Original Article

Acylated Ghrelin Administration Inhibits Sleeve Gastrectomy-Induced Hippocampal Oxidative Stress, Apoptosis and Tau-Hyperphosphorylation by Activating the PI3K/Akt Pathway

(ghrelin / gastrectomy / Tau / hippocampus / Alzheimer's disease)

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Abstract. This study investigated the impact of exogenous replacement therapy with acylated ghrelin (AG) post sleeve gastrectomy (SG) on the memory function in rats. In addition, we investigated the possible underlying mechanisms, including the effects on markers of oxidative stress, tau phosphorylation, and apoptosis. Adult male Wistar rats were divided into four groups (N = 18/group) as follows: sham (control), SG, SG+AG (100 µM), and SG+AG+LY294002 (0.25 µg/100 g). We continued all treatments daily for four weeks post-surgery. SG impaired the spatial, retention, and recognition memories as tested by the Morris water maze test, passive avoidance test, and novel object recognition test, respectively. Also, it enhanced the levels of reactive oxygen species and lipid peroxides, reduced glutathione and protein levels of Bcl-2, and increased the levels of Bax and cleaved caspase-3 in the hippocampus. In addition, SG reduced the hippocampal levels of acetylcholine and brain-derived neurotrophic factor. Concomitantly, it inhibited the hippocampal activity of Akt and increased the activity of glycogen synthase kinase 3β and tau protein phosphorylation. Exogenous administration of acylated ghrelin to rats that had undergone SG prevented memory deficits. Also, it prevented the alteration in the above-mentioned biochemical parameters, an effect that was abolished by co-administration of LY294002 (phosphoinositide 3-kinase inhibitor). In conclusion, AG replacement therapy after SG in rats protects them against memory deficits and hippocampal damage by suppressing tau protein phosphorylation, mediated by activating PI3K/Akt-induced inhibition of glycogen synthase kinase 3β.

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Abbreviations: Aβ – amyloid β, Ach – acetylcholine, AchE – acetylcholine esterase, AD – Alzheimer's disease, AG – acylated ghrelin, Akt – protein kinase B, BDNF – brain-derived neurotrophic factor, ChAT – choline acetyltransferase, DAG – des-acyl ghrelin, GH – growth hormone, GOAT – ghrelin-O-acyltransferase enzyme, GHS-R1a – secretagogue receptor type 1a, GSH – reduced glutathione, GSK3β – glycogen synthase kinase 3β, GSSG – glutathione disulphide, LY294002 – a phosphoinositide 3-kinase/Akt inhibitor, MDA – malondialdehyde, NFT – neurofibrillary tangles, NORT – novel object recognition test, PALT – passive avoidance learning test, PI3K – phosphoinositide 3-kinase, PP2A – phosphatase 2A, ROS – reactive oxygen species, SG – sleeve gastrectomy, MWM test –Morris water maze test.

Introduction

Alzheimer's disease (AD) is a common multi-factorial, progressive neurodegenerative disease that predominantly occurs in older individuals (Selkoe, 2002). It is characterized by severe cognitive deficits and a decrease in intellectual function (Weller and Budson, 2018). Extracellular accumulation of amyloid β (Aβ) in senile plaques and intracellular accumulation of neurofibrillary tangles (NFT) due to hyperphosphorylation of tau protein are the major pathological mechanisms. They lead to neural cell apoptosis and memory deficits during the progression of AD (Weller and Budson, 2018). This has been attributed to several mechanisms, including activation of microglial cells, stimulation of the synthesis and release of pro-inflammatory cytokines, and microtubule instability (Landrieu et al., 2011; Swomley et al., 2014; Weller and Budson, 2018).

Tau protein phosphorylation is a tightly regulated process in the brain of mammals. It requires a balance

between the activities of two enzymes, namely, phosphatase 2A (PP2A) and glycogen synthase kinase 3β (GSK3β), which dephosphorylates or phosphorylates the tau protein, respectively (Rankin et al., 2007; Landrieu et al., 2011; Zhao et al., 2011; Xiao et al., 2013; Iqbal et al., 2014; Ponce-Lopez et al., 2017). Therefore, an increase in the activity of GSK3β and/or inhibition of PP2A induces tau hyperphosphorylation and accumulation of NFT (Rankin et al., 2007; Landrieu et al., 2011; Zhao et al., 2011; Xiao et al., 2013; Iqbal et al., 2014; Ponce-Lopez et al., 2017). The major sites of GSK3-mediated phosphorylation of tau protein include Ser396, Ser199, Thr205, and Thr231. However, activation of protein kinase B (Akt) directly inhibits GSK3β by inducing phosphorylation at Ser9 (Zhao et al., 2011; Xiao et al., 2013; Iqbal et al., 2014).

Several studies have reported a cross-talk between the gastrointestinal tract hormones and cognitive function in several neurodegenerative diseases (Stoyanova, 2014; Seminara et al., 2018). Ghrelin, a peptide hormone primarily synthesised by gastric oxyntic cells, induces hunger and increases food intake (Ariyasu et al., 2001). It circulates in the blood in two forms, namely, unacylated ghrelin (des-acyl ghrelin or DAG, 90 %) and acylated ghrelin (AG, 10 %) (Stoyanova, 2014). While AG activates the growth hormone (GH) secretagogue receptor type 1a (GHS-R1a), DAG acts through undefined receptors (Stoyanova, 2014). The neuroprotective and memory protective effects of AG are explained by its ability to cross the blood-brain barrier (Howick et al., 2017). Furthermore, GHS-R1a receptors are widely distributed in different areas of the brain, including the hippocampus, sensory-motor cortex, and cingulated gyrus (Kojima et al., 1999; Cowley et al., 2003; Yagi et al., 2013; Stoyanova, 2014; Howick et al., 2017).

Several studies have shed light on the stimulatory effect of endogenous AG or administration of exogenous AG on memory and synaptic plasticity in rodents and humans (Stoyanova, 2014). Mean plasma AG concentrations are significantly reduced in older, normal-weight individuals compared to their younger counterparts (Rigamonti et al., 2002). Besides, the fasting levels of serum ghrelin are positively correlated with verbal learning and memory function (Bellar et al., 2013). Moreover, the circulating levels of ghrelin-O-acyltransferase (GOAT), the enzyme that converts DAG to AG, and GHS-R1a are reduced in patients with AD (Gahete et al., 2010, 2013). Furthermore, GHS-R1a knockout (KO) mice exhibit decreased spine synapses and neuroinflammation in their hippocampus, concomitant with impairment in their activity performance and behavioural memory (Diano et al., 2006; Santos et al., 2017). Similar findings were observed after deletion of the gene responsible for ghrelin expression in mice. However, the intra-cerebroventricular administration of AG reverses these developed drawbacks (Santos et al., 2017). In addition, MK-0677, an AG agonist, improves learning memory, reduces Aβ deposition, and prevents neuronal apoptosis and synaptic loss in the deep cortical layers and dentate gyrus of the hippocampus (Moon et al., 2014; Kang et al., 2015; Jeong et al., 2018).

Several studies have shed light on the inhibition of mitochondria-mediated apoptosis by AG. The latter inhibits ROS and increases B-cell lymphoma 2 (Bcl-2)/Bax (a member of the Bcl-2 gene family regulator) in hippocampal cells *in vitro* following exposure to Aβ oligomers or oxygen/glucose deprivation (Chung et al., 2007; Gomes et al., 2014). Interestingly, centrally-administered AG prevents the loss of memory and reduces tau phosphorylation in Aβ-induced AD (Kang et al., 2015). Despite these findings, there is limited literature on the association between the reduced AG level and tau phosphorylation during ageing. This necessitates further studies to investigate the role of AG and tau phosphorylation in the memory function to expand the scientific understanding of AD pathology and therapy.

Bariatric surgery is a highly recommended procedure for weight loss in obese individuals with a body mass index greater than 35 (Bužga et al., 2014). Sleeve gastrectomy (SG) is the predominant restrictive form of these bariatric surgeries. It involves the removal of a large part of the stomach with a minor curvature to produce a sleeve-like structure (Algahtani et al., 2016). Simultaneously, bariatric surgery was reported to associate with multiple neurological complications, in some cases including encephalopathy, behavioural abnormalities, seizures, cranial nerve palsies, ataxia, myelopathy, plexopathies, peripheral neuropathy, carpal tunnel syndrome, and neuralgia paresthetica (Algahtani et al., 2016; Çalapkorur and Köksal, 2017). However, the effect of SG from the perspective of AG deficiency in learning, memory, and tau phosphorylation has not yet been investigated.

In this unique study of a rat model of SG, we investigated the effects of lowering the basal AG level by SG on the spatial and retention memory of rats. Secondly, we explored the mechamism/s involved in the hippocampal ROS production, mitochondria-mediated cell apoptosis, and tau phosphorylation. Thirdly, we investigated whether the AG replacement therapy (at its physiological dose) could improve the intellectual deterioration and feeding behaviour.

Material and Methods

Animals

We procured healthy, age-matched, adult Sprague-Dawley male rats (200 ± 10 g, 15 weeks old) from the animal facility of the College of Medicine, King Khalid University, Abha, Kingdom of Saudi Arabia (KSA). We maintained all rats in a separate room at a controlled temperature of 23 ± 1°C, 60–62% humidity, and 12-h light/dark cycle. During the first experimental period, all rats were fed a purified diet (AIN-76A) (Dyets Inc., Bethlehem, PA). They had free access to drinking water. All procedures were approved by the animal's ethical committee at King Khalid University (KKU), which fol-

lows the guidelines established by the US National Institutes of Health (NIH publication No. 85-23, revised 1996).

Experimental design

We divided the rats into four groups (N = 18/each). First, the sham-operated group (control) underwent the same surgical procedure used to induce SG in the model group, without stapling and suturing the stomach. It received 0.2 ml of subcutaneous (s.c.) normal saline as a vehicle. Second, the SG model group underwent the SG protocol (as shown below) and received a daily dose of 0.2 ml of normal saline (s.c.). Third, the SG+AG-treated group underwent SG and received 100 µg/kg of body weight of ghrelin ($C_{147}H_{245}N_{45}O_{42}$, Cat. No. Ab 120231, Abcam, Cambridge, UK) at a final volume of 0.2 ml (s.c.). Fourth, the SG+AG+LY294002 – a phosphoinositide 3-kinase/Akt (PI3K/Akt) inhibitor-treated group – underwent the same procedure as group 3. Nonetheless, it received a concomitant i.p. injection of 50 µl LY294002 (0.25 µg/100 g) (Cat. No. 19-142, Sigma-Aldrich, Gillingham, UK)**.** The dose of LY294002 was adopted from the study by Shati and Alfaifi (2019). They reported on the sufficiency of this dose of LY294002 to completely inhibit the hippocampal activity of PI3K/Akt in rats. The selected dose of exogenous AG produces a physiological increase in circulatory AG in the control group when administered for four weeks. Moreover, it restores the levels of AG in SG-induced rats. We started all treatments immediately after surgery and continued them daily for four weeks. We recorded the daily food intake and alterations in the body weights of the rats (Morsy, 2020).

Anaesthesia and induction of SG

Anaesthesia, sham surgery, and SG were performed according to previous studies (Valentí et al., 2011; Morsy, 2020). Following fasting for 14 h, we anaesthetised all rats by isoflurane inhalation (4% in oxygen) using a vaporiser (Medical Supplies and Services International Ltd., Keighley, UK) at a flow rate of 0.5–0.7 l/min. We placed each rat on an operation table supplied with a nose cone. Isoflurane was delivered at a flow of 2.5 %. We injected each rat with a dose of 25 mg/kg enrofloxacin (i.m.) and conducted an upper median laparotomy (4 cm length) under sterile conditions. The stomach was extracted from the abdomen and placed on a saline-moistened gauze pad, after releasing all connective tissues between the greater curvature, spleen, and liver. We divided the great omentum to the level of the pylorus after ligation. We used the Auto-Suture TA automatic stapler supplied with TA30V3L (loading unit (three rows; 2.5 mm; DST Series, Tyco Healthcare Group LP, Norwalk, CT) for SG. It was positioned between 4 mm and 5 mm from the pylorus, followed by a reinforcement with a 6-0 hand-sewn polypropylene suture. This procedure leaves a tubular (sleeve) stomach and reduces the stomach volume by approximately 80 %, comprising most of the fore-stomach and glandular stomach. The control group underwent a similar surgical procedure, with the stomach remaining intact. We flushed the peritoneal cavity with normal saline and closed the incision with running 3/0 polyglycolic acid sutures. All animals were then administered 5 ml of normal saline (s.c.) to prevent dehydration. We administered 0.03 mg/kg buprenorphine as an analgesic measure (twice/day) for the next three days. The rats were fed a liquid diet consisting of 5% glucose during the first three days post-surgery, following which they were switched to their normal solid diet. All rats were administered 50 mg/kg ceftriaxone (i.m.) 30 min before the surgery and for the first three days post-surgery. We replaced the rats that died from the surgical procedure. All dead rats underwent necropsy.

NORT test

We analysed, by the novel object recognition test (NORT), the short- and long-term recognition memory functions by the end of the 28th and 29th days according to previously described procedures (Bevins and Besheer, 2006; Shati and Alfaifi, 2019). The test measures the spontaneous tendency of the rat to explore new objects at different time intervals. This task involved a black wooden apparatus (dimensions 40 × 50 × 50 cm), impermeable to light. It was comprised of two cuboidal (A1, A2), one cylindrical (B), and one triangle (C) glass blocks. Each rat was first exposed to a habituation training session (4 times) to explore the empty apparatus (10 min/each). After 12 h, the rat was again acclimated to the test room for 30 min before the training sessions. This facilitated exploration of two identical objects (A1 and A2). The rat was then returned to its cage and tested 3 h later (short-term recognition memory function). However, the examiner conducted the test in the presence of objects A1 and B instead of A2. The test was repeated after 21 h with objects A1 and C (long-term memory function), with an exploration time of 5 minutes for each trial. The room was always cleaned with alcohol between sessions and the total time to explore both objects was recorded. The examiner defined the discrimination ratio as the time spent exploring the familiar or novel object over the total time needed to explore both of them. A higher discrimination ratio indicates improved cognitive performance. Effective exploration was recognised only as directing or touching the nose with the object at a distance < 2 cm**.** Rearing up onto, turning around, and sitting on the object were not considered exploratory behaviours and were excluded. The examiner was blinded to the experimental groups.

PALT for retention memory

We tested the retention (reference) memory using the passive avoidance learning test (PALT) on day 30, as previously described by Barkur and Bairy (2015). This test measures the ability of each rat to remember a previously exposed electrical foot shock that had been delivered 2 h earlier (retention test). The examiner placed each rat in a passive avoidance apparatus that was com-

prised of a wooden box (50 × 50 × 35 cm), with a larger and brightly illuminated room and a smaller (15 × 15 × 15 cm) dark compartment. The dark compartment was supported with a grid floor, connected to the source of the electrical shock. A door separated the rooms. The test was divided into two stages as follows: (1) exploration and learning, and (2) the retention test. The door was kept open during the exploration phase. The examiner placed the rat in the illuminated large room and allowed it to explore the room freely for three trials. Each trial lasted 5 minutes, conducted at an interval of 30 minutes. At the end of the 3rd trial, the examiner closed the door when the rat had stepped into the dark compartment. Furthermore, a single foot shock (50 Hz, 1.5 mA, for 1 s) was delivered through the grid floor and the rat was kept in the dark for an additional 10 seconds. The rat was returned to its cage soon after and the test was repeated the next day (day 31). We recorded the time latency needed for the rats to step into the dark area. Normal rats usually avoid entering this dark area.

MWM test for spatial memory

We used the Morris water maze (MWM) test to measure the spatial memory on four consecutive days (days 32–35), as previously described by Morris (1984). The maze (a circular swimming pool, 180 cm in diameter) was filled with water (60 cm deep, 22 ± 2 °C). A circular platform (diameter of 10 cm) was submerged 2 cm below the surface of milky water in the middle of the SW quadrant (target quadrant). All animals were tested to find this hidden platform. Each rat was placed at a starting position (N, E, NE) and released to find the hidden platform. This procedure was repeated for four days with three trials/day (90 s/trial separated by an interval of 5 min), each conducted from a different starting position (N, E, or SE). The selected starting points created an equidistant path and reduced variation in the results. A rat unable to find the platform was directed by the investigator and left on the platform for 15 s. The examiner recorded the escape time, identified by the time spent by each rat to find the hidden platform. Furthermore, an extra probe trial was conducted for all rats an hour later, where the examiner removed the rescue platform and recorded the total number of times crossed by the rat over the site of the hidden platform.

Hippocampi collection

Directly after the MWM test, each rat was anaesthetised with 60–70 mg/kg sodium pentobarbital (i.p.) and killed by cervical dislocation. The brain was rapidly extracted on ice. An expert pathologist rapidly excised the hippocampi of the first six rats under a stereomicroscope. It was snap-frozen in liquid nitrogen and stored directly at −80°C for further use. The frozen hippocampi were homogenized in separate tubes in 200 µl of ice-cold phosphate-buffered saline plus a protease inhibitor mixture (Sigma-Aldrich) (PBS, pH 7.4) to prepare total cell homogenates. In addition, the supernatants were isolated and stored at −80°C and used later for biochemical analysis. The hippocampi of the other 12 rats were snap-frozen, grounded, stored in −80 °C, and used for Western blotting.

Biochemical analysis in the brain homogenates

The hippocampal levels of malondialdehyde (MDA) and reduced/oxidized glutathione (GSH and GSSG) were measured using a colorimetric assay kit (Cat. No. ab118970, Abcam, and Cat. No. 786-075, GBioscience, St Louis, MO). We measured the total levels of ROS and nitrogen reactive species using the OxiSelect™ *in vitro* ROS/RNS Assay Kit (Cat. No. STA-347, Cell Biolabs, Inc. San Diego, CA) and expressed them as a percentage of the control. We measured the hippocampal levels of acetylcholine esterase (AchE) (mU/mg protein), choline acetyltransferase (ChAT), and acetylcholine (Ach) using assay kits (Cat. No. ab138871, Abcam; Cat. No. SCB929Ra Cloud-Clone Corp. Houston, TX, and Cat. No. STA-603, Cell Biolabs, Inc., San Diego, CA). All procedures were performed in duplicate and in accordance with the manufacturer's instructions.

Western blotting

We extracted proteins from all hippocampus specimens with 200 µl RIPA buffer containing 150 mM sodium chloride, 1.0% NP-40 or Triton X-100, 0.5% sodium deoxycholate, 0.1% SDS, and 50 mM Tris-HCl (pH 8.0) plus protease inhibitor (Cat. No. P8340, Sigma-Aldrich). We determined the protein concentrations in each sample using a Bradford assay kit (Cat. No 23300, Thermo-Fisher Scientific, Waltham, MA) and subjected them to electrophoresis in SDS-polyacrylamide gel (40 µg protein/lane). The proteins were electro-transferred onto nitrocellulose membranes and immune-blotted with primary antibodies against PI3K, p-PI3K (Tyr607), total tau, p-tau (Ser199), p-tau (Ser396), Akt, p-Akt (Ser473) (Santa Cruz Biotechnology, Dallas, TX), GSK3β, p-GSK3β (Ser9), p-GSK3β (Tyr216), cleaved caspase-3, Bcl-2, Bax, and β-actin (Cell Signalling Technology, Danvers, MA), and p-GSK3β (Tyr216) (Abcam). While HRP-conjugated secondary antibodies facilitated band detection, Pierce ECL chemi-luminescence substrate (Cat. No. 32106, Pierce Biotechnology, Thermo-Fisher, Rockford, IL) enhanced them. We stripped one membrane up to four times and always detected the phosphorylated proteins first. The band intensities were photographed and quantified using a C-Digit Blot Scanner (LI-COR, Lincoln, NE), supplied with the Image Studio Digits software.

Statistical analysis

All data are presented as mean ± SD. We performed all analyses using one-way ANOVA, followed by Tukey's test using the GraphPad Prism statistical software package (version 6, Australia). A P value < 0.05 was considered statistically significant.

Results

Death rate

Two rats from the SG group and one rat from the SG+AG-treated group died. These rats were replaced with other rats and were treated like the rest of the rats in each group. The dead rats underwent necropsy. Histopathological examination revealed pancreatitis as the reason behind their death. It was predominantly caused by the compression of the pancreas and its duct. No deaths occurred in any other groups.

Exogenous AG prevents weight loss and stimulates food uptake in SG-induced rats in a PI3K-dependent manner

Compared to the sham-operated rats, the daily weight gain and food intake significantly decreased in the SG model rats, starting from day 4 post-surgery until the last day of the experiment (Fig. 1A, B). In contrast, the SG+AG-treated rats showed a significant increase in their daily body weight and food intake compared to the SG rats (Fig. 1A, B). Interestingly, co-treatment of the SG+AG-treated rats with LY294002 completely prevented the stimulatory effect of AG on the weight gain and food intake. There was no significant variation in the changes in their daily food intake and body weight between the SG and SG+AG+LY294002-treated rats (Fig. 1A, B).

Exogenous AG reverses SG-induced impairment in the memory and learning function in a PI3K-dependent manner

Studying the MWM indicated that rats with SG took a longer pathway and time to find the hidden platform during the 4-day trial phases. In addition, compared to the control group, they showed a reduced number of crossings above the site of the hidden platform during the probe phase, thus indicating impaired spatial memory (Fig. 2A, C). They also showed significantly lower latencies to enter the dark area during PALT, compared to their sham-operated counterparts, thus indicating impaired reference memory (Fig. 2D). Moreover, the SG model rats showed a decrease in the total time required to explore identical objects (A1, A2) (Fig. 3A). Compared to the control group, they showed a decrease in the total and individual time to explore two different objects, namely A and B (Fig. 3B, C) and A and C (Fig. 4A, B) 3 or 21 h following the training session, thus indicating an impairment in short-term and long-term memory function, respectively. In addition, there was a decreased discrimination ratio of both objects B and C (Figs. 3D, 4C). Nonetheless, these events were completely reversed in the SG+AG-treated rats compared to the SG rats. Furthermore, they were not significantly different from those of the control group (Figs. 2A, D, 3A, D, and 4A, C). Interestingly, the findings were not considerably different between the SG model rats and the SG+AG+LY294002 rats (Figs. 2A, D, 3A, D, and 4A, C).

Exogenous AG inhibits SG-induced oxidative stress in the hippocampi of SG rats in a PI3K-dependent manner

The rats exposed to SG had significantly lower plasma levels of both AG and DAG, compared to the control group (Fig. 5A). However, the plasma levels of AG and DAG in SG model rats that underwent daily exogenous administration of 100 µg/kg AG for four weeks were significantly increased when compared to their levels in the SG model rats. The AG and DAG levels in the SG+AG-treated rats were not significantly different from

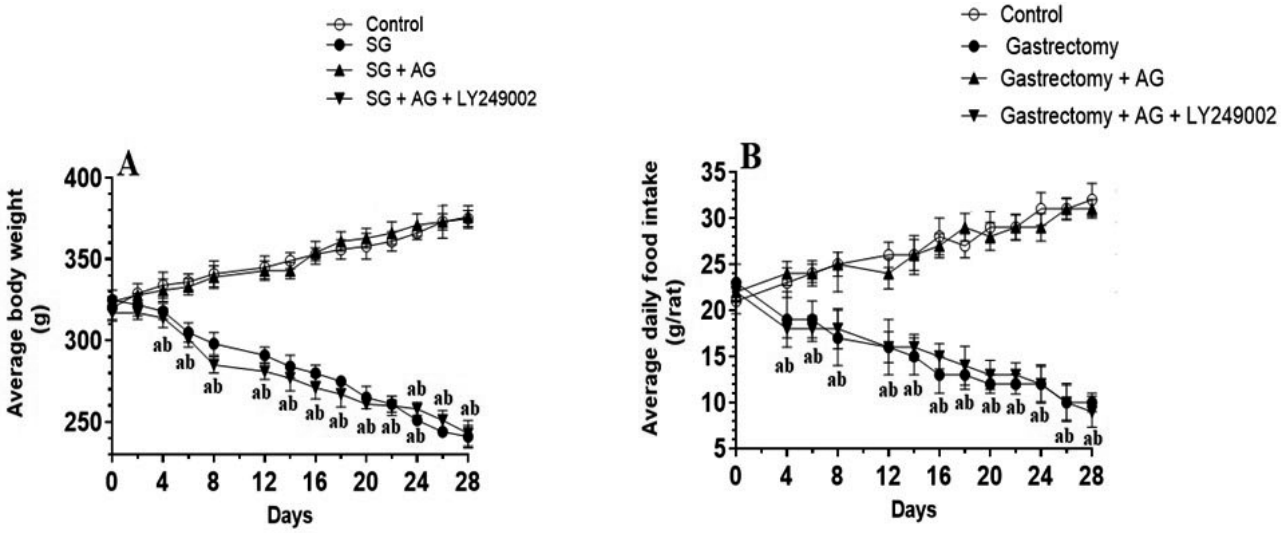

Fig. 1. Changes in body weight and food intake among all the experimental groups of rats. Data are presented as mean ± SD of N = 18 rats/group. [a]: significantly different as compared to the control sham-operated group. [b]: significantly different as compared to the sleeve gastrectomy (SG)-induced group.

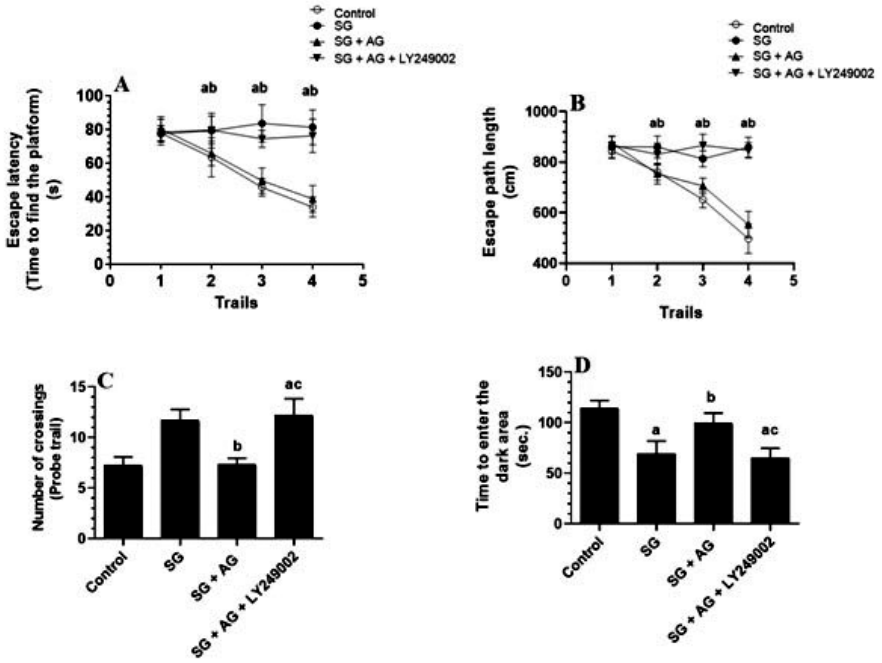

Fig. 2. The time to find the hidden platform (escape latency, **A**), escape path length (**B**), and the number of crossings over the hidden platform (**C**) during the Morris water maze test and the time latency needed for the rats to step into the dark area during the passive avoidance test (**D**). Data are presented as mean ± SD of N = 18 rats/group. In **A** and **B**: [a]: significantly different as compared to the control sham-operated group. [b]: significantly different as compared to the sleeve gastrectomy (SG)-induced group. In **C** and **D**: [a]: significantly different as compared to the control group. [b]: significantly different as compared to the SG-induced group. [c]: significantly different as compared to the SG+AG-treated group.

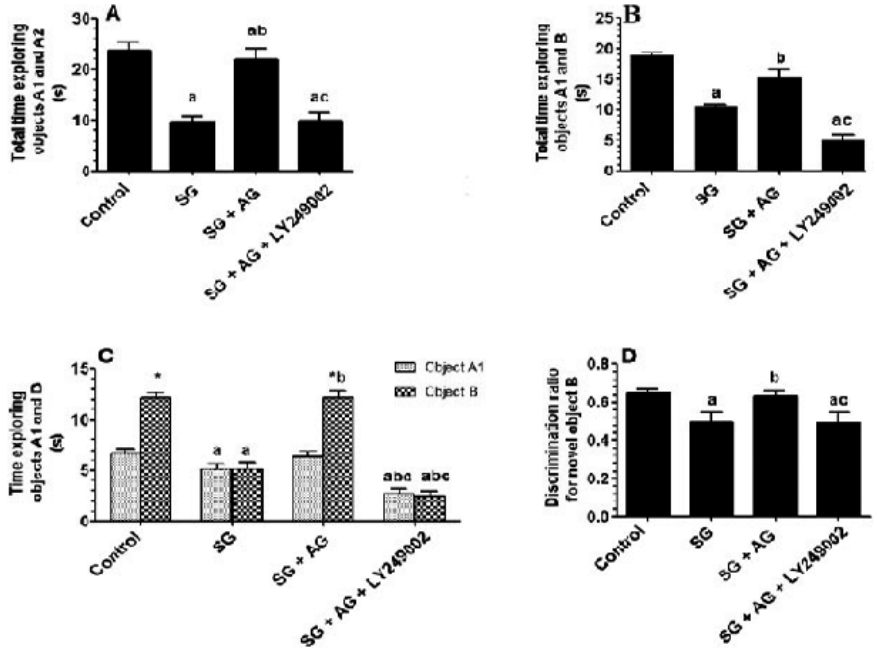

Fig. 3. Time intervals during the NORT for testing of basal and short-term memory (3 h post-training) in all groups of rats. **A**: total time needed to explore two identical objects (A1 and A2). **B**, **C**: total and individual times to explore objects A1 and B, respectively. **D**: The calculated discrimination ratio of object B (the time spent to explore object B divided by the total time to explore objects A1 and B). Data are presented as mean ± SD of N = 18 rats/group. [a]: significantly different as compared to the control group. [b]: significantly different as compared to the sleeve gastrectomy (SG)-induced group. [c]: significantly different as compared to the SG+AG-treated group. *: significantly different when compared to object A within the same group.

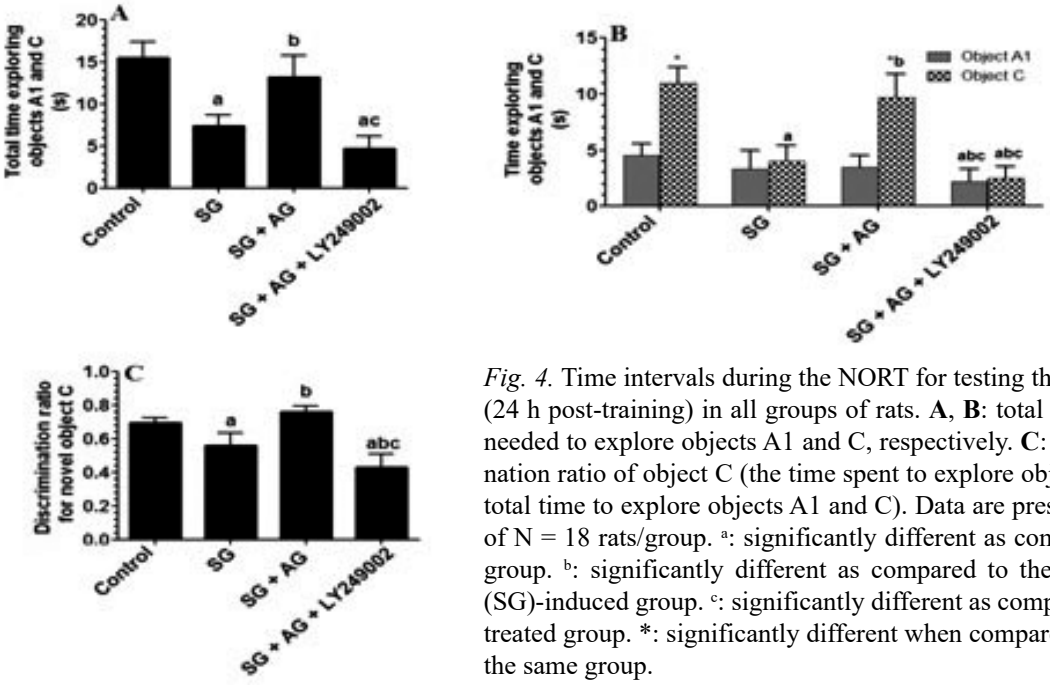

Fig. 4. Time intervals during the NORT for testing the long-term memory (24 h post-training) in all groups of rats. **A**, **B**: total and individual times needed to explore objects A1 and C, respectively. **C**: Calculated discrimination ratio of object C (the time spent to explore object C divided by the total time to explore objects A1 and C). Data are presented as mean ± SD of N = 18 rats/group. [a]: significantly different as compared to the control group. [b]: significantly different as compared to the sleeve gastrectomy (SG)-induced group. [c]: significantly different as compared to the SG+AG-treated group. *: significantly different when compared to object A within the same group.

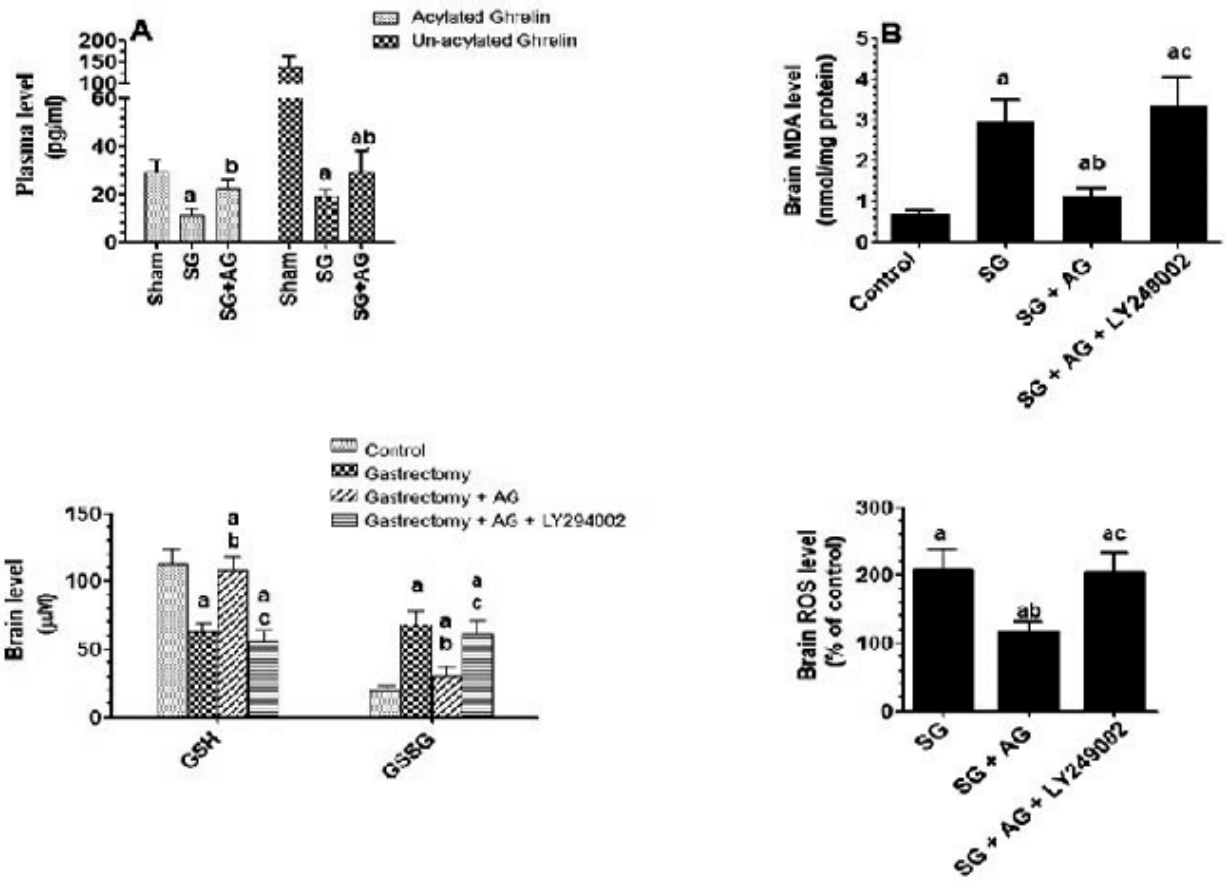

Fig. 5. Levels of acylated ghrelin (AG) and de-acylated ghrelin (DAG) (**A**) in the plasma and levels of malondialdehyde (MDA) (**B**), reduced glutathione (GSH) and oxidized glutathione (GSSG) (**C**), and reactive oxygen species (ROS) (**D**) in the hippocampi of all groups of rats. Data are presented as mean ± SD of N = 6 rats/group. [a]: significantly different as compared to the control group. [b]: significantly different as compared to the sleeve gastrectomy (SG)-induced group. [c]: significantly different as compared to the SG+AG-treated group.

their basal levels in the control group (Fig. 5A). This was concomitant with significantly higher levels of MDA, ROS and SGGS, and significantly lower levels of GSH, in the hippocampi of the SG model rats (Fig. 5B, C). While the levels of ROS, MDA and SGGS significantly decreased, those of GSH considerably increased in the SG+AG rats compared to those in the SG model rats (Fig. 5B, C). However, their levels were not significantly different between the SG model rats and the SG+AG+LY294002-treated rats (Fig. 5B, C).

Exogenous AG inhibits SG-induced intrinsic cell apoptosis in the hippocampi of rats in a PI3K-dependent manner

The protein levels of Bax and cleaved caspase-3 increased significantly, while those of Bcl-2 decreased considerably in the hippocampi of the SG model rats compared to the protein levels in the hippocampi of the control group (Fig. 6A, C). The protein levels of Bax and cleaved caspase-3 were significantly decreased, while those of Bcl-2 were significantly increased in the hippocampi of the SG+AG-treated rats compared to those in the hippocampi of the SG model rats (Fig. 6A, C). In contrast, there was no difference in the protein levels of these markers between the SG model rats and SG+AG+LY294002-treated rats (Fig. 6A, C).

Exogenous AG prevents the SG-induced decrease in the levels of Ach, activity of ChAT, and activation of AchE in the hippocampi of rats in a PI3K-dependent manner

Despite the substantial decrease in the levels of Ach and activity of ChAT, the activity of AchE significantly increased in the hippocampi of the SG model rats compared to that in the hippocampi of the control group (Fig. 7A, C). In contrast, the levels of cholinergic markers were normal in the hippocampi of SG+AG-treated rats compared to their levels in the hippocampi of the control group (Fig. 7A, C). However, the co-administration of LY294002 to the SG+AG-treated rats completely abolished the beneficial effects of AG on the hippocampal levels of the aforementioned cholinergic parameters.

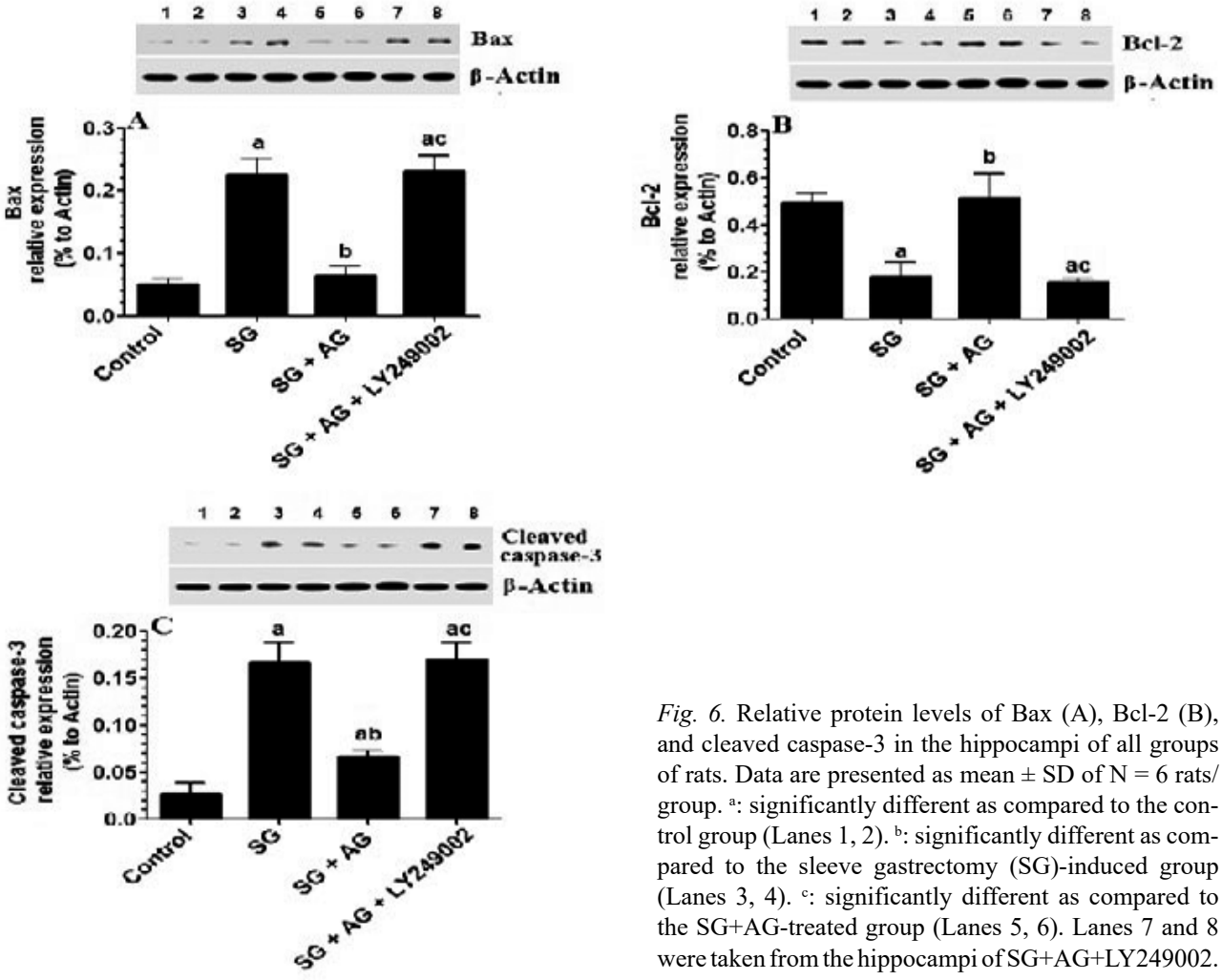

Fig. 6. Relative protein levels of Bax (A), Bcl-2 (B), and cleaved caspase-3 in the hippocampi of all groups of rats. Data are presented as mean ± SD of N = 6 rats/group. [a]: significantly different as compared to the control group (Lanes 1, 2). [b]: significantly different as compared to the sleeve gastrectomy (SG)-induced group (Lanes 3, 4). [c]: significantly different as compared to the SG+AG-treated group (Lanes 5, 6). Lanes 7 and 8 were taken from the hippocampi of SG+AG+LY249002.

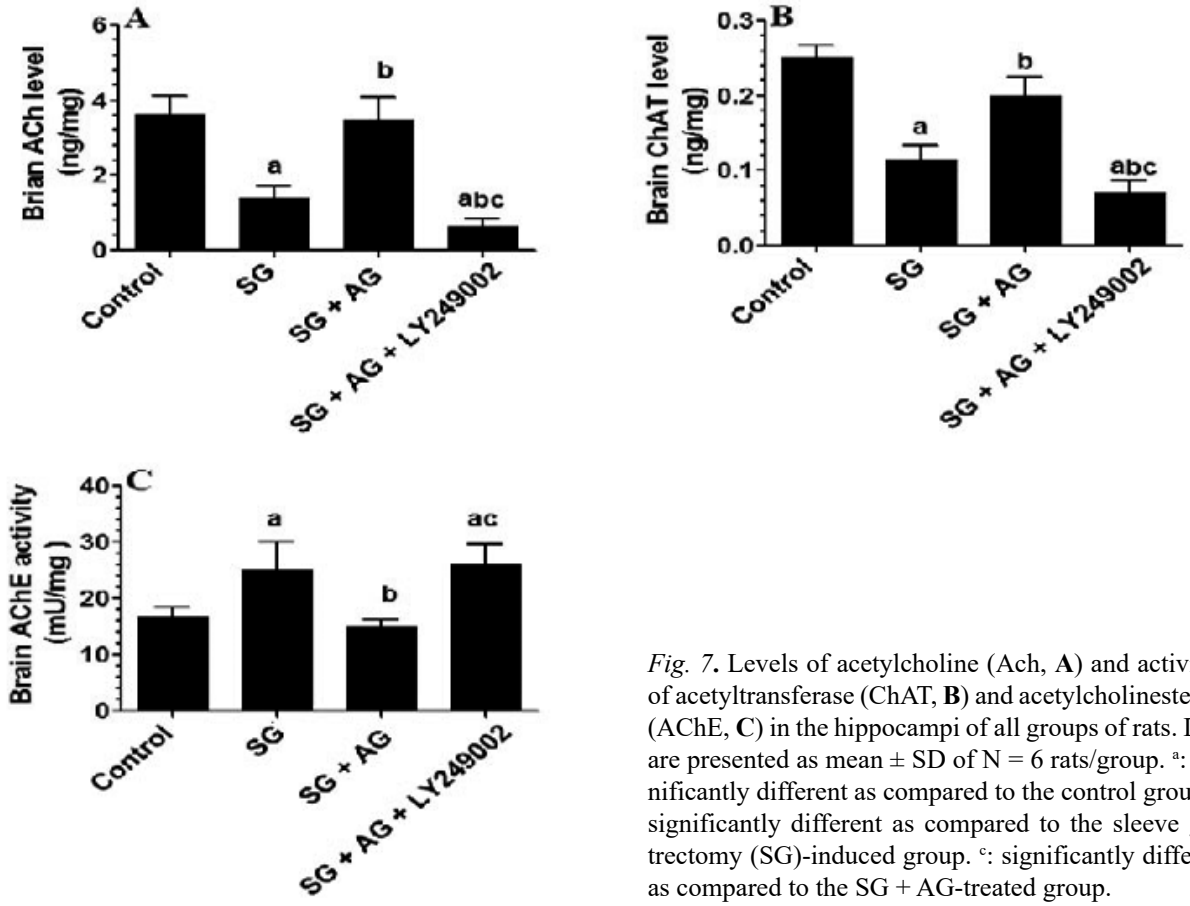

Fig. 7. Levels of acetylcholine (Ach, **A**) and activities of acetyltransferase (ChAT, **B**) and acetylcholinesterase (AChE, **C**) in the hippocampi of all groups of rats. Data are presented as mean ± SD of N = 6 rats/group. [a]: significantly different as compared to the control group. [b]: significantly different as compared to the sleeve gastrectomy (SG)-induced group. [c]: significantly different as compared to the SG + AG-treated group.

Thus, the levels were not significantly different between the SG model rats and the SG+AG+LY294002-treated rats (Fig. 7A, C).

Exogenous AG inhibits SG-induced phosphorylation of tau, reduction in phosphorylation of Akt, and decrease in the levels of BDNF in the hippocampi of rats

While the protein levels of both p-tau isoforms (Ser394 and Ser199) increased significantly, that of brain-derived neurotrophic factor (BDNF) decreased significantly in the hippocampi of the SG model rats compared to those in the hippocampi of the control group (Fig. 8A, D). However, co-administration of AG to the SG model rats significantly decreased the levels of both p-tau (Ser394 and Ser199) and BDNF in the hippocampi compared to those in the SG model rats (Fig. 8A, D). The protein expression levels of the above-mentioned markers were similar between the SG model rats and SG+AG-treated rats (Fig. 8A, D).

Discussion

The present study results revealed the possible link between SG and deterioration of some intellectual functions and AD-like symptoms postoperatively. We attributed these deteriorations to increased hippocampal oxidative stress and apoptosis and also to increased hippocampal tau phosphorylation mediated by suppression of Akt and activation of GSK3β. AG replacement therapy following SG surgery prevented these changes. Several studies have tried to explain the mechanisms of the intellectual deterioration after SG, but none of them was satisfactory.

The hippocampus is a major part of the brain that controls the motivational aspects of feeding, as well as the memory and cognitive function of mammals (Algahtani et al., 2016; Stevenson et al., 2017). The wide distribution of AG receptors in the region of the hippocampus in humans and rodents attracts attention of researchers to investigate the relation between the postoperative SG intellectual deteriorations and the AG deficiency (Howick et al., 2017). Our results showed a significant reduction in the food intake and body weights in rats, concomitant with reduction in their memory and intellectual functions after SG. However, the aforementioned events were prevented by exogenous administration of AG in SG+AG treated rats, which was not only able to stimulate food intake and body weight gain, but also improved the behaviour and memory function in these rats. Many other authors reported the orexigenic role of AG and its importance in regulating behaviour and memory in many animal models. Also, Santos et al. (2017) reported

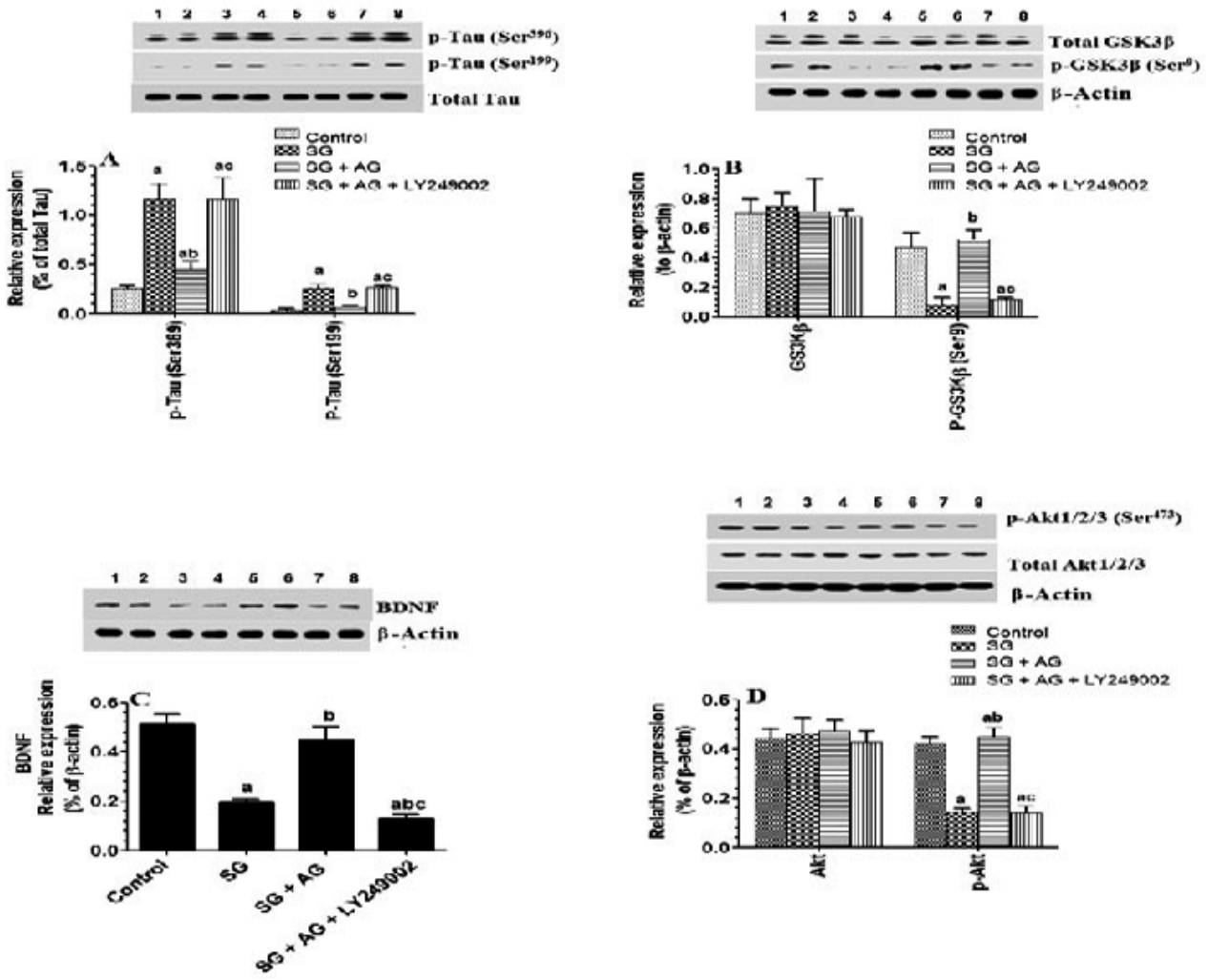

Fig. 8. Protein levels of total tau (A), phospho-tau (Ser^{394} and Ser^{199}) (**A**), total glycogen synthase kinase-3β (GSK3β) and phospho-GSK3β (Ser^{9}) (**B**), BDNF (**C**), and total Akt and phospho-Akt (Ser^{473}) (D) in the hippocampi of all groups of rats. Data are presented as mean ± SD of N = 6 rats/group. [a]: significantly different as compared to the control group (Lanes 1, 2). [b]: significantly different as compared to the sleeve gastrectomy (SG)-induced group (Lanes 3, 4). [c]: significantly different as compared to the SG + AG-treated group (Lanes 5, 6). Lanes 7 and 8 were taken from the hippocampi of SG + AG + LY249002 treated rats.

that the genetic deletion of AG in mice impairs their spatial and recognition memory, as measured by the Y-maze and novel object recognition tests, respectively (Santos et al., 2017). Furthermore, AG enhances the memory function in rats infused with Aβ (Kang et al., 2015), and similar results were also observed in an AD mouse model on a high-glycaemic index diet (Kunath et al., 2015). An intra-cerebroventricular infusion of AG reverses the impairment in object recognition memory in food-restricted mice (Carlini et al., 2008). In contrast, an intra-amygdaloid AG injection enhances the memory, as tested by PALT and MWM under aversive conditions (Tóth et al., 2009). While intra-hippocampal administration of AG improves long-term memory, it does not affect the short-term recognition memory (Carlini et al., 2010). The exact area of the hippocampus that is affected by AG cannot be determined from the published studies and from the present study as well.

The exact mechanisms mediating the protective effect of AG on memory remain largely unknown. Multiple hypotheses have been generated in this regard. While AG receptor knockout (GHSR1-KO) mice show reduced serotonin (5-HT) activity in their brain, acute intra-amygdaloid AG administration increases activity of the projecting serotoninergic neurons (Patterson et al., 2010; Hansson et al., 2014). In addition, AG promotes long-term potentiation by elevating activity of nitric oxide synthase in the hippocampus (Carlini et al., 2008). It improves learning and behaviour in rats by increasing the hippocampal spine density, which can be attributed to its ability to stimulate hippocampal neurogenesis (Abizaid et al., 2006; Berrout and Isokawa, 2012; Li et al., 2013).

A normal hippocampal level of Ach is crucial for the memory function and hippocampus-dependent learning. Also, a normal level of BDNF in the brain promotes neuronal differentiation, survival, and neurogenesis by up-regulation of antioxidant and anti-apoptotic genes, such as *Bcl2* (Haam and Yakel, 2017). Moreover, increased generation of ROS in the brain and hippocampus impairs synaptic plasticity and cognition by decreasing BDNF expression and inducing intrinsic cell apoptosis. The latter is achieved by up-regulation of Bax and suppression of Bcl-2 (Wu et al., 2004). However, reduced levels of BDNF and Ach increase ROS generation and oxidative stress, impair the cholinergic system, and enhance apoptosis. So, we can suggest a developing vicious circle between BDNF plus Ach and oxidative stress, which were considered the classical mechanism that links ROS with reduced cognitive function in ageing. The presented data confirmed these relations between oxidative stress, reduction in GSH levels, and impairment in both Ach and BDNF levels. Moreover, intrinsic cell apoptosis, mediated by reduced Bcl-2 levels and high Bax levels, were confirmed by our results in the hippocampus in rats after SG. On the other hand, AG replacement prevented the aforementioned effects, suggesting its antioxidant, neurotransmitter modulator, and anti-apoptotic roles in the hippocampus. Chung et al. (2007) and Zhang et al. (2013) attributed the neuroprotective effects of AG primarily to an increase in the expression of Bcl-2, reduction in the mitochondrial generation of ROS, suppression of cytochrome-c release, and inhibition of caspase-3 (Chung et al., 2007; Zhang et al., 2013).

The PI3K/Akt is the most common survival pathway in the majority of cells, including neurons, by promoting cell survival and inhibiting apoptosis by induction of Bcl-2 and down-regulation of Bax (Martinou et al., 2011). Also, Akt inhibits cell apoptosis by reducing the activity of GSK3β by phosphorylating its Ser9 residue (Zhao et al., 2011; Xiao et al., 2013; Iqbal et al., 2014). GSK3β induces tau phosphorylation at the Ser396, Ser199, Thr205, and Thr231 residues (Xiao et al., 2013; Iqbal et al., 2014; Ponce-Lopez et al., 2017). Interestingly, ROS directly inhibit the PI3K/Akt activity in numerous tissues, including neurons, thus inducing direct activation of GSK3β (Xiao et al., 2013; Ponce-Lopez et al., 2017). One of recent studies reported association between the SG induction and suppression of Akt activity in rats, with subsequently enhancing GS3Kβ that causes tau hyperphosphorylation in the hippocampus. The presented evidence confirms the impact of tau hyperphosphorylation on the adverse neurological disturbances and memory deficits after SG surgery. AG replacement therapy in SG+AG rats increased the activity of Akt and reduced the activity of both GSK3β and tau phosphorylation levels in the hippocampus. Another validation for our hypothesis is that co-administration of LY294002 to the rats abolished the beneficial effects of AG on all measured parameters. Also, Giordano et al. (2007) reported that the loss of normal AG signalling in the brain in AD could be one of the crucial mechanisms involved in the pathogenesis of the disease.

A major concern that can be raised in this study is the significant increase in food intake and body weight in the SG-induced rats after AG, which contradicts the major aim of SG in the patients. This could be explained by using a single daily dose of AG, which is considered a limitation in the study. Another limitation in this study is that the SG and replacement therapy were done on lean rats only, while the effects were not demonstrated in obese animals to resemble the real clinical situation where SG is applied.

In conclusion, SG surgery negatively affects memory and some other intellectual functions, as well as the hippocampal integrity, and leads to AD-like manifestations in the rat experimental model. The pathological background could be represented by increased oxidative stress, reduced Bcl-2 and Akt activity, and tau hyperphosphorylation with subsequent enhancement of intrahippocampal apoptotic activity. An early AG replacement therapy had a protective function against the oxidative stress-induced deterioration of intellectual functions, particularly the memory.

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Conflict of interest

The authors declare no conflict of interest.

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