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Effect of Intrahippocampal Ghrelin Agonist Administration on Passive Avoidance Learning and Anxiety in Rats

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Abstract: Hippocampus, Amygdale and dorsal raphe nucleus are the cerebral main structures involved in learning, memory and anxiety. Ghrelin increases the level of several hormones in these structures and affects learning, memory and anxiety-like behaviors. This study was performed to investigate the effect of ghrelin agonist on passive avoidance learning and anxiety in adult female rats in the presence and absence of ovary hormones. Five groups of rats, including control group with no injections, ovariectomized groups; one group receiving normal saline and other group receiving ghrelin agonist solution, surgery shocked (sham operated) groups; one group receiving saline and other group ghrelin agonist solution, were tested. Inside stereotaxis apparatus, two sided CA1 cannulae were used and 1 μL of saline or ghrelin agonist solution, at 3 nmol μL^{-1} concentration, was injected into each cannula. Passive avoidance learning was measured by using shuttle box and anxiety by elevated plus- maze. Ghrelin agonist increased the level of learning in surgery shocked group in comparison with control group. Anxiety-like behavior was seen in both ovariectomized and surgery shocked groups. Ghrelin agonist binds its own receptors in the hippocampus, thereby increases learning capability and induces anxiety-like behaviors. Proper management of these behaviors might be useful in controlling some forms of nervous system diseases in humans.

Key words: Ghrelin agonist, ovariectomy, alzheimer, learning, anxiety

INTRODUCTION

Peptide ghrelin was originally discovered in rat stomach in 1999 as a potent secretion increasing substance of growth hormone (Kojima *et al*., 1999). Ghrelin has also been demonstrated in many other organs such as testis, ovary, placenta (Gualillo *et al*., 2001), kidney, pituitary glands, small intestine, pancreas, lymphocytes (Hattori *et al*., 2001) and brain (Traebert *et al*., 2002). In central nervous system, the main site of ghrelin synthesis, of course at much lower levels than the stomach, is the hypothalamus (Ferrini *et al*., 2009). Intraperitoneal, intracerebroventricular or direct hypothalamic application of acylated ghrelin affects blood pressure (Carlini *et al*., 2007, 2008), food intake, gastro-intestinal motility, secretion of gastric acid (Carlini *et al*., 2002) and growth hormone (Cottrell and Ferguson, 2004).

Ghrelin also affects animals during peri-weaning period. In a study on goat kids, it was shown that weaning significantly increased plasma levels of ghrelin and authors concluded that ghrelin secretion may help minimize the negative consequences of the new diet on dry matter intake (Magistrelli *et al*., 2011). The fact that ghrelin has anti-inflammatory, para-sympathetic stimulatory and sympatho-inhibitory effects suggest that ghrelin may be of significant benefit in the management of sepsis and critically ill patients. Possible mechanisms regarding this new hope are discussed by Das (2011).

Hippocampus, amygdala and dorsal raphe nucleus are the main brain areas involved in learning and memory mechanisms. Recent studies show that acylated ghrelin may have influence on memory and learning processes, as well as anxiety (Ferrini *et al*., 2009).

Gonadal hormones are important factors which interfere with learning and anxiety in animals so that gonadectomy in both male and female rats affects these behaviors (Levinoff and Chertkow, 2002; Vazquez-Pereyra *et al*., 1995; Sato *et al*., 2003). Progestrone, as a neurosteroid, changes the irritability characteristics of central nervous system and alters learning and anxiety. Progesrtone receptors are present on

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several central nervous system structures such as frontal cortex, hippocampus and hypothalamus (Gould *et al*., 1990). One of the factors related to memory loss is the gradual decrease in the level of estrogen as a result of menopause. Brain areas such as hippocampus and cerebral cortex which are involved in learning and memory processes have also been shown to be extensively populated by estrogen receptors. Low levels of estrogen have been linked to increased incidence of neurodegenerative diseases and deterioration of cognitive functions in women (Toufexis *et al*., 2006).

In spite of several studies on the effects of ghrelin on learning and anxiety, there are rare investigations on female rats and the relation between these behaviors and gonadal hormones is not clearly understood. Moreover, in our country Iran, there has been no internationally published studies on the role of ghrelin in learning, memory and anxiety. Therefore, this study was planned to investigate the effect of intrahippocampal ghrelin agonist on passive avoidance learning and anxiety in adult female rats in the presence and absence of ovary hormones.

MATERIALS AND METHODS

Animals and test groups: This research was carried out in accordance with ethical principles originally laid down in the declaration of Helsinki, Finland 1964 and guidelines of the ICAC (1995). Female Wistar rats (180-240 g) receiving sufficient food and water ad libitum were used. The animals were placed in individual cages and colony room was maintained under controlled temperature (21-23°C) and light (12 h light, 12 h dark). Fifty rats were used in 5 groups, each group consisting 10 animals. Control group with no injection and surgery, 2 ovariectomized groups; one group receiving saline (ovs) and the other group ghrelin agonist (ovg), 2 surgery shocked (sham operated) groups; one group receiving saline (shs) and the other group ghrelin agonist (shg) were used. Surgery shock was performed only to mimic the ovariectomy procedure and ovaries were left intact in place.

Ovariectomy and surgery shock: The animals were anesthetized with 55 mg kg^{-1} ketamine HCl (Vetanarcol) and 11 mg kg^{-1} xylazine, both from Alfasan corp., Woerden, Netherland. Ovariectomy procedure was performed and ovaries were removed bilaterally (Azman *et al*., 2001). The surgery shock procedure was also performed similar to ovariectomy. These groups of animals were given normal diet for 1 week for the wounds to heal before starting next step.

Stereotaxic procedure: Ovariectomized and surgery shocked rats were anesthetized as before and were placed in stereotaxic apparatus (Stoelting Co., Wood Dale, IL, USA). These animals were implanted bilaterally into the hippocampus with steel guide cannulae, according to Paxinos and Watson (1986). The coordinates were, for hippocampus anterior; 4.3, lateral; 4.0 and vertical; 3.4. Cannulae were fixed to the skull surface with dental acrylic cement (Paxinos and Watson, 1986). Ghrelin agonist with a trademark of GHPR-2 (growth hormone releasing peptide-2) which has been supplied by LKT Lab. Inc., St.Paul, MN, USA, in the form of lyophilized powder was dissolved in normal saline to prepare a 3 nmol μL^{-1} working solution immediately before testing. After at least 7 days of recovery, 1 μL of saline or ghrelin agonist solution was injected into each cannula of conscious rats using a 10 μL Hamilton syringe connected by Pe-10 polyethylene tubing to a 30-gauge needle extended 0.75 mm beyond the guide cannulae.

Passive avoidance learning test: Learning was evaluated by one-trial step-through method (Chen *et al*., 2008). All animals, including control group, were kept in normal condition for at least 5 days before the experiments. The test was conducted using a custom-made two-compartment standard shuttle box (100×28×35 cm) supplied by Bionic Mobin Co., Tehran, Iran. First, the rats underwent training by being allowed to explore the apparatus without electric shock for 60 sec each. The test was conducted between 10 a.m. and 2 p.m. 1 day after the training. During the training session the sliding door was opened by the experimenter and the rat was allowed to cross-over into the dark compartment. Once the rat had entered the dark compartment with all four paws, the sliding door was closed and an electrical current (constant current, 1 mA for 2 sec) was delivered through the stainless-steel floor. Latency to cross into the dark compartment (i.e., pre-shock latency) was recorded. After exposure to the foot shock, the rat was removed from the apparatus to its home cage. Retention of passive avoidance learning performance was tested 2 min afterwards. The rat was placed in the lighted (safe) compartment again with access to the dark compartment without any shock. The latency to enter the dark compartment was measured (i.e., testing latency). One hundred and twenty seconds indicated full training. Test procedure was carried out 24 h later and 300 sec latency to enter the dark compartment considered full memory.

Anxiety behavior test: Elevated plus-maze was used in order to test anxiety behavior. A standard plus-maze was

made of wood and consisted of two open arms measuring 50-10 cm and two enclosed arms of 50-10 cm with walls 40 cm high. The arms extended from a central platform (10×10 cm); the whole apparatus was elevated 50 cm above the floor. To avoid rats from falling down, the open arms were bordered by transparent plastic 1 cm high. Individual rats were placed onto the central platform and observed for 10 min. The behavioral performances recorded were: the number of entries into the open arms; the number of entries into closed arms and time spent in closed arms; and the number of rearing, grooming and risk assessment. The test was performed as a single trial per animal (Carlini *et al*., 2002).

Statistics: Data were entered in Excel for Windows XP and then transferred to SPSS, version 17 (SPSS Inc., Chicago, IL, USA). For analysis, one way analysis of variance (ANOVA) followed by T-test was used. Significance for all statistical testing was set at $p<0.05$.

RESULTS

Effects on learning: One way analysis of variance was used for comparison of all four groups with control group and showed that only surgery shocked group which received ghrelin agonist had a significant difference (Fig. 1). Post hoc Tukey test also presented the same result. When t-test was used for comparison, a $p = 0.01$ was observed only for shg group. In order to examine the difference between ovg and shg groups with their respective saline groups, t-test was performed. For shg group, the difference was highly significant ($p = 0.004$), the ovg group also showed a significant difference, albeit to a very lower extent ($p = 0.015$). Whereas shs in comparison with ovs ($p = 0.797$) and shg in comparison with ovg ($p = 00346$) both showed no significant differences. Thus, ghrelin agonist increases learning especially in the presence of ovary hormones.

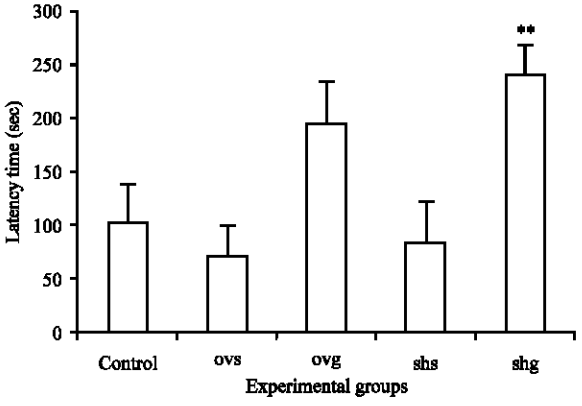

Fig. 1: Effects of ghrelin agonist on learning: Animals were administrated with ghrelin agonist 5 min before the test. Ghrelin agonist enhanced learning in surgery shocked ghrelin group (**$p<0.01$), n: 10 animals/group. For figures 1, 2, 3 and tables 1, 2: ovs: Ovariectomized saline group, ovg: Ovariectomized ghrelin agonist group, shs: Surgery shocked saline group and shg: Surgery shocked ghrelin agonist group

Effects on anxiety: Based on elevated plus maze experiment, using one way ANOVA and post hoc Tukey Tests, the time length spent in the open arms was significantly lower in ghrelin agonist groups compared to control group. Later, t-test was used for comparison and both ovariectomized and surgery shocked groups which received ghrelin agonist showed significant difference compared to control group using either open arms entire (Fig. 2) or open arms time (Fig. 3) suggesting effect on anxiety. Finally, comparison was performed by using t-test between ovg, shg and their respective saline groups. The results are presented in Table 1 and 2, respectively. As can be seen, no significant difference was observed in any case.

Table 1: Comparison of ghrelin agonist groups with saline groups using open arms entire

Groups	Mean	SE	t-value	t-test sig.
Ovs	37.78	6.23	-	-
Ovg	23.57	5.79	1.672	0.112
Ovs	37.78	6.23	-	-
Shs	26.16	6.56	1.285	0.215
Ovg	23.57	5.79	-	-
Shg	24.21	5.69	-0.079	0.938
Shs	26.16	6.56	-	-
Shg	24.21	5.69	0.225	0.825

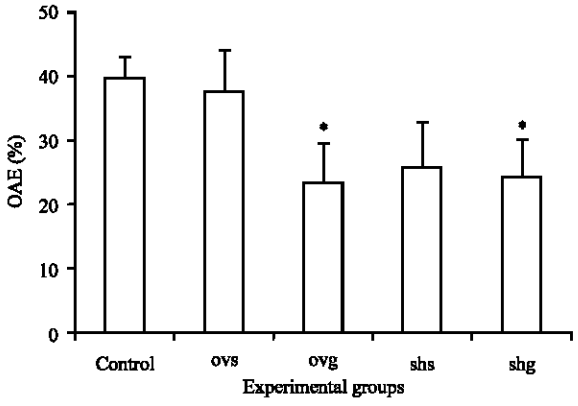

Fig. 2: Effects of ghrelin agonist on open arms entire, Animals were administrated with ghrelin agonist 10 min before test, for both ovg and shg groups, difference is significant (*$p<0.05$), showing anxiety. n = 10 animals/group

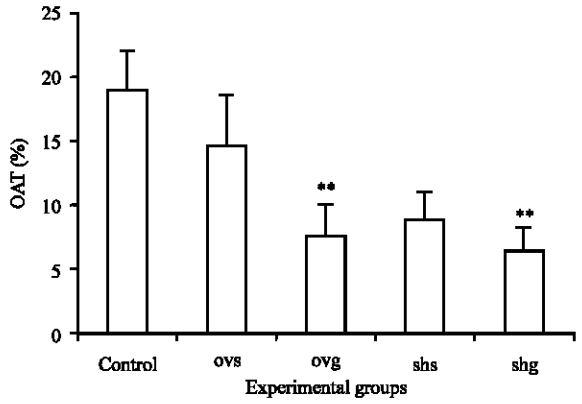

Fig. 3: Effects of ghrelin agonist on open arms time, Animals were administrated with ghrelin agonist 10 min before test, The difference is significant for both shg and ovg groups (**p<0.01), showing anxiety, n = 10 animals/group

Table 2: Comparison of ghrelin agonist groups with saline groups using open arms time

Groups	Mean	SE	t-value	t-test sig.
Ovs	14.73	3.89	-	-
Ovg	7.64	2.39	1.553	0.138
Ovs	14.73	3.89	-	-
Shs	6.41	1.82	1.936	0.069
Ovg	7.64	2.39	-	-
Shg	8.86	2.13	-0.381	0.707
Shs	6.41	1.82	-	-
Shg	8.86	2.13	-0.873	0.394

DISCUSSION

In this study, it was shown that ghrelin agonist increased learning in the presence of ovary hormones indicating synergism between ghrelin agonist and these hormones. Although, animals exhibited anxiety like behaviors, this might not be actually due to ghrelin agonist. Other studies, on both well nourished (Carlini *et al*., 2004) and food restricted animals (Carlini *et al*., 2008) have shown the similar findings. A study on well nourished animals which received ghrelin through intracerebroventicular and hippocampal routes showed memory improvement suggesting effects on consolidation. This effect was highest at 1 h after training. These effects could be related to an increase in hippocampal spine synapse density and to the long term potentiation (Carlini *et al*., 2004).

Another study on adult female mice which had been subjected to chronic food restriction also showed that acute central ghrelin administration restores learning and memory behaviors. Daily vaginal smears were taken and studied to evaluate estrous cycle and the majority of the animals exhibited alterations in the estrous cycle continuity (Carlini *et al*., 2008).

Clearly, several factors such as dose of drug, route of administration, animals conditions, etc, play roles in the outcome of these experiments. Therefore, in a study for evaluating effect of different doses of ghrelin, all three 0.3, 1.5 and 3.0 nmol μL^{-1} doses induced a marked increase in the latency time indicating that probably 0.3 nmol μL^{-1} was the dose of maximal effect and this was a ceiling effect. However, results also showed that the doses that could improve the memory retention did not have anxiogenic effect. As a consequence, the increase in the latency time observed in the step-down test could not be attributed to the anxiogenic effect of the drug because the increase in the memory retention was seen at doses of 0.3 and 1.5 that did not produce anxiogenic like behavior (Carlini *et al*., 2004).

To complete these findings, another study using step-down test showed that ghrelin did not induce any anxiety-like behavior 24 hrs after administration but it induced anxiogenesis only at the highest dose tested (3.0 nmol μL^{-1}) and in a short time of 15 min after administration (Carlini *et al*., 2010a).

The 3.0 nmol μL^{-1} dose of ghrelin agonist (GHRP-2) we used in this study showed to be effective on improving learning, memory retention and inducing anxiety like behaviors soon after administration, e.g., 10 min. This is in agreement with the fact that this molecule is pharmacologically similar to ghrelin and has a short half life with peak concentrations occurring around 15 min and no longer than 60 minutes after administration (Furuta *et al*., 2004).

It is well known that intracerebroventricular injection of ghrelin engages the activation of numerous subpopulations of growth hormone stimulating receptors while the direct administration of ghrelin into the different structures affects only the receptors present in this area. Thus, the results strongly suggest participation of extrahypothalamic areas in consummatory behavior. Moreover, findings also indicate that ghrelin acts centrally in these structures modulating ingestive behavior and influencing other processes such as memory and anxiety (Carlini *et al*., 2004). Similar effects were seen in both mice and rats when several structures in the brain such as hippocampus, amygdala and dorsal raphe nucleus were used for injections (Berry *et al*., 1997).

We therefore chose to inject directly into hippocampus CA1 which is believed to be one of the most proper sites for ghrelin administration in order to exhibit learning and anxiety behaviors.

Some mechanisms have been proposed with regard to the effect of ghrelin on learning, memory and anxiety. Drugs, such as fluoxetine that inhibit serotonin reuptake (Toth *et al*., 2010), are effective in reducing anxiety

symptoms; receptor agonists of serotonin impaired memory retention while antagonists improved retention (Mishkin, 1978). Another study showed that intrahippocampal (CA1) ghrelin administration in rats increased the nitric oxide activity but only for the highest dose such as 3 nmol μL^{-1} (Carlini *et al*., 2010b). Some forms of induced long term potentiation could be reduced by application of nitric oxide synthase inhibitors resulting in alteration of learning and memory (Bernabeu *et al*.,1995). Modifications of memory processes caused by acylated-ghrelin was also eliminated by pretreatment with equimolar amount of ghrelin antagonists (Toth *et al*., 2009).

Bearing in mind that ghrelin may help prevent the stress-induced depression and anxiety (Himmerich and Sheldrick., 2010), more specific and in-depth studies on ghrelin receptors and employing dose and time-dependent mechanisms can promote learning behaviors and control anxiety.

CONCLUSION

The proper management of these behaviors in animals might eventually lead to controlling nervous system disorders such as Alzheimer disease in humans.

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