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Research report

Exposure to GSM 900-MHz mobile radiation impaired inhibitory avoidance memory consolidation in rat: Involvements of opioidergic and nitrergic systems

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ABSTRACT

The use of mobile phones is increasing, and the main health concern is the possible deleterious effects of radiation on brain functioning. The present study aimed to examine the effects of exposure to a global system for mobile communication (GSM) with mobile phones on inhibitory avoidance (IA) memory performance as well as the involvement of endogenous opioids and nitric oxide (NO) in this task. Male Wistar rats, 10–12 *weeks* old, were used. The results showed that four weeks of mobile phone exposure impaired IA memory performance in rats. The results also revealed that post-training, but not pre-training, as well as pre-test intracerebroventricular (i.c.v.) injections of naloxone (0.4, 4 and 40 ng/rat), dose-dependently recovered the impairment of IA memory performance induced by GSM radiation. Additionally, the impairment of IA memory performance was completely recovered in the exposed animals with post-training treatment of naloxone (40 ng/rat) plus pre-test i.c.v. injections of L-arginine (100 and 200 nmol/rat). However, pre-test i.c.v. injections of L-NAME (10 and 20 nmol/rat), impaired IA memory performance in the animals receiving post-training naloxone (40 ng/rat). In the animals receiving post-training naloxone treatment, the impairment of IA memory performance due to pre-test i.c.v. injections of L-NAME was recovered by the pre-test co-administration of L-arginine. It was concluded that the recovery from impairment of IA memory in GSM-exposed animals with post-training naloxone treatment was the result of blockade of the opioidergic system in early memory consolidation as well as activation of the nitrergic system in the retrieval phase of memory.

1. Introduction

The radiation emitted from microwave instruments and global system for mobile communication (GSM) cell phones is in the range of electromagnetic radio-frequency (Narayanan et al., 2013). It has been reported that during a mobile conversation with common spatial holding of a mobile phone near an ear, the energy emitted from the mobile phone is mainly absorbed by the temporal lobe of a user (Cardis et al., 2011), which is the main substrate for memory formation (Dandolo and Schwabe, 2018). It has been shown that the radiation from mobile phones has some deleterious effects on human health (Velmurugan, 2017; Zhi et al., 2018) and cognitive function (Zhi et al., 2018), and, in some cases, it may increase the risk of cerebral tumor development (Falcioni et al., 2018). A large body of research has also proposed that exposure to electromagnetic radiation emitted from GSM and microwave gadgets may have interactions with cognition, learning and memory processes (Tang et al., 2015; Zhao et al., 2015).

Findings from a literature review on the effect of such electromagnetic exposure revealed impairment of learning and memory performance in different paradigms, including spatial tests (Li et al., 2015), object recognition tests (Son et al., 2016, 2018),

Abbreviations: ANOVA, analysis of variance; EMF, electromagnetic field; GSM, global system for mobile communications; Hz, hertz; h, hour; IA, inhibitory avoidance; i.c.v., intracerebroventricular; i.p., intraperitoneal; LTP, long term potentiation; L-NAME, NG-Nitro-L-arginine methyl ester hydrochloride; mA, milliampere; mm, millimeter; min, minute; ng/rat, nanogram/rat; nmol/rat, nanomole/rat; NO, nitric oxide; NOS, nitric oxide synthase; SAR, specific absorption rate; s, second; μl, microliter; μl/rat, microliter/rat.

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object location tasks (Ntzouni et al., 2013). However, some researchers have not found significant effects of electromagnetic exposure on memory at the sub-chronic and chronic levels (Ammari et al., 2008) or on cognition (Klose et al., 2014).

The use of mobile phones, especially among younger people, is increasing, and there are crucial concerns about potential adverse outcomes of GSM exposure on higher functions of the brain at the molecular and cellular levels (Velmurugan, 2017). It has been reported that electromagnetic fields (EMFs) and microwave irradiation alter the brain proteome (Fragopoulou et al., 2012), cause complete loss of hippocampal pyramidal cells, affect Ca^{+} binding protein and neuronal connections (Maskey et al., 2010), and alter cognitive function and oxidative stress in rats (Deshmukh et al., 2013). It has been reported that EMFs and microwave irradiation induce the release of endogenous opioids (Lai et al., 1986b, 1987; Lai et al., 1989; Kavaliers et al., 1996).

Findings from numerous studies have also revealed that electromagnetic radiation increases nitric oxide (NO) production, which in turn induces subsequent changes in gene and/or protein expression (Patruno et al., 2010). In particular, it has been shown that radiation from a 900-MHz mobile phone increases NO in the rat brain (Ilhan et al., 2004; Ozguner et al., 2005). Nitric oxide, as a neurotransmitter (dos Reis et al., 2018), cellular messenger (Pitsikas, 2015) and neuromodulator (Zhuo et al., 1994; Luo et al., 2014), is involved in different forms of synaptic plasticity (Anaeigoudari et al., 2016b) and long-term potentiation (LTP) (Anaeigoudari et al., 2016a, Kamii et al., 2017). In addition, interactions between opioidergic and nitrergic systems on memory performance have been previously reported (Nasehi et al., 2013). In particular, the involvement of central opioid receptors in the regulation of NO production has been reported (Someya et al., 2017). Therefore, it is logical to hypothesize that influences of GSM exposure on memory performance could be mediated, at least partly, through the interaction of the opioidergic and nitrergic systems.

Despite several studies that have suggested adverse effects of GSM exposure on learning and memory, the possible involved mechanisms are still controversial (Hietanen, 2006). Since the previously published reports suggest the stimulation of endogenous opioids under the influence of EMF and microwave irradiation (see above), the main objectives of the present study are as follows. First, does blocking opioid receptors attenuate the impairment of IA memory performance induced by GSM radiation? Second, is this attenuation mediated through the NO pathway? In the first series of experiments, we examined intracerebroventricular (i.c.v.) injections of naloxone at pre-training, post-training and pre-test phases of an IA task to examine the involvement of the opioidergic system in different phases of IA memory, including acquisition, consolidation and retrieval, respectively, which were impaired by the GSM exposure. Then, to verify the involvement of NO in opioid-mediated memory processes under the influence of GSM exposure, we examined the effects of pre-test i.c.v. injections of an NO donor or blocker (L-arginine and L-NAME, respectively), alone or in combination, in the presence of GSM exposure and the post-training i.c.v. administration of naloxone on IA memory performance.

The novelty of the current study is the fact that, for the first time, we showed the involvement of two important neurotransmitter systems in the impairment of IA memory following exposure to GSM radiation emitted from a real cell phone. In addition, we used a conventional cell phone with a typical frequency range (E-GSM: Up-link frequency 880–915 MHz) instead of a signal generator that only partially simulates a 900 MHz situation of which the findings are not completely applicable to the real world. We also used a single trial IA model of memory to observe the effects of the exposure on the different phases of long term memory, including acquisition, consolidation and retrieval. In addition, we used a missed (alternative/non-continuous) call type in silent non-vibratory mode of exposure instead of a continuous one to prevent any distractive effect of a ring tone and background sounds on animal learning and memory processes.

2. Results

2.1. GSM irradiation impaired IA memory performance

One-way ANOVA showed a significant difference in the IA memory performance among the control, sham and exposed animals after four weeks of GSM exposure [$F(2, 21) = 23.83$, $P < 0.001$]. Post hoc testing revealed that GSM radiation impaired IA memory performance compared to both the sham and control groups; however, the sham and control groups showed no differences in their IA memory performance (Fig. 1).

2.2. Pre-training naloxone treatment had no effect on IA memory performance in the animals with a history of GSM radiation

Fig. 2 shows the effects of the pre-training administration of naloxone on IA memory performance in the animals with four weeks of exposure to GSM radiation. One-way ANOVA revealed significant differences among the groups [$F(4, 35) = 47.81$, $P < 0.001$]; however, post hoc testing showed that pre-training naloxone (0.4, 4 and 40 ng/rat, i.c.v.) failed to reverse the impairment of IA memory performance induced by GSM irradiation compared to the exposed group that received pre-training saline treatment.

2.3. Post-training naloxone treatment improved the impairment of IA memory performance in the animals that received GSM irradiation

Fig. 3 shows the effects of the post-training administration of naloxone on IA memory performance after four weeks of GSM irradiation. One-way ANOVA revealed significant differences among the groups [$F(4, 35) = 44.02$, $P < 0.001$]. Post hoc testing also showed that post-training naloxone (0.4, 4 and 40 ng/rat, i.c.v.) dose-dependently recovered the impairment of the IA memory

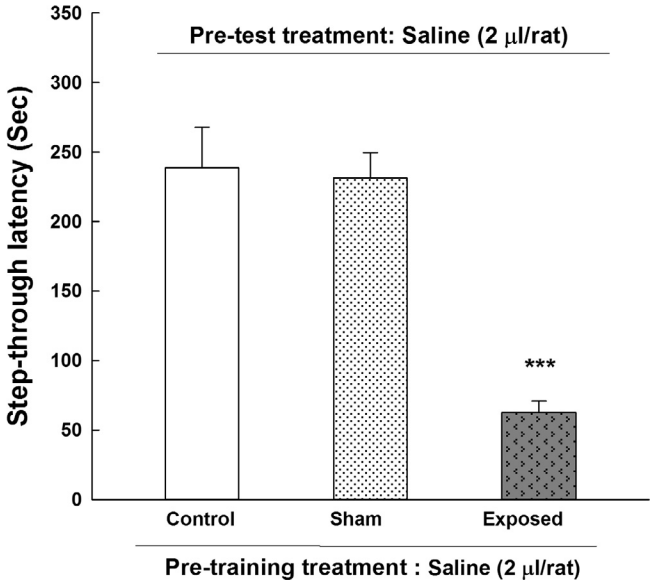

Fig. 1. The effects of four weeks GSM irradiation on IA memory performance. Three groups of animals were used. The control and sham groups had no history of GSM irradiation but the exposed group was received GSM irradiation from a silent mobile phone for four weeks. All groups received pre-training and pre-test saline (2 μl/rat) treatments. ***P < 0.001 compared to the control and sham groups.

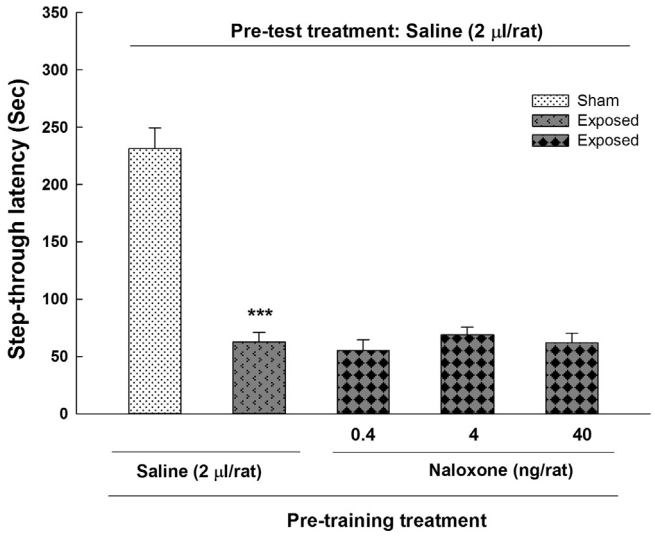

Fig. 2. The effects of pre-training i.c.v. injections of naloxone on IA memory performance in the animals exposed to GSM radiation. Five groups of rats were used. The sham group with no history of GSM exposure received pre-training and pre-test injections of saline (2 μl/rat). The other four groups after four weeks GSM irradiation on the training day of IA task received pre-training saline (2 μl/rat) or naloxone at different doses (0.4, 4, and 40 ng/rat) 5 min before training. On the test day, all the groups received pre-test saline (2 μl/rat) treatments 5 min prior to the test. $^{***}P < 0.001$ compared to the control group.

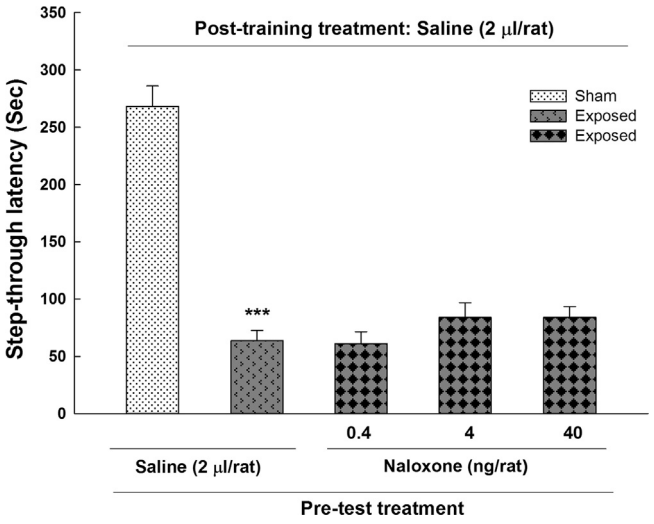

Fig. 4. The effects of pre-test i.c.v. administration of naloxone on IA memory performance in the animals exposed to four weeks of GSM radiation. Five groups of the animals were used. On the training day, all of the groups received saline (2 μl/rat) immediately after training. On the test day, the sham group with no history of GSM irradiation received pre-test injections of saline (2 μl/rat) but the other four groups with a history of GSM radiation received the pre-test injections of either saline or naloxone at different doses (0.4, 4, and 40 ng/rat) 5 min prior to the test. $^{***}P < 0.001$ compared to the control group.

performance induced by GSM irradiation compared to the exposed group that received post-training saline treatment.

2.4. Pre-test naloxone treatment did not affect IA memory performance in the presence of GSM irradiation

Fig. 4 shows the effects of the pre-test administration of naloxone on IA memory performance after four weeks of GSM irradiation. One-way ANOVA revealed significant differences among the groups [$F(4, 35) = 50.81$, $P < 0.001$]. However, post hoc testing showed that pre-test naloxone (0.4, 4 and 40 ng/rat, i.c.v.) had no effect on the impairment of IA memory performance induced by GSM radiation compared to the exposed group that received pre-test saline treatment.

2.5. Effect of pre-test injections of L-arginine on IA memory performance in the GSM-exposed animals with post-training administration of saline or naloxone

We considered the post-training treatment as factor A with two levels (saline or naloxone) and the pre-test treatment of L-arginine with three levels (0, 100 and 200 nmol/rat) as factor B. Two-way ANOVA indicated significant effects for factor A [$F(1, 47) = 496.76$,

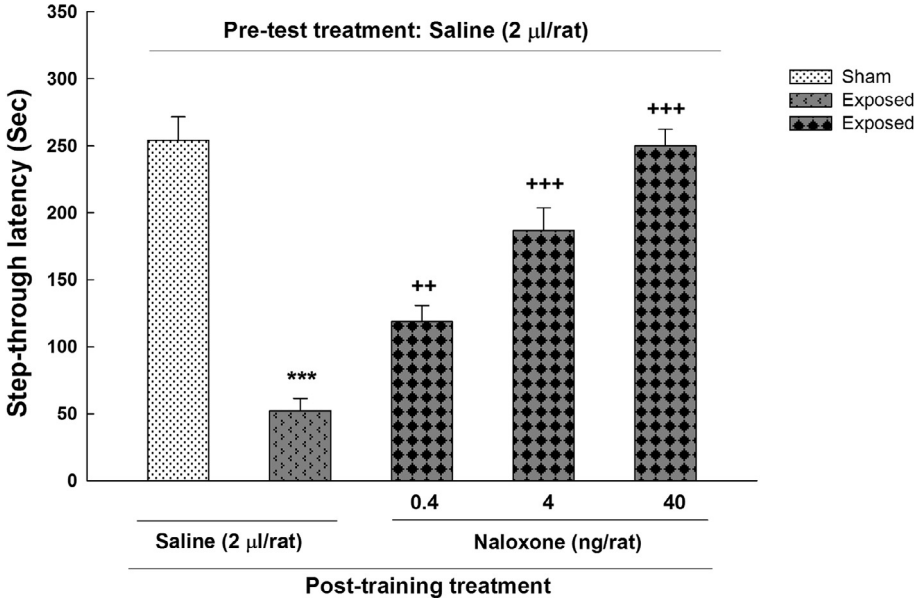

Fig. 3. The effects of post-training i.c.v. administration of naloxone on IA memory performance in the presence of GSM radiation. Five groups of rats were used. The sham group with no history of GSM exposure received post-training and pre-test injections of saline (2 μl/rat). The other four groups after four weeks of GSM radiation on the training day of the IA task immediately received post-training injections of saline (2 μl/rat) or naloxone at different doses (0.4, 4, and 40 ng/rat). On the test day, all of the groups received pre-test saline (2 μl/rat) treatments 5 min before testing. $^{***}P < 0.001$ compared to the control group. $^{++}P < 0.01$ and $^{+++}P < 0.001$ compared to the sham group.

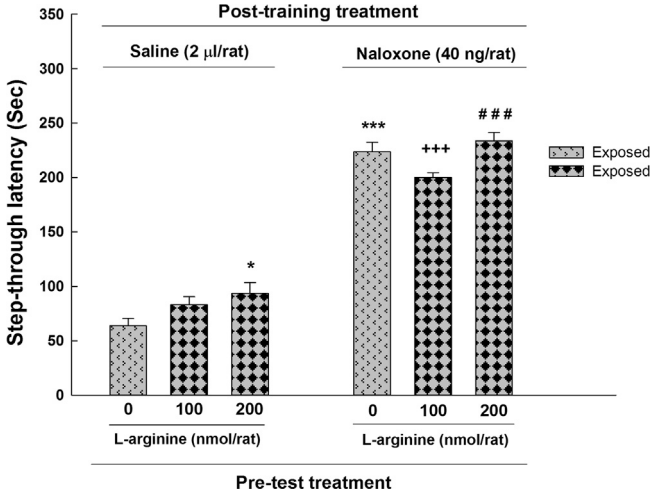

Fig. 5. The effects of pre-test i.c.v. administration of L-arginine on IA memory performance in the exposed animals that received either the post-training saline (2 μl/rat) or naloxone 40 (ng/rat) treatments. Six groups of the animals with a history of four weeks of GSM radiation were used and divided into two sets of three groups. On the training day, all three groups of the first set received instantly post-training saline (2 μl/rat) treatment but all groups in the second set received immediately post-training naloxone (40 ng/rat) treatments. On the testing day, the groups of each set received either saline (2 μl/rat) or L-arginine (100 and 200 nmol/rat) 5 min prior to the test. $^{*}P < 0.05$ compared to the group with post-training and pre-test saline treatments. $^{***}P < 0.001$, $^{+++}P < 0.001$ and $^{\#\#\#}P < 0.001$ compared to the group in the first set of the animals with the same pre-test treatment.

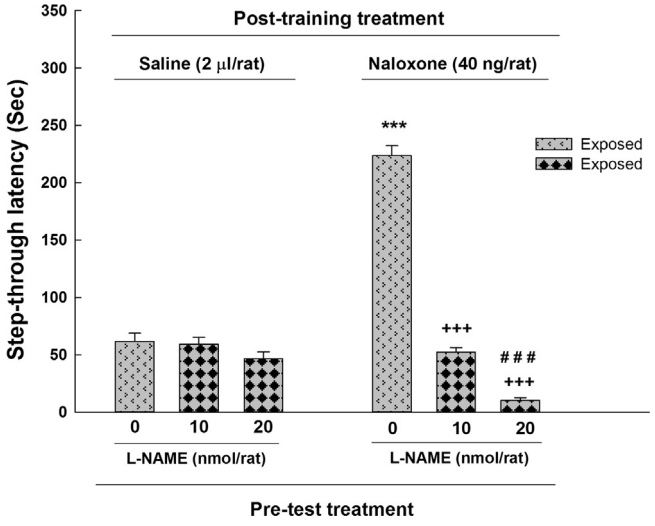

Fig. 6. The effects of pre-test i.c.v. injections of L-NAME on memory performance in the GSM-exposed animals that received either post-training saline (2 μl/rat) or naloxone (40 ng/rat) treatments. Six groups of animals were used and divided into two sets of three groups. On the training day, all three groups of the first set received post-training saline (2 μl/rat) treatment instantly but all groups in the second set received post-training naloxone (40 ng/rat) treatment. On the testing day, three groups in the both set of the animals received either saline or L-NAME at different doses (10, and 20 nmol/rat) 5 min before testing. $^{***}P < 0.001$ and $^{\#\#\#}P < 0.001$ compared to the group in the first set of the animals with the same pre-test treatment. $^{+++}P < 0.001$ compared to the group in the second set of the animals with the post-training naloxone (40 ng/rat) and pre-test saline treatments.

P < 0.001], factor B [F (2, 42) = 5.09, P < 0.05] and the interactions of both factors [F (2, 42) = 3.95, P < 0.05] on IA memory performance. Post hoc testing showed that the pre-test i.c.v. injection of L-arginine at a dose of 200 nmol/rat by itself slightly reversed the impairment of IA memory performance in the exposed animals with post-training saline treatment. Post hoc testing also showed that IA memory performance in the animals with either pre-test i.c.v. injection of saline or L-arginine was significantly different in the exposed animals with post-training naloxone treatment compared to the respective groups with post-training saline treatment (Fig. 5).

2.6. Effect of pre-test injections of L-NAME on IA memory performance in the GSM-exposed animals with post-training administration of saline or naloxone

In this experiment, the post-training treatment was considered as factor A with two levels (saline or naloxone) and the pre-test treatment of L-NAME with three levels (0, 10 and 20 nmol/rat) as factor B. Two-way ANOVA indicated a significant effect for factor A [F (1, 47) = 63.94, P < 0.001], factor B [F (2, 42) = 193.68, P < 0.001] and the interaction of both factors [F (2, 42) = 155.46, P < 0.001] on IA memory performance. Post hoc testing revealed that pre-test i.c.v. injection of L-NAME by itself had no effect on the impairment of IA memory performance in the GSM-exposed animals with post-training saline treatment. However, post hoc testing also showed significant impairment of IA memory performance in the animals with post-training naloxone and pre-test L-NAME (20 nmol/rat) treatments compared to the respective group with post-training saline and pre-test L-NAME (20 nmol/rat) treatments (Fig. 6).

2.7. 2.7. Effects of the pre-test co-administration of L-NAME and L-arginine on IA memory performance in the GSM-exposed animals with the post-training naloxone treatment

Fig. 7 shows the effect of pre-test i.c.v. coadministration of L-arginine and L-NAME on IA memory performance in the

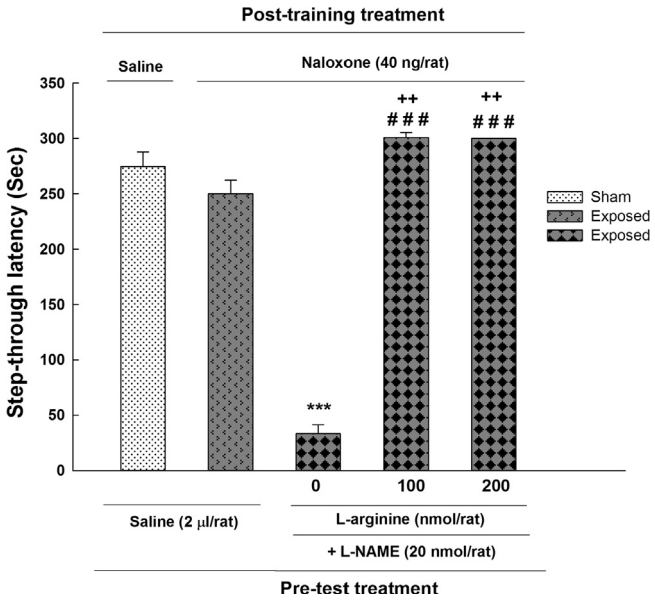

Fig. 7. The effects of pre-test i.c.v. co-administration of L-arginine and L-NAME on IA memory performance in the GSM-exposed animals in the presence of post-training injections of naloxone (40 ng/rat). Five groups of animals were used. Sham group with no history of GSM irradiation received both post-training and pre-test injections of saline (2 μl/rat). The other four groups with a history of four weeks of the GSM exposure immediately received post-training injection of naloxone (40 ng/rat); and either pre-test saline (2 μl/rat) in the first group or co-administration of L-NAME (20 nmol/rat) along with L-arginine at different doses (0, 100, and 200 nmol/rat) 10 min prior to the test with 5 min interval in the other three groups. $^{***}P < 0.001$ compared to the sham group. $^{\#\#\#}P < 0.001$ compared to the group with the post-training naloxone (40 ng/rat) and pre-test L-NAME (20 nmol/rat) treatments. $^{+}$ $^{+}P < 0.001$ compared to the group with the post-training naloxone (40 ng/rat) and pre-test saline treatments.

GSM-exposed animals with post-training naloxone treatment. One-way ANOVA indicated significant differences among the groups on IA memory performance [$F(4, 35) = 155.39$, $P < 0.001$]. Post hoc analysis showed that the i.c.v. administration of L-arginine (100 and 200 nmol/rat) significantly reversed the L-NAME-induced inhibition of the naloxone response and improved IA memory performance compared to the group that received post-training naloxone (40 nmol/rat) and pre-test saline treatments.

3. Discussion

Memory is often considered a process with several phases including acquisition, consolidation and retrieval (McGaugh, 2000; Abel and Lattal, 2001). The IA task is typically used in behavioral studies on learning and memory in rodents to explore the effects of various drugs on different phases of memory (Azizbeigi et al., 2013; Zarrindast et al., 2013; Fabbri et al., 2016). This task consists of a training session followed, after 24 h or more, by a test session (Fabbri et al., 2016). It has been reported that drug administration before training of the IA task mainly affects the acquisition as well as the early stages of memory consolidation. However, the injection of drugs after training generally affects the consolidation and somewhat the retrieval of memory but not the acquisition process. Finally, administration of drugs before testing may primarily affect the retrieval of memory as well as the later phase of consolidation (McGaugh, 2000; Abel and Lattal, 2001).

The results of the present study revealed that four weeks of exposure to GSM radiation impaired IA memory performance in rats. Similar to the present findings, impaired consolidation and/or retrieval of the spatial memory has been seen following GSM exposure in other studies (Sienkiewicz et al., 1998; Jadidi et al., 2007). The present results also revealed that the pre-training blockade of mu-opioid receptors using an i.c.v. injection of naloxone failed to alter the impairment of IA memory performance induced by GSM exposure. However, the post-training i.c.v. injection of naloxone dose-dependently recovered the impairment of IA memory performance. In addition, the pre-test i.c.v. injection of naloxone failed to reverse the impaired IA memory performance. According to these results, one may propose that GSM irradiation for four weeks induces the activation of the opioidergic system with an impairing effect on IA memory consolidation but not on the acquisition and retrieval phases. In support of this finding, other investigators have also reported that the activation of cerebral endogenous opioids following microwave radiation can be reversed by naloxone (Lai et al., 1986a, Lai et al., 1988) or naltrexone (Lai et al., 1994) in rats. In the current study, the GSM-exposed group with pre-training and pre-test injections of saline was a control for state-dependent learning. This group received four weeks of GSM irradiation. Then, while performing the IA memory task, the conditions under which the IA response was learned (5 min after pre-training saline treatment) remained the same as the conditions under which the IA memory performance was tested (5 min after pre-test saline treatment). However, this group showed impairment of IA memory performance, supporting the impairing effect of GSM exposure on IA memory performance and excluding a possible role for state-dependent learning in this study.

The present findings also revealed that, in the GSM-exposed animals with post-training treatment of saline and pre-test injections of L-arginine as an NO precursor, IA memory performance was slightly recovered. Intriguingly, the impairment of IA memory performance was completely recovered in the GSM-exposed animals with post-training treatment of naloxone (40 ng/rat) and pre-test injections of L-arginine. These results verify a synergistic effect between the blockade of opioidergic and the activation of nitrergic systems on IA memory performance, which has been previously reported by other researchers (Nasehi et al., 2013). Additionally, pre-test injections of L-NAME as an NO synthase (NOS) inhibitor did not alter IA memory performance in the GSM-exposed animals with the post-training saline treatment but impaired IA memory performance in the animals receiving post-training naloxone treatment. According to the above findings, it seems logical to propose that the recovery from the impairment of IA memory in the GSM-exposed animals with post-training naloxone treatment was a result of a blockade of the opioidergic system in an early consolidation phase of memory and activation of the nitrergic system in a later phase of consolidation or retrieval of memory. The findings of the present research also revealed that in the animals with post-training naloxone treatment, the impairment of IA memory performance with the pre-test i.c.v. injections of L-NAME was recovered by the pre-test co-administration of L-arginine and L-NAME. According to these results, an interaction between opioidergic and nitrergic systems on the later phase of the IA memory consolidation or retrieval phase in the GSM-exposed animals has been further verified. An interaction between the opioidergic and nitrergic systems in the mediation of learning and memory functions has been previously reported (Cuellar et al., 2000; Sahraei et al., 2004; Rezayof et al., 2006; Karami et al., 2014).

According to previous researchers, systemic (Hosseini et al., 2010) or i.c.v. injection of L-arginine (Baratti and Kopf, 1996; Telegdy and Kokavszky, 1997) improved memory performance in the Morris water maze and the IA model of memory, respectively. However, there are some reports that L-NAME impairs memory in similar learning paradigms (Zou et al., 1998; Majlessi et al., 2008). In addition, a few studies have demonstrated no effect on memory after a pre-test administration of L-arginine or L-NAME (Khavandgar et al., 2003). This discrepancy in the reported effects of NO on memory may be a result of the modulatory function of NO and possibly of the differences in the doses of the drugs used in different studies (Nasehi et al., 2013). We used doses of these drugs at a nanoscale level to obtain more specific effects. The fact that blockade of nitric oxide synthase with L-NAME also blocks IA memory performance indicates a facilitating effect of NO on the later phases of consolidation and/or retrieval of IA memory (Holscher and Rose, 1992; Baratti and Kopf, 1996; Holscher et al., 1996; Meyer et al., 1998; Plech et al., 2003; Paul and Ekambaram, 2011). In addition, other investigators have reported that systemic application of L-arginine increases NO release (Salter et al., 1996; Yamada and Nabeshima, 1997) and improves memory consolidation in rats (Plech et al., 2003). Likewise, a number of researchers have shown increased and decreased NO release in L-arginine- and L-NAME-treated rats, respectively, as well as in GSM-exposed animals (Ahmed and Wieraszko, 2008; Paul and Ekambaram, 2011; Salunke et al., 2014). IA memory consolidation relies mainly on the functions of some brain areas including the hippocampus, amygdala and medial prefrontal cortex (de Bock et al., 2012). The opioidergic and nitrergic systems in these areas of the brain have important roles in IA memory processing (Nasehi et al., 2013). Therefore, we propose that GSM irradiation may activate the opioidergic system in these areas that, in turn, may decrease NO release, which finally may result in the impairment of IA memory performance.

In addition to the diverse and complex effects of GSM irradiation on learning and memory mediated by putative changes in neurotransmitter systems, one may also presume that some of the adverse effects may be related to the thermal effect of electromagnetic irradiation emitted from cell phones. The specific absorption rate (SAR) is a measure of the rate at which energy is absorbed by the human body when exposed to a radio frequency EMF such

as GSM irradiation. The SAR is defined as the power absorbed per mass of tissue and has units of watts per kilogram (W/kg). Moreover, identifying the peak SAR value does not provide any information about the intracerebral structures that are under the influence of mobile phone radiation (Boutry et al., 2008). Based on the guidelines of the International Commission on Non-Ionizing Radiation Protection (2009), to ensure that thermal effects are avoided, a safety factor has been incorporated for exposure in controlled environments, resulting in a whole-body-averaged SAR limit of 0.4 W/kg. As such, the peak SAR limits for exposure in controlled environments are 20 W/kg for the limbs and 8 W/kg for the head, neck and trunk. The cell phone used in this study was a 900 MHz frequency Nokia 5110, with a maximum SAR value of 0.69 W/kg as reported by the manufacturer (Sieron-Stoltny et al., 2015). However, the SAR value related to the use of a mobile phone is not expected to affect the animal's body temperature, and the adverse effects of the exposure to GSM radiation on IA memory in the present experiments can be considered a non-thermal effect.

Regardless of the conflicts between the findings of studies carried out with real cell phone emissions and those with simulated emissions from generators, as well as conflicts within the findings taken from simulated emissions per se, almost complete consistency has been seen in real exposure studies (Panagopoulos et al., 2015). Given the variability of real EMF emission by commercial cell phones, we reported this measured variability as the average intensity to better quantify the real-life exposure. Contrary to the simulated radiation with the least similarity to the real condition experienced by cell phone users (Czyz et al., 2004), exposure to non-continuous electromagnetic radiation had greater bioactivity (Hoyto et al., 2008; Campisi et al., 2010; Franzellitti et al., 2010), and this is also the case for exposure to intermittent cell phone radiation with short intermittent durations (Chavdoula et al., 2010).

Nonetheless, a lack of measurement of body temperature, locomotion and anxiety tests may be considered one of the limitations of the present study. We did not measure the body temperature due to some technical limitations. A caveat of cell phone exposure studies is the consideration of dosimetry. Methodologically, using computer simulation and phantom models is the best dosimetry approach in this regard. However, conducting such a thorough method is not practical for every cell phone (Cardis et al., 2011). Another point of consideration is the inclusion of both male and female animals in mobile phone studies, as the NIH guidelines suggest. However, in addition to the mobile studies that used only male (Aynali et al., 2013) or female animals (Sonmez et al., 2010), there are some studies that used both sexes (Nittby et al., 2008). After considering the differences in performance, locomotion and other behaviors between males and females, we only used male rats to more easily interpret the results (Nittby et al., 2008). However, using only male animals may also be considered an addition limitation of the present study.

In addition to its limitations, the current study has some strengths. For the first time, the involvement of two important neurotransmitter signaling pathways (opioids and NO) in learning and memory under the influence of GSM exposure was reported. To our knowledge, only a few prior studies have investigated the effects of real mobile phone radiation in a long-term memory model. An additional point and a suggestion for future studies is to take these signaling pathways into account and provide extra supporting proof in terms of molecular detection of the neurotransmitters. We also used real mobile exposure with a silent non-vibratory mode for freely moving animals to prevent any stress or disturbing effects induced by ring tones or speaking modes.

In summary, exposure to four weeks of GSM radiation impaired IA memory performance mainly in the memory consolidation phase in rats. Interactions between the adverse effects of GSM radiation and both opioidergic and nitrergic systems on IA memory consolidation were verified. Taken together, these results emphasize that the effects of GSM 900-MHz mobile phone radiation on IA memory performance are mediated, at least partly, through opioid and NO signaling. This suggestion may potentially be important and needs to be considered in future investigations of memory disorders caused by the routine use of cell phones.

4. Methods

4.1. Animals

Four hundred male Wistar rats (10–12 weeks old and weighing 200–220 g at the time of surgery) were used in the study; however, due to surgery-induced mortality and incorrect insertion of the cannula, only data from 280 rats were included in the dataset. At the end of the experiments, the rats weighed 260 ± 21 g. The sham, GSM-exposed and cage control groups did not significantly differ in weight. Animals that were subjected to GSM and sham exposure were handled once a week when the animals were transported from the animal house to the experimental laboratory.

Four rats were housed in a polycarbonate cage (58 × 38 × 20 cm, Tajhiz Gostar Omid Iranian Co., Tehran, Iran) inside a well-ventilated room at 22 ± 2 °C with a 12-h light/dark cycle. Free access to standard diet and water was provided for all animals. All experiments were done during the light phase, from 8 (a.m.) to 14 (p.m.). Each group of the animals included eight rats, each rat was used only once, and independent subjects were used for each experiment. The Animal Ethics Committee of Kashan University of Medical Sciences (Kashan, Iran) approved all the procedures of the study (# 9273), which was in accordance with the National Institutes of Health (NIH) Guidelines for the Care and Use of Laboratory Animals (NIH Publications No. 8023, revised Edition 1978).

4.2. Stereotaxic surgery

Animals were anesthetized with intraperitoneal (i.p.) injection of a ketamine/xylazine mixture (100 mg/kg and 10 mg/kg, respectively) and placed in a stereotaxic frame (Stoelting Co., Wood Dale, Illinois, USA) with a flat skull position. A midline incision was made in the skin of the skull, the underlying periosteum was retracted, and a stainless steel guide cannula (22 gauge) was implanted above the right lateral ventricle. The stereotaxic coordinates were 0.8 mm posterior to bregma, 1.6 mm lateral to the midline and 3.4 mm below the top of the skull (Fig. 8) (Paxinos and Watson,

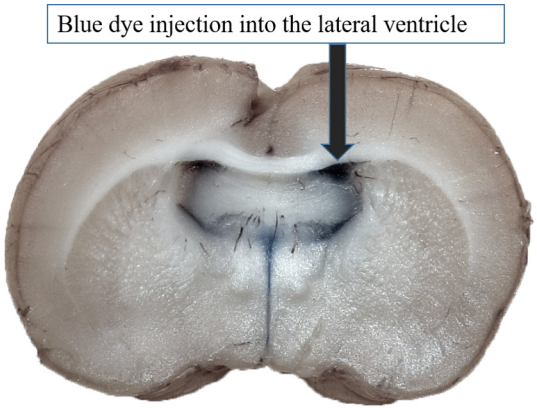

Fig. 8. A coronal section of the rat brain showing the blue dye microinjection site in the lateral ventricle of a representative animal.

2007). The cannula was anchored to the skull with dental cement and one jewelry screw. After the surgery, one stainless steel stylet (27 gauge) was inserted into the guide cannula to maintain patency prior to the drug injections. The animals were allowed two days of recovery, and, on the third day after the surgery, they were subjected to radiation exposure.

4.3. Exposure procedure

The grouping of the animals in the current study included the cage-control (control), sham control (sham), and GSM-exposed (exposed) groups. The rats (8 per group) were habituated for one hour in the "exposure room" and then were exposed or sham exposed within their home standard cages. The rats in the control group were simply kept in their cages during the four weeks of the study. Some other similar studies used the same period of exposure (Aynali et al., 2013; Narayanan et al., 2013; Wang et al., 2015). On a daily basis, the animals in the exposed group were irradiated with 50 missed calls (missed call duration 35 s) separated by 15 s intervals on the silent non-vibratory mode per day for a period of four weeks (Narayanan et al., 2013, 2010, 2009). In all experiments, only one cell phone (Nokia 5110, GSM 900, SAR = 0.69 W/kg) was used, and it was individually placed in all cages. The animals in the sham group were placed in the cages, and the same mobile phone was applied to their cage as in the exposed group, except that the mobile phone was turned off with the SIM card present.

According to the ICNIRP guidelines, the emitted field strength of the mobile phone was measured over a 6-min period (Bakker et al., 2012). The electric field strength (E) at the center of the exposure cage was measured by an Electrosmog meter (TES-92, Taiwan) in triaxial mode (X, Y and Z axis). The maximum average of the electric field strength in the turned-off mode was 16.94 ± 1.22 mV/m, which increased to 416.9 ± 57.3 mV/m during a missed call duration. The animals were free to move in the cages, and the mobile phone was fixed in a hanging position one cm above the cages using a commercial holder to avoid animal contact or damage to the hardware (Ferreira et al., 2006; Narayanan et al., 2009). In both cases, during the exposure, the experimenter's position in relation to the mobile phone and the cages was the same. The placement of the phone was also the same as described above for the sham-exposed group. The animals were exposed and tested at the same time of day at fixed time intervals. Although some authors have previously studied the effect of radio stations or similar sources on animal memory (Fragopoulou et al., 2010), in the present study, we used the missed-call type of radiation to evaluate only the effects of the emitted radiation from a mobile phone during 50 missed calls without stress from sounds on IA memory in rats. Therefore, we used the silent non-vibratory mode to prevent any stress and disturbing effects of the background sounds or ring tone-induced vibrations on animal learning and memory.

4.4. IA apparatus

The IA apparatus consisted of two compartments with an equal size ($20 \times 20 \times 30$ cm^3) separated by a wall with a guillotine door placed in the middle that could be lifted automatically using a remote system (Ahmadi et al., 2007a; Zarrindast et al., 2012). The walls and floor of one compartment consisted of white opaque Plexiglas and were lit with a 20 W electric bulb placed approximately 50 cm above the floor of the apparatus. The walls of the other compartment were dark, but the floor of the compartment consisted of parallel stainless steel grids (3 mm in diameter), located at 1-cm intervals. Intermittent electric shocks (frequency 50 Hz, duration 3 s, intensity 1 mA) were delivered to the grid floor of the dark compartment using an isolated stimulator (Maze Router Co, Tabriz, Iran).

4.5. IA memory performance test

The IA memory performance test was performed according to previous studies (Ahmadi et al., 2007b; Azizbeigi et al., 2013; Zarrindast et al., 2013). The test consisted of a two-day task with 24 h intervals between the training and testing sessions. On the training day of the IA task (one day after the four weeks of GSM irradiation), the animals were moved to the experimental room 30 min prior to the experiments. During the first acquisition trial, each animal was gently placed in the brightly lit compartment of the apparatus. After five seconds, the guillotine door was opened, and the animal was allowed to enter the dark compartment. Once the animal crossed with all four paws to the next compartment, the guillotine door was closed, and the rat was immediately withdrawn from the compartment. The second trial was repeated 30 min later, the same as in the first trial, where, after 5 s, the guillotine door was opened; as soon as the animal crossed to the dark (shock) compartment, the door was closed, and a foot shock (50 Hz, 1 mA and 3 s) was immediately delivered to the grid floor of the dark room. Twenty seconds after the shock, the rat was temporarily returned to its home cage. The animal was retested in the same way as in the trials, and, if the rat did not enter the dark compartment over 120 s, a successful acquisition of an IA response was recorded. Otherwise, when the rat entered the dark compartment (before 120 s), a non-successful acquisition of an IA response was recorded. In this case, the door was closed, and the animal received the shock again to learn a successful IA response. On the second day, the testing session was performed 24 h after the training, in which the latency of each animal entering the dark compartment was recorded as its IA memory performance.

4.6. Drugs and treatments

Naloxone hydrochloride, a potent mu-opioid receptor antagonist, was purchased from Sigma-Aldrich (St. Louis, USA). L-arginine, a nitric oxide (NO) precursor, and L-NAME hydrochloride (NG-Nitro-L-arginine methyl ester hydrochloride), an NO synthase inhibitor, were gifts from Tocris (Tocris Bioscience, Cookson Ltd, UK). All drugs were dissolved and diluted to the required volume using sterile saline (0.9%). Intracerebroventricular (i.c.v.) injections of fresh drugs with different doses were delivered in a final volume of 2 μl/rat in the intervention groups, but the control groups only received i.c.v. injections of 2 μl/ratsaline. Other investigators have also used saline as a vehicle for i.c.v. injections (Zarrindast et al., 2002; Kahveci et al., 2006; Zarrindast et al., 2007). The i.c.v. injections were administered by lowering a 27-gauge injection cannula, 1 mm longer than the guide cannula and attached to a 5 μl Hamilton syringe, with a piece of polyethylene tube. Each injection was carried out over 60 s, followed by an additional 60 s to facilitate the diffusion of the drugs from the tip of the injection cannula. On the training day of the IA task, pre-training injections of saline or drugs were performed 5 min before the first acquisition trial, but post-training injections were administered immediately after the successful learning of the IA response. A pre-test single injection was administered 5 min before testing on the test day, and, in the case of the two pre-test injections, the first one was given10 min before testing, followed by the second injection after a 5 min interval; finally, the animals were tested 5 min after the last injection. The timing of the drug injections was based on some previous reports (Zarrindast et al., 2007).

4.7. Experimental design

4.7.1. Experiment 1

This experiment examined the effects of four weeks of exposure to GSM radiation on IA memory performance. Three groups of rats

were used. On the training day, the control and sham groups, with no history of GSM exposure, received pre-training injections of saline (2 μl/rat), and the last group after four weeks of GSM radiation received saline (2 μl/rat). On the test day, all of the groups received pre-test saline (2 μl/rat) treatment.

4.7.2. Experiment 2

This experiment examined the effects of pre-training i.c.v. injections of an opioid antagonist, naloxone, on IA memory performance in animals exposed to GSM radiation. Five groups of animals were used. The sham group, with no history of GSM exposure, received pre-training and pre-test injections of saline (2 μl/rat). The other four groups, after four weeks of GSM radiation, received pre-training saline (2 μl/rat) or naloxone at different doses (0.4, 4, and 40 ng/rat) 5 min before the first acquisition trial. On the test day, all the groups received pre-test saline (2 μl/rat) treatment 5 min before testing.

4.7.3. Experiment 3

This experiment examined the effects of post-training i.c.v. administration of naloxone on IA memory performance after four weeks exposure to GSM radiation. Five groups of the animals were used. The sham group with no history of GSM exposure received post-training and pre-test injections of saline (2 μl/rat).The other four groups after four weeks of GSM radiation received post-training injections of saline (2 μl/rat) or naloxone at different doses (0.4, 4, and 40 ng/rat). On the test day, all the groups received pre-test saline (2 μl/rat) treatment 5 min before testing.

4.7.4. Experiment 4

The effects of the pre-test i.c.v. administration of naloxone on IA memory performance in animals with a history of exposure to GSM radiation were evaluated. Five groups of animals were used. On the training day, all groups received saline (2 μl/rat) after training. On the test day, the sham group, with no history of GSM radiation, received pre-test injections of saline (2 μl/rat), but the other four groups, with a history of four weeks of GSM radiation, received pre-test injections of either saline (2 μl/rat) or naloxone at different doses (0.4, 4, and 40 ng/rat) 5 min before testing.

4.7.5. Experiment 5

This experiment evaluated the effects of pre-test i.c.v. administration of L-arginine (a nitric oxide precursor) on IA memory performance in the GSM-exposed animals that received either post-training saline (2 μl/rat) or naloxone (40 ng/rat). Six groups of animals, with a history of GSM radiation, were used and divided into two sets of three groups. On the training day, all three groups of the first set immediately received post-training saline (2 μl/rat) treatment, but the groups in the second set received post-training naloxone (40 ng/rat). On the testing day, three groups of both sets received either saline (2 μl/rat) or L-arginine (100 and 200 nmol/rat) 5 min prior to the test.

4.7.6. Experiment 6

This experiment evaluated the effects of pre-test i.c.v. administration of L-NAME (a NOS inhibitor) on IA memory performance in the GSM exposed animals that received either post-training saline (2 μl/rat) or naloxone (40 ng/rat). Six groups of animals were used and divided into two sets of three groups. On the training day, all three groups of the first set immediately received post-training saline (2 μl/rat) treatment but all groups in the second set received post-training naloxone (40 ng/rat) treatment. On the testing day, three groups of the animals in each set received either saline or L-NAME at different doses (10, and 20 nmol/rat) 5 min before testing.

4.7.7. Experiment 7

This experiment examined the effects of pre-test i.c.v. coadministration of L-arginine and L-NAME on IA memory performance in the GSM exposed animals in the presence of post-training injections of naloxone (40 ng/rat). Five groups of animals were used. The sham group with no history of GSM exposure received both post-training and pre-test injections of saline (2 μl/rat). The other four groups with history of four weeks GSM irradiation received post-training injection of naloxone and pre-test coadministration of L-NAME (20 nmol/rat) along with L-arginine at different doses (0, 100, and 200 nmol/rat) at 10 and 5 min before testing, respectively.

4.8. Histological verification

After completion of the experimental sessions, each rat was deeply anesthetized with an overdose of chloroform, and 2 μl of a methylene-blue solution (1%) was slowly infused into the lateral ventricle via the same cannula through which the drugs were administered. After decapitation, each rat brain was removed and placed in a formalin solution (10%). After several days, the fixed brains were sliced in a rat brain matrix, and the sites of injection were verified according to the atlas of Paxinos and Watson (2007). Only data from animals with correct cannula implantations were included in the statistical analyses (Fig. 8).

4.9. Statistical analysis

The results were statistically evaluated using one- or two-way analysis of variance (ANOVA) where appropriate. Further analyses for paired group comparisons were carried out with post hoc Tukey's tests. A statistical significance level of $P < 0.05$ was used throughout.

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