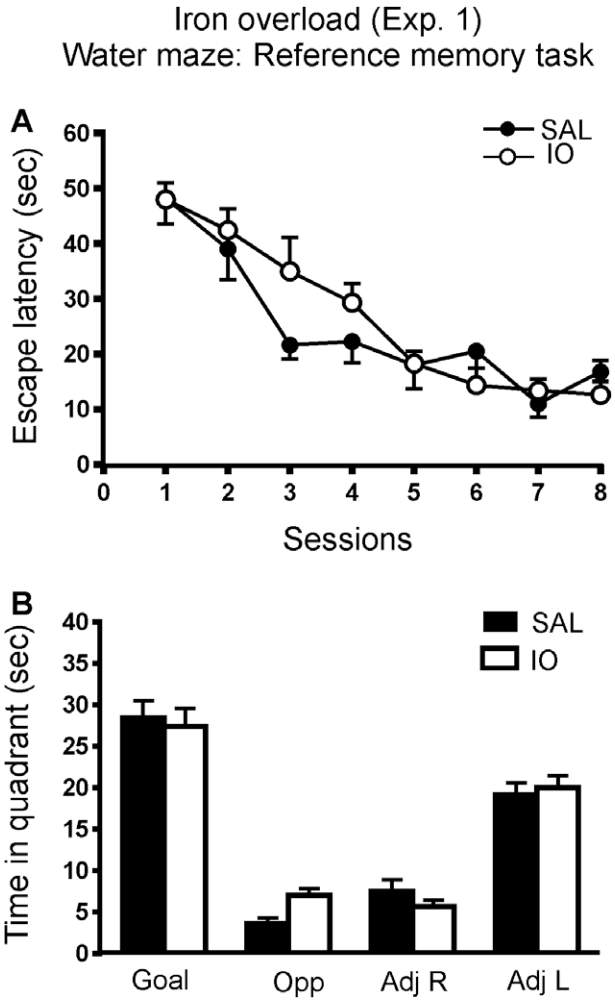

Fig. 3. (A) Escape latency to reach the platform during acquisition. (B) Time spent in each quadrant during the probe test in IO and SAL groups. Opp: opposite, AdjR: adjacent right, AdL: adjacent left quadrants.

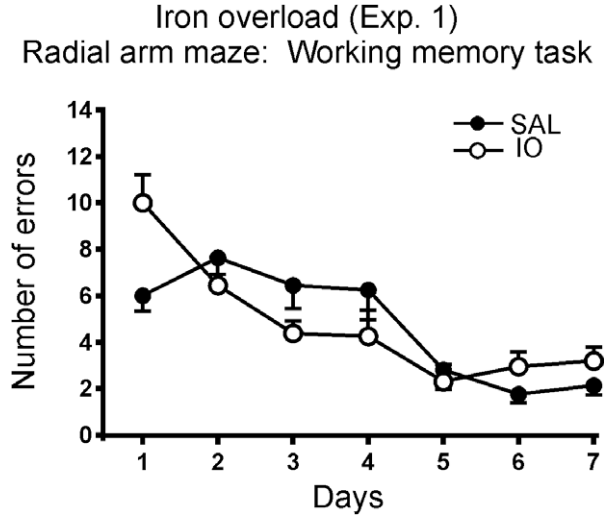

Fig. 4. Number of errors (visits to an already visited arm) during acquisition in IO and SAL groups. Opp: opposite, AdjR: adjacent right, AdjL: adjacent left quadrants.

3.1.2. Eight-arm radial maze: working memory task

Fig. 4 shows the number of errors across days for the two groups. A two-way ANOVA with repeated measures revealed no effect of group ($F(1, 14) = 1.62$; $P > 0.05$), an effect of day ($F(6, 84) = 12.20$; $P < 0.001$) and no group × day interaction ($F(6, 84) = 1.26$; $P > 0.05$), thus indicating that the two groups showed similar learning rate and performance.

3.1.3. Object exploration: spatial vs. non-spatial memory

3.1.3.1. Locomotor activity. Fig. 5A shows the time-course of locomotor activity throughout sessions in SAL and IO groups. As confirmed by a two-way ANOVA with repeated measures, IO rats showed lower activity levels than SAL rats (group effect: $F(1, 14) = 7.53$; $P < 0.05$). However, both groups exhibited a decrease of locomotor activity across sessions (session effect: $F(4, 56) = 28.39$; $P < 0.001$; group × session interaction: $F(4, 56) = 0.33$; $P > 0.05$), indicating that although iron overload impacted locomotor activity, it did not affect locomotor habituation.

3.1.3.2. Exploratory activity. Habituation (sessions 1–5). Fig. 5B displays the time-course of object exploration between-session habituation index in IO and SAL groups. The results show that IO rats

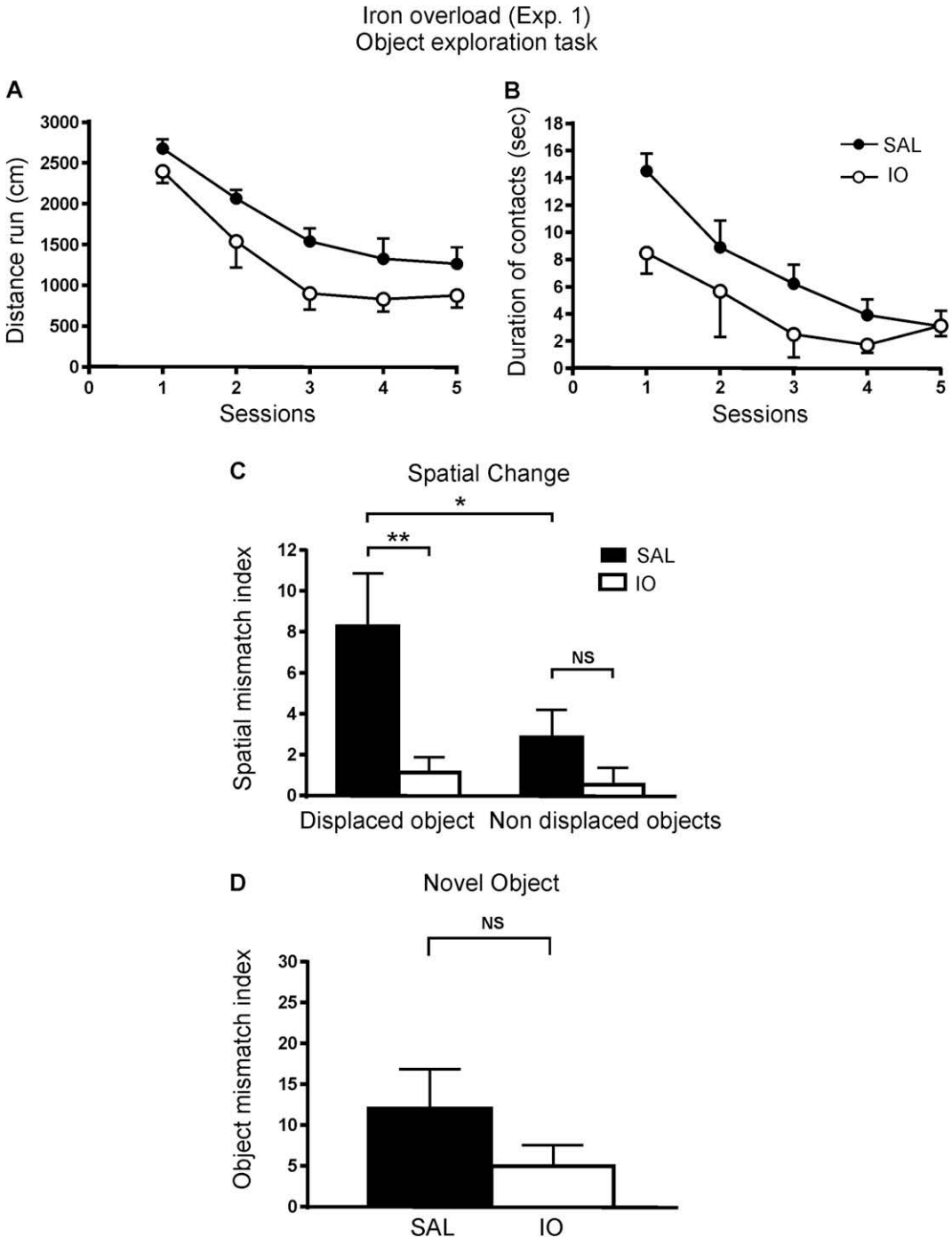

Fig. 5. (A) Time-course of locomotor activity across habituation sessions. (B) Time-course of contact duration across habituation sessions. (C) Spatial mismatch index for displaced and non-displaced objects in the spatial change sessions. (D) Object mismatch index for the novel object in the non-spatial change session in IO and SAL groups. $^{*}P < 0.05$; $^{**}P < 0.01$; NS non-significant.

had lower exploratory activity than SAL rats but eventually habituated (ANOVA: significant effect of group $F(1, 14) = 5.35$; $P < 0.05$, session $F(4, 56) = 26.85$; $P < 0.001$, no group × session interaction $F(4, 56) = 2.49$; $P > 0.05$). At the end of habituation (session 5), the two groups showed similar exploratory activity (Newman–Keuls, $P > 0.05$). Thus, habituation was not affected in IO rats.

Spatial change (session 6). Fig. 5C shows the spatial mismatch index in IO and SAL groups when the animals were exposed to a new object arrangement. The index was calculated for displaced and non-displaced objects. A group × object (displaced vs. non-displaced) factorial ANOVA revealed an effect of group $F(1, 28) = 9.18$; $P < 0.01$, but no effect of object $F(1, 28) = 3.69$; $P > 0.05$, and no group × object interaction $F(4, 56) = 2.35$; $P > 0.05$). These results show that the SAL rats displayed more object re-exploration than IO rats. In addition, Newman–Keuls tests indicated that SAL rats re-explored the displaced object more than the non-displaced objects ($P < 0.05$). IO rats did not display such a selective reaction. Overall, the results show that IO rats were impaired in detecting the spatial change.

Non-spatial change (sessions 8). Fig. 5D shows the object mismatch index in IO and SAL groups when a familiar object was replaced by a novel object. There was no difference between SAL and IO groups (unpaired t tests, $t(14) = 1.30$; $P > 0.05$), showing that IO rats were not impaired in detecting the non-spatial change.

Overall, the object exploration results show that the IO group displayed normal habituation, was impaired in the detection of spatial change but was able to detect the non-spatial change.

3.1.4. Iron overload and monoamine levels in brain tissue

Table 1 shows the levels of Dopamine (DA), Serotonin (5HT) and their catabolites (3, 4-dihydroxyphenylacetic acid (DOPAC), and 5-hydroxyindolacetic acid (5HIAA), respectively) in various brain regions. The ratios 5HIAA/5HT and DOPAC/DA expressing the rate of metabolism relative to their primary catabolite was calculated. Iron overload induced an increase of 5HT especially in the cerebellum (unpaired t tests $t = -5.034$; $P < 0.001$), hippocampus ($t = -3.046$; $P < 0.01$) and in the prefrontal cortex ($t = -4.526$; $P < 0.001$). In addition, the ratio 5HIAA/5HT was found to be decreased in the cerebellum ($t = 5.006$; $P < 0.001$), hippocampus ($t = 2.808$; $P < 0.05$) and in the prefrontal cortex ($t = 2.604$; $P < 0.05$), therefore indicating a lower rate of 5HT turnover. The contents in DA and DOPAC were not modified.

3.2. Experiment 2: effects of combined iron overload and low frequency EMF exposure

3.2.1. Morris water maze: reference memory task

Fig. 6A shows the time-course of escape latency throughout place learning in SHAM-EMF, EMF and EMF-IO groups. A two-way ANOVA with repeated measures showed no effect of group

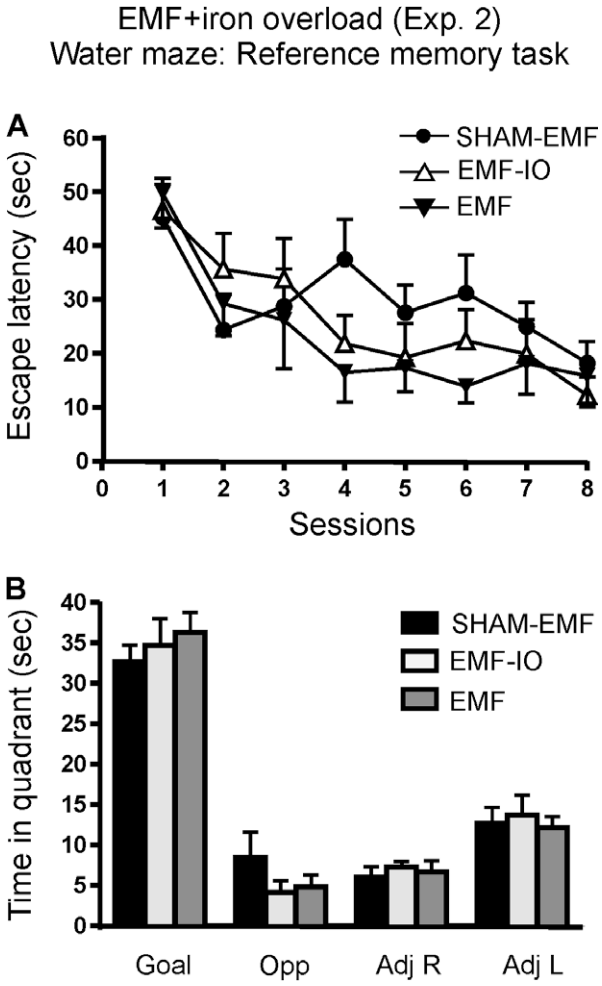

Fig. 6. (A) Escape latency to reach the platform during acquisition. (B) Time spent in each quadrant during the probe test in EMF, EMF-IO and SHAM-EMF groups. Opp: opposite, AdjR: adjacent right, AdjL: adjacent left quadrants.

($F(2, 15) = 2.26$, $P > 0.05$), an effect of session ($F(7, 105) = 14.49$, $P < 0.001$) and no group × session interaction ($F(14, 105) = 1.63$, $P > 0.05$). All groups reached similar performance level in session 8 (Newman–Keuls, all $Ps > 0.05$). As shown in Fig. 6B, the three groups showed a preference for the goal area during the probe trial. A factorial ANOVA indicated a significant effect of quadrant ($F(3, 60) = 125.03$; $P < 0.001$) and no effect of group ($F(2, 60) = 0.0001$; $P > 0.05$) and group × quadrant interaction ($F(6, 60) = 0.82$; $P > 0.05$). Newman–Keuls post-hoc tests showed

Table 1
Experiment 1: content of monoamines (pmol/mg of tissue, mean + s.e.m.) in various brain regions in SAL and IO groups. $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$. DA: dopamine; DOPAC: 3, 4-dihydroxyphenylacetic acid; 5HT: serotonin; 5HIAA: 5-hydroxyindolacetic acid. DOPAC/DA and 5HIAA/5HT are measures of the metabolism of the dopaminergic and serotoninergic systems, respectively.

pmol/mg of tissue	DA	DOPAC	DOPAC/DA	5HT	5HIAA	5HIAA/5HT
SAL						
Cerebellum	0.05 ± 0,02	0.05 ± 0.02	1.23 ± 0.44	0.44 ± 0.13	1.82 ± 0.27	4.81 ± 0.62
Striatum	57.72 ± 10.40	11.21 ± 1.93	0.20 ± 0.02	1.39 ± 0.38	3.06 ± 0.82	2.31 ± 0.20
Hippocampus	0.90 ± 0.24	0.30 ± 0.10	0.35 ± 0.07	1,57 ± 0.30	4.90 ± 0.46	3.47 ± 0.61
Prefrontal cortex	0.49 ± 0.07	0.19 ± 0.03	0.40 ± 0.09	2.08 ± 0.16	2.69 ± 0.21	1.32 ± 0.11
IO						
Cerebellum	0.12 ± 0.03	0.03 ± 0.01	0.32 ± 0.01	1.19 ± 0.08***	2.49 ± 0.23	2.10 ± 0.14***
Striatum	53.72 ± 6.40	9.02 ± 1.00	0.19 ± 0.04	1.54 ± 0.26	3.63 ± 0.27	2.44 ± 0.61
Hippocampus	0.91 ± 0.24	0.19 ± 0.02	0.25 ± 0.07	2.58 ± 0.18**	5.32 ± 0.22	2.09 ± 0.08*
Prefrontal cortex	0.71 ± 0.13	0.19 ± 0.03	0.29 ± 0.04	2.86 ± 0.06***	2.87 ± 0.12	1.01 ± 0.05*

that in the three groups, rats spent more time searching in the goal quadrant than in the other quadrants (SAL: all *P*s < 0.001; IO: all *P*s < 0.001).

3.2.2. Eight-arm radial maze: working memory task

Fig. 7 shows the number of errors across days in EMF, EMF-IO, and SHAM-EMF groups. An ANOVA with repeated measures revealed no effect of group ($F(2, 15) = 0.96$; $P > 0.05$), an effect of day ($F(6, 90) = 6.48$; $P < 0.001$) and a significant group × day interaction ($F(12, 90) = 2.07$; $P < 0.05$). Thus, all groups improved their performance across days. SHAM-EMF rats exhibited faster learning rate than both EMF-IO and EMF rats but all groups reach similar level of performance at the end of training (all *P*s > 0.05).

3.2.3. Object exploration: spatial vs. non-spatial memory

3.2.3.1. Locomotor activity. A two-way ANOVA with repeated measures revealed no effect of group ($F(2, 15) = 0.30$; $P > 0.05$), a significant effect of session ($F(4, 60) = 30.74$; $P < 0.001$) and no group × session interaction ($F(8, 60) = 0.68$; $P > 0.05$), indicating that the treatments did not affect locomotion and that all groups exhibited locomotor habituation.

3.2.3.2. Exploratory activity. Habituation (sessions 1–5). Fig. 8A shows the between-session habituation index in EMF, EMF-IO, and SHAM-EMF groups. All groups displayed habituation (ANOVA: no effect of group $F(2, 15) = 1.31$; $P > 0.05$, significant effect of session, $F(4, 60) = 28.96$; $P < 0.001$). EMF rats displayed faster habituation (group × session interaction, $F(8, 60) = 2.13$; $P < 0.05$) but all groups reach similar exploratory activity in session 5 (Newman–Keuls, $P > 0.05$) Thus, EMF and EMF + iron overload treatments did not have a detrimental effect on habituation.

Spatial change (session 6). Fig. 8B shows the spatial mismatch index in EMF, EMF-IO, and SHAM-EMF groups for the displaced and non-displaced objects. A group × object (displaced vs. non-displaced) factorial ANOVA revealed an effect of group $F(2, 30) = 14.13$; $P < 0.001$, an effect of object $F(1, 30) = 42.20$; $P < 0.001$, but no group × object interaction $F(2, 30) = 1.01$; $P > 0.05$). Newman–Keuls tests indicated that SHAM-EMF rats displayed more object re-exploration than both EMF-IO and EMF rats ($P < 0.001$). All groups re-explored more the displaced object than the non-displaced objects (all *P*s < 0.05).

Non-spatial change (session 8). Fig. 8C shows the object mismatch index in EMF, EMF-IO and SHAM-EMF groups when a familiar object was replaced by a novel object. One sample *t* tests revealed that all three groups significantly re-explored the novel object (SHAM-EMF: $t(5) = 8.03$; $P < 0.001$; EMF: $t(5) = 3.67$; $P < 0.05$; EMF-IO: $t(5) = 3.70$; $P < 0.05$). No difference in magnitude of re-exploration between groups was found (ANOVA: $F(2, 15) = 0.51$; $P > 0.05$).

Overall, all groups exhibited habituation. EMF-IO and EMF groups were mildly impaired in the detection of the spatial change and were not affected in the detection of non-spatial change.

3.2.4. EMF-Iron overload and monoamine levels in brain tissue

Table 2 shows the levels of Dopamine (DA), Serotonin (5HT) and their metabolites (3, 4-dihydroxyphenylacetic acid (DOPAC), and 5-hydroxyindolacetic acid (5HIAA), respectively) in various brain regions including the cerebellum, striatum, hippocampus and prefrontal cortex. No modifications in monoamine levels were found in treated groups, indicating that the combination of EMF and iron overload had no effect on monoamines content in the brain.

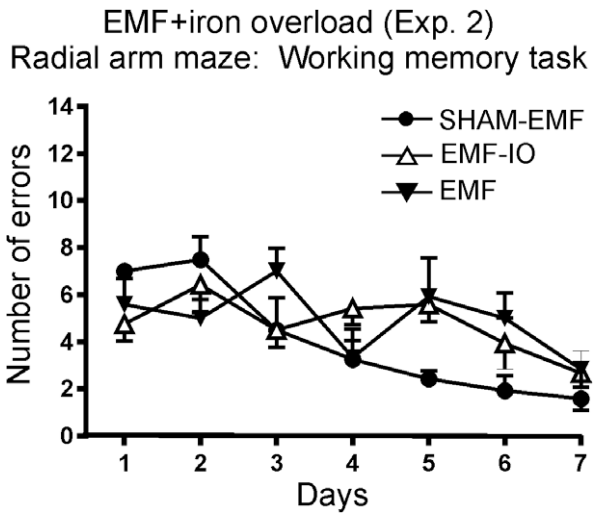

Fig. 7. Number of errors (visits to an already visited arm) during acquisition in EMF, EMF-IO and SHAM-EMF groups.

4. Discussion

The purpose of the study was to examine the impact of iron overload and EMF exposure on spatial learning and memory in young rats. First, we tested the effects of iron supplementation (Experiment 1) and second, the effect of combining iron supplementation and EMF (Experiment 2). In each experiment, the rats were trained in a number of spatial tasks including a reference memory task in the water maze, a working memory task in the radial arm maze and an object configuration memory task in an open field. In Experiment 1, we found that iron overload did not affect the performance in the water maze and radial maze tasks but affected the reaction to spatial change in the object exploration task. In Experiment 2, EMF exposure and combined EMF and iron overload both did not affect the detection of spatial novelty but attenuated the exploratory reaction.

4.1. Effects of iron overload

Administration of iron in young rats has been previously shown to induce a number of behavioral deficits. Spontaneous locomotor activity, habituation, and re-activity to environmental stimuli as measured by startle reflex and prepulse inhibition of startle were diminished (Fredriksson et al., 1999, 2000; Fredriksson, Schröder, & Archer, 2003; Schröder et al., 2001; Sobotka et al., 1996). In addition, iron-treated rats and mice were also impaired in the acquisition of a radial maze task (Fredriksson et al., 1999, 2000; Schröder et al., 2001) suggesting a working memory deficit. Note that in most of these studies, deficits were observed when iron was administrated on postnatal days 10–12, therefore suggesting a critical brain sensitivity period to iron overload. In the present study, we found that even when administrated outside this critical period (4–5 weeks old rats), excess iron produced deficits in the detection of spatial change in the object exploration task. Thus, the effects of iron overload may not be limited to those observed following treatment in the postnatal 10–12 day period but occur when iron is administrated at later periods.

An important issue is whether behavioral alterations observed in the present study are a non-specific consequence of iron accumulation in peripheral tissues during the 21-day-treatment or result from a cognitive impairment. Our results support the latter hypothesis since we found that rats exhibited deficits in the object configuration memory task only. No alterations in the water maze and radial maze tasks were seen. Another selective effect was that in the object configuration memory task, rats were impaired in the

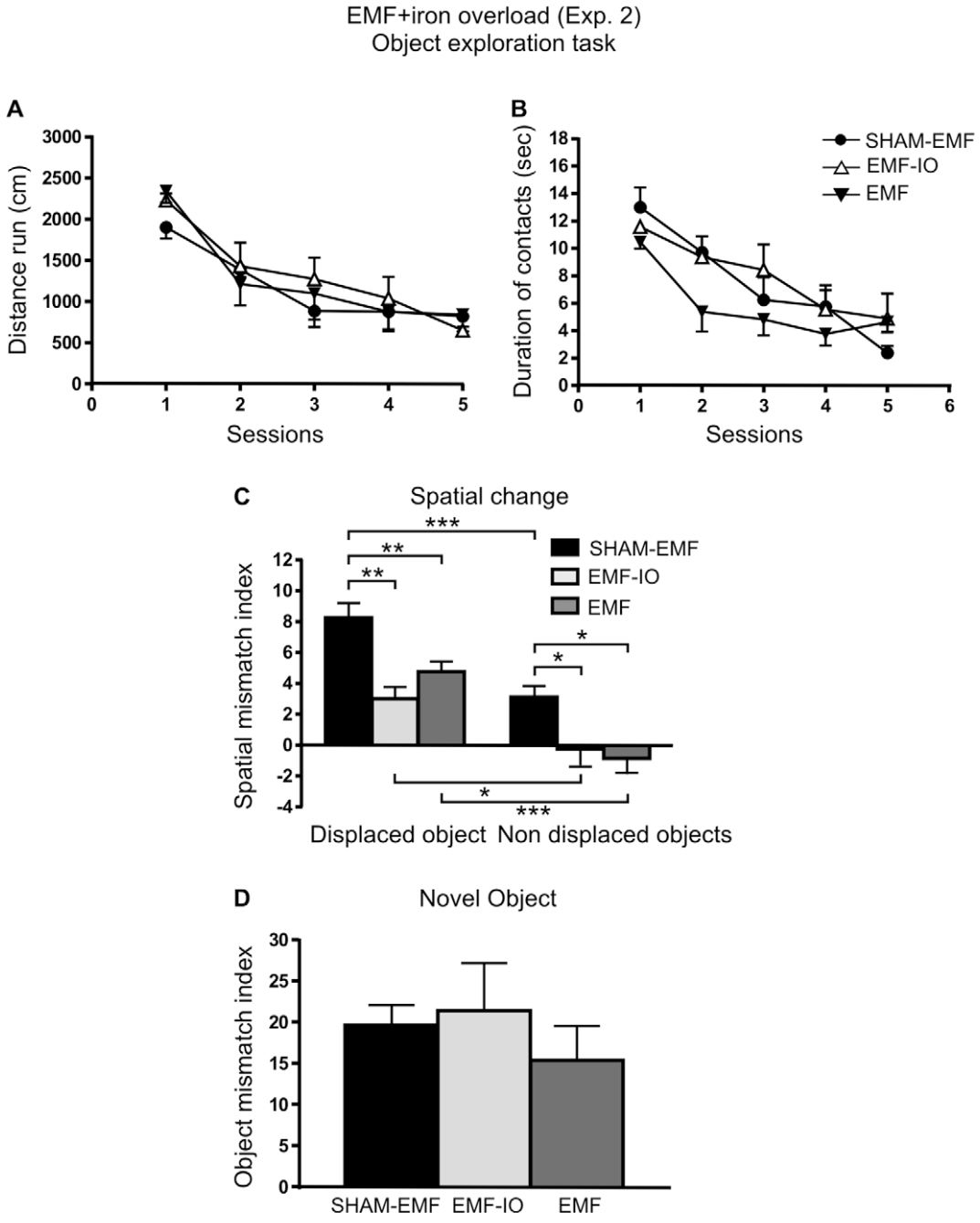

Fig. 8. (A) Time-course of locomotor activity across habituation sessions. (B) Time-course of contact duration across habituation sessions. (C) Spatial mismatch index for displaced and non-displaced objects in the spatial change sessions. (D) Object mismatch index for the novel object in the non-spatial change session in EMF, EMF-IO and SHAM-EMF groups. $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$.

detection of the spatial change but not in the detection of non-spatial change. Note that although treated rats exhibited less locomotor and exploratory activity than controls, they displayed habituation, therefore indicating that they were able to process environmental information (Save et al., 1992). Thus, iron overload specifically interfered with spatial information processing. However, altered processes were different from those necessary for water maze or radial maze learning since rats were still able to perform both tasks. We also measured a number of biochemical markers to determine whether iron overload had a direct impact on the brain. Previous work has shown that monoaminergic systems are sensitive to iron brain content changes. Iron deficiency induces a decrease of central dopamine and serotoninergic neurotransmission (Chen, Bear, & Jones, 1995; Beard & Connor, 2003; Burhans et al., 2005; Nelson, Erikson, Pinero, & Beard, 1997; Shukla, Agarwal, Chansuria, & Taneja, 1989; Youdim, Ben Sachar, & Yehuda, 1989; Youdim et al., 1980). We found indeed changes in monoamines: 5HT and 5HIAA, but not DA and DOPAC, contents were increased. Contrary to a number of studies however we did not find modifications of the dopaminergic system. Note that in the literature, changes in the dopaminergic system have been found following iron deficiency. Perhaps does iron supplementation promote different mechanisms from those produced by iron deficiency? Nevertheless, iron overload may have extended biochemical effects due to the reciprocal functional interactions between the serotoninergic and the dopaminergic systems (Jenner, Sheehy,

Table 2
Experiment 2: content of monoamines (pmol/mg of tissue, mean + s.e.m.) in various brain regions in SHAM-EMF, EMF-IO and EMF groups. $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$. DA: dopamine; DOPAC: 3, 4-dihydroxyphenylacetic acid; 5HT: serotonin; 5HIAA: 5-hydroxyindolacetic acid. DOPAC/DA and 5HIAA/5HT are measures of the metabolism of the dopaminergic and serotoninergic systems, respectively.

pmol/mg of tissue	DA	DOPAC	DOPAC/DA	5HT	5HIAA	5HIAA/5HT
SHAM-EMF						
Cerebellum	0.05 ± 0.02	0.16 ± 0.01	3.0 ± 0.32	0.42 ± 0.08	1.76 ± 0.15	4.64 ± 0.69
Striatum	50.21 ± 5.51	9.65 ± 1.06	0.20 ± 0.01	1.69 ± 0.11	3.41 ± 0.15	2.07 ± 0.17
Hippocampus	1,08 ± 0,61	0,34 ± 0,15	0,52 ± 0,14	1,50 ± 0,25	4,37 ± 0,21	3,26 ± 0,45
Prefrontal cortex	0.52 ± 0.07	0.26 ± 0.05	0.54 ± 0.10	1.94 ± 0.25	2.61 ± 0.08	1.45 ± 0.21
EMF-IO						
Cerebellum	0.06 ± 0.01	0.14 ± 0.01	2.62 ± 0.18	0.40 ± 0.05	1.47 ± 0.18	3.76 ± 0.32
Striatum	49.49 ± 7.17	8.42 ± 1.06	0.17 ± 0.01	1.66 ± 0.02	3.61 ± 0.32	2.18 ± 0.20
Hippocampus	0.63 ± 0.30	0.23 ± 0.07	0.67 ± 0.16	1.14 ± 0.15	3.38 ± 0.20	3.06 ± 0.18
Prefrontal cortex	0.34 ± 0.04	0.15 ± 0.01	0.45 ± 0.05	1.34 ± 0.16	2.29 ± 0.12	1.76 ± 0.15
EMF						
Cerebellum	0.05 ± 0.01	0.13 ± 0.01	3.09 ± 0.29	0.31 ± 0.02	1.45 ± 0.14	4.83 ± 0.81
Striatum	48.36 ± 4.20	8.02 ± 0.74	0.17 ± 0.01	1.52 ± 0.23	3.39 ± 0.19	2.39 ± 0.26
Hippocampus	1.15 ± 0.37	0.43 ± 0.12	0.45 ± 0.08	1.35 ± 0.18	3.83 ± 0.42	2.89 ± 0.10
Prefrontal cortex	0.37 ± 0.06	0.20 ± 0.03	0.58 ± 0.06	1.53 ± 0.12	2.45 ± 0.10	1.64 ± 0.13

& Marsden, 1983; Di Matteo, Di Giovanni, Pierucci, & Esposito, 2008; Guiard, El Mansari, Merali, & Blier, 2008). For example, activation of 5HT2C receptors has been shown to exert an inhibitory effect on the basal electrical activity of dopaminergic neurons and on DA release (Esposito, Di Matteo, & Di Giovanni, 2008). It is therefore important to take these interactions into consideration in order to understand the global biochemical effects of iron overload.

An increase in 5HT levels parallel with a decrease in turnover was shown in the cerebellum, hippocampus and prefrontal cortex, three structures known to be involved in learning and memory. The hippocampus and the prefrontal cortex are main targets of serotoninergic neurons of the raphe nuclei. Thus, because the serotoninergic system has been demonstrated to modulate memory processes (Buhot et al., 2008), there may be a link between our biochemical findings and the behavioral deficits. Interestingly, stimulating 5HT1A and 5HT1B receptors affects object exploration in a task similar to that we used (Buhot & Naïli, 1995). Moreover, working and reference memory in a radial maze task is not disrupted by an intrahippocampal injection of a 5HT1A agonist but is affected by an injection of a 5HT1B agonist (Buhot, Patra, & Naïli, 1995). The serotoninergic system may also exert an influence over learning and memory via modulation of the cholinergic system (Cassel & Jeltsch, 1995).

Overall, our results are compatible with the hypothesis that prolonged iron overload has a detrimental effect on spatial cognition in young rats (Sobotka et al., 1996). They suggest that iron overload has a direct effect on the brain and induces serotoninergic modifications that might be associated with selective deficits in spatial learning and memory.

4.2. Effects of combining EMF and iron overload

Rats exposed to low-frequency EMF or to a combination of low-frequency EMF and iron overload were not impaired in the water maze reference memory task. A moderate deficit was found in the radial arm maze working memory task since both exposed groups displayed a lower learning rate. However, all groups were eventually able to learn the task. In the object configuration task, exposed groups, like the control group, discriminated displaced and non-displaced objects, therefore indicating that they were able to process and discriminate the spatial change. The re-activation of exploratory activity was nevertheless weaker than in controls. This cannot be accounted for a neophobic reaction since they were able to detect the non-spatial change when challenged with a novel object. Thus, EMF exposure attenuated the reaction to spatial novelty.

Previous work has yielded variable effects of EMF exposure. It has been shown that acute exposure to low frequency EMF has a detrimental effect on learning and memory in rodents (Lai, 1996; Lai, Carino, & Ushijima, 1998; Jadidi et al., 2007; Sienkiewicz, Haylock, & Saunders, 1998). However, other work suggests that chronic or sub-chronic exposure to EMF produces different effects from acute exposure. Janac and co-workers (2005) found that a 7-day exposure (but not 1-day or 3-day exposures) to 50 Hz EMF attenuated amphetamine-induced hyperactivity. More strikingly, 4 weeks of daily 4-h exposure to a 50 Hz EMF produced an improvement in acquisition and long-term retention performance of rats in the water maze navigation task. Our results are consistent with the hypothesis that chronic low-frequency EMF exposure yields weaker effects than acute exposure. First, EMF exposure did not impede the detection of the spatial change. Second, combining EMF exposure and iron overload did not result in a greater impairment. In Experiment 1, rats with iron overload were not able to discriminate the displaced from the non-displaced object indicating that they failed to recognize the spatial change. In contrast, in Experiment 2, exposed and iron-administrated rats were able to perform such recognition. Thus, not only EMF did not potentiate the effects of iron overload as previously hypothesized, but actually attenuated these effects. In a follow-up study it would be interesting to examine the effects of shorter EMF exposures in the spatial tasks we used. According to the hypothesis, a 1-day exposure would be more disruptive than a 21-day exposure. As proposed by Liu, Wang, He, and Ye (2008), several possible compensatory mechanisms may account for the paradoxical effect of prolonged low-frequency EMF exposure but they have not been evidenced so far. Thus, an important issue for future work is to identify the changes in the brain that may be brought in play during prolonged EMF-induced adaptive mechanisms. It would be interesting to determine the dynamics of these changes and whether they can be reversed.

In Experiment 2, the biochemical results are consistent with the behavioral results. Indeed, no modifications of the dopaminergic and serotoninergic systems were found. It is nevertheless of interest that low frequency EMF appeared to have a protective effect on 5HT metabolism with respect to iron overload effects (Experiment 1). Because to our knowledge they are no data reporting similar effect, hypotheses remains speculative. One possibility is that EMF would activate some time-dependent regulatory mechanism resulting in restoration of 5HT level. This hypothesis could be tested by analyzing 5HT metabolism at different delays during treatment. Another possibility is that EMF would enhance the potential mechanisms for iron export from the brain. The existence of

such mechanisms has not been demonstrated, however (Rouault & Cooperman, 2006). Overall, the interaction between EMF and neurotransmitter systems is poorly documented and needs further studies.

One possible interpretation is that there would be some regulatory mechanism that would compensate for the disruptive effect of iron overload.

4.3. Conclusion

The impact of EMF exposure on cognitive processing probably depends on many parameters including the characteristics of EMF (intensity, low vs. high frequency, specific absorption rate, etc.), exposure (duration of exposure in one day and over days, time of exposure, etc.), and subjects (species, young vs. old, etc.). A critical aspect regarding the effect of low frequency EMF may be the duration of exposure over days. Prolonged exposure may stimulate various regulatory mechanisms which remain to be identified that eventually allow maintaining cognitive processes. These mechanisms would be powerful enough to limit or compensate the impact of iron overload in young rats. In contrast, such mechanisms would not be brought into play following acute exposure. Our results also point out that it is important to test the animal's spatial cognitive abilities in various memory tasks. In particular, the object configuration memory task, a non-associative task may be more sensitive to treatments than associative tasks like the Morris water maze navigation task and the radial arm-maze task (Parron et al., 2006).

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