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# Elevated Ghrelin Promotes Hippocampal Ghrelin Receptor Defects in Humanized Amyloid-β Knockin Mice During Aging

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## Abstract

**Background:** Emerging evidence has revealed that dysregulation of the hormone ghrelin and its receptor, growth hormone secretagogue receptor (GHSR), contributes to the pathogenesis of Alzheimer's disease (AD). Specifically, defective GHSR function and resultant hippocampal ghrelin resistance are linked to hippocampal synaptic injury in AD paradigms. Also, AD patients exhibit elevated ghrelin activation. However, the detailed molecular mechanisms of hippocampal GHSR dysfunction and the relevance of ghrelin elevation to hippocampal ghrelin resistance in AD-relevant pathological settings are not fully understood.

**Objective:** In the current study, we employed a recently established mouse line of AD risk [humanized amyloid beta knockin (hAβ KI mice), also referred to as a mouse model of late-onset AD in previous literature] to further define the role of ghrelin system dysregulation in the development of AD.

**Methods:** We employed multidisciplinary techniques to determine the change of plasma ghrelin and the functional status of GHSR in hAβ KI mice as well as primary neuron cultures.

**Results:** We observed concurrent plasma ghrelin elevation and hippocampal GHSR desensitization with disease progression. Further examination excluded the possibility that ghrelin elevation is a compensatory change in response to GHSR dysfunction. In contrast, further *in vitro* and *in vivo* results show that agonist-mediated overstimulation potentiates GHSR desensitization through enhanced GHSR internalization.

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CONFLICT OF INTEREST

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SUPPLEMENTARY MATERIAL

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**Conclusions:** These findings suggest that circulating ghrelin elevation is a pathological event underlying hippocampal GHSR dysfunction, culminating in hippocampal ghrelin resistance and resultant synaptic injury in late-onset AD-related settings.

### Keywords

Alzheimer's disease; amyloid-β; ghrelin; growth hormone secretagogue receptor; hippocampal synaptic injury

## INTRODUCTION

Patients with Alzheimer's disease (AD) demonstrate loss in hippocampal synaptic strength, resulting in progressive memory deficits [1, 2]. A recently identified hippocampal pathology associated with AD is blunted response of growth hormone secretagogue receptor (GHSR, also known as ghrelin receptor) to agonist-induced activity [3, 4]. This functional defect of GHSR causes hippocampal ghrelin resistance, leading to impaired synaptic plasticity and transmission. The significance of hippocampal GHSR signaling dysfunction in AD pathogenesis is not only endorsed by its contribution to hippocampal failure in AD paradigms but also supported by AD-like hippocampal synaptic injury and cognitive impairment in mice deficient in GHSR [3] or ghrelin [5]. So far, despite the deleterious effect of amyloid-β (Aβ) on GHSR [3], the precise molecular mechanisms of hippocampal GHSR dysfunction and resultant ghrelin resistance in AD-relevant pathological settings are not yet fully understood.

The hormone ghrelin, also referred to as acyl-ghrelin in the literature, is a mainly stomach-produced acylated polypeptide that passes the blood-brain barrier (BBB) to activate GHSR in multiple brain regions including the hippocampus [5, 6], where GHSR is abundantly expressed [7, 8]. Despite its best-known actions to stimulate food intake and body weight gain [9], ghrelin-dependent GHSR signaling is pivotal in modulating hippocampal synaptic strength [5, 10, 11] and neurogenesis [12–14], thus regulating multiple types of hippocampal functions including spatial and aversive memories as well as conditioned feeding behavior [3, 5, 15]. In contrast to dysfunctional GHSR, a previous clinical study reported elevated ghrelin and its negative association with cognitive performance in patients even at the early stage of AD [16]. At face value, simultaneous high ghrelin levels and impaired GHSR function in AD-relevant pathological settings, represents a conundrum. Yet, the irresponsiveness of GHSR to its agonist may support a state of ghrelin resistance, which underpins a hypothesis that ghrelin elevation may reflect an adaptive change to compensate for GHSR dysfunction in AD [3]. However, it also can be argued that the high ghrelin has a harmful influence on GHSR, leading to GHSR exhaustion due to loss of vacillatory ligand stimulation. Because both ideas sound plausible but lack support from experimental evidence, it is, therefore, of great interest to delineate the impact of circulating ghrelin elevation on hippocampal GHSR function and the relevance of circulating ghrelin dysregulation to hippocampal ghrelin resistance in AD-related conditions.

Here, we report circulating ghrelin elevation and hippocampal GHSR defects that concurred with disease progression in humanized amyloid beta knockin (hAβKI) mice, a recently

established mouse model of late-onset AD [17]. In contrast to no influence of functional GHSR availability on ghrelin regulation, further results show that agonist-mediated overstimulation promotes GHSR desensitization through enhanced GHSR internalization. Therefore, these findings suggest that circulating ghrelin elevation is a pathological event underlying hippocampal GHSR dysfunction, culminating in hippocampal ghrelin resistance and resultant synaptic injury in late-onset AD-related settings.

## MATERIALS AND METHODS

### Animal studies

Mice studies were approved and performed following the guidelines of the University of Kansas Institutional Animal Care and Use Committee (IACUC) and National Institutes of Health (NIH). B6N(Cg)-App$^{tm1.1Aduci/J}$ (hAβ KI) and nontransgenic (nonTg) C57BL/6NJ mice were purchased from Jackson Laboratory. GHSR-null mice were obtained from UT Southwestern Medical Center [18]. GHSR null mice on a C57BL/6N genetic background were backcrossed with C57BL/6NJ mice at least 10–12 times to generate GHSR null mice on a C57BL/6NJ genetic background, which were used in this study. Genotypes of mice were confirmed by PCR. Mouse numbers used in this study were calculated based on previous results and power analysis.

Non-transgenic (nonTg) mice were treated with vehicle or 1 mg/kg MK 0677 (Tocris, #5272) via intraperitoneal (IP) injection for 30 days and then proceeded to GHSR and synaptic function analysis.

Mice at desired ages were fasted for 8 h and then proceeded to whole blood collection and brain dissection. Submandibular blood collection was performed, and the blood were collected into ice-cold EDTA-coated tubes. Plasma was prepared by centrifuging the whole blood samples for 15 min at 1,500×g, at 4°C. Protease inhibitor cocktail (Millipore Sigma, #20–201) and PMSF (Fisher Scientific) were added to all plasma samples. Plasma samples for ghrelin assays were further prepared by adding HCl to a final concentration of 0.1 N HCl and stored in −80°C for later use.

Fourteen-month-old and 24-month-old hAβ KI mice, adult GHSR null mice and age- and gender-matched nonTg mice were subjected to ELISA and chemical tests. HCl-free plasma samples were used for assays including LEAP2 ELISA (EK-075-50, Phoenix Pharmaceuticals) and Glucose (TR15421, Thermo Fisher). Plasma with 0.1N HCl were used for ghrelin assays: total ghrelin ELISA (EZRGRT-91K, Millipore Sigma) and acylated ghrelin ELISA (EZRGRA-90K, Millipore Sigma). All assays were performed according to the user manual. Data were collected and analyzed using Biotek Neo2 microplate reader.

### Neuron culture and treatment

Mouse hippocampal neurons were cultured as previously described [19]. Whole mouse hippocampi were dissected from postnatal day 0–1 pups in cold HBSS (Corning). Cells were dissociated using 0.025% trypsin with 37°C 15 min incubation followed by 10 times homogenization in ice-cold HBSS. Dissociated cells were then passed through a 100 μm cell strainer (Corning) and centrifuged for 5 min at 210×g. The pellet was gently resuspended

in neuron culture medium (Neurobasal A with 2% B27 supplement, 0.5 mM L-glutamine, Invitrogen) and plated on poly-D-lysine (Sigma-Aldrich) coated Lab-Tek chamber slides (Nunc, 177445) with appropriate densities.

At 21 days *in vitro* (DIV), hippocampal neurons were exposed to synthetic mouse ghrelin (Phoenix Pharmaceuticals) for 5 min or 24 h. The exposure was followed by immunostaining to examine the effects of GHSR short-term or long-term activation on synaptic function as described in the immunocytochemistry section.

## Immunocytochemistry

Frozen tissue sections were prepared as previously described [4]. Mouse brains were dissected and fixed in 4% paraformaldehyde (PFA, Sigma-Aldrich) overnight at 4°C. Brain blocks were prepared using Leica cryostat and stored in −80°C until using. Primary cultured hippocampal neurons on a Lab-Tek chamber slides were fixed in 4% PFA for 30 min at 37°C. After blocking (5% goat or donkey serum (Sigma-Aldrich), 0.3% Triton X-100 (Fisher Scientific) in PBS, pH 7.4), brain slices or cultured neurons were incubated with our previously validated primary antibodies against GHSR (Santa Cruz Biotechnology, #sc-10359, 1 : 100), DRD1 (Abcam, #ab81296, 1 : 200), PSD 95 (CST, #3450, 1 : 400), VGLUT1 (SYNAPTIC SYSTEMS, #135304, 1 : 400), Phospho-CaMKII (Thr286) (CST, #12716, 1 : 200), MAP2 (Sigma-Aldrich, #M4403, 1 : 300) in mixture or separately as we previously described [3, 20]. After washing with PBS, the slices or cultured neurons were probed with appropriate cross-adsorbed secondary antibodies conjugated to Alexa Fluor 488, Alexa Fluor 594, or Alexa Fluor 647 (Thermo Fisher Scientific, 1 : 500). Nuclear were labeled with diamidino-2-phenylindole (DAPI, Thermo Fisher, #62248). Images were collected on a Nikon confocal microscope. Mean intensity or the overlap of different staining were analyzed using Nikon-Elements Advanced Research software accordingly.

Cell surface GHSR in cultured hippocampal neurons was labeled with 15 min light fixation in 2% PFA at 4°C, followed by overnight GHSR1a antibody (Santa Cruz Biotechnology, #sc-10359, 1 : 100) incubation at 4°C. Images were then collected on a Nikon confocal microscope.

## Duolink in situ assay

Protein interactions between GHSR/DRD1 and GHSR/β-arrestin 2 in mouse brain slices and hippocampal neuron cultures were detected using Duolink Proximity Ligation Assay (PLA) detection kits (Sigma-Aldrich, #DUO92008) following manufacturer's instructions. The following primary antibodies were used in proper combinations: goat-anti-GHSR (Santa Cruz Biotechnology, #sc-10359, 1 : 100), rabbit-anti-DRD1 (Abcam, #ab81296, 1 : 200), mouse-anti -arrestin 2 (Santa Cruz, #sc-13140). The specificity of antibodies to GHSR and DRD1 was validated as previously described [3]. The following Duolink *in Situ* PLA Probes were used: anti-Rabbit PLUS (Sigma-Aldrich, #DUO92002), anti-Goat MINUS (Sigma-Aldrich, #DUO92006), anti-Mouse PLUS (Sigma-Aldrich, #DUO92001). Images were collected on a Nikon confocal microscope. PLA-positive dot number was counted using Nikon-Elements Advanced Research software.

### Cell membrane isolation and membrane blotting

Mouse hippocampal cell membrane blot was performed using a previously published protocol [21]. Mouse hippocampal tissues from 14- and 24-month-old hAβ KI and nonTg were homogenized and incubated in ice-cold isolation buffer (50 mM Tris-HCl, pH 7.4, 1 mM $MgCl_2$, 0.5 U/μl benzonase) for 10 min. Hippocampal cell membranes were isolated and washed in PBS for three times 10 min centrifugation at 16,500×g. Purified hippocampal cell membranes were then fixed in 4% PFA for 0.5 h followed by 1 h blocking (5% donkey serum, 0.3% Trition-X-100, PBS, pH 7.4). Hippocampal cell membrane was incubated in primary goat-anti-GHSR1a antibody (Santa Cruz Biotechnology, #sc-10359, 1 : 100) overnight at 4°C. Cell membranes were washed with PBST (PBS containing 0.05% Tween-20) for three times and then incubated with anti-goat HRP-conjugated secondary antibody (Sant Cruz) at room temperature for 1 h. Cell membrane proteins were then extracted using urea buffer (50 mM Tris-HCl, 8 M urea, 2% SDS, 10% glycerol, pH 6.8) and loaded onto a nitrocellulose membrane (NC, Bio-Rad) and allowed to dry completely before imaging. The dried NC membrane was subjected to imaging immediately using Bio-Rad Chemidoc Imaging System with signal developed using enhanced chemiluminescent substrate (ECL, Thermo Fisher). The membrane was re-probed with Rabbit anti-pan cadherin (CST, #4068, 1 : 1,000) to normalize protein level.

### Statistical analysis

Statistical analyses were performed using Graph-Pad Prism 9 software. Unpaired two-way Student's *t* test was applied in data analysis. The data collected from mouse studies were presented as interleaved box & whiskers box graphs displaying median as a line within the box, interquartile range (IQR) as the box, 95% CI as bars flanking the box, all data points showed on the graphs. Significance was concluded when the $p$ value was less than 0.05. The statistical significance was indicated by $*p < 0.05$, $**p < 0.01$, $***p < 0.001$.

## RESULTS

### Circulating ghrelin is elevated in aged hAβ KI mice

Plasma samples were collected from hAβ KI mice and their nontransgenic (nonTg) controls at 14 and 24 months old as hAβ KI mice demonstrate no cognitive deficits at 14 months old and evident cognitive deficits at 24 months old, respectively [17]. In contrast to the comparable ghrelin levels in hAβ KI and nonTg mice at 14 months old (Fig. 1A), increased plasma ghrelin was detected in old hAβ KI mice as compared with their nonTg counterparts (Fig. 1B). In contrast to the age-dependent changes of ghrelin, the levels of plasma total ghrelin, which is composed of both ghrelin and its deacylated form, remained constant in hAβ KI mice throughout the tested ages (Fig. 1C, D). Liver-expressed antimicrobial peptide 2 (LEAP2) is a recently identified endogenous antagonist of GHSR that counterbalances ghrelin's effect [22, 23]. In view of the importance of LEAP2 and ghrelin balance to ghrelin sensitivity [24], we next performed ELISA assays for plasma LEAP2 and determined no difference in LEAP2 between hAβ KI mice and their nonTg controls at either tested age (Fig. 1E, F). Of note, comparable body weight (Supplementary Figure 1A, B) and blood glucose (Supplementary Figure 1C, D) were determined in hAβ KI mice and their nonTg counterparts at both tested ages, ruling out the impact of metabolic status on ghrelin

regulation. Moreover, in addition to comparable levels of ghrelin, total ghrelin, and LEAP2 between female and male nonTg mice, hAβ mice did not display any sex effect on these parameters at the tested ages (Supplementary Figure 2A–C). Collectively, these results suggest ghrelin elevation is a phenotypic change in a late-onset AD-related setting, which agrees with a previous report of increased ghrelin in patients in the early stage of late-onset AD [16].

## Hippocampal GHSR is impaired in aged hAβ KI mice

To determine whether ghrelin changes accompany GHSR deregulation in hAβ KI mice, hippocampal slices from age- and sex-matched hAβ KI and nonTg mice at 14 and 24 months old were subjected to immunostaining for GHSR expression in neurons in the CA1 area, which is an AD-sensitive brain region [25]. Further analysis showed no quantitative difference in GHSR between the two types of mice at a younger age (Fig. 2A). However, a reduction in hippocampal GHSR expression was determined in aged hAβ KI mice as compared with their nonTg counterparts (Fig. 2B). Like many other G protein-coupled receptors (GPCRs), ligand-stimulated GHSR undergoes β-arrestin 2-mediated internalization for recycling or degradation [26]. To this end, we performed Duolink proximity ligation assay (PLA) for the interaction of GHSR with β-arrestin 2. In contrast to no genotypic change in young hAβ KI mice (Fig. 2C), augmented β-arrestin 2/GHSR complexation demonstrated by increased Duolink PLA-positive dots in hippocampal CA1 neurons was determined in 24-month-old hAβ KI mice (Fig. 2D). To determine whether loss of GHSR has a pathological consequence that undermines GHSR's function in modulating synaptic strength, we examined GHSR heteromerization with hippocampal dopamine receptor D1 (DRD1), a pivotal mechanism of GHSR-related regulation of hippocampal synaptic activity via activating calcium/calmodulin-dependent protein kinase type II (CamKII) [10]. Duolink PLA assay for GHSR/DRD1 complexes in the hippocampal CA1 region was performed using hippocampal slices from hAβ KI and nonTg mice at 14 and 24 months old. Consistent with the changes of GHSR expression in hAβ KI mice, loss of hippocampal GHSR/DRD1 complexes was not determined in young hAβ KI mice (Fig. 2E) but became evident with mouse aging (Fig. 2F). Moreover, the unchanged expression of DRD1 in the hippocampal CA1 regions in hAβ KI mice at either tested age (Supplementary Figure 3A, B) indicates that the impaired GHSR/DRD1 heteromerization is possibly, to a large extent, due to GHSR deregulation. Echoing the disrupted hippocampal GHSR/DRD1 interaction, an age-dependent decrease in hippocampal neuronal CamKII activation demonstrated by reduced phosphorylation modification was determined (Fig. 2G, H) alongside reduced synaptic density in the CA1 region (Fig. 2I, J) in hAβ KI mice. Therefore, the concurrent deregulation of ghrelin and hippocampal GHSR indicates attenuated GHSR response to its agonist and implicates a potential association between ghrelin elevation and hippocampal ghrelin resistance with disease progression in the mouse model of late-onset AD.

## GHSR loss-of-function has no impact on ghrelin

If we can draw an analogy between ghrelin and insulin resistance, we anticipate seeing increased circulating ghrelin in mice devoid of GHSR function, alike compensatory hyperinsulinemia in response to peripheral insulin receptor dysfunction [27]. To this end,

we adopted adult mice with GHSR deficiency (GHSR null mice) (Fig. 3A) and performed ELISA assays for ghrelin, total ghrelin, and LEAP2 in plasma samples from GHSR null mice and their age- and sex-matched nonTg littermates. Data analysis showed no difference in the tested parameters including ghrelin (Fig. 3B), total ghrelin (Fig. 3C), or LEAP2 (Fig. 3D) between the two groups of mice. The unchanged ghrelin in GHSR-deficient mice agrees with previous findings [28, 29], indicating no impact of GHSR functional status on ghrelin. Of note, previous studies have determined that diet-induced obesity is associated with reductions in plasma ghrelin [24, 30–32]. Yet, we measured mouse body weight and found that the tested GHSR-null and nonTg mice demonstrated comparable body weights (Fig. 3E), corroborating a previous observation with standard chow-fed male GHSR null mice [18]. These results together contradict the hypothesis that ghrelin elevation is a compensatory change in response to GHSR loss-of-function in AD-related conditions.

## Persistent ghrelin stimulation induces GHSR desensitization in hippocampal neurons

To further delineate the relationship between ghrelin and GHSR deregulation in AD-relevant pathological settings, we then asked whether plasma ghrelin elevation is a cause of GHSR loss-of-function. It is a well-documented notion that short-term ligand exposure regulates GPCRs in a multi-step process including internalization, desensitization, and resensitization; while long-term intense ligand-mediated activation leads to GPCR internalization followed by degradation [26]. Therefore, it would be of great interest to determine the impact of such a ghrelin-mediated overstimulation on the regulation and function of GHSR in hippocampal neurons. Primary hippocampal neuronal cultures exposed to transient 5-min treatment of ghrelin at 0, 1, and 10 μM exhibited a dose-dependent reduction in their surface GHSR determined by immunostaining (Fig. 4A). Further Duolink PLA for the interaction of GHSR with β-arrestin 2 showed increased GHSR/β-arrestin 2 complexation (Fig. 4B), implicating neuronal GHSR's response to transient ghrelin stimulation. To determine whether transient ligand-mediated activation promotes the synapse-modulating effect of GHSR, we examined the activation status of CamKII by immunostaining and detected ghrelin-induced CamKII activation demonstrated by increased CamKII phosphorylation modification (Fig. 4C). Accordingly, primary cultured hippocampal neurons showed increased synaptic density (Fig. 4D), which agrees with the well-defined synaptogenesis-promoting effect of ghrelin [5, 33]. When challenged by 24 h exposure of ghrelin at varying doses including 0, 1, and 10 μM, hippocampal neurons displayed greater responses to ghrelin-induced reduction in neuronal surface GHSR (Fig. 4E) and an increase in GHSR complexation with β-arrestin 2 (Fig. 4F). However, long-term ghrelin-challenged neurons exhibited irresponsiveness to ghrelin-induced CamKII activation (Fig. 4G), which concurred with impaired ghrelin-elicited synaptogenesis (Fig. 4H), implicating blunted GHSR signaling. To test the detrimental influence of agonist-mediated overstimulation on hippocampal GHSR in an *in vivo* setting, we treated wildtype (wt) mice with vehicle or 1 mg/kg MK 0677, a synthetic mimetic of ghrelin, via daily intraperitoneal (i.p.) injection for 30 days. Dot blotting assay using isolated cell membrane fractions from hippocampal tissues showed a reduction in cell membrane-bound GHSR in MK 0677-treated mice (Fig. 4I), indicating GHSR overstimulation-induced GHSR loss. Further immunostaining for synaptic density hippocampal CA1 region showed a marginal decrease in MK 0677-treated mice as compared with their vehicle-treated counterparts (Fig. 4J), suggesting diminished response of hippocampal neurons to GHSR

agonist-induced synaptogenesis. Put together, these findings from *in vitro* and *in vivo* settings support the deleterious impact of persistent ghrelin stimulation on GHSR regulation, leading to GHSR desensitization and ghrelin resistance in hippocampal neurons.

## DISCUSSION

The hippocampus is a pivotal brain region for memory storage and processing [34]. Accordingly, hippocampal synaptic injury constitutes a characteristic pathology underlying memory loss in multiple types of dementias including AD [35, 36]. So far, the detailed molecular mechanisms of AD-related hippocampal deficits are not fully understood. In addition to their well-documented function in maintaining energy homeostasis [6, 37–39], ghrelin and GHSR are critical for the regulation hippocampal synaptic physiology [5, 10, 11]. Emerging evidence suggests deregulation of hippocampal GHSR signaling and its close association with hippocampus-related memory deficits in AD-relevant pathological settings [3, 4]. A prominent defect of hippocampal GHSR in AD-related conditions is blunted response of GHSR to its agonist-induced activation, supporting the presence of ghrelin resistance in AD hippocampi [3, 4]. However, other than the deleterious impact of the interaction between GHSR and Aβ, a key mediator of AD [40] on GHSR signaling [3, 4], the precise pathways causing hippocampal ghrelin resistance in this neurodegenerative disorder have not yet been fully depicted. In this study, we newly determined a detrimental effect of high circulating ghrelin in promoting hippocampal GHSR desensitization via enhanced internalization and degradation in humanized Aβ knockin mice, a mouse model of late-onset AD [17]. These findings corroborate our previous report of increased ghrelin in patients at the early stage of late-onset AD [16], implicating ghrelin dysregulation is a phenotypic change accompanying AD, at least, in a late-onset AD-related setting. Notably, despite their similarities in clinical and pathological features, the late-onset sporadic and early-onset familial AD exhibit differences in the course of disease and several key components including genetics, aging, and other risk factors in the etiopathogenesis [41–45]. To this end, it would be of great interest to examine whether ghrelin dysregulation follows suit in early-onset familial AD and animal models carrying familial AD-associated genetic causes, which requires further investigation. In addition, a sex difference has been implicated in the development of AD [46]. Previous studies have shown a sex-related difference in the baseline levels of ghrelin in adults [47] and a sex-related difference in the response to sex hormone-elicited ghrelin changes in peripubertal children [48], indicating the sex effect on ghrelin regulation. However, postmenopausal women, with the sharp decrease in female sex hormones, demonstrate a close association of ghrelin with testosterone, which diminishes the sex difference in circulating ghrelin [49]. These findings indicate an age effect on the patterns of sex-related ghrelin regulation and further address our observations of no sex effect on ghrelin in the tested mice at old ages. In this regard, we cautiously postulate that ghrelin dysregulation may have deleterious impact on patients with late-onset AD regardless of their sexes, which also instigates our interest to investigate the impact of ghrelin dysregulation on patients with early-onset AD, who develop AD symptoms early in life, in our future study.

Our current observations of the deleterious impact of ghrelin elevation on GHSR agree with previous reports of GHSR desensitization due to agonist-induced GHSR overstimulation

[50] and thus have further deepened our understanding of ghrelin system deregulation in AD pathogenesis. In fact, such an agonist-induced receptor deactivation is not unique to GHSR and frequently occurs to many other types of GPCRs. For instance, it is a well-recognized clinical problem in the management of Parkinson's disease (PD) that PD patients under the long-term treatment of dopamine receptor agonists such as levodopa demonstrate the "wearing-off" phenomenon due to increased dopamine receptor desensitization via internalization, resulting in the reemergence of symptoms [51–53]. Indeed, most GPCRs follow a sequence of agonist-induced activation, internalization/desensitization, and resensitization in physiology [26]. However, the deleterious impact of ghrelin overactivation-induced GHSR defects determined in this study together with the aforementioned levodopa-induced "wearing-off" phenomenon [51–53] support the previously documented notion that the physiological regulation of GPCRs is disrupted when they are persistently activated by loss of vacillatory ligand stimulation, eventually leading to receptor exhaustion [26]. Another outstanding example to reflect the different regulation of GPCRs in response to agonist-induced transient activation versus continuous overstimulation is the clinical applications of leuprolide, a synthetic analogue of gonadotropin-releasing hormone (GnRH) [54]. In contrast to the luteinizing hormone (LH)- and follicle stimulating hormone (FSH)-promoting effects of transient use of the drug, long-term administration of leuprolide, which demonstrates clinical benefits for the management of sex hormone-sensitive cancers such as the breast and prostate cancers, desensitizes gonadotropin-releasing hormone receptor (GnRHR), thus leading to LH and FSH suppression [54–57]. Therefore, together with the deleterious impact of ghrelin deficiency on hippocampal function [5], our findings support that ghrelin overactivation is an AD-associated pathological event that adversely induces GHSR desensitization, at least, in the hippocampus and further highlight the importance of ghrelin homeostasis to hippocampal fitness.

Of note, previous findings [16] and our observation have raised an interesting question of the mechanisms of disrupted ghrelin homeostasis towards overactivation in AD-related conditions. Consistent with the previous report in patients [16], we did not find any change in total ghrelin in aged hAβ KI mice, suggesting unaffected production of ghrelin. To this end, the most possible reasons for ghrelin abnormality are increased ghrelin activation via acylation or decreased ghrelin deactivation via deacylation or both in AD-related conditions. GOAT, also known as membrane bound O-acyltransferase domain containing 4, which is encoded by the *MBOAT4* gene, is so far the only determined specific enzyme that activates ghrelin through a post-translational acylation modification [58–60]. However, whether the expression and/or activity of GOAT in AD is changed is, to date, poorly investigated and thus endorses our future studies on the functional status of GOAT in this neurodegenerative disorder. In contrast to the explicit pathway of ghrelin activation, the mechanisms of ghrelin deacylation still remain elusive. Butyrylcholinesterase (BChE) is a determined major enzyme responsible for ghrelin deacylation [61]. An association of BChE with AD pathogenesis has been suggested. In addition to the intertwined relationship between BChE, amyloid plaques [62], iron deregulation [63] and apolipoprotein E (ApoE) [64] in AD-relevant pathological settings, a genetic variant of *BChE*, which impairs BChE expression and activity, has been arguably associated with AD risk [65–67]. In this context, we cannot fully exclude the possibility that BChE may play a role in promoting ghrelin

deregulation in AD. Furthermore, there is increasing recognition of the interactions between ghrelin system and AD-associated pathological molecules including Aβ and pathological tau [3, 68, 69]. Although whether Aβ and/or tau may affect ghrelin regulation remains uncharacterized, given the presence of circulating Aβ and pathological tau in AD patients [70–72], we cannot refute the possibility that Aβ and/or pathological tau may exert influence on ghrelin activation and deactivation in direct or indirect manners. All these outstanding questions need to be addressed in further comprehensive studies.

Lastly, the neurotrophic property of ghrelin [5, 6, 13, 33, 73] has promoted the research interest in the therapeutic potential of ghrelin or its synthetic mimetics for the management of AD. [74, 75]. However, current attempts to treat AD using ghrelin or its mimetics have shown mixed results. In contrast to a protective effect of ghrelin or its mimetics in mitigating brain pathology in some mouse experiments [76–79], studies of both ours and others reported opposite results [3, 4, 80]. Indeed, debate on the therapeutic potential of ghrelin for AD treatment may have been resolved by a 12-month large-scale clinical trial which used MK 0677, a synthetic ghrelin mimetic to treat AD patients in different stages and reported no clinical benefit [81]. Our current findings of ghrelin elevation and its deleterious impact on hippocampal GHSR have provided further experimental evidence against the usefulness of ghrelin and its mimetics for AD management. In fact, previous reports of the poor performance of ghrelin mimetics supplementation [3, 4, 81] and our current study support that ghrelin system-targeting strategies solely using ghrelin or its mimetics should be avoided from AD treatment regimen.

In summary, our current study suggests that ghrelin elevation is not a beneficial but rather a pathological phenomenon that undesirably contributes to hippocampal GHSR dysfunction and resultant ghrelin resistance, leading to impaired GHSR signaling-dependent hippocampal synaptic modulation in AD-relevant pathological settings. The unaffected ghrelin regulation in mice with loss of GHSR function further refutes the possibility that ghrelin elevation is an adaptive change to compensate for GHSR dysfunction in AD. It should be noted that our recent study showed imbalanced ghrelin/LEAP2 towards LEAP2 effect and its association with age-associated cognitive decline in nondemented elderly and aging mice [20], representing another form of ghrelin system dysregulation in an aging setting. However, such a normal aging-related phenotype was not seen with hAβ KI mice although aged hAβ KI mice displayed a slight but not statistically significant increase in their LEAP2 levels. A possible explanation is that the mice used in this study were not adequately aged to 30 months old as we used before to demonstrate the age effect. In addition, we cannot rule out the possibility that ghrelin elevation, which is not an age-but AD-related change, may interfere with LEAP2 and break ghrelin/LEAP2 balance towards ghrelin effect in AD-related conditions. In this regard, although normal aging and dementia share hippocampal GHSR dysfunction in common, the two scenarios may have distinct mechanisms causing GHSR changes. This symbolizes the difference in the pathophysiology between normal and pathological aging and signifies the diagnostic potential of ghrelin and LEAP2 measurement in differentiating age-associated cognitive decline and memory loss in AD, especially in its early or prodromal stage. Nevertheless, our findings support the contribution of ghrelin system dysregulation, to be specific, ghrelin elevation to AD pathogenesis. Therefore, it would be of paramount importance to delineate the molecular

mechanisms of ghrelin deregulation in AD-related conditions, which will not only foster us a better understanding of ghrelin system in AD pathogenesis but also have the potential to advance the development of practical avenues to restore ghrelin homeostasis for the prevention and treatment of this devastating neurological disorder.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

## FUNDING

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## DATA AVAILABILITY

Data sharing is not applicable to this article as no datasets were generated or analyzed during this study.

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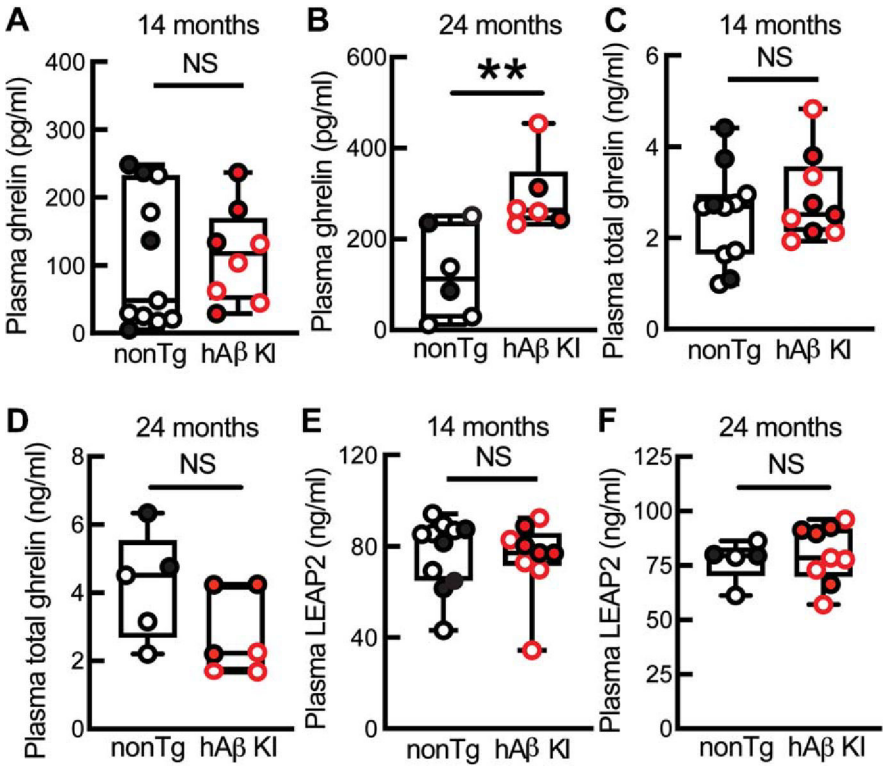


**Fig. 1.**

Elevated plasma ghrelin in 24-month-old hAβ KI mice. A, B) Plasma ghrelin level in (A) 14- and (B) 24-month-old hAβ KI mice and the nonTg controls. Unpaired two-way Student's *t* test. 14-month-old nonTg $n = 7$ males, 4 females, hAβ KI $n = 4$ males, 4 females; 24-month-old nonTg $n = 4$ males, 2 females, hAβ KI $n = 4$ males, 2 females. C, D) Plasma total ghrelin level in (C) 14- and (D) 24-month-old hAβ KI mice and the nonTg controls. Unpaired two-way Student's *t* test. 14-month-old nonTg $n = 7$ males, 4 females, hAβ KI $n = 5$ males, 4 females; 24-month-old nonTg $n = 3$ males, 2 females, hAβ KI $n = 3$ males, 3 females. E, F) Plasma LEAP2 level in (E) 14- and (F) 24-month-old hAβ KI mice and the nonTg controls. Unpaired two-way Student's *t* test. 14-month-old nonTg $n = 7$ males, 4 females, hAβ KI $n = 5$ males, 4 females; 24-month-old nonTg $n = 3$ males, 2 females, hAβ KI $n = 5$ males, 4 females. NS = not significant, $^{**}p < 0.01$. Females: filled circles, males: open circles.

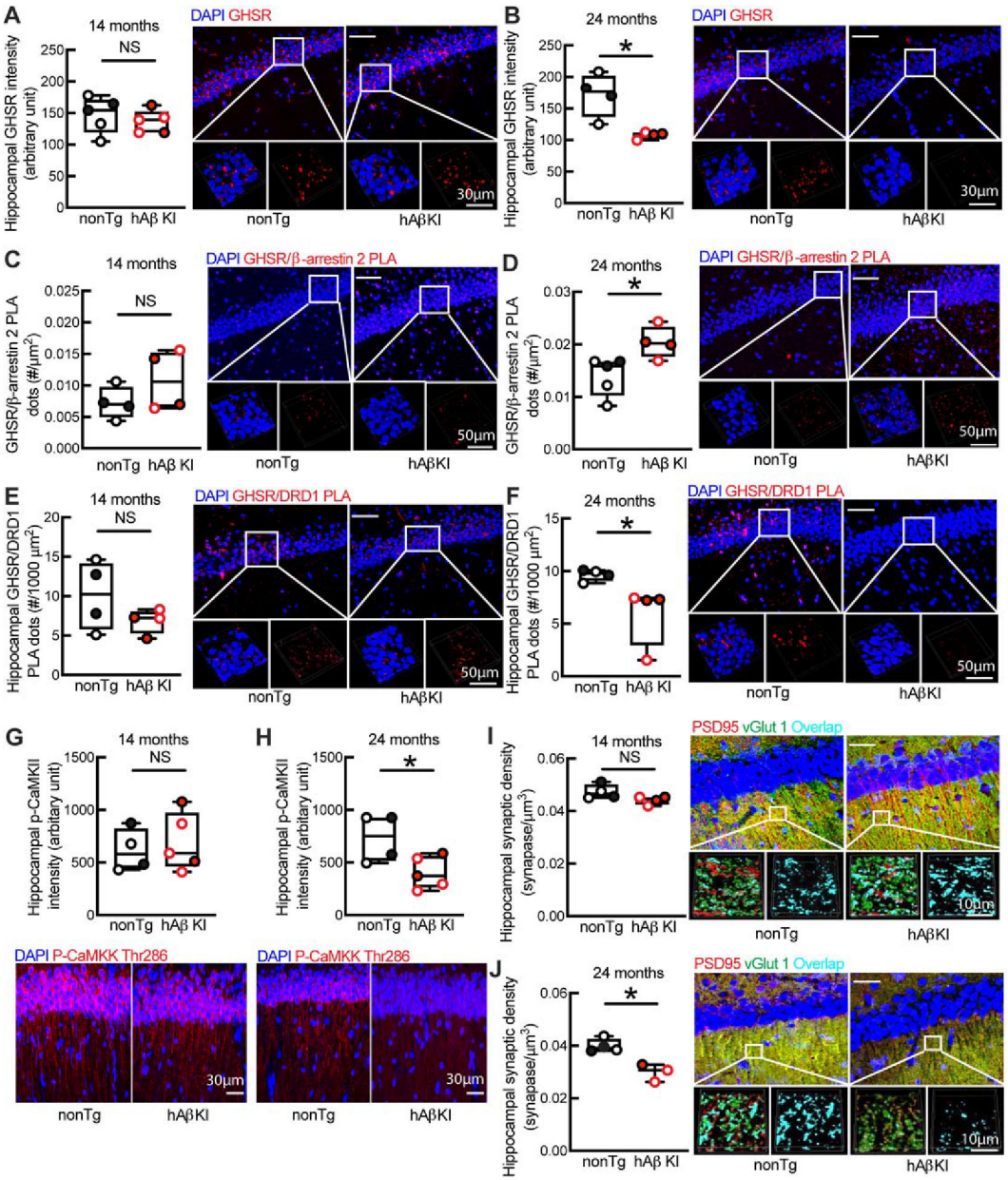


**Fig. 2.**

Deregulated hippocampal GHSR and synaptic function in 24-month-old hAβ KI mice. A, B) Hippocampal GHSR level in (A) 14- and (B) 24-month-old hAβ KI mice and the nonTg controls. Unpaired two-way Student's *t* test. 14-month-old $n = 3$ males, 2 females; 24-month-old $n = 2$ males, 2 females. Bottom panels are the representative images, scale bar = 200 μm (inset scale = 30 μm). C, D) Hippocampal GHSR/β-arrestin 2 complex level in (C) 14- and (D) 24-month-old hAβ KI mice and the nonTg controls. Unpaired two-way Student's *t* test. 14-month-old $n = 2$ males, 2 females; 24-month-old nonTg $n = 3$ males, 2 females, hAβ KI $n = 2$ males, 2 females. Bottom panels are the representative images, scale bar = 250 μm (inset scale = 50 μm). E, F) Hippocampal GHSR/DRD1 complex level in (E) 14- and (F) 24-month-old hAβ KI mice and the nonTg controls. Unpaired two-way

Student's *t* test. 14-month-old *n* = 2 males, 2 females; 24-month-old *n* = 2 males, 2 females. Bottom panels are the representative images, scale bar = 250 μm (inset scale = 50 μm). G, H) Hippocampal CamKII activation in (G) 14- and (H) 24-month-old hAβ KI mice and the nonTg controls represented by CamKII Thr286 phosphorylation. Unpaired two-way Student's *t* test. 14-month-old nonTg *n* = 2 males, 2 females, hAβ KI *n* = 3 males, 2 females; 24-month-old nonTg *n* = 2 males, 2 females, hAβ KI *n* = 3 males, 2 females. Bottom panels are the representative images, scale bar = 30 μm. I, J) Hippocampal synaptic density in (I) 14- and (J) 24-month-old hAβ KI mice and the nonTg controls represented by the overlap of presynapse marker vGlut1 and postsynapse marker PSD95. Unpaired two-way Student's *t* test. 14-month-old nonTg *n* = 2 males, 2 females, hAβ KI *n* = 2 males, 2 females; 24-month-old nonTg *n* = 2 males, 2 females; hAβ KI *n* = 2 males, 1 female. Bottom panels are the representative images, scale bar = 100 μm (inset scale = 10 μm). NS = not significant, $*p < 0.05$. Females: filled circles, males: open circles.

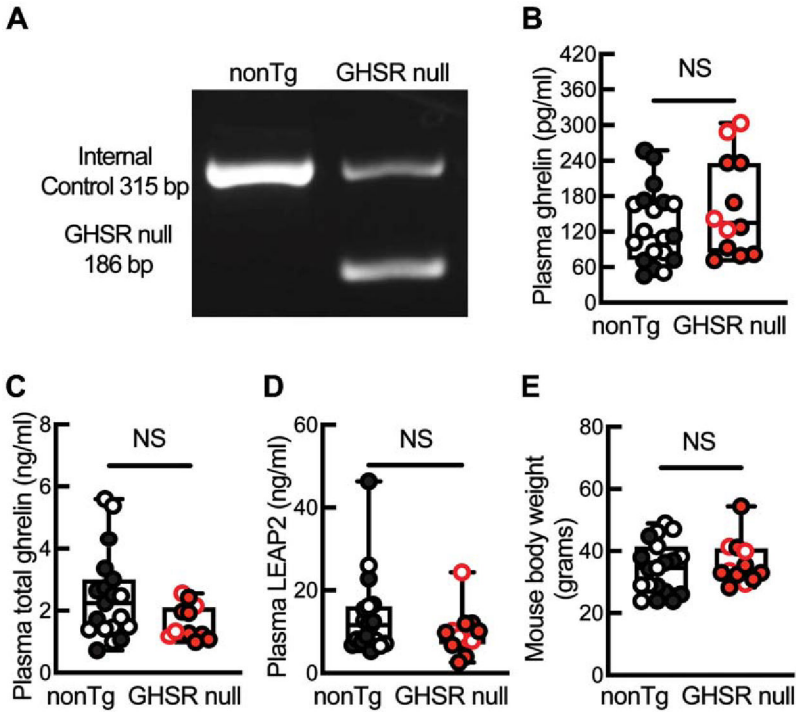


**Fig. 3.**

Unaltered ghrelin activation in GHSR null mice. A) Representative genotyping result of adult GHSR null mice and the nonTg littermate. B) Plasma activated ghrelin in GHSR null and nonTg control mice. Unpaired two-way Student's *t* test. nonTg $n = 9$ males, 10 females, GHSR null $n = 4$ males, 8 females. C) Plasma total ghrelin in GHSR null and nonTg control mice. Unpaired two-way Student's *t* test. nonTg $n = 9$ males, 10 females, GHSR null $n = 4$ males, 8 females. D) Plasma LEAP2 level in GHSR null and nonTg control mice. Unpaired two-way Student's *t* test. nonTg nonTg $n = 9$ males, 10 females, GHSR null $n = 4$ males, 8 females. E) Body weight of GHSR null and nonTg control mice. Unpaired two-way Student's *t* test. nonTg $n = 9$ males, 10 females, GHSR null $n = 4$ males, 8 females. NS = not significant. Females: filled circles, males: open circles.

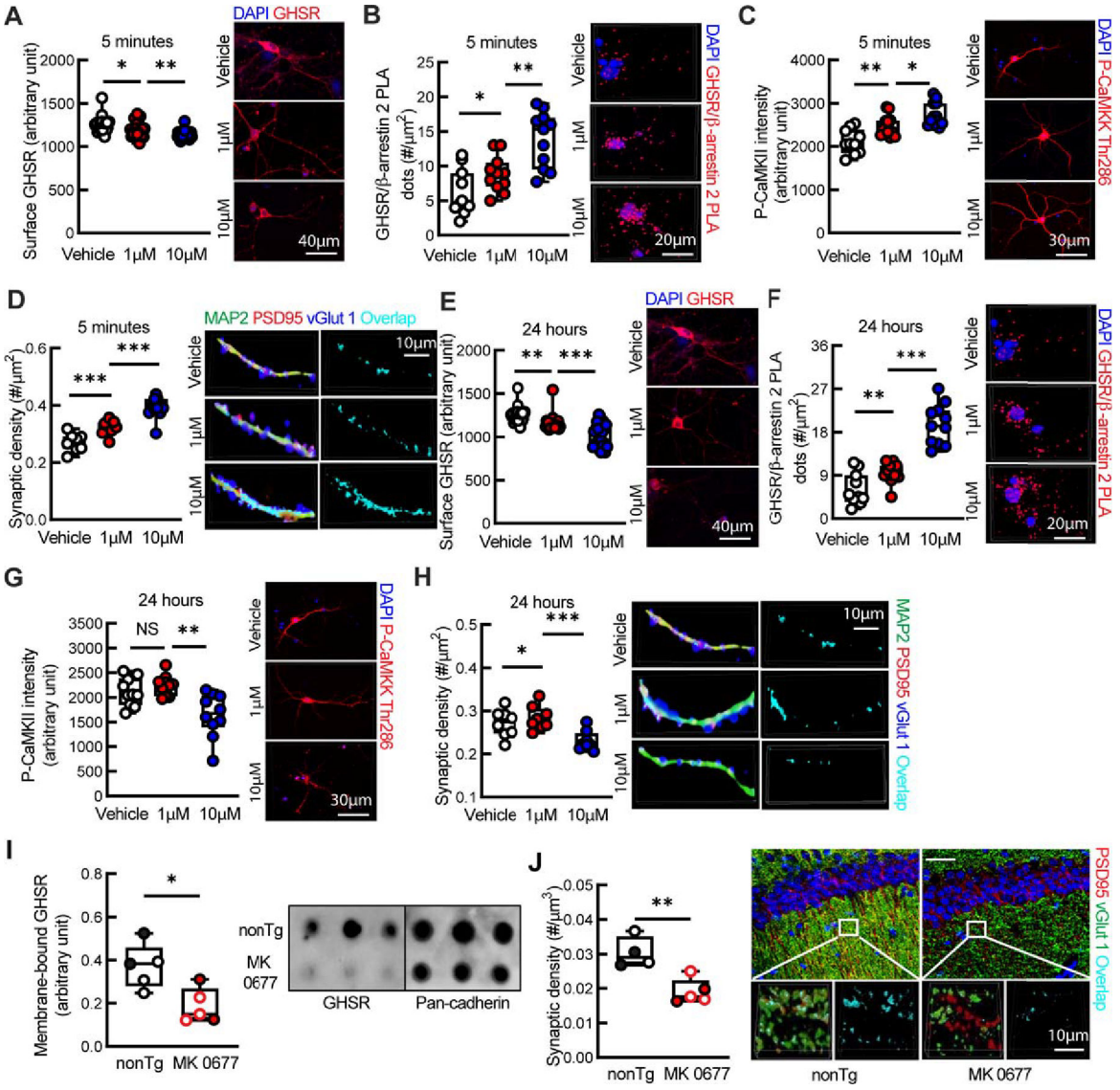


**Fig. 4.**

Long-term ghrelin treatment-induced GHSR desensitization in hippocampal neurons. A) Cell surface GHSR intensity in 5 min 1 μM or 10 μM ghrelin-treated hippocampal neurons. Unpaired two-way Student's *t* test. vehicle $n = 17$, 1 μM $n = 20$, 1 μM $n = 20$ neurons. Bottom panels are the representative images, scale bar = 40 μm. B) GHSR/β-arrestin 2 complex level in 5min 1 μM or 10 μM ghrelin-treated hippocampal neurons. Unpaired two-way Student's *t* test. $n = 11$ neurons each group. Bottom panels are the representative images, scale bar = 20 μm. C) CamKII activation in 5 min 1 μM or 10 μM ghrelin-treated hippocampal neurons. Unpaired two-way Student's *t* test. $n = 10$ neurons each group. Bottom panels are the representative images, scale bar = 30 μm. D) Synaptogenesis in 5min 1 μM or 10 μM ghrelin-treated hippocampal neurons. Unpaired two-way Student's *t* test. vehicle $n = 8$, 1 μM $n = 8$, 1 μM $n = 10$ neurons. Bottom panels are the representative images, scale bar=10 μm. E) Cell surface GHSR intensity in 24h 1 μM or 10 μM ghrelin-treated hippocampal neurons. Unpaired two-way Student's *t* test. vehicle $n = 17$, 1 μM $n =$

23, 1 μM $n = 23$ neurons. Bottom panels are the representative images, scale bar = 40 μm. F) GHSR/β-arrestin 2 complex level in 24h 1 μM or 10 μM ghrelin-treated hippocampal neurons. Unpaired two-way Student's *t* test. $n = 11$ neurons each group. Bottom panels are the representative images, scale bar = 20 μm. G) CamKII activation in 24h 1 μM or 10 μM ghrelin-treated hippocampal neurons. Unpaired two-way Student's *t* test. $n = 10$ neurons each group. Bottom panels are the representative images, scale bar = 30 μm. H) Synaptogenesis in 24h 1 μM or 10 μM ghrelin-treated hippocampal neurons. Unpaired two-way Student's *t* test. $n = 8$ neurons each group. Bottom panels are the representative images, scale bar = 10 μm. I) Hippocampal cell membrane GHSR of MK 0677- and vehicle-treated nonTg mice. Unpaired two-way Student's *t* test. $n = 3$ males, 2 females each group. Bottom panels are the representative images. J) Hippocampal synaptic density of MK 0677- and vehicle-treated nonTg mice. Unpaired two-way Student's *t* test. nonTg $n =$ 2 males, 2 females, MK 0677 $n = 3$ males, 2 females. Bottom panels are the representative images, scale bar = 100 μm (inset scale = 10 μm). NS, not significant; $*p < 0.05$, $**p < 0.01$, $***p < 0.001$. Females: filled circles, males: open circles.