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Acyl ghrelin improves cognition, synaptic plasticity deficits and neuroinflammation following amyloid beta (Aβ1-40) administration in mice

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ABSTRACT

Ghrelin is a metabolic hormone that has neuroprotective actions in a number of neurological conditions including Parkinson's disease (PD), stroke and traumatic brain injury. Acyl ghrelin treatment *in vivo* and *in vitro* also shows protective capacity in Alzheimer's disease (AD). In this study, we used ghrelin knockout (KO) and their wildtype (WT) littermates to test whether or not endogenous ghrelin is protective in a mouse model of AD, in which human amyloid beta peptide ($A\beta_{1\text{-}40}$) was injected into the lateral ventricles (icv). Recognition memory, using the novel object recognition task, was significantly impaired in ghrelin KO mice and after icv $A\beta_{1\text{-}40}$ treatment. These deficits could be prevented by acyl ghrelin injections for 7 days. Spatial orientation, as assessed by the Y-maze task, was also significantly impaired in ghrelin KO mice and after icv $A\beta_{1\text{-}40}$ treatment. These deficits could be prevented by acyl ghrelin injections for 7 days. Ghrelin KO mice had deficits in olfactory discrimination, however, neither icv $A\beta_{1\text{-}40}$ treatment nor acyl ghrelin injections affected olfactory discrimination. We used stereology to show that ghrelin KO and $A\beta_{1\text{-}40}$ increased the total number of glial fibrillary acidic protein expressing astrocytes and ionized calcium-binding adapter expressing microglial in the rostral hippocampus. Finally, $A\beta_{1\text{-}40}$ blocked long-term potentiation induced by high frequency stimulation and this effect could be acutely blocked

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with co-administration of acyl ghrelin. Collectively, our studies highlight ghrelin deletion affects memory performance and that acyl ghrelin treatment may delay the onset of early events of AD. This supports the idea that acyl ghrelin treatment may be therapeutically beneficial to restrict disease progression in AD.

INTRODUCTION

The stomach hormone ghrelin is best known for its effects on metabolism, including food intake, blood glucose regulation and adiposity (1, 2). However, additional studies show that ghrelin plays an important role in non-metabolic functions, including learning, memory, stress, anxiety, fear, pain, vascular function and neuroprotection. In the plasma, ghrelin exists in an acylated and des-acylated form with des-acyl ghrelin being the most abundant. Acyl ghrelin, but not des-acyl ghrelin, activates the only known ghrelin receptor (GHSR; Growth Hormone Secretagogue Receptor), which is located in several regions throughout the brain, including the hypothalamus, substantia nigra, olfactory bulb, brain stem and hippocampus (3).

In terms of neuroprotection, numerous studies have identified that ghrelin prevents degeneration in animal models of Parkinson's disease (PD). Recently, we demonstrated that ghrelin mediates the neuroprotective benefit of calorie restriction in PD by controlling AMPK in substantia nigra dopamine neurons (4). Of course, calorie restriction will impact upon numerous physiological systems and recent evidence shows that ghrelin may link calorie restriction with increased neurogenesis (5), blood glucose production (6, 7) and mood (8). Collectively these studies show that ghrelin is a signal of energy deficit to the brain and that the effective actions of ghrelin are greater in states of negative energy balance (2, 9).

Alzheimer's disease (AD) is another neurological disease that may benefit from forms of calorie restriction (10, 11). If ghrelin was a common neuroendocrine feedback signal linking metabolic state to neuroprotection, then it is expected that ghrelin would also promote neuronal resilience in different models of AD. Indeed, human males with mild stage AD show significantly lower plasma ghrelin than aged matched controls, although no difference in females was observed (12). A single nucleotide polymorphism (Leu90Gln) in the ghrelin gene was associated with age of AD onset in a Japanese population (13). Moreover, a number of *in vivo* and *in vitro* studies show that ghrelin or GHSR agonists improve cognition and neuronal function in models of AD. For example, peripheral ghrelin improved T-maze footshock avoidance in a U-shaped dose response curve in young and old SAMP8 KO mice (14), which exhibit an age-related increase in amyloid beta (Aβ), the peptide involved in brain

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amyloid plaques in AD and impaired learning and memory (14). Moon *et al*. reported that peripheral ghrelin injections rescue memory deficits, prevent microgliosis and neuronal or synaptic degeneration in an AD mouse model induced by intrahippocampal injections of Aβ (15). These results have been confirmed with a central infusion of acyl ghrelin, which improved memory function, hippocampal AMPK activation and decreased Aβ deposition (16). In cultured hippocampal neurons, ghrelin ameliorated Aβ-induced cell death by preventing mitochondrial dysfunction (17).

In the 5XFAD AD mouse model, hippocampal neuroblast number is significantly reduced compared to WT controls and peripheral ghrelin injections restore this neurogenic capacity; whether or not this produced any functional recovery was not examined (18). However, these results are consistent with acyl-ghrelin-induced adult hippocampal neurogenesis via hippocampal GHSR activation in healthy lean mice (5) and rats (19). Long-term treatment with the GHSR agonist, LY444711, improves performance in a water maze and reduces microglial activation and amyloid beta (Aβ) (20). However, recent work from the same group using the same agonist failed to replicate this finding (21).

While the studies above demonstrate that administration ghrelin improves outcomes in mouse or rat models of AD, or *in vitro*, there is no evidence to show that endogenous ghrelin provides a protective effect that prevents or restricts cognitive loss in models of AD. Therefore, in this study we have used ghrelin wild type (WT) and ghrelin knockout (KO) mice in order to determine whether or not endogenous ghrelin prevents cognitive decline after intracerebroventricular (icv) Aβ administration. We further examined the protective actions of acyl ghrelin administration in ghrelin WT and KO mice and explored the possible impact of ghrelin and Aβ in two processes known to critically impact on memory performance, namely neuroinflammation and synaptic plasticity.

METHODS

Animals

All experiments were conducted in compliance with the guidelines of the Animal Ethics Committees of the Monash University and of the Center for Neuroscience and Cell Biology of the University of Coimbra. Mice were kept at standard laboratory conditions with free access to food and water at 23 °C in a 12-hour light/dark cycle unless otherwise stated. Male ghrelin KO on a C57/Bl6 background were obtained from Regeneron Pharmaceuticals (Tarrytown,

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NY) and bred at the Monash Animal Services. All mice were group-housed to prevent isolation stress. All mice were 10-12 weeks of age at time of experimentation.

Treatment

Ghrelin KO mice that produce neither acylated nor des-acylated ghrelin (22, 23) were used to examine whether endogenous ghrelin prevents cognitive decline and neuroinflammation after icv injection of human amyloid-beta peptide 1-40 ($A\beta_{1\text{-}40}$) (cat # A1075, Sigma, USA). $A\beta_{1\text{-}40}$ was prepared as a stock solution at 1 mg/ml in sterile phosphate buffered saline (PBS, 0.1 M; pH 7.4) and $A\beta_{1\text{-}40}$ protein fragments were aggregated for 4 days at 37°C, as previously described (24). Mice were anaesthetised with isofluorane/oxygen mixture and mounted in a stereotaxic apparatus (Stoelting Co, Wood Dale, IL, USA). Mice were injected icv with 400 pmol of $A\beta_{1\text{-}40}$ or PBS (1 µl injection volume) using the following coordinates; bregma anterior/posterior 0.2 mm, medial/lateral 0.8 mm and dorsal/ventral 2.1 mm according to a mouse brain atlas as a guide (Franklin and Paxinos). Acyl-ghrelin (0.3 mg/kg) was injected ip daily for 7 days after $A\beta_{1\text{-}40}$ injection at approximately 9 am each morning. Food was removed immediately after ip injection for 6 hours after the injections, as performed previously to prevent immediate food intake (4, 25, 26), which alters metabolic feedback and negates ghrelin signaling properties.

Behavioural testing

Behaviour testing occurred between days 6-17 since the start of ghrelin injections and mice were tested for novel objection recognition, Y-maze performance and olfactory discrimination according to the timeline established in figure 1. All behavioural tests were conducted at least 2 days apart to reduce any potential stress associated with behavioural testing. All behavioural testing was conducted between 9 am and 2 pm.

All tests were performed in an experimental room with sound isolation and dim light. The animals were carried to the test room for at least 1 hour of acclimation. Behaviour was monitored using a video camera positioned above the apparatuses and the videos were later analyzed by an experienced blinded researcher using video tracking software (CleverSys Inc, Reston, VA, USA).

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Novel object recognition (NOR): The NOR task exploits a mouse's natural tendency to explore a novel object after previous exposure to two identical objects. At 10-12 weeks of age, mice (n=8) were habituated for 3 periods of 10 minutes each separated with 10 minutes in its home cage to reduce anxiety associated with the novel arena (plastic arena 30 x 30 x 50 cm). After habituation, mice were ready for the NOR task, which was conduct using two trials (familarisation trial [T1] and test trial [T2]) separated by 30 minutes. During T1, mice were allowed to explore for 10 minutes two identical objects (plastic screw-top tubes) secured to the floor using a small amount of Blu Tack in habituated arenas. For T2, one identical object from T1 was replaced with a novel object (small green flask) and mice were allowed to freely explore for 5 minutes. T1 and T2 were recorded using a video camera and analyzed for the time spent interacting with the novel object. All arenas were washed and dried and then sprayed with 80% ethanol prior to experimentation. Novel object exploration was calculated in T2 by $(T_{novel} \times 100)/(T_{novel} + T_{identical})$ with exploration defined as the nose being less than 1 cm from the object when facing the object or actively engaging with the object by sniffing or paw touching. Climbing on the object was not considered exploration.

Modified Y-maze: The modified Y-maze measures spatial memory, as spatial orientation cues facilitate rodents to explore a novel arm rather than returning to a previously visited arm. We used a Y-shaped grey Perspex maze (30 cm x 10 cm x 16 cm) and each arm could be isolated by blocking entry with a sliding door. Saw dust from a mouse's home cage lined the maze during the trials and extra maze cues on the walls were placed 30-40 cm from the end of the arms to provide spatial orientation cues. Behaviour was tested across two trials, the first of which had one arm of the maze randomly blocked off. Mice were allowed to explore the reduced maze for 10 minutes and then returned to their home cage. The second trial was conducted 30 minutes after the first trial and both arms of the maze were opened. Mice were placed in the start arm and allowed to explore the full maze for 5 minutes. All behaviours were recorded and analyzed using tracking software. Novel arm exploration was record when all 4 feet of each mouse entered the novel arm. The apparatus was cleaned with 80% ethanol between each trial and each animal.

Olfactory discrimination: The task is based on the fact that mice prefer places with their own odor (familiar compartments) instead of places with unfamiliar odors. In this test, mice have access to 2 adjacent identical chambers separated by an intermediate zone. One chamber contained familiar bedding from its home cage over the last 48 hours (familiar) whereas the other contained fresh bedding (non-familiar). Mice were placed into the intermediate zone and allowed to freely explore each chamber. Rodents are capable of discriminating familiar versus non-familiar chambers since they prefer their odour to no odour at all. The time spent in each chamber was recorded and analyzed. An olfactory discrimination index was

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generated according to the following $t_{familiar}/(t_{familiar} + t_{non\text{-}familiar})$, where t equal time and 0.5 equals no preference.

Immunohistochemistry and stereology

We collected every section (30 µm thickness) in sets of 4 through the rostral hippocampus from bregma -0.9 mm to -2.5 mm. Every 4th section was washed in 0.1 M PB and incubated with 1% hydrogen peroxide (H_2O_2) for 15 minutes to prevent endogenous peroxidase activity and blocked for 1 hour with 5% normal horse serum (NHS) in 0.1 M PB with 0.3% Triton. In order to assess neuroinflammation, we quantified astrocytosis by immunostaining for glial fibrillary acidic protein (GFAP; rabbit anti-GFAP antibody from Abcam, USA, Cat no. 7260) and the microglial marker ionized calcium-binding adapter molecule (Iba1; rabbit anti-Iba1 antibody from Wako, Japan Cat no. #019-19741) all at 1:1000 in PB 0.1 M with 1% NHS and 0.3% Triton. After incubation with the primary antibodies, the sections were washed and incubated with biotin-SP-conjugated goat anti-rabbit IgG (Jackson ImmunoResearch Laboratories, Inc. West Grove, PA, USA) at 1:200 in 0.1 M PB. Sections were then washed and incubated with avidin-biotin complex (ABC, 1:200) for 90 minutes. To visualise immunoreactivity, sections were incubated with a solution containing 1% nickel ammonium sulfate, 1% Diaminobenzidine and 4 µl of 30% H_2O_2. Sections were then washed in 0.1 M PB, mounted and coverslipped. The non-specific staining of our antibodies used was confirmed with controls where we omitted the addition of the primary antibody.

Stereology: We employed a design-based approach to quantify GFAP- and Iba1-positive cells in the rostral hippocampus using the Stereoinvestigator software (MicroBrightField, Williston, VT, USA). We used a Zeiss microscope with a motorised stage coupled with a MicroFibre digital camera to a computer. Cells were counted using the optical fractionator probe on both sides of the brain, controlled by Stereoinvestigator software in randomly positioned grids in a defined region to create a collection of 3 dimensional counting areas. Guard zones were set at 10% of the section thickness to account for damage during the staining procedure and to prevent overcounting. The counting frame width (X) and height (Y) was 40.2 µm producing a counting frame area (XY) of 1616 μm^2. The dissector height (Z) was 20 um creating a dissector volume (XYZ) of 32320 μm^3. With this counting frame area we discovered that we needed to sample approximately 150-200 sites throughout the entire rostral hippocampus and count approximately 200-250 labeled cells throughout the rostral hippocampus to obtain a coefficient of error (using the Gunderson method) of 0.1 using a smoothness factor m=1. Cells were only counted if they touched the inclusion border or did not touch the exclusion border of the sampling grid.

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Electrophysiology

Electrophysiological recordings were carried out as previously described (27). Briefly, mice (C57Bl/6) were deeply anesthetized under a halothane-saturated atmosphere (Sigma-Aldrich, St Louis, MO, USA) before decapitation. Brains were quickly removed and placed in ice-cold standard artificial cerebrospinal fluid (aCSF) containing (in mM); 124 NaCl, 4.5 KCl, 2 $CaCl_2$, 1 $MgCl_2$, 26 $NaHCO_3$, 1.2 NaH_2PO_4 and 10 D-glucose, gassed with 95% O_2 and 5% CO_2. The hippocampi were cut in 400 µm thick transverse slices using a McIlwain tissue chopper (Mickle Lab Engineering, Guildford, UK) and kept in oxygenated aCSF at room temperature for at least 60 minutes, before being used. Individual slices were transferred to a recording chamber and superfused with oxygenated aCSF at 30.5 ^{o}C at a flow rate of 3 mL/min. Bipolar stainless steel electrodes were placed on the Shaffer collateral/commissural fibers and test stimuli were delivered via a S44 stimulator (Grass Instruments, West Warwick, RI) with a stimulus isolation unit (PSIU6, Grass Instruments) at a frequency of 0.06 Hz. Glass microelectrodes (1–2 MΩ) filled with 4 M NaCl were used to record field excitatory postsynaptic potentials (fEPSPs) in the stratum radiatum of the CA1 region of hippocampus. Recordings were obtained using an ISO-80 amplifier (World Precision Instruments, Hertfordshire, UK) and digitized using an ADC-42 board (Pico Technologies, Pelham, NY, USA). Averages of 4 consecutive responses were continuously monitored on a personal computer with the LTP 1.0.1 software (28).

An input-output curve was first carried out to evaluate the threshold to the maximum response and the working stimulus intensity was adjusted to evoke fEPSPs of half maximal amplitude (50%). Long-term potentiation (LTP) was induced with a protocol of high-frequency stimulation (HFS) with pulses delivered at 100 Hz during 1 second; the fEPSPs were recorded for an additional 60 minutes. The average slope of the fEPSP at baseline was set at 100%, and changes of the fEPSP slope calculated 50-60 minutes after delivery of the HFS train were expressed as percent of change from baseline to estimate the amplitude of LTP. $A\beta_{1-40}$ (200 or 500 nM) and ghrelin (1 nM or 1 µM) were added through the superfusion system 30 minutes before LTP induction and kept in the superfusate until the end of the experiment. When testing the interaction between ghrelin (1 nM) and $A\beta_{1-40}$ (200 nM) both were added simultaneously to the superfusion system 30 minutes before LTP induction.

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Statistical analysis

All data are represented as mean ± standard error of the mean (SEM). A three-way analysis of variance (ANOVA), or a two-way ANOVA, with Tukey's *post hoc* tests for multiple comparsions were used to determine statistical significance between genotype, peptide and hormone treatment. $p<0.05$ was considered statistically significant. Data were plotted and analysed using Prism 7.0b for Mac OS X.

RESULTS

NOR

The NOR is a highly-validated test for recognition memory and exploits a mouse's natural tendency to explore a novel object after previous exposure to two identical objects. To examine the effect of icv $A\beta_{1-40}$ on recognition memory, we measured NOR in ghrelin WT or KO mice injected with aCSF or $A\beta_{1-40}$ icv and then injected daily ip with either saline or acyl ghrelin (Fig 2). A three-way ANOVA revealed a main effect of hormone treatment ($F=12.33$; $p=0.001$), genotype ($F=22.03$; $p<0.0001$) and peptide treatment ($F=9.449$; $p=0.0035$). There was a significant interaction between hormone treatment and genotype ($F=4.05$; $p=0.0498$) and a significant interaction between hormone treatment x genotype x peptide treatment ($F=4.751$; $p=0.0342$) (Fig 2).

NOR was significantly reduced in ghrelin WT mice injected icv with $A\beta_{1-40}$ and saline (ip), compared to ghrelin WT mice injected with icv aCSF and saline (ip) (Fig 2), indicating that $A\beta_{1-40}$ reduces recognition memory. Daily acyl ghrelin injections for 7 days significantly improved NOR in ghrelin WT mice treated with icv $A\beta_{1-40}$ and ghrelin KO mice treated with either aCSF or icv $A\beta_{1-40}$ (main effect of hormone treatment), although it did not improve NOR in WT mice treated with icv aCSF (Fig 2). These results indicate acyl ghrelin can overcome the deficit in NOR caused by $A\beta_{1-40}$, but it does not potentiate NOR in ghrelin WT mice without $A\beta_{1-40}$-induced pathology (i.e. treated icv with aCSF). Ghrelin KO mice treated with either icv aCSF or $A\beta_{1-40}$ and daily ip saline injections could not discriminate between novel and familiar objects based on 50% exploration time of both objects (Fig 2). However, daily acyl ghrelin injections for 7 days to ghrelin KO mice resulted in significantly increased recognition memory (NOR) in both ghrelin KO mice treated icv with aCSF or $A\beta_{1-40}$. NOR was significantly reduced in ghrelin KO compared to ghrelin WT mice injected daily with saline, indicating that genotype alone significantly affects NOR (main effect of genotype). $A\beta_{1-40}$ reduced NOR to 50% in both ghrelin WT and KO mice (main effect of peptide treatment),

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where 50% represents no ability to discriminate between novel and familiar objects. Thus, deletion of ghrelin or $A\beta_{1-40}$ alone is sufficient to reduce recognition memory to chance, which can be overcome with acyl ghrelin treatment. This floor effect means that the combination of ghrelin KO mice and $A\beta_{1-40}$ administration cannot further impair NOR.

Modified Y-maze

The modified Y-maze is a highly-validated test for spatial memory, which utilizes spatially orientated visual cues to help rodents explore a previously unexplored novel arm. To examine the effect of icv $A\beta_{1-40}$ on spatial memory, we measured novel arm exploration (seconds) in ghrelin WT or KO mice injected icv with either aCSF or $A\beta_{1-40}$ and then injected daily ip with either saline or acyl ghrelin (Fig 3). A three-way ANOVA revealed a main effect of hormone treatment ($F=15.8$; $p=0.0002$), genotype ($F=16.79$; $p=0.0002$) and peptide treatment ($F=14.66$; $p=0.0004$). There was a significant interaction between hormone treatment and genotype ($F=5.232$; $p=0.0266$), genotype and peptide treatment ($F=13.56$; $p=0.0006$) and a significant interaction between hormone treatment x genotype x peptide treatment ($F=8.404$; $p=0.0056$) (Fig 2).

Novel arm exploration was significantly reduced in ghrelin WT mice injected icv with $A\beta_{1-40}$ and saline ip, compared to ghrelin WT mice injected icv with aCSF and saline ip (Fig 3), indicating that $A\beta_{1-40}$ impairs spatial memory. Daily acyl ghrelin injections for 7 days significantly improved novel arm exploration in ghrelin WT mice treated icv with $A\beta_{1-40}$ and ghrelin KO mice treated with either aCSF or icv $A\beta_{1-40}$ (main effect of hormone treatment) but not in mice treated icv with aCSF (Fig 3). Similar to results from the NOR tests, these results suggest acyl ghrelin overcomes the deficit in novel arm exploration caused by $A\beta_{1-40}$, but it cannot enhance novel arm exploration in ghrelin WT mice without $A\beta_{1-40}$-induced pathology.

Ghrelin KO mice treated icv with either aCSF or $A\beta_{1-40}$ and injected daily with ip saline could not differentiate between the novel arm and the previously explored arm (Fig 3). However, daily ip acyl ghrelin increased novel arm exploration in both icv aCSF and $A\beta_{1-40}$ treated ghrelin KO mice (Fig 3). Novel arm exploration was reduced in ghrelin KO mice without $A\beta_{1-40}$ treatment compared to ghrelin WT mice treated icv with $A\beta_{1-40}$ (main effect of genotype; Fig 3). $A\beta_{1-40}$ reduces novel arm exploration in ghrelin WT mice to ghrelin KO mouse levels (main effect of peptide treatment), although $A\beta_{1-40}$ has does not further reduce novel arm exploration when compared to ghrelin KO mice treated icv with aCSF (Fig 3C). Thus,

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deletion of ghrelin or Aβ_{1-40} alone reduces novel arm exploration to a similar degree, which cannot be reduced further in ghrelin KO mice after Aβ_{1-40}.

Olfactory discrimination

As deficits in olfactory processing are a common feature in early stages of AD (29) and ghrelin regulates olfactory processing in mice and humans (30), we used an olfactory discrimination test to determine whether Aβ_{1-40} affects olfactory discrimination in ghrelin WT and KO mice. We also tested whether exogenous acyl ghrelin could improve olfactory discrimination in ghrelin WT and KO mice. Specifically we chose to examine olfactory behaviour 10 days after the final acyl ghrelin ip injection since olfactory discrimination is reported to require olfactory neurogenesis (31-34) and this time frame influences hippocampal neurogenesis (35). A three-way ANOVA revealed a main effect of genotype ($F=30.91$; $p<0.0001$) and no effects of hormone treatment or peptide treatment were observed. Moreover, no significant interactions were observed. All WT groups of mice could discriminate between chambers paired with familiar and fresh bedding (i.e. discrimination index > 0.5; Fig 4). In contrast, ghrelin KO groups of mice could not discriminate between familiar and fresh bedding, as indicated by an index of 0.5, regardless of icv treatment or daily injection of saline or acyl ghrelin (main effect of genotype; Fig 4). These results indicate that ghrelin KO mice exhibit a deficit in olfactory discrimination irrespective of hormone or peptide treatment.

Stereological analysis of GFAP and Iba1 in the rostral hippocampus

In order to examine the effect of Aβ_{1-40} on neuroinflammation in ghrelin WT and KO mice, we performed a stereological analysis of GFAP and Iba1 cell number in the rostral hippocampus. Ghrelin KO mice had greater GFAP-positive cells compared to WT mice treated icv with aCSF (Fig 5A). Aβ_{1-40} administration significantly increased GFAP-positive cell number in ghrelin WT mice, whereas there was no further increase in GFAP-positive cell number in ghrelin KO mice treated icv with Aβ_{1-40} compared to ghrelin KO mice treated icv with aCSF (Fig 5A). A similar effect was seen in Iba1 microglial number, although absolute numbers of Iba1-positive cells were lower than GFAP-positive cells. The number of Iba1-positive cells was larger in ghrelin KO mice treated icv with aCSF compared to ghrelin WT mice treated icv with aCSF. Furthermore, Aβ_{1-40} treatment significantly increased Iba1-positive microglia cells in ghrelin WT mice, but not in ghrelin KO mice (Fig 5B). This indicates that ghrelin deletion

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increases the number of GFAP-positive and Iba1-positive cells in the rostral hipoocampus. Likewise, icv treatment with $A\beta_{1-40}$ also increases the number of GFAP-positive Iba1-positive cells in the rostral hippocampus in the presence, but not in the absence, of ghrelin.

Hippocampal LTP

The electrophysiological analysis in hippocampal slices revealed that $A\beta_{1-40}$ (200 and 500 nM) did not significantly modify basal (i.e. low frequency) synaptic transmission ($P > 0.05$) (data not shown), but decreased LTP amplitude ($P < 0.05$) (Fig 6A and B). Similarly, the administration of a low (1 nM) or a high concentration (1 µM) of ghrelin did not significantly modify basal synaptic transmission but increased ($P < 0.05$) the amplitude of HFS-induced LTP (Fig 6C and D).

The possible interaction between the effects of ghrelin and $A\beta_{1-40}$ on hippocampal LTP was investigated using the lower effective concentration of $A\beta_{1-40}$ (200 nM) and ghrelin (1 nM). We found that the concomitant administration of ghrelin blunted ($P < 0.05$) the depressive effect of $A\beta_{1-40}$ on the amplitude of HFS-induced LTP (Fig 6E and F).

DISCUSSION

Our results indicate 5 key findings; 1) ghrelin KO mice have deficits in spatial, recognition and olfactory memory based on the results from Y-maze, NOR and olfactory discrimination tests, respectively; 2) icv $A\beta_{1-40}$ treatment reduces spatial and recognition memory performance in ghrelin WT mice, but not in ghrelin KO mice since memory performance is already impaired; 3) ip acyl ghrelin treatment recovers memory deficits in ghrelin KO treated icv with aCSF or $A\beta_{1-40}$ and in ghrelin WT treated icv with $A\beta_{1-40}$, but it does not enhance memory performance in control mice (ghrelin WT mice treated icv with aCSF); 4) ghrelin KO mice treated icv with aCSF or $A\beta_{1-40}$ and ghrelin WT treated icv with $A\beta_{1-40}$ show neuroinflammation, as indicated by an increased number of GFAP- and Iba1-positive cells in the rostral hippocampus; 5) acute $A\beta_{1-40}$ depresses HFS-induced LTP, an effect prevented by acyl ghrelin.

These findings indicate that the genetic deletion of endogenous ghrelin influences memory performance in a manner similar to $A\beta_{1-40}$ icv injection and that the administration of exogenous acyl ghrelin prevented the decrease in NOR and Y-maze spatial memory in both $A\beta_{1-40}$-treated mice and in mice lacking ghrelin. The combination of $A\beta_{1-40}$ administration to

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ghrelin KO mice did not worsen spatial and recognition memory performance beyond $A\beta_{1-40}$ administration alone. This is likely to represent a floor effect since ghrelin KO mice were already performing at 50% novel object exploration, where 50% represents no ability to discriminate between novel and familiar objects. Moreover, the equipotent effect of ghrelin deletion and $A\beta_{1-40}$ on neuroinflammation and the ability of acyl ghrelin to prevent $A\beta_{1-40}$-induced hippocampal synaptic plasticity highlight that these two processes are tightly linked to memory performance. It is likely that these mechanisms underlie the observed effects of ghrelin and $A\beta_{1-40}$ on memory performance. The particular ability of ghrelin deletion to dampen memory performance and, conversely, of exogenous acyl ghrelin to restore memory selectively when is it perturbed, strongly suggests a critical role of ghrelin to prevent memory deterioration rather than acting as a memory enhancer. This is supported by the observations that acyl ghrelin did not enhance memory performance in ghrelin WT mice without $A\beta_{1-40}$ administration.

Furthermore, based on the proposed detrimental role of soluble Aβ in early AD (36, 37), the reported findings consolidate the therapeutic potential of manipulating ghrelin signaling in the brain to manage early AD (15-17, 38). This is further heralded by the ability of ghrelin to control two key processes that we have previously shown to critically mediate the early memory dysfunction caused by the icv administration of Aβ, namely neuroinflammation (39) and synaptic dysfunction (40, 41). It still remains to be determined if ghrelin might also affect classical pathological features of late phases of AD such as the formation of amyloid plaques and the aggregation of phosphorylated tau since these two features are not recapitulated in the Aβ1-40 icv injection model (36, 37), which mostly mimics early AD, as validated in numerous studies (24, 36, 39, 42, 43).

The spatial Y maze memory test is highly validated and depends on hippocampal function, whereas recognition memory is largely perirhinal-dependent but with a hippocampal component (44-46). Our studies and the work by Diano and colleagues (14) supports a role for ghrelin in the hippocampus in object recognition, however the role of ghrelin in the perirhinal cortex, a key site for object recognition (47), needs to be addressed. We show for the first time that spatial memory is also dependent on endogenous ghrelin production, as ghrelin KO mice showed deficits in Y-maze (spatial memory) performance that could be reversed with acyl ghrelin treatment. These results are consistent with data obtained from GHSR KO mice or GHSR antagonism in the hippocampus, showing that ghrelin signaling requires the GHSR in the hippocampus to promote spatial memory performance (48, 49). It should be noted however, that GHSR deficient rats showed a deficit in radial arm maze food

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motivated task but not in the water maze (50), suggesting that ghrelin may control context-dependent spatial memory via GHSR signaling (5), which are highly expressed in the hippocampus, particularly the rostral dentate gyrus (3, 19, 51). Considering the important role of ghrelin and GHSR signaling in conveying energy deficit, it is likely that ghrelin/GHSR signaling in the hippocampus enhances spatial learning to promote re-feeding and restoration of energy balance (52, 53). However, this ghrelin/GHSR signaling may operate differently in different brain regions, since contextual fear memory is also dependent on GHSR (5), but is independent of energy balance (54).

$A\beta_{1-40}$ treatment reduced both spatial and recognition memory performance in ghrelin WT mice, which was reversed by acyl ghrelin treatment, consistent with previous reports showing the neuroprotective potential of acyl ghrelin in AD (15-17, 20, 38). One possible mechanism through which ghrelin deletion or $A\beta_{1-40}$ affects memory may be neuroinflammation. Indeed, our results show that the elimination of endogenous ghrelin and the exposure to icv $A\beta_{1-40}$ increases the number of GFAP- and Iba1-positive cells in the hippocampus, as assessed by unbiased stereological approach, the most reliable and robust methods to accurate estimate cell number within a large area (55). These results are consistent with the known roles of ghrelin in preventing neuroinflammation in models of AD and other neurological disorders (26, 56) and the ability of Aβ to induce neuroinflammation (57). The control of synaptic plasticity, which is the best neurophysiological correlate of memory (58), is another mechanism likely involved in the effects of $A\beta_{1-40}$ and ghrelin on hippocampal-dependent memory. Indeed, the onset of memory impairment in early AD seems to depend on synaptic dysfunction (59) and we now report that the known deleterious effect of $A\beta_{1-40}$ on hippocampal LTP (60) is prevented by the addition of ghrelin. This provides a neurophysiological basis for the ability of ghrelin to prevent $A\beta_{1-40}$-induced dysfunction.

We also showed that deletion of ghrelin significantly impairs olfactory discrimination, which has significant implication in AD, as deficits in olfactory processing are a common feature in early stage AD (29). To the best of our knowledge this is the first demonstration that endogenous ghrelin modulates olfactory function. Our results are consistent with the fact that ghrelin influences olfactory responsiveness to odors, which can be blocked by ghrelin receptor antagonism (61) and ghrelin increases sniffing frequency in mice and humans (30). Together with the high expression of the GHSR in the olfactory bulb (3), these results suggest endogenous ghrelin plays an important role in olfactory discrimination and learning. Nevertheless, neither icv Aβ1-40 treatment nor ip acyl ghrelin injections influenced olfactory learning in our experimental conditions. One possibility is that icv Aβ1-40 treatment was not

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sufficiently localized to the regions of the brain processing olfactory information, such as the olfactory bulb and piriform cortex.

Given the expression of the GHSR in the olfactory bulb and the strong effects of ghrelin in the olfactory responsiveness and sniffing frequency, the lack of olfactory learning in response to ip acyl ghrelin treatment was initially surprising. This is likely due to the different mechanisms of action of the ghrelin/GHSR signaling to control different memory domains encoded by different brain circuits. In contrast to the control of hippocampal-dependent spatial memory, which may involve neuroinflammation and synaptic plasticity based on our data, the ghrelin/GHSR-mediated control of the consolidation of contextual fear memory instead requires the down-regulation of GHSR in the amygdala (54). The effect of endogenous ghrelin on olfactory memory might involve a different mechanism, such as ghrelin-induced neurogenesis (5, 62), which is known to affect olfactory discrimination (31, 32). This would explain the observed lack of effect of ip acyl ghrelin since the generation of functioning new adult born olfactory bulb neurons takes ~28 days (62) and acyl ghrelin would be ineffective following 11 days of treatment (63), when we analyzed olfactory memory. (5, 31-34, 62). Future studies are required to address the relationship between ghrelin-induced olfactory bulb neurogenesis and olfactory learning.

In this study, we have tested the cognitive effects of $A\beta_{1-40}$ icv treatment in ghrelin WT and KO mice with or without acyl ghrelin treatment. Importantly, ghrelin KO mice lack both des-acyl ghrelin and acyl ghrelin, as both forms are derived from the same proghrelin precursor. Thus, it is possible that the lack of des-acyl ghrelin may also contribute to the memory reported in this study. However, Kang et al, recently showed that acyl ghrelin rather than des-acyl ghrelin is neuroprotective in AD by activating AMPK (16), a result consistent with the neuroprotective actions of acyl ghrelin, and not des-acyl ghrelin, via AMPK signaling in a mouse model of PD (26, 64, 65). The activation of AMPK may drive downstream intracellular mitochondrial pathways to prevent degeneration in AD. Indeed, acyl ghrelin treatment reduces ROS production and prevents mitochondrial membrane depolarization in hippocampal and hypothalamic cells treated with Aβ oligomers (17, 38). These results are similar to the actions of acyl ghrelin in the hypothalamus, substantia nigra and in stroke models (25, 66, 67) suggesting common neuroprotective actions of acyl ghrelin involving the maintenance of mitochondrial homeostasis regardless of the neurological disease.

In conclusion, we show that ghrelin deletion impairs spatial and recognition memory as well as olfactory discrimination. Treatment with $A\beta_{1-40}$ icv significantly impairs spatial and recognition memory in ghrelin WT mice but does not further exacerbate the memory deficit in ghrelin KO mice suggesting that the loss of endogenous ghrelin signaling may be associated

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with $A\beta_{1-40}$-induced memory deficits. Furthermore, the ability of daily ip injections of acyl ghrelin to restore spatial and recognition memory performance as well as hippocampal synaptic plasticity highlight that enhancing ghrelin secretion may preserve cognitive ability and delay the onset of AD and further support the idea that acyl ghrelin treatment may be therapeutically beneficial to restrict disease progression in early AD.

Figure Legends

Figure 1. Experimental timeline. Mice were injected icv with $A\beta_{1-40}$ (400 pmol) or vehicle (aCSF) on day 0 and then received ip acyl ghrelin (0.3 mg/kg) at 9 am daily, for 7 days. Novel object recognition (NOR), modified Y-maze, and olfactory discrimination behavioural task were evaluated on days 6, 8 or 17 after icv injection and mice were sacrificed on day 18.

Figure 2. Novel object recognition (NOR). Novel object exploration is the time spent exploring the novel object in the test trial, where 50% represents no difference in exploratory time between the novel and familiar object. $A\beta_{1-40}$ reduces novel object exploration in ghrelin WT mice treated with saline, however this reduction is prevented by daily administration with acyl ghrelin. A main effect of genotype was observed with ghrelin KO mice performing worse than ghrelin WT in NOR. Daily ip injection of acyl ghrelin increased novel object exploration in both ghrelin WT and KO mice (main effect of hormone treatment). In addition, a main effect of peptide treatment (icv $A\beta_{1-40}$ treatment) was also observed indicating that icv $A\beta_{1-40}$ treatment worsened recognition memory performance. Significant interactions between hormone treatment and genotype, and between hormone treatment, genotype and peptide treatment were observed. Data are presented as mean +/- SEM, n=10 per group, three-way ANOVA with Tukey's post hoc analysis to identify significant difference between groups. a, significant difference ($p<0.05$) between aCSF and $A\beta_{1-40}$ treatment. The dotted line indicates 50% in novel object recognition, which represents no ability to discriminate between novel and familiar objects (chance).

Figure 3. Modified Y-maze where we quantified the time spent in the novel arm in the second visit to the Y-maze 30 minutes after a previous visit where this arm was closed. $A\beta_{1-40}$ reduces the time spent in the novel arm in ghrelin WT mice treated with saline, and this reduction is prevented by daily administration of acyl ghrelin. Daily ip acyl ghrelin treatment increased time spent in the novel arm relative to ip saline injections (main effect of hormone

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treatment) and ghrelin KO mice performed worse in the Y-maze task compared to ghrelin WT mice (main effect of genotype). A main effect of peptide treatment (icv $A\beta_{1-40}$ treatment) was also observed indicating that icv $A\beta_{1-40}$ treatment worsened spatial memory performance. Significant interactions between hormone treatment and genotype; between genotype and peptide treatment; and between hormone treatment, genotype and peptide treatment were observed. Data are presented as mean +/- SEM, n=10 per group, three-way ANOVA with Tukey's post hoc analysis to identify significant difference between groups. a, significant difference ($p<0.05$) between aCSF and $A\beta_{1-40}$ treatment.

Figure 4. Olfactory discrimination was determined by comparing the amount of time mice spent in a chamber with familiar bedding compared to the time spent in an adjoining chamber with non-familiar bedding. The olfactory discrimination index measures the preference for the familiar bedding with 0.5 being equal to no preference, as indicated by the dotted line. We observed a main effect of genotype on olfactory discrimination, but no effect of hormone or peptide treatment, indicating ghrelin KO mice performed worse than WT mice, independent of acyl ghrelin or $A\beta_{1-40}$ treatment. Data are presented as mean +/- SEM, n=10 per group, three-way ANOVA with Tukey's post hoc analysis to identify significant difference between groups.

Figure 5. Stereological estimation of the number of GFAP- and Iba1-positive cells in the rostral hippocampus. (A) Ghrelin KO mice exhibited a significantly greater number of GFAP-positive cells in the rostral hippocampus compared to ghrelin WT mice, both groups treated icv with aCSF. $A\beta_{1-40}$ icv treatment increased the number of GFAP-positive cells in ghrelin WT but not in ghrelin KO mice. (B) Ghrelin KO mice exhibited a significantly greater number of Iba1-positive cells in the rostral hippocampus compared to ghrelin WT mice, both groups treated icv with aCSF. $A\beta_{1-40}$ icv treatment increased the number of GFAP-positive cells in ghrelin WT but not in ghrelin KO mice. Data are presented as mean +/- SEM, n=10 per group, two-way ANOVA with Newman-Keuls post hoc analysis to identify significant difference between groups. a, significant difference ($p<0.05$) between groups, as indicated by the horizontal lines.

Figure 6. Hippocampal LTP. Bath application of $A\beta_{1-40}$ (either 200 or 500 nM) prevents LTP induced by HFS (A-B). Acyl ghrelin increases HFS-induced LTP as measured by the percent

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increase in fEPSPs before and 60 minutes after HFS in the absence and presence of acyl ghrelin (1 nM or 1 μM; C-D). Co-application of acyl ghrelin with $A\beta_{1-40}$ prevents the $A\beta_{1-40}$-induced suppression of hippocampal LTP. Data are presented as mean +/- SEM, n=4 per group, one-way ANOVA with Newman-Keuls post hoc analysis to identify significant difference between groups. a, significantly different ($p<0.05$) from control group; b, significantly different ($p<0.05$) from ghrelin 1nM + $A\beta_{1-40}$ 200 nM.

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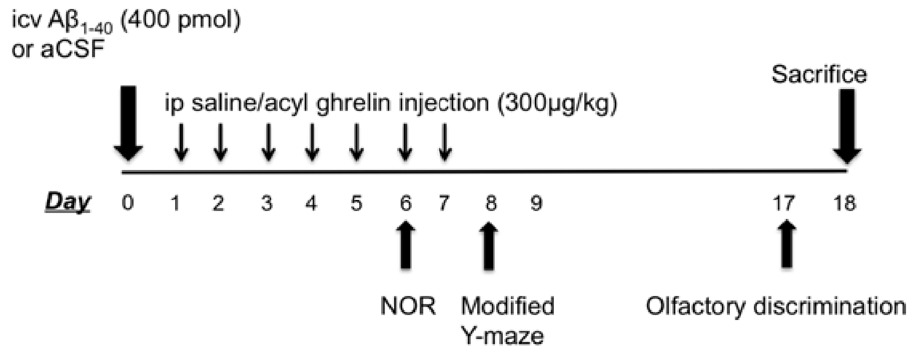

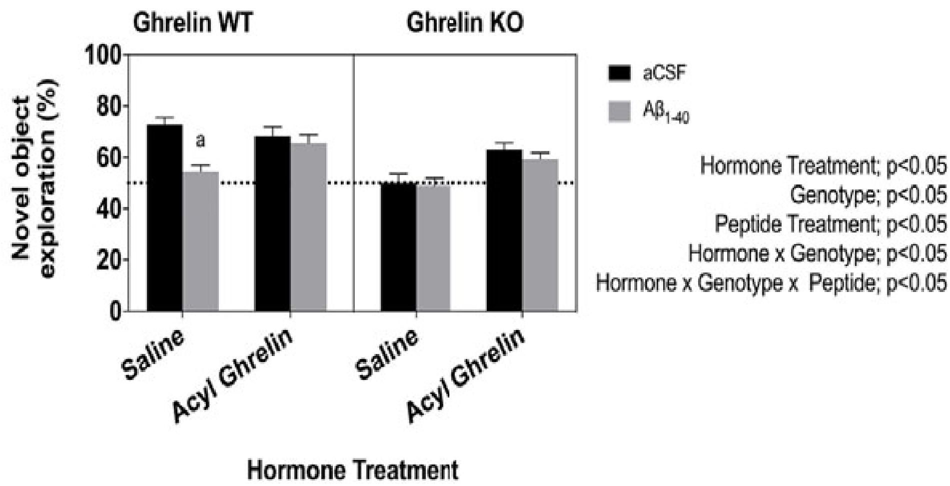

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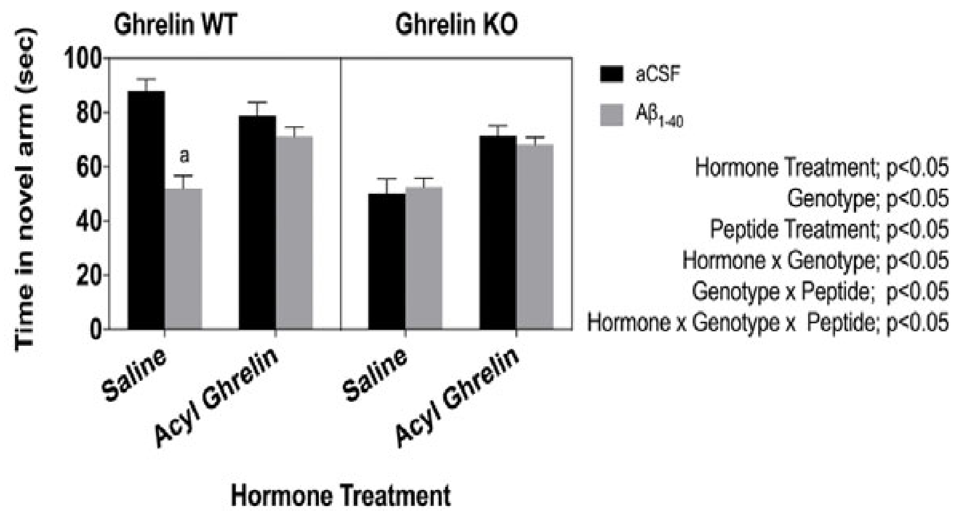

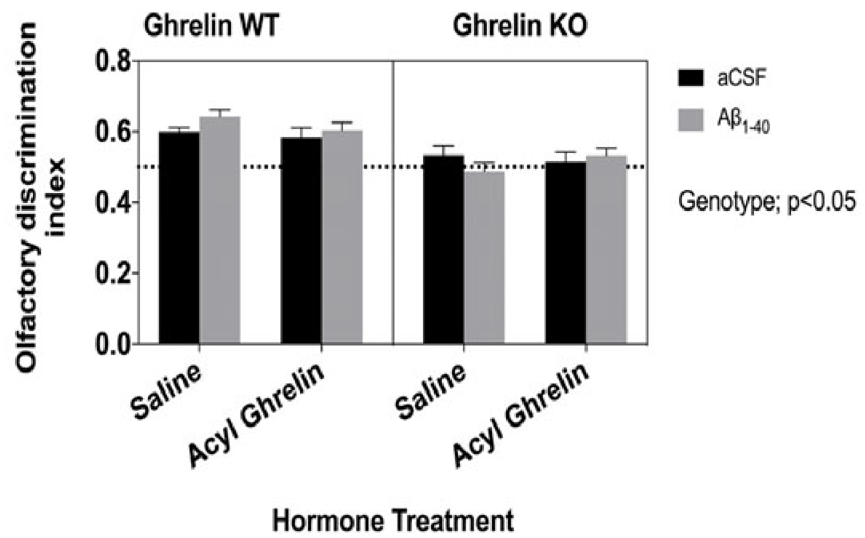

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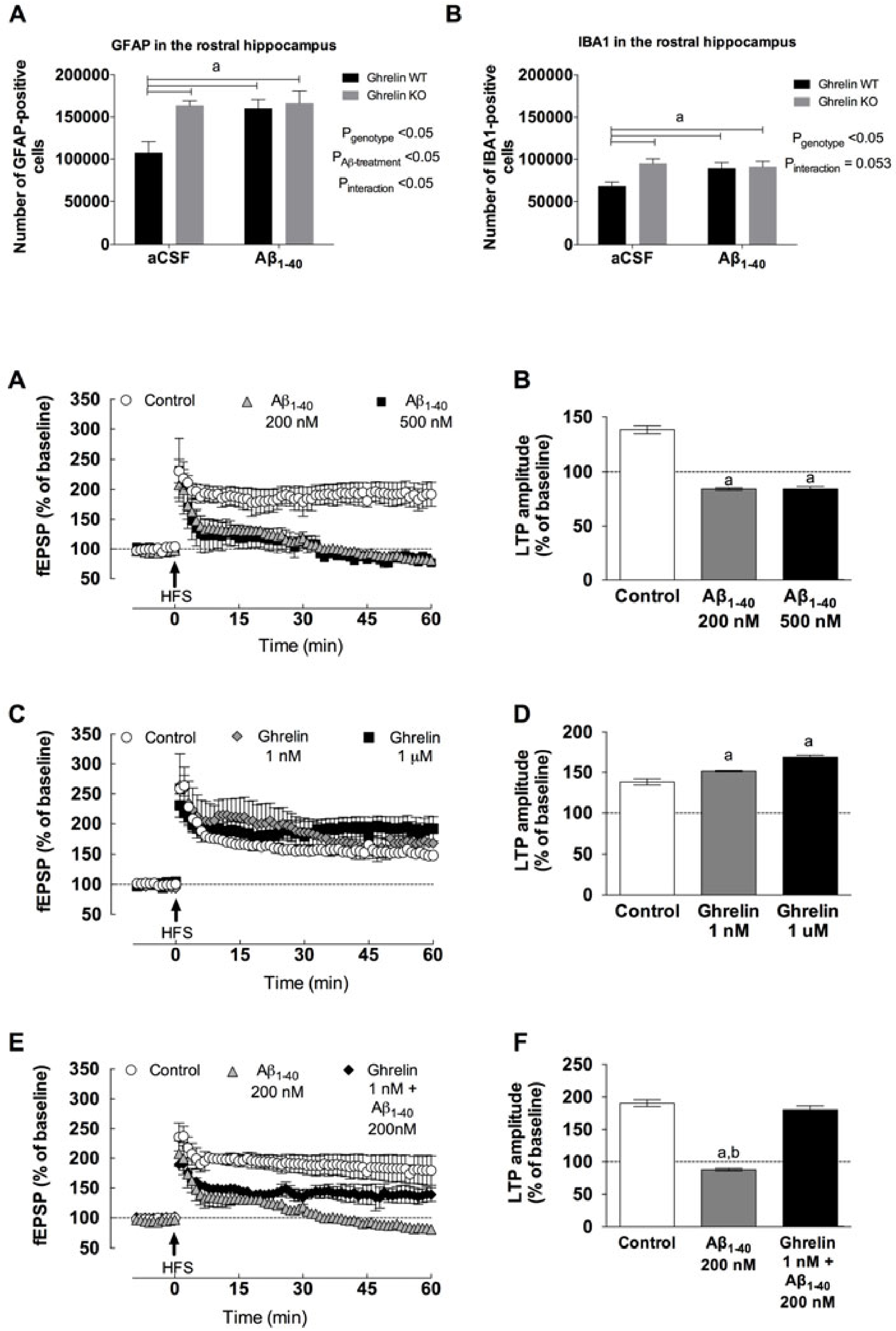

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