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Research Paper

Investigating the Co-administration of Ghrelin and Nicotine Into the Medial Septum and Its Influencing on Morphine Amnestic Effect

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ABSTRACT

Introduction: Evidence indicates that medial septum nicotinic receptors regulate cognitive processes. Ghrelin is a gut hormone that regulates energy homeostasis. Ghrelin is also produced in the brain and is involved in cognitive function. This study aims to evaluate the effects of medial septal administration of ghrelin on the amnestic effect of morphine in rats. In addition, the possible relationship between the medial septal ghrelin and acetylcholine nicotinic receptors on the amnestic effect of morphine is evaluated.

Methods: The rats were implanted at the medial septum area and were microinjected with ghrelin and nicotinic receptor agents. The step-through type inhibitory avoidance apparatus was used for memory retrieval assessment.

Results: The results showed that the administration of morphine after the training phase impaired memory consolidation. Post-training intra-septal injection of the same doses of either ghrelin or nicotine did not change memory performance; however, their co-application with morphine (significant dose: 7.5 mg/kg subcutaneous injection) increased the step-through latency and improved memory consolidation. Moreover, post-training co-application of low doses of the two agonists could not affect morphine-induced memory impairment.

Conclusion: These results indicated no interaction between medial septal ghrelin and nicotinic receptors on the amnestic effect of morphine in rats.

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Highlights

- Post-training morphine administration impaired memory performance.
- Intra-septal injection of ghrelin or nicotine alone did not affect memory performance.
- Co-application of either ghrelin or nicotine with morphine improved memory.
- Co-application of the two agents could not affect morphine-induced memory impairment.

Plain Language Summary

Morphine abuse has been associated with memory disturbance. Ghrelin is a gastrointestinal hormone known as hunger hormone. It also affects cognitive performance via binding ghrelin receptors in central nervous system. On the other hand, the medial septum nicotinic receptors improve memory-associated behavior. Hence, we hypothesized that septal ghrelin receptors could affect the effect of nicotine on morphine-induced memory deficit. We examined this hypothesis in avoidance memory task. We found that subcutaneous administration of morphine inhibited avoidance memory. The effect of morphine was blocked by intra-medial septum injection of nicotine or ghrelin. However, co-infusion of ghrelin with nicotine into the medial septum area had no effect on morphine amnesia. Overall, the study results suggest no interaction between ghrelin and cholinergic nicotinic receptors in morphine amnesia.

1. Introduction

The medial septum (MS) is a sub-region of the basal forebrain and provides heterogeneous projections to the hippocampus (Khakpai et al., 2013; Müller & Remy, 2018). These projections contribute to the generation of hippocampal theta rhythm (Nunez & Buno, 2021) and have a critical role in cognitive processes (Buzsaki & Moser, 2013). MS also receives cholinergic input from the pedunculopontine tegmental nucleus. Meanwhile, acetylcholine nicotinic receptors are highly expressed in the medial septum (Hallanger & Wainer, 1988). 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 acts as a potent regulator of numerous physiological functions. Circulating ghrelin can penetrate the blood–brain barrier and affect higher brain functions (Muller et al., 2015). In addition, ghrelin can also be synthesized in some brain regions (Cabral et al., 2017). It has received considerable attention because of its capacity for the 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, 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 that is widely used in memory studies in rodents (Atucha & 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 & Rezayof, 2017; Tavassoli et al., 2020). In our recent studies, we showed that intra-hippocampal or anterior port of ventral tegmental area (VTA) microinjection of ghrelin improved the amnestic effect of morphine and this improvement was

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

2. Materials and Methods

Study subjects

Male Wistar rats (with a weight range of 220 to 240 grams at the time of surgery) were caged together (4 per cage) at a constant-temperature environment (22±2°C; lights on 07:00–19:00) under 12-h light/dark cycles. All animals had ad libitum access to food and water. The experimental procedures were performed between 9:00 and 14:00.

Stereotaxic surgery

Cannulas implantations were performed according to our previous study (Nazari-Serenjeh et al., 2019). In brief, the animals were anesthetized with an intraperitoneal injection of ketamine hydrochloride: Xylazine (50 mg/kg: 5 mg/kg) and then placed in a stereotaxic instrument (Stoelting Co., USA). According to the atlas of Watson, one cannula was implanted into the medial septum with the cannula tips 1 mm above the site (anterior posterior=+1.2 mm, medial lateral=±0.1 mm and dorsal ventral=−6.5 mm). The cannula was secured by denture powder. All animals were allowed a 7-day recovery to recover from surgery and the remnant effect of anesthetic agents. The animals were handled 2 times for 1 min each during this recovery period.

Drugs and micro injection procedures

The morphine sulfate (Daroopakhsh, Tehran, Iran), nicotine hydrogen tartrate (Sigma, Poole, Dorset, UK), and ghrelin (Abcam, UK) were used. Morphine and ghrelin were dissolved in saline (0.09%). Nicotine was initially dissolved in saline; then, the pH needed to be adjusted to 7.2 with NaOH (0.1 N solution). The morphine was injected subcutaneously after the 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 the guide cannula) was attached to a 2-μL Hamilton syringe by polyethylene tube. Subsequently, 1 μL volumes of the drugs were injected in the MS region over a 60 s period. The injection needle remained inside the injection site for an additional 60 s to avoid drug reflux. Drug doses were based on our previous experiments along with a pilot study (Yadegari et al., 2020).

Passive avoidance task

To investigate memory performance, 2 days of passive avoidance tests were carried out according to our previous study (Nazari-Serenjeh et al., 2019). In brief, the passive avoidance task consisted of two light and dark compartments connected through a removable guillotine door (same size, diameter: 20×20×30 cm high). The floor of the dark compartment consisted of stainless-steel bars used to deliver electric shocks (50 Hz, 1 mA, and 3 s).

On the 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 the rat did not enter the dark compartment for more than 100 s, 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 the dark compartment was recorded (step-through latency). Once the animal completely crossed 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. Then, 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 s with no movement to the dark compartment, successful training was achieved; however, if the animal reentered the dark chamber before 120 s, the guillotine door was released and the animal received a second shock (maximum of trials was 3 times).

The testing phase was 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 of 300 s was established.

Experimental design

Experiment 1. A total of 5 groups (7 rats in each group) were used to evaluate morphine-induced-memory consolidation impairment. Accordingly, 4 groups received various doses of morphine (0.5, 2.5, 5, and 7.5 mg/kg, subcutaneously) immediately after the training phase. The control group received subcutaneous administration of saline (1 mL/kg, subcutaneously). 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, 8 groups of animals were used. Immediately after successful training, the groups received various doses of ghrelin (0, 0.1, 0.3, and 1 nmol/

μL, intra-medial septum) plus saline (1 mL/kg, subcutaneously) or an effective dose of morphine (7.5 mg/kg, subcutaneously) with a 5-min interval. The step-through latency was recorded 24 h after the 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, 8 groups of rats received post-training intra-medial septum microinjection of saline (1 μL/rat) plus different doses of nicotine (0, 1, 1.25, and 1.5 μg/rat) or morphine (7.5 mg/kg, subcutaneously) with a 5-min interval. The step-through latency was recorded 24 h after the last injection.

Experiment 4. In this experiment, the effects of post-training injection of an ineffective dose of ghrelin (0.1 nmol/μL, intra-MS) plus an ineffective dose of nicotine (1 μg/μL) on morphine-induced amnesia were evaluated. The rats were assigned into 5 separate groups. Two groups of animals received saline (1 mL/kg) or morphine (7.5 mg/kg). Treatment groups received ineffective doses of ghrelin (0.1 nmol/μL, intra-MS) or/plus nicotine (1 μg/rat, intra-MS) and trained under morphine (7.5 mg/kg) treatment. On the testing day, the step-through latency was measured.

Histology

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

Statistical analysis

The results were presented as Mean±SE of the mean. The data were analyzed using SPSS software, version 22; SPSS Inc., Chicago, IL, USA) with one or two-way analysis of variance, followed by the Tukey post hoc comparisons to determine the source of the detected significances, when appropriate. A $P<0.05$ was considered statistically significant.

3. Results

Effects of subcutaneous administration of morphine on step-through latency

Figure 1 indicates the effects of subcutaneous administration of morphine (0, 0.5, 2.5, 5, and 7.5 mg/kg, subcutaneously) on step-through latency of passive avoid-ance apparatus (one-way analysis of variance: $F_{(4, 31)}=15.82$, $P<0.001$). The retrieval test performed after 24 h indicated a decreased latency time taken by the mor-phine-treated group (5 and 7.5 mg/kg, subcutaneously) to enter the dark compartment in comparison with the saline-control group ($P<0.01$ and $P<0.001$, respective-ly).

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

Figure 2 shows the effect of post-training intra-medial septum microinjection of ghrelin with or without morphine on memory consolidation. The two-way analysis of variance revealed a significant difference between the groups of animals that received ghrelin (0, 0.1, 0.3, and 1 nmol/μL, intra-medial septum) plus saline (1 mL/kg, subcutaneously) and the animals that received the same doses of ghrelin plus morphine (7.5 mg/kg, subcutaneously) (for treatment $F_{(1, 56)}=38.72$, $P<0.001$; dose, $F_{(3, 56)}=20.04$, $P<0.001$; treatment×dose interaction, $F_{(3, 56)}=21.35$, $P<0.001$). The one-way analysis of variance indicated that the post-training intra-medial septum microinjection of ghrelin was not significant on the effect on the step-through latency, compared to the control group (left panel of Figure 2; $F_{(3, 24)}=0.593$, $P>0.05$). However, ghrelin at a dose of 1 nmol/μL 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 the step-through latency ($P<0.001$; right panel of Figure 2).

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

Figure 3 shows the effects of intra-medial septum microinjection of nicotine with or without morphine on memory consolidation. The two-way analysis of variance revealed a significant difference in memory consolidation between the groups of animals that received nicotine (1, 1.25, and 1.5 μg/rat, intra- medial septum) plus saline (1 mg/kg, subcutaneously) and those that received the same doses of nicotine plus an effective dose of morphine (7.5 mg/kg, subcutaneously) (for treatment, $F_{(1, 56)}=175.24$,

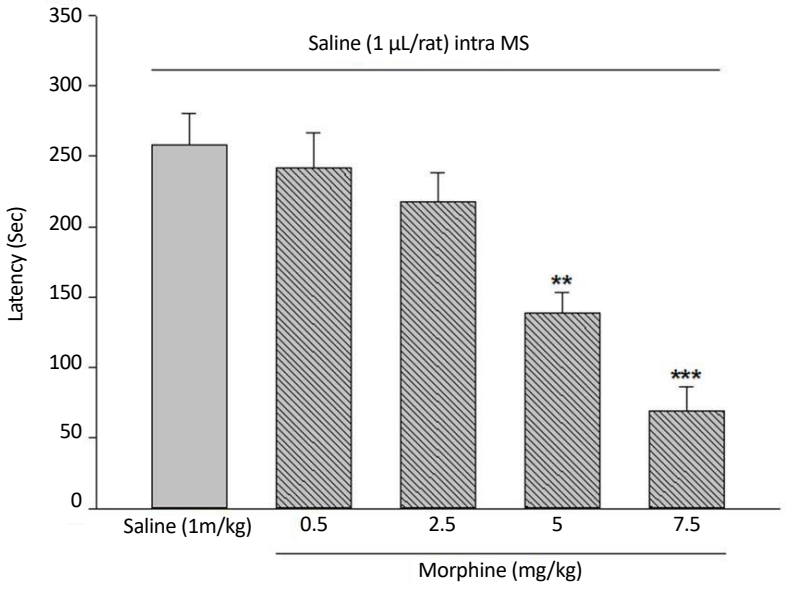

Figure 1. The effects of morphine administration on memory consolidation

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Notes: The animals were trained in the step-through latency apparatus. Immediately after training, the animals received an intra-medial-septum injection of saline (1 µL/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 the Mean±SE of the mean of seven rats per group.

**P<0.01, ***P<0.001 compared to saline control group.

P<0.001; dose, $F_{(3, 56)}$=1.38, P>0.05; treatment×dose interaction, $F_{(3, 56)}$=3.43, P<0.05). The left panel of Figure 3 shows that following post-training microinjection of saline (1 µL/rat, intra-medial septum) plus nicotine (1, 1.25, and 1.5 µg/rat, intra-medial septum), no significant change was observed in the step-through latencies as compared with the saline/saline control group (one-way analysis of variance: $F_{(3, 24)}$=2.51, P>0.05). As shown in the right panel of Figure 3, post-training microinjection of nicotine (1, 1.25, and 1.5 µg/rat, intra-medial septum) plus morphine (7.5 mg/kg, subcutaneously) improved the amnesic effect of morphine in the passive avoidance apparatus ($F_{(3, 24)}$=5.21, P<0.001).

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

Figure 4 shows the effects of intra-MS injection of ineffective doses of ghrelin (0.1 nmol/µL) with nicotine (1 µg/rat) on morphine-induced memory impairment. The one–way analysis of variance on latencies data indicated no significant interaction between drugs at these concentrations (P>0.05).

4. Discussion

As mentioned in the literature review, morphine induces cognitive impairment. In this study, we found that ghrelin or nicotine microinjection into the medial septum improved the morphine induced-amnesic effect, however, their co-injection did not affect 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 & Rezayof, 2017; Tavassoli et al., 2020), in the current study, morphine administration caused passive avoidance memory consolidation impairment. Previously, morphine–induced memory impairment results from changes in CAMKII/CREB and also BDNF/cFos signaling pathways was demonstrated (Ghasemzadeh & Rezayof, 2016; Ghasemzadeh & Rezayof, 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 was mediated by *MOP* receptors (Kitanaka et al., 2015). Given that central or systemic injections of morphine increase the level of GABA neurotransmitters in the different brain areas (Caillé & Parsons, 2006; Ramshini et al., 2019; Sun et al., 2011), this may explain the amnesic effect of morphine; however, morphine-induced memory impairment is a more complex process. Thus, considering the modulatory effect of medial septum and also ghrelin on morphine-related effects, the evaluation

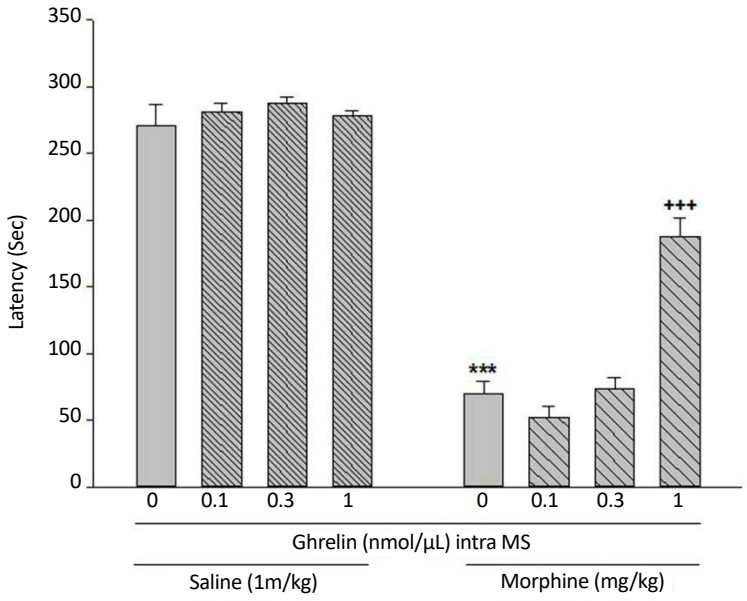

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Figure 2. The effect of post-train administration of ghrelin with or without morphine on memory consolidation

Notes: Four groups of animals received intra- medial-septum injection of ghrelin and after 5 min injected with saline (1 mL/kg). The other 4 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 animals were tested to measure the step-through latency. The data is expressed as Mean±SE of the mean of 7 animals per group.

***P<0.001 compared to the saline/saline control group, +++P<0.001 compared to the saline/morphine group.

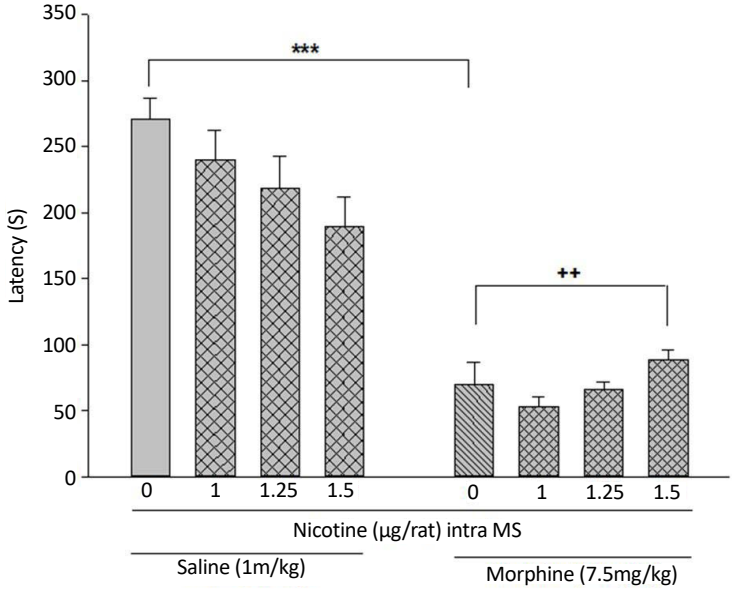

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Figure 3. The effect of intra-media-septum injection of nicotine on memory formation in the presence or absence of morphine

Notes: After successful training, the animals received nicotine injections (0, 1, 1.25, and 1.5 µg/rat) plus saline (1 mL/kg) or morphine (7.5 mg/kg, subcutaneously) at 5 min intervals. The data are expressed as Mean±SE of the mean of 7 rats per group.

***P<0.001 compared to the saline/saline control group, ++P<0.001 compared with the morphine group.

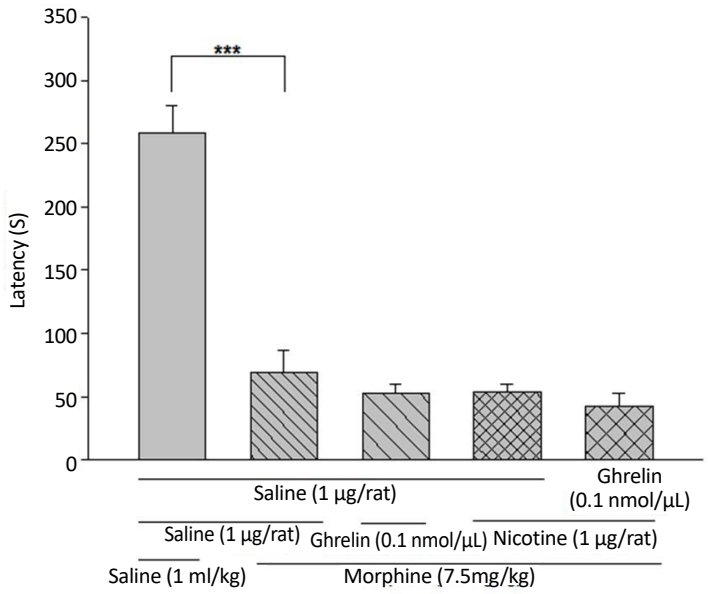

Figure 4. Effect of intra-medial-septum co-administration of ineffective doses of ghrelin plus nicotine on memory in rats trained under morphine

Notes: Five groups of animals were used. After successful training, the animals received saline (1 mL/kg, subcutaneously), morphine (7.5 mg/kg, subcutaneously) or ineffective doses of mg/kg, subcutaneously) with 5 min intervals. The data are expressed as Mean±SE of the mean of 7 rats per group.

***P<0.001 compared to the saline/saline/saline control group.

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 could not affect memory in avoidance tasks. In numerous studies, improving/inhibitory properties of ghrelin were evaluated using multiple tasks and animal strains (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 the Morris water maze test and the fear-conditioning paradigm, Albarran-Zeckler et al. (2012) reported that GHS-R1A knockout mice have improved spatial memory and also disrupted contextual memory. This inconsistency may be due to different routes of administration, drug doses, types of tasks, and also animal strain.

In this study, intra-septal ghrelin significantly increased the retrieval of memory in rats treated with morphine, indicating the improving effect of ghrelin. The blockade of *GHS-R1A* ghrelin receptors attenuate the dopamine release, the stereotypic behaviors (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. The co-localization of *GHS-R1A* receptors with other neurotransmitters, such as dopaminergic and cholinergic receptors have been determined (Ferrini et al., 2009; Guan et al., 1997). The co-expression of GHS-R1A with other receptors proposes the potential interaction between ghrelin and the neurotransmitters to affect brain functions. On the other hand, ghrelin affects 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 & Isokawa, 2015). One suggestion is that ghrelin may affect the balance between neurotransmitter release and causes improvement in memory impairment induced by morphine. However, the MS mechanisms that underpin ghrelin's modulation of the memory process under morphine treatment are unclear. 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 the MS region on morphine-dependent memory.

Our analysis showed that the microinjection of nicotine into the medial septum inhibited morphine-induced memory impairment. Moreover, intra-medial septum microinjection of the drug by itself did not affect passive avoidance memory consolidation. A previous study conducted by Alijanpour and Rezayof has shown that intra-MS microinjection of low doses of nicotine-induced did not affect the step-down type passive avoidance apparatus (Alijanpour & Rezayof, 2013). However, the co-administration of an ineffective dose of nicotine (1 µg/rat) plus ghrelin (0.1 nmol/µL) could not affect morphine-induced memory consolidation. It has been reported that nicotine administration (oral and smoked) led to an elevation in peripheral ghrelin concentrations (Ali et al., 2015). An increase in ghrelin concentrations was also reported following smoking one filtered cigarette (in both smokers and nonsmokers; Bouros et al., 2006). The cross-talk between ghrelin and nicotine has been confirmed. In this regard, Palotai and colleagues reported that ghrelin causes an 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 the 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 the learning process. However, in the present study, a 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 attenuated corticosterone release (Chuang et al., 2011) Thus, the activation of the ghrelin receptor causes an increase in corticosterone release. On the other hand, the administration of nicotine increased the secretion of corticosterone (Bugajski et al., 2002). One may suggest that the potentiation of corticosterone release in ghrelin-treated rats by intra-MS microinjection of nicotine may cause the differential occupation of corticosteroid receptors which affects the impact of the drugs on memory formation (de Kloet et al., 1999). Reuland et al. reported that high corticosteroid levels provide a potential issue for both mineralocorticoid and glucocorticoid receptors response in amnesia (Reul & de Kloet, 1985); therefore, it is suggested further studies with more focus on this hypothesis.

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

Ethical Considerations

Compliance with ethical guidelines

This study was approved by the Research and Ethics Committee of Arak University of Medical Sciences (Code: IR.ARAKMU.REC.1397.215). The procedures were in accordance with the guideline for the care and use of laboratory animals (NIH publications No. 80–23).

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Authors' contributions

Conceptualization and supervision: Niloufar Darbandi; Data collection: Soudabe Dastjanifarahani; Data analysis: Niloufar Darbandi, Writing–review & editing: Farzaneh Nazari-Serenjeh and Zahra Ghasemzadeh.

Conflict of interest

The authors declared no conflict of interest.

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