International Journal of Sciences

Research Article Volume 6 – January 2017 (01)

The Ghrelin/GHS-R1a Pathway is Involved in the Mechanism of the CTA-regulated Neuronal Loop

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Abstract: Objective: We aimed to investigate the effects of ghrelin and its receptor GHS-R1a on the regulation of taste aversion-associated emotional memory. Methods: We studied the effect of selective and reversible inactivation of emotion-associated neurons on emotions processed by the local neuronal loop of the lateral amygdala and insular cortex. We used intraperitoneal clozapine-N oxide (CNO) injection, local brain microinjection, and taste aversion test. Results: Microinjection of ghrelin into the lateral amygdala blocked the acquisition of taste aversion memory in mice. However, microinjection of ghrelin did not affect memory consolidation. Adeno-associated virus (AAV) microinjection into the lateral amygdaloid nucleus blocked the acquisition of taste aversion memory in mice. Moreover, intraperitoneal injection of CNO inhibited taste aversion memory formation, altering the aversion index. Conclusion: Microinjection of ghrelin and AAV-CaMKII-hM4Di-2A-GHS-R1a-GFP into the lateral amygdala inhibited the acquisition of conditioned taste aversion in mice. The inhibition of the memory formation was achieved through the activation of growth hormone secretagogue receptor 1a (GHS-R1a).

Keywords: Ghrelin, insular cortex, CNO, CTA, GI-DREADD System

1 Introduction

Ghrelin is a 28 amino acid protein. Ghrelin was first reported by Kojima *et al.* in 1999 in mice, human gastric mucosa cells, and human hypothalamic arcuate nucleus. Ghrelin strongly promotes the secretion of growth hormone[1]. The physiological function of ghrelin is exerted through the activation of the ghrelin receptor[2-3]—growth hormone secretagogue receptor 1a (GHS-R1a). GHS-R1a is a G protein-coupled receptor comprised of seven transmembrane regions. Both ghrelin and GHS-R1a are involved in the regulation of memory, learning, motivation and reward, and anxiety and depression. Ghrelin and GHS-R1a are associated with the pathology of many neuropsychiatric diseases involving emotional and cognitive disorders, such as schizophrenia, depression, epilepsy, addiction, and neurodegeneration[4-6].

The amygdala plays an important role in the acquisition, consolidation, and extraction of emotional memory[7]. It has been shown that the amygdala receives projections from ghrelin neurons. Moreover, expression levels of GHS-R1a mRNA in the lateral amygdala were higher than in the central nucleus of the amygdala in mice. These results suggest that ghrelin/GHS-R1a and the corresponding downstream signaling pathways might regulate neuronal activity in the lateral amygdala. Signaling in the lateral amygdala affects the formation and maintenance of emotional memory. Neurons can be selectively reversibly inactivated using the

Published at: http://www.ijsciences.com/pub/issue/2017-01/
DOI: 10.18483/ijSci.1182; Online ISSN: 2305-3925; Print ISSN: 2410-4477

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GI-DREADD system. After injection of AAV-CaMKII-hM4Di-2A-GHS-R1a-GFP into a specific brain region, infected neurons co-express GHS-R1a, hM4Di, and GFP. Subsequent intraperitoneal injection of clozapine-N-oxide (CNO)[8-11] specifically activates hM4Di after 45 min and results in neuronal inactivation due to hyperpolarization. Conditioned taste aversion (CTA) is an experimental model commonly used in studies of memory and learning. In addition, CTA is widely used in studies on acquisition, consolidation, and subsidence processes and mechanisms of emotional memory[8]. The lateral amygdala and insular cortex (IC) are important brain regions involved in the acquisition and storage of CTA-associated emotion and memory[12-13].

In this study, we explored the effects of ghrelin and its receptor GHS-R1a on the regulation and the underlying molecular mechanism of taste aversion-associated emotional memory by using the DREADD system[14]. The DREADD system allows selective and reversible functional regulation of specific neuron types. We studied the effect of selective and reversible inactivation of emotion-associated neurons on emotions associated with the neuronal circuit of the lateral amygdala and IC using intraperitoneal injection of CNO, microinjection, and taste aversion behavior tests.

2. Material and Methods

2.1 Animals and reagents

Adult male Jac mice (2–3 months of age, weighing 25–30 g) were obtained from Beijing Vital River Laboratory Animal Technology Co., Ltd. Ghrelin was purchased from Tocris Bioscience (USA), and was prepared as 1 mmol/L stock solution by using sterile saline. Ghrelin stocks were divided into aliquots and stored at -80°C. Sterile saline was used to dilute ghrelin stocks.

2.2 Construction of HSV and AAV viral plasmids

The hM4Di plasmid was purchased from Addgene. The GHS-R1a plasmid was a gift from Helen Wise (University of Hong Kong). The AAV-CaMKII-hM4Di-2A-GHS-R1a-GFP vector was constructed on the basis of an existing viral backbone plasmid (lab stock). Viral packaging was completed by Neuron Biotech Co., Ltd.

2.3 Animal surgery

IC and BLA microinjection: A syringe (23 G) was stereotactically placed on the lateral amygdala and IC bilaterally. The coordinates for the bilateral IC were: fontanelle 1.3 mm, bilateral opening 3.7 mm, depth 2.5 mm; fontanelle 0.3 mm, bilateral opening 3.9 mm, depth 2.5 mm. The coordinates for the amygdala were: fontanelle= -1.3, bilateral opening = ±3.3, depth = -4.8 mm. AAV-CaMKII-hM4Di-2A-GHS-R1a and AAV-CON viruses were injected using a microinjector (including a locator and a micropusher). The injection needle (28 G; the needle exceeded the syringe casing by 0.8 mm) was connected to a Hamilton syringe through a PE50 polyethylene tube. Using a micro peristaltic pump (Stoelting Co., USA), chemicals were slowly injected into the brain at a constant rate of 0.25 µl/min. Virus (0.5 µl) were administered for 10 min. After the injection, the needle remained at the injection site for another 5 min before being slowly removed to prevent spilling. After surgery, mice recovered for 4-6 weeks before undergoing behavioral tests.

2.4 Histological examination

mices were anesthetized, and the tissue was fixed by perfusing 4% paraformaldehyde into the heart. After perfusion, the brain was removed, and fixed with paraformaldehyde for another 12 h. Subsequently, the brains were transferred to 30% sucrose for 2 days. The brains were frozen and sectioned into 40-µm sections. After staining with crystal violet, locations where the syringe and needles had been placed were examined by using LEICA microscope.

2.5 CTA experiment

LiCl consumption test: Mice were water-deprived for 24 h and were allowed to adapt to the following

schedule for 3 days. One bottle contained 15 ml of tap water and another bottle contained equal body volumes of 50 mM NaCl. Mice were allowed to drink from each bottle for 10 min successively. On the fourth day, mice were first allowed to drink tap water and equal body volumes of 50 mM NaCl for 10 min. Then, mice were allowed to drink 180 mM LiCl and 180 mM NaCl for 10 min. After 24 h, perform mice behavioral test. Prior to the test, mice were intraperitoneally injected with CNO. After 30 min, mice were allowed to drink tap water and 50 mM NaCl freely for 20 min. The remaining liquid volume in each bottle was measured. The related index of the record was tested again after 24 h.

LiCl injection test: Mice were water-deprived for 24 h and were allowed to adapt to the same schedule as in the LiCl consumption test schedule for 3 days. On the fourth day, mice were allowed to drink 0.2% sweetener (Saccharin sodium salt hydrate, Sigma) from the two bottles for 10 min successively. After 20 min, mice were injected intraperitoneally with 100 mM LiCl (2 ml/100 g). After 24 h, perform mice behavioral test. Prior to the test, the mice were intraperitoneally injected with CNO. After 30 min, mice were allowed to drink tap water and 0.2% sweetener freely for 20 min. The remaining liquid volume in each bottle was measured. The results were recorded again after 24 h. Taste-aversion memory was evaluated using the aversion index (AI):

$$AI = \frac{\text{Volume of water intake}}{\text{Volume of water and NaCl (or sweetener) intake}} * 100$$

. The cutoff was set at 50%. A higher AI indicates a better taste-aversion memory.

2.6 Statistical analysis

Data are expressed as mean ± standard error. GraphPad Prism4 was used for statistical analysis and plotting. One-sample t-tests were performed to compare AI to chance level with a fixed value of 50%. Two-tailed t-tests were performed for comparison between two groups. One-way ANOVA was used for univariate comparison of two or more groups, followed by Newman-Keuls test for multiple comparisons. Two-way ANOVA and repeated measures ANOVA were used for multivariate comparisons. $p<0.05$ was considered statistically significant.

3. Results

3.1 Successful transfection of AAV viruses and neuronal overexpression of GHS-R1a and hM4Di

After microinjection of AAV-CaMKII-hM4Di-2A-GHS-R1a-GFP into the IC, eGFP was detected using immunohistochemistry. eGFP staining indicates successful transection of the virus and overexpression of GHS-R1a and hM4Di proteins in the neurons (**Figure 1**).

3.2 Microinjection of ghrelin into the amygdala blocked the acquisition, but not the formation, of aversion memory

Twenty min prior to CTA training, 12 ng of ghrelin (0.5 µl/side) were microinjected into the lateral amygdala. After 24 h, the AI of the ghrelin-treated group (n = 10) was significantly lower than the AI of the control group (n = 10; **Figure 2A**). The taste AI of the ghrelin-treated group did not differ significantly from chance (50% cutoff, $p>0.05$, **Figure 2A**). Low doses of (12 µg/0.5 µl) ghrelin microinjected into the lateral amygdala inhibited the acquisition of CTA memory in mice. In contrast, microinjection of ghrelin into the amygdala did not affect the liquid uptake of mice during CTA training and memory tests (**Figures 2B** and **2C**).

3.3 Microinjection of AAV into the IC blocked the acquisition of CTA memory in mice

Prior to the start of CTA training, AAV-hM4DGi-GHS-R1a and the corresponding viral AAV-CON were microinjected into the IC. After 24 h, the AI of the mice injected with AAV-hM4DGi-GHS-R1a (n = 10) was smaller than the AI of the AAV-CON group (n = 10) (**Figure 3C**). However, virus microinjection into the IC did not

significantly affect liquid uptake of mice during CTA training and memory tests (**Figures 3A** and **3B**).

3.4 Microinjection of AAV into the IC blocked the acquisition of CTA memory in mice; concurrent intraperitoneal injection of CNO altered the formation of CTA memory

Microinjection of AAV into the IC blocked the acquisition of CTA memory in mice (Figure **4 C**). However, liquid uptake during CTA training and memory tests was not significantly affected (**Figures 4A** and **4B**). Intraperitoneal injection of CNO prior to the start of the test revealed that the specific activation of hM4DGi receptors by CNO led to hyperpolarization and neuronal inactivation. The AI of the AAV-hM4DGi-GHS-R1a group (n = 10) was reduced fter CNO injection (**Figure 4 D**). After 24 h, when CNO was fully metabolized, hM4DGi receptors were no longer activated, and the AI recovered.

3.5 Microinjection of AAV into the amygdala blocked the acquisition of CTA memory in mice; concurrent injection of CNO inhibited the formation of CTA memory

Microinjection of the AAV-hM4DGi-GHS-R1a into the amygdala blocked the acquisition of CTA memory in mice (**Figure 5 C, test 1**), and did not significantly affect liquid uptake during CTA training and memory tests (**Figures 5A** and **5B**). However, intraperitoneal injection of CNO prior to the test revealed that the AAV-CON and AAV-HA/hM4DGi were activated and triggered the silencing of neuronal inactivation. Thus, AI decreased and memory formation was inhibited (**Figure 5 C, test 2**). The AI of the AAV-hM4DGi-GHS-R1a group (n = 10) was significantly lower than the AI of the AAV-CON group (n = 10; **Figure 5 C test 2**). After 24 h, when CNO was fully metabolized, the neuronal activity and the acquisition of memory recovered (n = 10, **p $<$ 0.01, *p $<$ 0.05). Accordingly, we observed increased AI (**Figure 5 C test 3**).

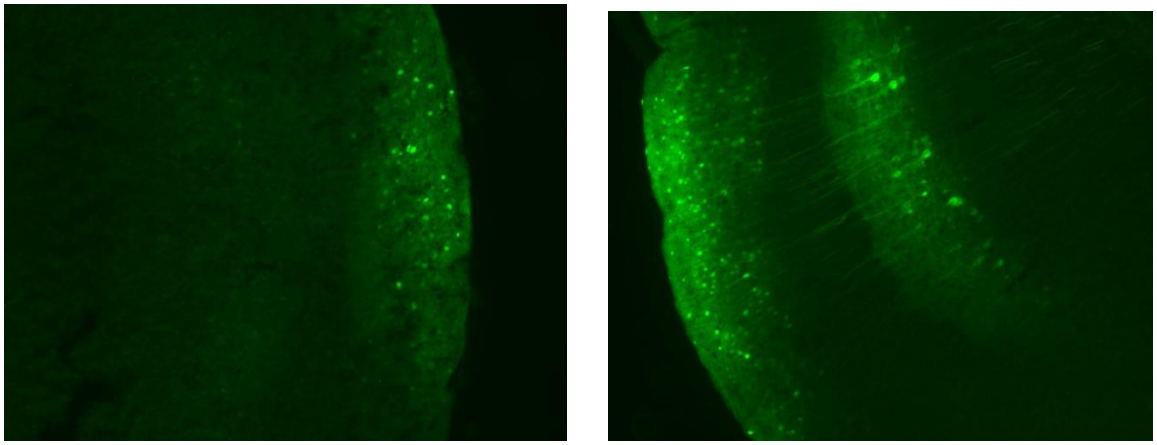

Figure 1. Successful transfection of AAV viruses and overexpression of GHS-R1a and hM4Di in neurons.

GFP antibody

A. AAV-HA/hM4Di-GHS-R1a/eGFP **B.** AAV-CON

Figure 1. (A) Immunohistochemical assay of the expression of eGFP in the insular cortex after injection of AAV-hM4Di. (B) AAV-CON also showed minimal eGFP expression in the insular cortex.

Figure 2. Microinjection of ghrelin into the amygdala blocked the acquisition, but did not affect the formation, of CTA memory.

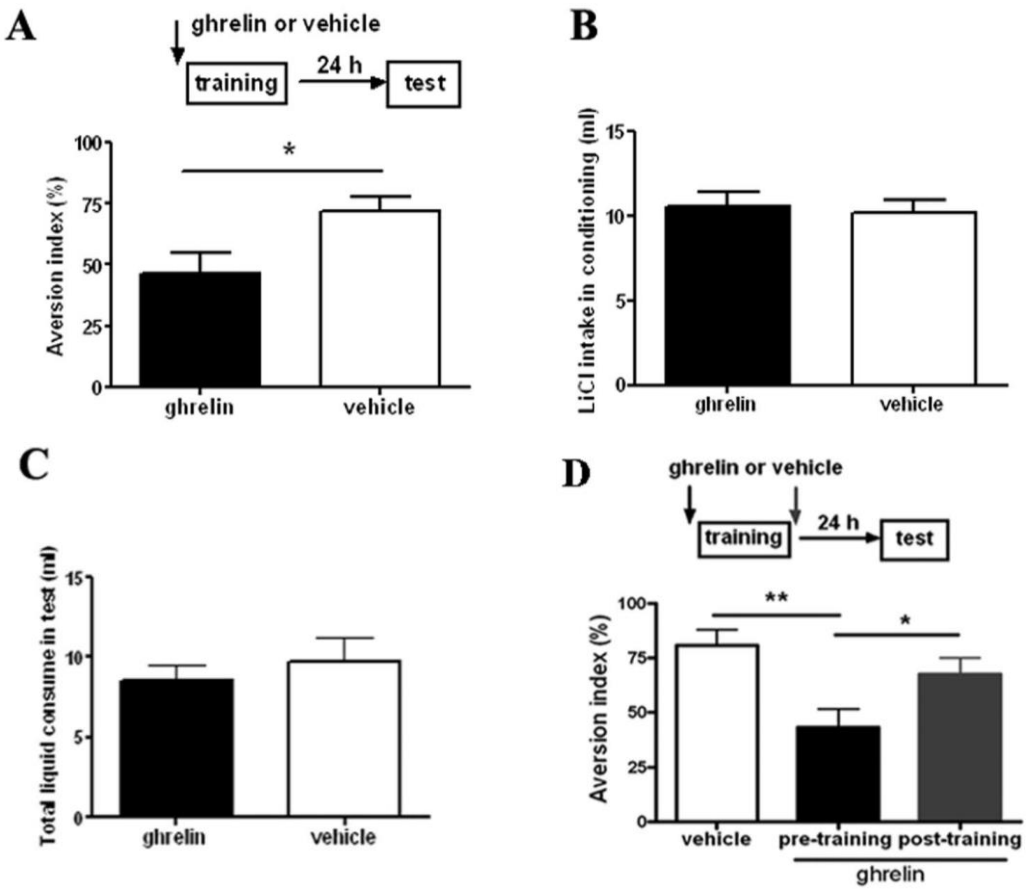

Figure 2. (A) Microinjection of ghrelin (12 ng, 0.5 μl) into the lateral amygdala blocked the acquisition of CTA memory in mice. (B) Microinjection of ghrelin into the lateral amygdala did not affect the total intake of LiCl during CTA training. (C) Microinjection of ghrelin into the lateral amygdala did not affect the total fluid intake during CTA training. (D) Microinjection of ghrelin into the lateral amygdala did not affect the consolidation of CTA memory in mice ($n = 10$, $^{**}p < 0.01$, $^{*}p < 0.05$). Pre-training denotes microinjection of ghrelin before CTA training. Post-training denotes microinjection of ghrelin immediately after CTA training.

Figure 3. Microinjection of AAV viruses into the insular cortex blocked the acquisition of CTA memory in mice.

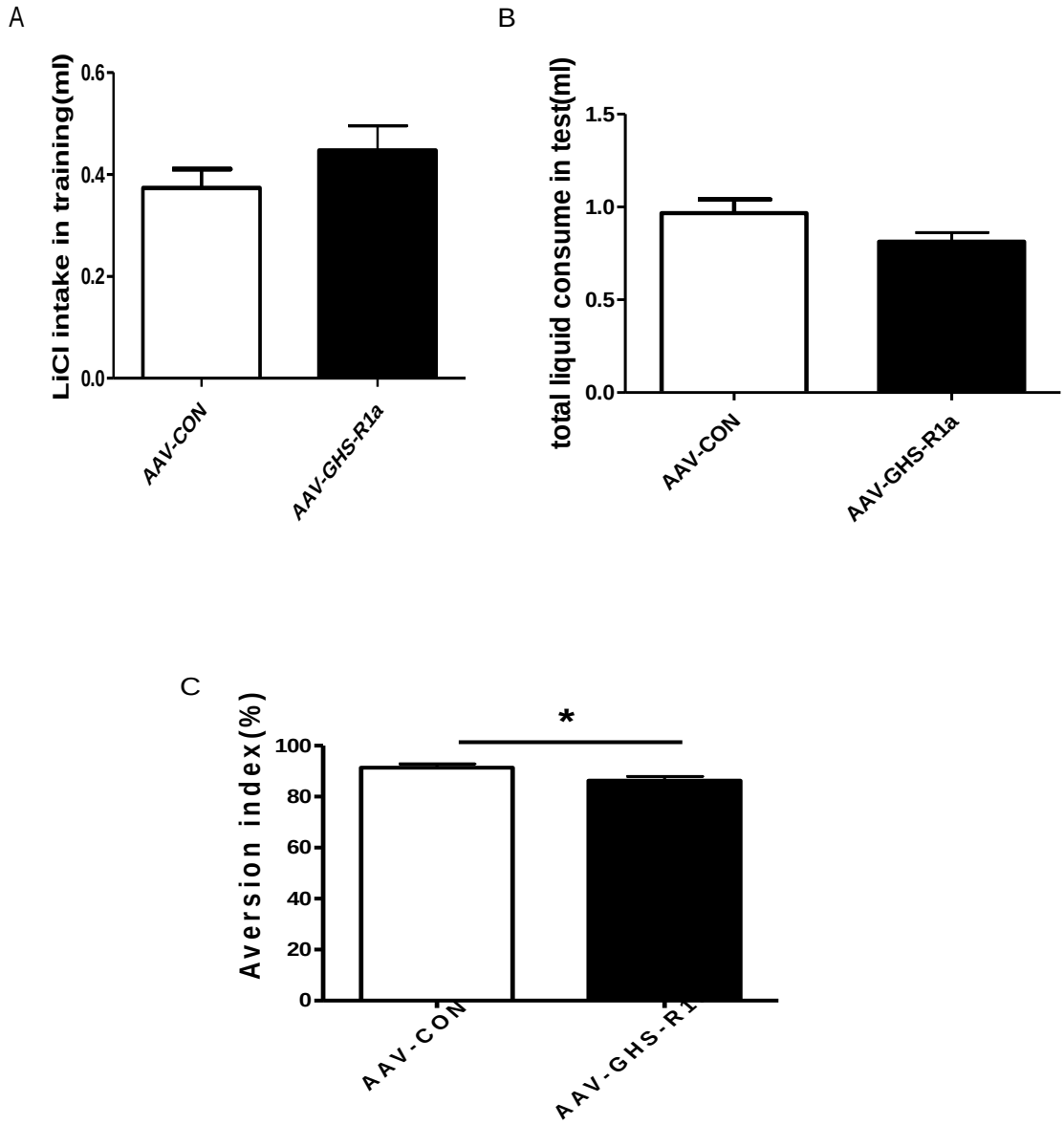

Figure 3. (A) Viral microinjection into the insular cortex of mice did not significantly affect the total LiCl intake during CTA training. (B) Microinjection of AAV into the insular cortex of mice had no significant effect on the total fluid intake during CTA training. (C) Microinjection of AAV viruses into the insular cortex blocked the acquisition and inhibited the formation of CTA memory in mice (n = 10, *p＜0.05).

Figure 4. Intraperitoneal injection of CNO altered the formation of CTA memory.

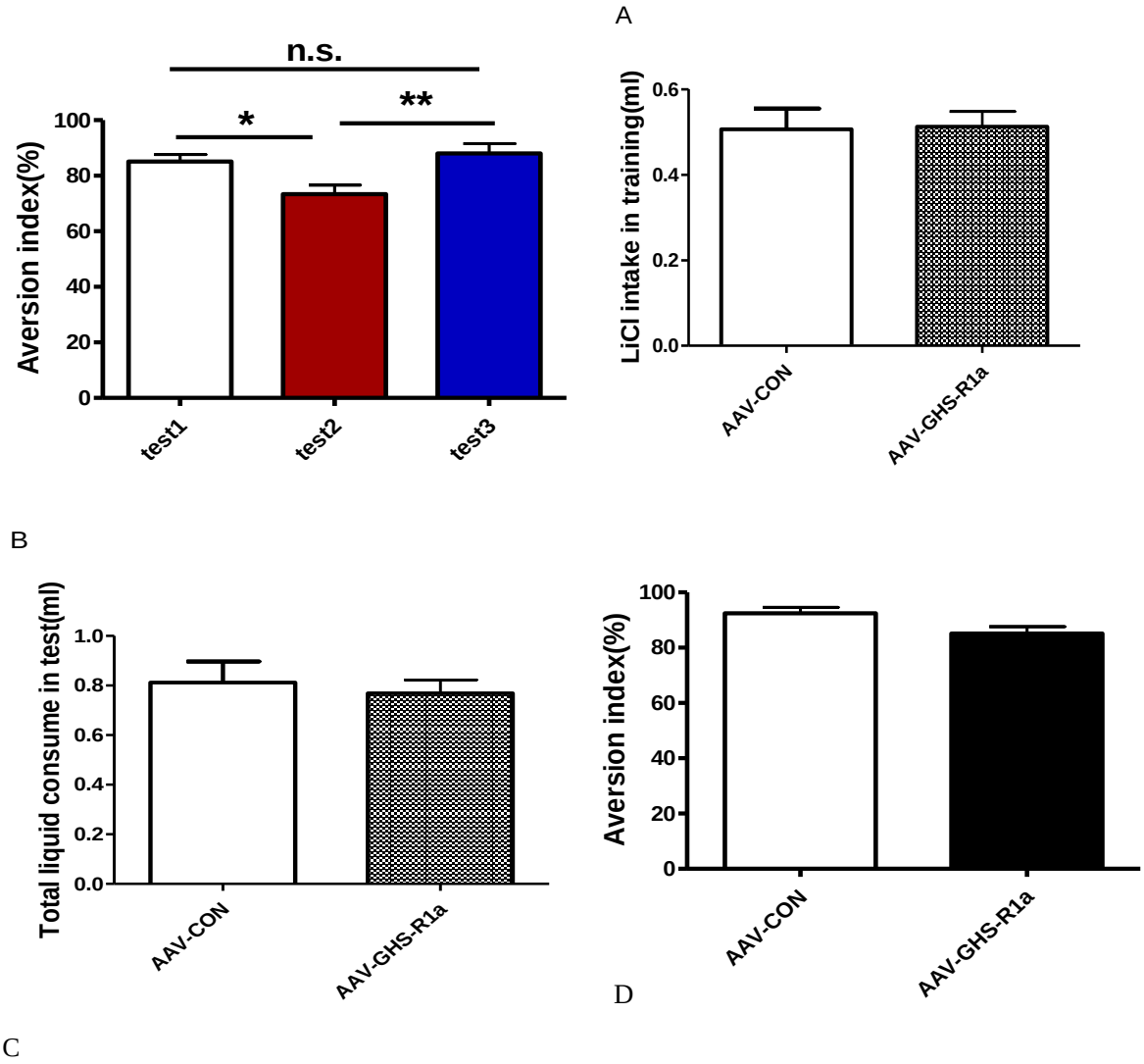

Figure 4. (A) Viral microinjection into the insular cortex of mice had no significant effect on LiCl intake during CTA training. (B) AAV microinjection into the insular cortex of mice had no significant effect on the total liquid intake during CTA training. (C) Microinjection of AAV viruses into the insular cortex blocked the acquisition of CTA memory in mice (test 1, n = 10, *$p<0.05$). (D) Memory formation in mice was inhibited after injection of CNO as evidenced by decreased aversion indices (test 2). After 24 h, when CNO was fully metabolized, memory acquisition recovered (n = 10, **$p<0.01$, *$p<0.05$).

Figure 5. Microinjection of AAV into the amygdala blocked the acquisition of CTA memory in mice; concurrent injection of CNO inhibited the formation of memory.

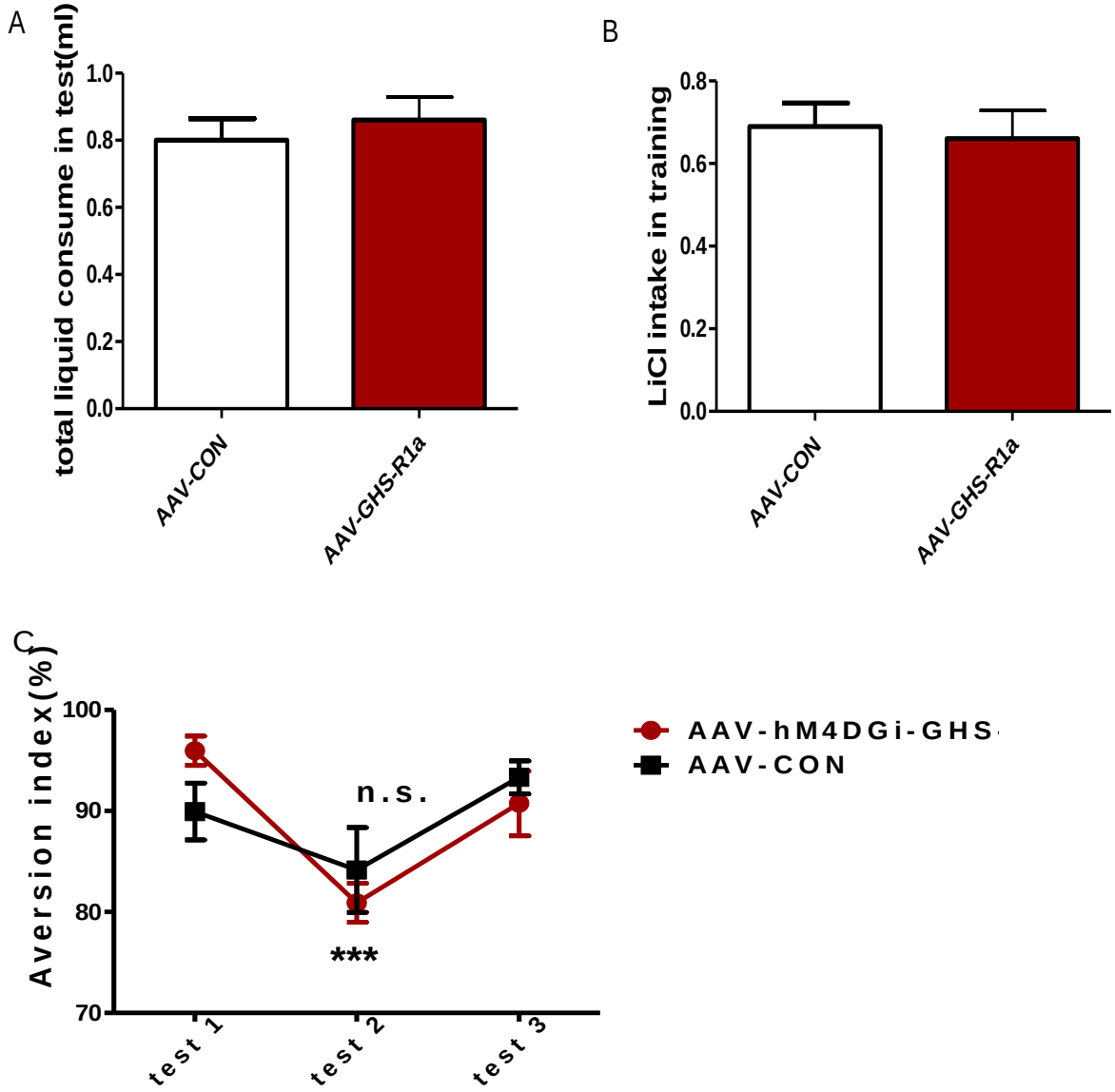

Figure 5. (A) Microinjection of AAV-hM4DGi-GHS-R1a into the insular cortex did not significantly affect the total fluid intake in mice during CTA training. (B) Microinjection of AAV had no significant effect on total LiCl intake during CTA training in mice. (C) Microinjection of AAV-hM4DGi-GHS-R1a blocked the acquisition of CTA memory in mice. After injection of CNO, memory formation was inhibited and AI decreased (test 2). After 24 h, when CNO was fully metabolized, neuronal activity and memory acquisition recovered (n = 10, **p＜0.01, *p＜ 0.05) as evidenced by increased AI.

4. Discussion

In the present study, we demonstrated that microinjection of ghrelin into the amygdala inhibited the acquisition of CTA memory in mice. This inhibition was achieved through the activation of GHS-R1a. GHS-R1a overexpression also inhibited CTA memory acquisition. Microinjection of AAV-CaMKII-hM4Di-2A-GHS-R1a-GFP into the IC further increased expression levels of GHS-R1a, resulting in altered CTA memory formation. Moreover, AAV injection led to hM4Di expression. Interestingly, intraperitoneal injection of CNO prior

to CTA training specifically activated hM4DGi, resulting in hyperpolarized inactivated neurons. This reversibly led to the silencing of the GHS-R1a expression in neurons, altering CTA memory acquisition and inhibiting CTA memory formation.

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