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Title: The Effect of Intra-Medial Septum Co-Injection of Ghrelin and Nicotine on Morphine Induced Memory Impairment

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Abstract

Evidence indicates that medial septum nicotinic receptors regulate cognitive process. Ghrelin is a gut hormone that regulates energy homeostasis. Ghrelin also is produced in brain and known to be involved in cognitive function. The purpose of the study was to evaluate the effects of medial septal injection of ghrelin on morphine-induced memory impairment in rats. Also, the possible relationship between the medial septal ghrelin and acetylcholine nicotinic receptors on morphine-induced memory impairment was evaluated. Male Wistar rats implanted at the medial septum area and microinjected with ghrelin and nicotinic receptor agents. Memory retrieval was measured in a step-through type inhibitory avoidance apparatus. The results indicated the impairment of memory consolidation by posttraining administration of morphine. Posttraining intra-septal injection of either ghrelin or nicotine alone had no significant influence on memory performance, but their co-application with significant dose of morphine (7.5 mg/kg.s.c) improved memory consolidation. Moreover, posttraining co-application of low doses of the two agonists couldn't affect morphine-induced memory impairment. These results indicated no interaction between medial septal ghrelin and nicotinic receptors on morphine-induced memory impairments in rats.

Keywords: Ghrelin, Inhibitory Avoidance Learning, Medial Septum, Nicotine

1. Introduction

Medial septum (MS) is a sub-region of the basal forebrain and provides heterogeneous projections to the hippocampus (Khakpai et al., 2013; Müller and Remy, 2018). These projections contribute to generation of hippocampal theta rhythm (Nunez and Buno, 2021) and play a critical role in cognitive function (Buzsaki and Moser, 2013). MS also receives cholinergic input from the pedunculopontine tegmental nucleus and acetylcholine nicotinic receptors are highly expressed in the medial septum (Hallanger and Wainer, 1988). It is well established that nicotinic receptors are involved in the regulation of MS neurons activity (Cheng-biao et al, 2013).

Ghrelin is a small peptide produced in the gastrointestinal tract and act as a potent regulator of numerous physiological functions. Circulating ghrelin can cross the blood–brain barrier and affect higher brain functions (Muller et al., 2015). In addition, some studies have reported that ghrelin could also be synthesized in some brain regions (Cabral et al., 2017). It has received considerable attention due to its capacity in neuromodulation of cognitive functions (Seminara et al, 2018). In the hippocampus, ghrelin interferes with neurogenesis, spine synapse density and long-term memory (Chanyang et al., 2017; Chen et al., 2011; Diano et al., 2006; Eslami et al., 2018). In situ hybridization histochemistry methods indicated that ghrelin receptors, namely the growth hormone secretagogue receptor (GHSR) are expressed in rat MS (Jeffrey et al, 2006). Moreover, ghrelin mRNA is expressed in MS (Cowley et al., 2003). Recently it has been suggested that MS may be a source of ghrelin release in the hippocampus (Muniz et al, 2015).

Passive avoidance learning is a hippocampus-dependent simple task which widely use in memory studies in rodents (Atucha and Roozendaal et al., 2015). According to previous studies, acute exposure to morphine could affect various aspects of memory formation in avoidance learning (Adedayo et al., 2018; Ghasemzadeh and Rezayof, 2017; Tavassoli et al., 2020). Our previous studies showed that injection of ghrelin into the hippocampus or anterior port of ventral tegmental area (VTA) improved morphine-induced memory impairment and this improvement is

associated with the acetylcholine nicotinic receptor activity (Nazari-Serenjeh et al., 2019; Yadegay et al., 2020). Based on the evidence which shows that (1) the activation of the brain GHS-R1A receptors can improved morphine amnesic effects (Nazari-Serenjeh et al., 2019; Yadegay et al., 2020) and potentiate nicotine effect on morphine-induced memory impairment (Darbandi et al., 2008; Rezayof et al., 2008) and that (2) systemic administration of ghrelin modulates the hippocampal spine synapses density and cholinergic fibers (Farzin et al., 2021) and considering to the expression of ghrelin receptors in the MS and the effect of MS on hippocampal-dependent behavior, the present study set out to evaluate the effect of the MS ghrelin/ nicotine interaction on the memory impairing effect of morphine in avoidance learning.

2. Materials and Methods

2.1. Subjects

Male Wistar rats (a weight range between 220–240 grams at time of surgery) were caged together (4 per cage) at constant temperature environment (22 ± 2 °C; lights on 07:00–19:00 h) under 12-h light/dark cycles. All animals had *ad libitum* access to food and water. Experimental procedures were performed between 9:00 and 14:00 h. All procedures were approved by the Research and Ethics Committee of the Arak University of Medical Sciences and were performed in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals (NIH publications No. 80–23).

2.2. Stereotaxic surgery

Cannulas implantations were performed according to our previous study (Nazari-Serenjeh et al., 2019). In brief, the animals were anesthetized with intraperitoneal (i.p) administration of ketamine hydrochloride: xylazine (50 mg/kg:5 mg/kg) and then placed in a stereotaxic instrument (Stoelting Co., USA). According to atlas of Paxinos and Watson (Paxinos and Watson 2007), one cannulas was implanted into the medial septum with the cannula tips 1 mm above the site (AP = +1.2 mm, ML = ± 0.1 mm, and DV = -6.5 mm). The cannula was secured by denture powder. All animals were allowed for a minimum 7 days to recover from surgery and the remnant effect of anesthetic agents. The animals were handled two times for 1 min each during this recovery period.

2.3. *Drugs and microinjection procedures*

The drugs used in the study were morphine sulfate (Daroopaksh, Tehran, Iran), nicotine hydrogen tartrate (Sigma, Poole, Dorset, UK) and ghrelin (Abcam, UK). Morphine and ghrelin were dissolved in saline (0.09%). Nicotine was initially dissolved in saline and then the pH needed to be adjusted to 7.2 with NaOH (0.1 N solution). The morphine was injected subcutaneously (s.c) after training phase. Nicotine (5 min before morphine administration), and ghrelin (10 min before morphine administration) were microinjected in the medial septum. The injection needle (gauge 27; 1 mm longer than guide cannula) is connected to a 2- μ L Hamilton syringe by polyethylene tube. Volumes of 1 μ L of the drugs were injected in MS region over a 60 s period. The injection needle was remaining inside the guide cannula for an additional 60 s to allow complete diffusion of the solution and to reduce the possibility of reflux. Drugs doses are based on our previous experiments and a pilot study (Yadegari et al., 2020).

2.4. *Passive avoidance task*

To investigate the memory performance, two days passive avoidance tests were carried out according to our previous study (Nazari-Serenjeh et al., 2019). In brief, the passive avoidance task consists of two light and dark compartments connected through a removable guillotine door (same size, diameter: 20 $\times$ 20 $\times$ 30 cm high). The floor of dark compartment consists of stainless-steel bars used to deliver electric shocks (50 Hz, 1 mA, and 3 s).

In the first day (training day), the rat was placed in the light compartment with the open guillotine door and let to explore both compartments for 5 min. If a rat did not enter the dark compartment at all, it was excluded from the study. After 5 min, the rat was located in the light compartment of the apparatus. After 10 s, the guillotine door was elevated. The latency of the rat to enter to the dark compartment was recorded (step-through latency). Once the animal completely crossed to the dark compartment (with all four paws), the guillotine door was closed and a foot shock was delivered. After 20 s, the rat was returned to its home cage. 2 min later, the procedure was repeated and the latency of entrance to the dark compartment was measured. If the rat stayed in the light compartment for 120 with no movement to the dark compartment, successful training of passive avoidance task was achieved. However, if an animal moved to the

dark chamber before 120 s, the guillotine door was released and another shock was applied to the animal (maximum of three trials).

Testing phase done 24 h after the acquisition trial. The rat was placed in the light compartment and after 10 s the guillotine door was elevated and the step-through latency for entrance to the dark compartment was recorded. The cut-off time 300 s was established.

3. Experimental design

Experiment 1. In this experiment, five groups (7 rats in each group) were used to evaluate morphine induced-memory consolidation impairment. Four groups received various doses of morphine (0.5, 2.5, 5 and 7.5 mg/kg, s.c.) immediately after training phase. Another group received subcutaneous administration of saline (1 ml/kg, s.c.) as a control group. On the testing day, the latency to enter the dark compartment was recorded.

Experiment 2. To evaluate the effects of post-training intra-medial septum microinjection of ghrelin on morphine-induced amnesia, eight groups of animals were used. Immediately after successful training, the groups received various doses of ghrelin (0, 0.1, 0.3 and 1 nmol/ μ l, intra-medial septum) plus saline (1 ml/kg, s.c) or effective dose of morphine (7.5 mg/kg, s.c.) with 5 min interval. The step-through latency was recorded 24 h after last injection.

Experiment 3. The effect of post-training intra-medial septum microinjection of nicotine with or without morphine was evaluated on memory consolidation in 8 groups of animals. After successful training, eight groups of rats received post-training intra-medial septum microinjection of saline (1 μ l/rat) plus different doses of nicotine (0, 1, 1.25 and 1.5 μ g/rat) or morphine (7.5 mg/kg, s.c.) with 5 min interval. The step-through latency was recorded 24 h after last injection.

Experiment 4: In this experiment, effects of post-training injection of ineffective dose of ghrelin (0.1 nmol/ μ l, intra-MS) plus ineffective dose of nicotine (1 μ g/ μ l) on morphine-induced amnesia in passive avoidance task was evaluated in five groups of animals. After successful training, two groups of animals received saline (1 ml/kg) or morphine (7.5 mg/kg). Other groups of animals received ineffective doses of ghrelin (0.1 nmol/ μ l,intra-MS) or/plus nicotine (1 μ g/rat, intra-MS) and trained under morphine (7.5 mg/kg) treatment with 5 min interval. On the testing day, the step-through latency was measured.

3.1. *Histology*

At the end of experiments, histological verification of the guide cannulas placement within the medial septum was performed on anesthetized rats. After decapitation, the brain was removed and stored in 10% formalin for at least 7 days. Afterward, the injection sites were determined according to rat brain atlas of Paxinos and Watson (2007).

3.2. *Statistical analysis*

The results were presented as mean $\pm$ SEM. Data were analyzed (using SPSS 22.0 software; SPSS Inc., Chicago, IL, USA) with two- or three-way ANOVAs followed by Tukey's post hoc comparisons to determine the source of the detected significances, when appropriate. A P-value of less than 0.05 was considered as statistical significance.

4. **Results**

Effects of subcutaneous administration of morphine on step-through latency

Fig.1 shows the effects of subcutaneous administration of morphine (0, 0.5, 2.5, 5 and 7.5 mg/kg, s.c.) on step-through latency of passive avoidance apparatus [One way ANOVA: $F(4, 31) = 15.82$, $P < 0.001$]. Retrieval test performed after 24 h indicated a decreased latency time taken by the morphine-treated group (5 and 7.5 mg/kg, s.c.) to enter the dark compartment in comparison with saline-control group ($P < 0.01$ and $P < 0.001$ respectively).

Effects of post-training intra-medial septum microinjection of ghrelin on morphine-induced amnesia

Fig. 2 shows the effect of post-training intra-medial septum microinjection of ghrelin with or without morphine on memory consolidation. Two-way ANOVA revealed a significant difference between the groups of animals that received ghrelin (0, 0.1, 0.3 and 1 nmol/ μ l, intra-medial septum) plus saline (1 ml/kg, s.c.) and those that received the same doses of ghrelin plus morphine (7.5 mg/kg, s.c.) [for Treatment, $F(1, 56) = 38.72$, $P < 0.001$; Dose, $F(3, 56) = 20.04$, $P < 0.001$; and Treatment $\times$ Dose interaction, $F(3, 56) = 21.35$, $P < 0.001$]. One-way ANOVA indicated that the post-training intra-medial septum microinjection of ghrelin had no significant

effect on the step-through latency, compared with the control group (left panel of Fig. 2; [F (3, 24) = 0.593, $P > 0.05$]). However, ghrelin at dose of 1 nmol/ μ l significantly improved the morphine induced-amnesia, in comparison with the control group [F (3, 24) = 36.00, $P < 0.001$]. Therefore, ghrelin treatment significantly increased step-through latency ($P < 0.001$, right panel of Fig. 2).

The effect of post-training intra-medial septum microinjection of nicotine with or without morphine memory consolidation

Fig. 3 shows the effect of intra-medial septum microinjection of nicotine with or without morphine on memory consolidation. Two-way ANOVA revealed a significant difference in memory consolidation between the groups of animals that received nicotine (1, 1.25, and 1.5 μ g/rat, intra- medial septum) plus saline (1 mg/kg, s.c) and those that received the same doses of nicotine plus an effective dose of morphine (7.5 mg/kg, s.c.) [for Treatment, F (1, 56) = 175.24, $P < 0.001$; Dose, F (3, 56) = 1.38, $P > 0.05$; and Treatment $\times$ Dose interaction, F (3, 56) = 3.43, $P < 0.05$]. The left panel of Fig. 3 shows that following post-training microinjection of saline (1 μ l/rat, intra-medial septum) plus nicotine (1, 1.25, and 1.5 μ g/rat, intra-medial septum) no significant change was observed in the step-through latencies as compared with the saline/saline control group (One-way ANOVA: [F(3, 24) = 2.51, $P > 0.05$]). As shown in right panel of Fig. 3, post-training microinjection of nicotine (1, 1.25, and 1.5 μ g/rat, intra-medial septum) plus morphine (7.5 mg/kg, s.c.), improved the amnesic effect of morphine in passive avoidance apparatus [F (3, 24) = 5.21, $P < 0.001$].

Effect of ghrelin and nicotine combination on morphine-induced response on avoidance memory

Fig. 4. shows the effects of intra-MS injection of ineffective doses of ghrelin (0.1 nmol/ μ l) with nicotine (1 μ g/rat) on morphine-induced memory impairment. One –way ANOVA on latencies data indicated there is no significant interaction between drugs at these concentrations ($P > 0.05$).

5. Discussion

As mentioned in the literature review, it has been found that morphine-induced cognitive impairment. In this study, we found that ghrelin or nicotine microinjection into the medial

septum improved the morphine induced- memory consolidation impairment, but co-injection of them had no effect on morphine-induced memory consolidation impairment. We revealed for the first time that there is no interaction between medial septum nACh receptors and ghrelin on morphine-induced memory consolidation impairment.

Consistent with prior results (Adedayo et al., 2018; Ghasemzadeh and Rezayof, 2017; Tavassoli et al., 2020), in the present study, morphine administration cause to passive avoidance memory consolidation impairment. Previously, it has been shown that morphine-induced memory impairment results from changes in CAMKII/CREB and also BDNF/cFos signaling pathways (Ghasemzadeh and Rezayof, 2016, 2018). In addition, using Y-maze and Morris Water Maze tasks, it was shown that morphine administration led to short-term spatial memory and retrieval impairment which mediated by MOP receptors (Kitanaka et al., 2015). The fact that central or systemic injections of morphine increase the level of GABA neurotransmitter in the different brain areas (Caillé and Parsons, 2006; Ramshini et al., 2019; Sun et Al., 2011), may explain the amnesic effect of morphine. However, morphine-induced memory impairment is a more complex process. Thus, considering to modulatory effect of medial septum and also ghelin on morphine-related effects, the evaluation of medial septal ghrelin injection on morphine amnesia provides more information regarding the potential therapeutic effect of ghrelin on side effects of drug abuse such as morphine.

In the present study, acute ghrelin injected into the medial septum had no effect on the passive avoidance memory. In numerous studies, improving/inhibitory properties of ghrelin were evaluated using multiple task and animal strain (Diano et al., 2006; Davis et al., 2011; Song et al., 2013; Zhao et al., 2014). Our findings are inconsistent with previously published results showing that intra-hippocampus, -amygdala and -dorsal raphe nucleus injection of ghrelin increases memory retention (Carlini et al., 2004). Using Morris water maze test and the fear-conditioning paradigm, Albarran-Zeckler and co-workers (2012) reported that GHS-R1A knockout mice have an improved spatial memory and also a disrupted contextual memory. This inconsistency may be due to different rout of administration, the drug doses, kind of task and also animal strain.

In this study, intra-septal ghrelin significantly increased the step-through latency of rat treated with morphine, indicating the improving effect of ghrelin. The blockade of GHS-R1A

ghrelin receptors attenuates the dopamine release, the stereotypic behaviours (Sustkova-Fiserova et al., 2014), locomotor stimulation, as well as conditioned place preference (Engel et al., 2015) caused by morphine treatment supporting the involvement of ghrelin on morphine induced-memory consolidation impairment. Co-localization of GHS-R1A receptors with other neurotransmitters such as dopaminergic and cholinergic receptors has been reported (Ferrini et al., 2009; Guan et al., 1997). Co-expression of GHS-R1A with other receptors proposing the potential interaction between ghrelin and the neurotransmitters to affect brain functions. On the other hand, it has become apparent that ghrelin affect neurotransmitter release. For example, dopamine release increased in response to ghrelin administration (Palotai et al., 2013a). Ghrelin also decreases GABA release (Mir et al., 2018) and regulates glutamatergic transmission (Muniz and Isokawa, 2015). One possible suggestion is that ghrelin may affect the balance between neurotransmitter release and causes to improvement in memory impairment induced by morphine. However, the MS mechanisms that underpin ghrelin's modulation on memory process under morphine treatment are not clear. Considering the active role of nicotinic receptors on the activation of MS and consequently hippocampus, we investigated whether there is any interaction between nicotine and ghrelin within MS region on morphine-dependent memory. Our analysis showed that intra-medial septum microinjection of the nicotine inhibited morphine-induced memory impairing. Moreover, intra-medial septum microinjection of the drug by itself had no effect on passive avoidance memory consolidation. Previous study conducted by Alijanpour and Rezayof has been shown that intra-MS microinjection of low doses of nicotine induced no effect on step-down type passive avoidance apparatus (Alijanpour and Rezayof, 2013). However, co-administration of an ineffective dose of nicotine (1µg/rat) plus ghrelin (0.1 nmol/µl) couldn't affect morphine-induced memory consolidation. It has been reported that nicotine administration (oral and smoked) led to elevation in peripheral ghrelin concentrations (Ali et al., 2015). Increase in ghrelin concentrations also reported following smoking one filtered cigarette (in both smokers and nonsmokers; Bouros et al, 2006). The previous studies confirm the cross-talk between the ghrelin and nicotine. In this regards, Palotai and colleagues reported that ghrelin cause to increase in nicotine-induced dopamine release in the striatum (Palotai et al., 2013b). Also, in our previous studies we reported that hippocampal or anterior VTA nicotinic receptors are involved in ghrelin-mediated effect on morphine-related memory (Nazari-Serenjeh

et al., 2019; Yadegay et al., 2020). Since ghrelin and nicotine alone or in co-administration elevated dopamine release (Zallar et al, 2017; Palotai et al., 2013b), one may suggest that the dopaminergic mechanism may be directly or indirectly associated with ghrelin/nicotine interaction in memory formation. However, in the present study synergistic effect between ghrelin and nicotine has not been observed. An explanation can be that other receptors may be involved in ghrelin/ nicotine interaction. It has been shown that in response to stressors, ghrelin receptor knockout mice had attenuated corticosterone release (Chuang et al., 2011; Currie et al., 2012). Thus it seems that activation of ghrelin receptor cause to increase in corticosterone release. On the other hand, administration of nicotine also increased the secretion of corticosterone (Bugajski et al., 2002). One may suggest that the potentiation of corticosterone release in ghrelin- treated rat by intra-MS microinjection of nicotine may be cause to the differential occupation of corticosteroid receptors which affects the impact of the drugs on memory formation (de Kloet et al., 1999). Reul and co-workers reported that in the presence of high corticosteroid levels, both mineralocorticoid and glucocorticoid receptors mediate response which may be involved in amnesia (Reul et al., 1985). Further studies with more focus on this hypothesis are therefore suggested.

On the other hand, the present results may suggest that the involvement of nicotine receptor in ghrelin effect on morphine-induced memory consolidation may be site specific in the brain. In support of this suggestion, previously it has been shown that ghrelin has regionally specific effect on neurotransmitter release within the brain (Bouros et al., 2006; Mir et al., 2018). Taken together, these results indicate substantial participation of the medial septal ghrelin receptors on morphine-induced memory consolidation impairment.

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Conflict of interest

The authors report no conflict of interest.

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Legends:

Fig.1. Effects of morphine administration on memory consolidation. The animals were trained in step-through latency apparatus. Immediately after training, the animals received intra-MS injection of saline (1µl/rat) plus subcutaneous administration of different doses of morphine. The memory retrieval test was carried out 24 h after the training phase. Each bar represents mean $\pm$ S.E.M. of seven rats per group. **P<0.01 and ***P < 0.001 compared to saline control group.

Fig.2. The effect of post-train administration of ghrelin with or without morphine on memory consolidation. Four groups of animals received intra-MS injection of ghrelin () and after 5 min, injected with saline (1ml/kg). The other four groups received the same doses of ghrelin and after 5 min they were treated with an effective dose of morphine (7.5 mg/kg). After 24 h, all of animals were tested to measure step-through latency. Data is expressed as mean $\pm$ S.E.M. of seven animals per group. *** $P < 0.001$ compared to saline/saline control group. +++ $P < 0.001$ compared to saline/morphine group.

Fig. 3. Effect of intra-MS injection of nicotine on memory formation in the presence or absence of morphine. After successful training, the animals received nicotine injection (0, 1, 1.25 and 1.5 µg/rat) plus saline (1 ml/kg) or morphine (7.5 mg/kg, s.c.) with 5 min interval. Data are expressed as mean $\pm$ S.E.M. of 7 rats per group. *** $P < 0.001$ compared to saline/saline control group ++ $P < .001$ compared with the morphine group.

Fig. 4. Effect of intra-MS co-administration of ineffective doses of ghrelin plus nicotine on memory in rats trained under morphine. Five groups of animals were used. After successful training, the animals received saline (1 ml/kg, s.c.), morphine (7.5 mg/kg, s.c.) or ineffective doses of ghrelin (0.1 nmol/µl, intra-MS) or/whit nicotine (1 µg/rat, intra-MS) plus morphine (7.5 mg/kg, s.c.) with 5 min interval. Data are expressed as mean $\pm$ S.E.M. of 7 rats per group. ***P<0.001 compared to saline/saline/saline control group.

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