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NEUROSCIENCE

Ligand-independent activity of the ghrelin receptor modulates AMPA receptor trafficking and supports memory formation

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The biological signals of hunger, satiety, and memory are interconnected. The role of the hormone ghrelin in regulating feeding and memory makes ghrelin receptors attractive targets for associated disorders. We investigated the effects of the high ligand-independent activity of the ghrelin receptor GHS-R1a on the physiology of excitatory synapses in the hippocampus. Blocking this activity produced a decrease in the synaptic content of AMPA receptors in hippocampal neurons and a reduction in GluA1 phosphorylation at Ser⁸⁴⁵. Reducing the ligand-independent activity of GHS-R1a increased the surface diffusion of AMPA receptors and impaired AMPA receptor-dependent synaptic delivery induced by chemical long-term potentiation. Accordingly, we found that blocking this GHS-R1a activity impaired spatial and recognition memory in mice. These observations support a role for the ligand-independent activity of GHS-R1a in regulating AMPA receptor trafficking under basal conditions and in the context of synaptic plasticity that underlies learning.

INTRODUCTION

Ghrelin is a peptide hormone believed to signal meal initiation (1, 2) and is found at the highest concentration in human plasma immediately before each meal (3). It is mainly secreted by X/A-like cells in the oxyntic glands of the stomach and intestine (4). Apart from effects on food intake (5) and feeding behavior (6, 7), ghrelin influences several other physiological systems (8). For example, it is well established that ghrelin improves learning and memory (9, 10), facilitates reward through its action on the mesolimbic dopamine system (11), modulates anxiety-like (9) and depressive-like (12) behaviors, and enhances long-term fear memory (13).

The actions of ghrelin are mediated by the growth hormone secretagogue receptor type 1a (GHS-R1a), a G protein-coupled receptor, whose activation by ghrelin regulates gene expression, neuronal excitability, and AMPA-type glutamate receptor (AMPA)

trafficking (10, 14–16). In the brain, GHS-R1a is highly expressed in the hypothalamus, pituitary gland, and hippocampus (17), and its expression levels increase during fasting (18–20). GHS-R1a displays unusually high constitutive activity, corresponding to about 50% of its maximal activity (21), which results from a natural shift in the equilibrium between its inactive and active conformations (22), in the absence of ligand. The ligand-independent GHS-R1a activity plays a role in the control of food intake and regulation of body weight (18, 20, 23–25) and in the acquisition of conditioned taste aversion (26). Human mutations that lead to a selective loss of constitutive activity of GHS-R1a, but that do not interfere with ghrelin-induced activation, are associated with familial short stature (27–30). A study in mice expressing a human mutation in the GHSR that impairs constitutive GHSR activity revealed that this activity contributes to the native depolarizing conductance of hypothalamic neurons and to growth hormone release (31). The recently described liver-expressed antimicrobial peptide 2 (LEAP2) is an endogenous antagonist of GHS-R1a (32), which also exhibits inverse agonist activity, blocking the ligand-independent activity of GHS-R1a (33, 34). LEAP2 plasmatic levels are lower in fasted states (32, 33), increase with body mass and blood glucose, and are higher in obesity (33), in a manner that is opposite to that of plasma acyl-ghrelin. These observations indicate that acyl-ghrelin and LEAP2 bidirectionally control ligand-dependent activity of GHS-R1a and that LEAP2 might exert endogenous control of the ligand-independent activity of GHS-R1a, which is physiologically relevant (35).

GHS-R1a knockout (KO) animals display spatial and contextual memory impairments (36, 37), which can be attributed not only to the absence of ghrelin-triggered effects but also to the loss of the ligand-independent activity. Here, using a combination of imaging, biochemical and electrophysiological approaches, and behavior analysis, we investigated the role for the ligand-independent activity of GHS-R1a and found that it provides tonic control for the regulation of AMPAR trafficking, influencing synaptic plasticity in the hippocampus and interfering with learning and memory in vivo. These

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findings should be taken into account given that inverse agonists of GHS-R1a are presently being tested in humans to treat alcohol use disorder (38, 39).

RESULTS

Ligand-independent activity of GHS-R1a in hippocampal neurons

In the absence of ligand, GHS-R1a displays unusually high constitutive activity (21), which has been shown to control food intake and body weight (18, 20, 23–25) and the acquisition of conditioned taste aversion (26). Because the activity of GHS-R1a in the absence of agonist has been associated with the G_q protein/phospholipase C (PLC)/inositol-1,4,5-trisphosphate (IP_3) pathway (21), to directly evaluate the ligand-independent activity of GHS-R1a in hippocampal neurons, we visualized PLC activation using a construct consisting of the PLC δ pleckstrin homology domain (PLC δ PH) fused to green fluorescent protein (PLC δ PH-GFP), as previously described (40). PLC δ PH-GFP favors phosphatidylinositol 4,5-bisphosphate (PIP_2) over other inositol phospholipids but has higher affinity for IP_3 than for PIP_2 (41). Therefore, PIP_2 hydrolysis by PLC causes PLC δ PH-GFP to translocate from the plasma membrane to the cytosol, where it binds respectively to PIP_2 or IP_3 . Under basal conditions, PLC δ PH-GFP is localized at the plasma membrane and along the dendritic shaft in primary cultured hippocampal neurons (*t0*; Fig. 1, A and B). Acute bath application of the nonpeptidyl GHS-R1a agonist MK-0677 caused a robust translocation of PLC δ PH-GFP into the cytosol (Fig. 1, A and C, and movie S1), reflecting the generation of IP_3 , and which was accompanied by a decrease in the plasma membrane levels of the probe (Fig. 1, A and C, and movie S1). These observations show activation of GHS-R1a-mediated signaling by the receptor agonist in hippocampal neurons. To evaluate the basal constitutive signaling of GHS-R1a, we first used [D-Arg¹, D-Phe⁵, D-Trp^{7,9}, Leu¹¹]-substance P (SP-A), a well-established GHS-R1a inverse agonist (20, 21), to block the receptor ligand-independent activity. Acute application of SP-A led to an increase in the plasma membrane levels of PLC δ PH-GFP, with no detectable changes in the cytoplasmic fluorescent signal (Fig. 1, B and C, and movie S2). To evaluate the long-term effect of the ligand-independent activity of GHS-R1a, we blocked it in PLC δ PH-GFP-transfected hippocampal neurons for 20 hours with either SP-A or with a recently described blood-brain barrier (BBB)-permeable inverse agonist of GHS-R1a, AZ12861903 (AZ), which decreases the ligand-independent activity of the receptor (25). Neurons were fixed, and the spine and dendritic shaft distribution of PLC δ PH-GFP was evaluated (Fig. 1, D and E). We observed that in neurons treated with either of the GHS-R1a inverse agonists, SP-A or AZ, there was a redistribution of PLC δ PH-GFP from the dendritic shaft to spines (Fig. 1, D and E), suggestive of spine accumulation of PIP_2 . These results indicate that the basal GHS-R1a activity in the absence of the ligand contributes to baseline hydrolysis of PIP_2 , which is blocked by SP-A and AZ, and support ligand-independent activity of GHS-R1a in hippocampal neurons.

The surface and synaptic localization of AMPA receptors is regulated by ligand-independent activity of GHS-R1a

To test whether the ligand-independent activity of GHS-R1a could provide a tonic signal in the hippocampus and regulate AMPAR trafficking, we evaluated whether inverse agonists of GHS-R1a

affect the synaptic content of AMPARs in cultured hippocampal neurons. Incubation of 15-day in vitro (DIV) hippocampal neurons with either SP-A or AZ decreased the total cell surface levels of GluA1 and the levels of cell surface GluA1-containing AMPARs colocalized with the postsynaptic protein PSD95 and the presynaptic protein VGluT1 (Fig. 2, A to D). In contrast, incubation with GHS-R1a antagonist JMV2959 did not significantly affect the total or synaptic levels of surface GluA1 (fig. S1, A and B). Similarly to GluA1, GluA2 synaptic levels were decreased in neurons incubated with SP-A (Fig. 2, E and F). However, the incubation with SP-A did not affect synapse density in 15-DIV cultured hippocampal neurons (fig. S1C), measured by the colocalization of PSD95 and VGluT1 puncta. SP-A also decreased the total surface and synaptic levels of GluA1, as well as synapse density in older neurons (20 DIV; Fig. 2, G and H, and fig. S1C), but not in 7 DIV neurons (Fig. 2, I and J, and fig. S1C), which, at this age, present lower expression levels of GHS-R1a (15).

To test whether the ligand-independent activity of GHS-R1a functionally modulates CA3-CA1 hippocampal excitatory transmission, organotypic hippocampal slices were treated with SP-A for 20 hours, and electrophysiological recordings were performed. Compared with that in control neurons, the AMPA:NMDA (*N*-methyl-D-aspartate) ratio of synaptic responses decreased significantly after treatment with SP-A but not with GHS-R1a antagonist [D-Lys³]-GHRP-6 (Fig. 2, K and L), in agreement with low levels of ghrelin in the culture and a specific role for the GHS-R1a inverse agonist in inhibiting CA3-CA1 synaptic transmission. Together, these results suggest that the ligand-independent activity of GHS-R1a regulates AMPAR levels and excitatory synaptic transmission under basal conditions.

We then silenced the expression of GHS-R1a in cultured hippocampal neurons using a short hairpin RNA (shRNA) containing a previously validated sequence (42) and tested for surface levels of GluA1. Knockdown of GHS-R1a decreased the levels of surface and synaptic GluA1, which were rescued when the shRNA was coexpressed with an shRNA-insensitive human form of the receptor (hGHS-R1a; Fig. 3, A to C). Treatment with either SP-A or AZ did not affect the total surface or synaptic levels of GluA1 in neurons where the expression of GHS-R1a was silenced (Fig. 3, A to C), confirming the specificity of SP-A and AZ in targeting the GHS-R1a. We have also tested whether a mutant form of GHS-R1a that lacks ligand-independent activity can rescue the phenotype found in neurons depleted for GHS-R1a. We have used GHS-R1a F279L, a mutant form of the GHS-R1a that was identified in a child with short stature (30). Phe²⁷⁹ in GHS-R1a was found to be critical for the constitutive signaling activity of the receptor (43). Whereas reintroduction of wild-type (WT) GHS-R1a rescued normal synaptic levels of GluA1 in neurons depleted of endogenous GHS-R1a, expression of GHS-R1a F279L did not (Fig. 3, D and E). These observations confirm the idea that the ligand-independent activity of GHS-R1a regulates the baseline synaptic level of AMPARs.

Ligand-independent GHS-R1a activity controls its ligand-dependent effect on AMPAR surface and synaptic localization

Because the ligand-independent activation of GHS-R1a promotes its own basal internalization (44), we reasoned that it could limit the agonist-induced effects in the hippocampus (15). To test this, we used 15-DIV cultured hippocampal neurons, for which no effect of

Fig. 1. Ligand-independent activity of GHS-R1a regulates PIP_2 hydrolysis in hippocampal cultured neurons. (A to C) Analysis of hippocampal neurons cotransfected with PLC δ PH-GFP and mCherry (DIV 13 to 14) and then imaged for 60 min on DIV 15 to 16 upon application (at $t=6$ min, ■ in the graph) with either GHS-R1a agonist MK-0677 [1 μM ; $n=4$ neurons (A and C)] or inverse agonist SP-A [1 μM ; $n=5$ neurons (B and C)]. Scale bars, 5 μm . Data are means $\pm$ SEM PLC δ PH-GFP fluorescence (F/F_0) in the dendritic cytoplasm and membrane, in three independent experiments. Differences in the 30- to 35.5-min period (pink) to the baseline (0 to 5.5 min) assessed by paired t test, $*P < 0.05$ and $**P < 0.01$ (cytosol_MK: $t = 2.104$ and $df = 3$; cytosol_SP-A: $t = 1.905$ and $df = 4$; membrane_MK: $t = 6.599$ and $df = 3$; membrane_SP-A: $t = 2.846$ and $df = 4$). See also movies S1 and S2. (D and E) Imaging of DIV 15 hippocampal neurons that were cotransfected at DIV 13 with PLC δ PH-GFP and mCherry and incubated at DIV 14 with SP-A (D) (1 μM) or AZ12861903 (E) (AZ; 50 nM) for 20 hours. Scale bars, 5 μm . Data are means ($\pm$ SD) spine/shaft ratio of GFP intensity from two independent experiments, each 19 to 20 neurons per condition. $***P < 0.0001$ by unpaired t test ($t = 5.45$ and $df = 38$).

ligand-dependent activation of GHS-R1a on AMPAR surface expression was detected (Fig. 4, A and B). We found that upon blockade of the ligand-independent activity of GHS-R1a using SP-A, subsequent activation of GHS-R1a with the agonist MK-0677 increased the surface and synaptic expression of GluA1-containing AMPARs (Fig. 4). Together, these observations indicate that the hippocampal ligand-independent GHS-R1a activity promotes tonic expression of synaptic AMPARs (Figs. 2 and 3) and, on the other hand, limits the agonist-mediated effects of GHS-R1a on AMPAR trafficking (Fig. 4).

Ligand-independent activity of GHS-R1a regulates AMPAR surface mobility

To determine how the ligand-independent activity of GHS-R1a may regulate AMPARs, we tested for effects on the cell surface diffusion of GluA1 AMPAR subunit. We expressed super ecliptic pHluorin (SEP)-GluA1 in cultured hippocampal neurons and took advantage of the single-particle tracking approach to monitor individual AMPAR complexes (Fig. 5, A to E). Hippocampal neurons were exposed to

SP-A for 1 hour, and single-particle imaging of SEP-GluA1 was performed thereafter. SP-A exposure significantly increased the surface diffusion of GluA1 [both the mean square displacement (MSD) and diffusion coefficient were increased; Fig. 5, A and B], decreased the fraction of synaptic immobile receptors (Fig. 5C), and decreased the synaptic residence time of GluA1-containing AMPARs (Fig. 5D).

Fig. 2. Ligand-independent activity of GHS-R1a controls synaptic levels of AMPARs and excitatory synaptic transmission in the hippocampus. (A to J) Representative images and quantitative analysis of DIV 7 (I), DIV 15 (A, C, and E) or DIV 20 (G) hippocampal neurons incubated with AZ (50 nM) (A) or SP-A (1 μ M) (C, E, G, and I) for 20 hours and immunostained for surface GluA1 or GluA2 (green), PSD95 (red), and VGluT1 (blue). Total fluorescence intensity of GluA1 or GluA2 cell surface puncta (left) and synaptic clusters (VGluT1/PSD95-colocalized, right) normalized to synapse density. Results are medians relative to controls from either two independent experiments (B) ($n = 24$ neurons per group) or four to five independent experiments (D, F, H, and J) ($n = 59$ to 75 neurons per group). Mann-Whitney test: $*P < 0.05$, $**P < 0.01$, and $***P < 0.0001$. Scale bars, 5 μ m. Arrowheads indicate synaptic GluA1- or GluA2-AMPA receptors. (K and L) Representative traces and quantitative analysis of evoked synaptic AMPAR and NMDAR currents in DIV 7 organotypic hippocampal slices under control condition and upon treatment for 20 hours with SP-A (1 μ M) or [D-Lys3]-GHRP-6 (Atg; 100 μ M). Scale bars, vertical, 50 pA; horizontal, 20 ms. AMPA/NMDA ratios are presented as means $\pm$ SD from 13 to 19 neurons, compared by one-way ANOVA followed by Dunnett's multiple comparison test ($F = 4.158$), $*P < 0.05$. See also fig. S1.

We repeated this experiment now applying AZ and using quantum dot (QD)-labeled antibodies against an extracellular region in GluA1 to follow endogenous AMPARs (Fig. 5, F to J). Endogenous GluA1 also showed increased MSD and diffusion coefficient and decreased synaptic residence time in neurons treated with AZ. Similarly, SP-A treatment increased the surface diffusion of endogenous GluA2 (fig. S2, A to D). These observations provide evidence that the ligand-independent activity of GHS-R1a contributes to decrease the surface diffusion of synaptic AMPARs, thereby increasing the synaptic content of AMPARs under basal conditions in hippocampal neurons.

Effects of ligand-independent activity of GHS-R1a on activity-induced synaptic incorporation of AMPARs

We then assessed whether activity-induced synaptic incorporation of AMPARs is regulated by the ligand-independent activity of GHS-R1a. We used a neuronal culture model of chemical long-term potentiation (cLTP), in which activation of NMDA receptors triggers an increase in the expression of surface and synaptic AMPARs (45). In agreement

Fig. 3. Inverse agonists AZ12861903 and SP-A specifically target GHS-R1a to control synaptic levels of AMPARs. (A to E) Representative images (A, B, and D) and quantitative analysis (C and E) of surface GluA1 (green) and VGlut1 (blue) immunostaining in DIV 15 hippocampal neurons transfected with constructs encoding luciferase shRNA-GFP (control), GHS-R1a shRNA-GFP (KD), GHS-R1a shRNA-GFP + hGHS-R1a (rescue), or GHS-R1a shRNA-GFP + hGHS-R1a F279L (rescue*) and, where indicated (A to C), treated with 1 μ M SP-A or 50 nM AZ for 20 hours. Scale bars, 5 μ m. Arrowheads indicate VGlut1-colocalized GluA1-AMPARs. Total fluorescence intensity of GluA1 cell surface puncta (C and E) (left) and total fluorescence intensity of GluA1 synaptic clusters (VGlut1-colocalized) (C and E) (right) were normalized to density of VGlut1 clusters and presented as the median relative to controls. $N = 2$ to 4 (C) and 3 or 4 (E) independent experiments, wherein $n = 50$ to 51 (C) (SP-A), 36 (C) (KD + SP-A), 21 to 22 (C) (AZ), and 52 to 54 (E) neurons. Differences between groups were assessed by Kruskal-Wallis analysis of variance followed by Dunn's multiple comparison test (Kruskal-Wallis statistic = 26.2, 7.899, 23.64, 17.65, 20.09, and 14.79, respectively); * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.0001$.

with previous reports, application of glycine (co-agonist of NMDA receptors), in the absence of Mg^{2+} , led to a notable increase in the synaptic expression of GluA1-containing AMPARs compared to control cells (Fig. 5, K to N). However, this effect was blocked in neurons preincubated with either SP-A (Fig. 5, K and L) or AZ (Fig. 5, M and N), indicating that the ligand-independent GHS-R1a activity is necessary for AMPAR synaptic insertion upon cLTP.

Intracellular signaling of hippocampal ligand-independent GHS-R1a activity

We tested cell signaling pathways downstream of the ligand-independent GHS-R1a activity that could result in altered trafficking of AMPARs. We found that upon blockade of the ligand-independent activity of GHS-R1a in organotypic hippocampal slices, there was a decrease in the phosphorylation of GluA1 at Ser⁸⁴⁵ (Fig. 6A), a protein kinase A (PKA) phosphorylation site critical for priming AMPARs for synaptic insertion (46). In addition, we detected a decrease in the phosphorylation of Ca^{2+} /calmodulin-dependent protein kinase type IV (CaMKIV) (Fig. 6B), whereas no changes were found in the phosphorylation of GluA1 at Ser⁸³¹, stargazin, and the kinase Akt (Fig. 6, C to E). These results suggest that PKA activation downstream of the ligand-independent activity of GHS-R1a may result in phosphorylation of GluA1 at Ser⁸⁴⁵ and contribute to maintaining a population of AMPARs available for synaptic insertion.

To test whether the ligand-independent GHS-R1a activity contributes to AMPAR trafficking through effects on GluA1 phosphorylation at Ser⁸⁴⁵, we evaluated whether SP-A affects the cell surface and synaptic levels of phospho-deficient and phosphomimetic mutants of GluA1 at Ser⁸⁴⁵ (S845A and S845D, respectively). We found that, contrary to WT GluA1, SP-A treatment did not alter the cell surface or synaptic levels of either of these mutants (Fig. 6, F and G), suggesting GluA1 phosphorylation at Ser⁸⁴⁵ as one mechanism through which the ligand-independent activity of GHS-R1a regulates AMPAR trafficking.

Ligand-independent activity of GHS-R1a is relevant for memory formation

Given the role of the hippocampus and excitatory transmission in spatial memory (47) and that GHS-R1a KO mice present memory impairments (36, 37), we tested whether the ligand-independent activity of GHS-R1a plays a role in memory formation by evaluating performance in the novel object recognition test (48) in mice injected with the BBB-permeable inverse agonist of GHS-R1a AZ. During the familiarization session, animals were allowed to explore two identical objects for 10 min. After 6 hours, one of the objects was replaced with a novel object, and the percentage of time exploring either object was measured (test session; Fig. 7A). Whereas control animals explored the novel object a higher number of times and for longer, animals treated with the GHS-R1a inverse agonist before the familiarization session did not show a preference for either object, as measured by the number of explorations of each object (Fig. 7B) or time spent with each object (Fig. 7C).

To test whether the ligand-independent activity of GHS-R1a is relevant for spatial memory, we used the object displacement recognition test (Fig. 7D) (49). During the habituation session, the animals explored an open-field arena in the absence of objects for 6 min; immediately after, the animals were treated with GHS-R1a inverse agonist or vehicle injection. The animals returned to the open field 10 min after injection, and two different objects were present in specific locations.

This familiarization session lasted 6 min and was repeated twice. After 24 hours, the animals were tested in the open field with one of the objects displaced to a different location. Our results show that animals injected with vehicle preferentially explored the moved object, whereas animals injected with the inverse agonist did not show such preference (Fig. 7, E and F). Total distance traveled by the animals in both memory tests was not significantly affected by injection of GHS-R1a inverse agonist (fig. S3, A and B). Because anxiolytic effects have been observed in mice administered with ghrelin (12), we tested performance in the elevated plus maze after blocking ligand-independent activity of GHS-R1a. Consistent with prior results using KO mice for GHS-R1a (12), blockade of the ligand-independent activity of GHS-R1a did not affect performance in the elevated plus maze (Fig. 7G and fig. S3, C and D). The total distance traveled by animals in this test was also not affected by injections (fig. S3E). Our results indicate that acute blockade of the ligand-independent GHS-R1a activity impairs performance during the novel object recognition task and in the object displacement recognition task, which suggests that tonic activity of GHS-R1a is important for learning and memory.

The ligand-independent activity of GHS-R1a has been previously described to regulate food intake and body weight (18, 20). Here, we provide strong evidence for the presence of ligand-independent activity of GHS-R1a in the hippocampus and that it regulates AMPAR levels at the synapse and the formation of spatial memories. Overall, our results show a dual role for the ligand-independent GHS-R1a activity: On one hand, it promotes the synaptic accumulation of

We found that in organotypic hippocampal slices, blockade of the ligand-independent activity of GHS-R1a led to decreased phosphorylation in CaMKIV and GluA1 Ser⁸⁴⁵, but not to changes in the PKC phosphorylation site in GluA1 (Ser⁸³¹), despite increased PIP₂ levels in hippocampal neurons incubated with the GHS-R1 inverse agonists. Ligand-independent activity of GHS-R1a has been demonstrated both by measuring IP₃ turnover and by using assays for transcription activity controlled by adenosine 3',5'-monophosphate-responsive element (CRE) (21, 43). Blockade of basal signaling from GHS-R1a in cultured mouse hypothalamic cells using SP-A led to decreased CRE-binding protein (CREB) phosphorylation (20), but signaling downstream of the ligand-independent activity of GHS-R1a

Fig. 5. Blocking ligand-independent activity of GHS-R1a increases the cell surface diffusion of AMPARs and prevents the activity-triggered delivery of AMPARs to synaptic sites. (A to E) Single-particle tracking analysis of SEP-GluA1 in DIV 15 hippocampal neurons cotransfected with SEP-GluA1 and Homer1C-DsRed (at DIV 11) and incubated with SP-A (1 μM for 1 hour) using quantum dot-labeled antibodies for GFP (QD-GluA1). GluA1 mean square displacement (MSD) versus time plots for control and SP-A-treated cells (A). Surface diffusion coefficient of synaptic (left) and global (right) single QD-GluA1. Median diffusion [± 25 to 75% interquartile range (IQR)] of 8816 to 8607 trajectories; Mann-Whitney test ($U = 803,490$ and $17,834,511$, respectively), $***P < 0.0001$ (B). Mean percentage ($\pm$ SD) of synaptic immobile GluA1-AMPARs in control and SP-A-treated cells. Ctr ($n = 38$ neurons) and SP-A ($n = 37$); unpaired t test ($t = 2.113$, $df = 73$), $*P < 0.05$ (C). Synaptic residence time (median) of GluA1-AMPARs in control and SP-A-treated cells. Mann-Whitney test ($U = 1,799,767,896$), $***P < 0.0001$ (D). Reconstructed GluA1 trajectories in the synaptic (red) and extrasynaptic compartments (blue). $N \geq 37$ cells in three independent experiments. Scale bar, 1 μm (E). (F to J) Single-particle tracking analysis of GluA1 in DIV 15 hippocampal neurons transfected with Homer1C-DsRed (at DIV 11) and incubated with AZ (50 nM) for 1 hour by using quantum dot-labeled antibodies for GluA1 (QD-GluA1). GluA1 mean square displacement (MSD) versus time plots for control and AZ-treated cells (F). Surface diffusion coefficient of synaptic (left) and global (right) single QD-GluA1. Median diffusion [± 25 to 75% IQR] of 1770 to 1303 trajectories; Mann-Whitney test ($U = 15,250$ and $212,277$, respectively), $***P < 0.0001$ (G). Mean percentage ($\pm$ SD) of synaptic immobile GluA1-AMPARs in control and AZ-treated cells ($n = 40$ to 41 neurons); unpaired t test ($t = 1.269$, $df = 79$), $P = 0.2081$ (H). Synaptic residence time (median) of GluA1-AMPARs in control and AZ-treated cells. Mann-Whitney test ($U = 39,940,707$), $***P < 0.0001$ (I). Reconstructed GluA1 trajectories in the synaptic (red) and extrasynaptic compartments (blue). $N \geq 40$ cells in three independent experiments. Scale bar, 1 μm (J). See also fig. S2. (K to N) Representative images and quantitative analysis of DIV 19 to 20 hippocampal neurons treated with SP-A (1 μM) (K) or AZ (50 nM) (M) for 20 hours, subjected to cLTP or pretreated with SP-A (K) or AZ (M) and submitted to cLTP. Neurons were immunostained for surface GluA1 (green), PSD95 (red), and VGLUT1 (blue) (K) or surface GluA1 (green) and VGLUT1 (blue) (M). Scale bar, 5 μm . Arrowheads indicate synaptic GluA1-AMPARs (K) or VGLUT1-colocalized GluA1-AMPARs (M). Total fluorescence intensity of GluA1 synaptic clusters (VGLUT1/PSD95-colocalized) (K) or (VGLUT1-colocalized) (M) normalized to synapse density or VGLUT1 clusters, respectively. Data are medians relative to controls from ≥ 3 independent experiments, $n = 40$ to 60 neurons, compared by Kruskal-Wallis analysis of variance followed by Dunn's multiple comparison test (Kruskal-Wallis statistic = 44.11 and 35.87 , respectively), $*P < 0.05$, $**P < 0.01$, and $***P = 0.0004$.

Fig. 6. Signaling pathways downstream of the ligand-independent GHS-R1a activity. (A to E) Western blot analysis of DIV 7 organotypic hippocampal slices nontreated or treated with 1 μ M SP-A for 20 hours. Primary antibodies detected: phospho-Ser⁸⁴⁵ at GluA1 (A), phospho-Thr¹⁹⁶ at CaMKIV (B), phospho-Ser⁸³¹ at GluA1 (C), phospho-Ser^{239/240} at stargazin (D), phospho-Ser⁴⁷³ at Akt, total GluA1 (A and C), total CaMKIV (B), and total Akt (E). Tubulin was used as a loading control. The graphs represent the quantification of band intensities (means $\pm$ SD) relative to control extracts in eight (A and C to E) or six (B) independent experiments. The statistical significance was calculated using the unpaired *t* test: Ser⁸⁴⁵ ($t = 3.011$, $df = 14$, and $**P = 0.0093$), Thr¹⁹⁶ ($t = 5.012$, $df = 10$, and $***P = 0.0005$), Ser⁸³¹ ($t = 0.3006$, $df = 14$, and $P = 0.7681$), Ser^{239/240} ($t = 0.3211$, $df = 14$, and $P = 0.7529$), and Ser⁴⁷³ ($t = 0.1313$, $df = 14$, and $P = 0.8974$). (F and G) Representative images and quantitative analysis of surface GFP (green) and VGlut1 (blue) immunostaining in DIV 15 hippocampal neurons expressing SEP-GluA1^{WT}, SEP-GluA1^{S845A}, or SEP-GluA1^{S845D} (nontreated or treated with SP-A 1 μ M for 20 hours). Scale bar, 5 μ m. Arrowheads indicate VGlut1-colocalized GluA1-AMPA receptors. Total fluorescence intensity of SEP-GluA1 cell surface puncta (G) (left) and total fluorescence intensity of SEP-GluA1 synaptic clusters (VGlut1-colocalized, right) normalized to density of VGlut1 clusters. Data are median relative to control cells from three independent experiments, $n = 37$ to 39 neurons each group. Differences were assessed by Kruskal-Wallis analysis of variance followed by Dunn's multiple comparison test (Kruskal-Wallis statistic = 14.87 and 16.29, respectively), $*P < 0.05$ and $**P < 0.01$.

in the hippocampus has not been explored before. Our data now suggest that the ligand-independent activity of GHS-R1a may affect G_q/PLC/IP₃-, CaMKIV-, and PKA-dependent pathways and lead to changes in phosphorylation levels of GluA1 in the hippocampus.

Besides signaling in response to ghrelin and in the absence of the ligand, GHS-R1a has recently been shown to modulate dopamine

signaling through heterodimerization with dopamine receptors DRD1 and DRD2 (52, 53). In the hippocampus, GHS-R1a and DRD1 form heteromers that are activated by DRD1 agonists to induce intracellular Ca²⁺ mobilization, activation of early synaptic plasticity markers, and to modulate memory (53). The dopamine-induced effect on Ca²⁺ signaling is independent of the ligand-independent activity of

Fig. 7. Administration of an inverse agonist of GHS-R1a impairs recognition and spatial memory. (A to C) Male C57/BL6 mice of 8 to 15 weeks of age received injections of AZ (100 mg/kg i.p.) or vehicle and underwent the novel object recognition test (A) (schematic of the test with the “habituation,” “drug injection,” “familiarization,” and “test session” timeline; details are in Materials and Methods). The number of interactions mice had with objects during test session (B), expressed as a percentage of the total of interactions with both objects during test session (median; $n = 11$ to 13) and assessed by Wilcoxon matched-pairs signed-rank test, $*P < 0.05$. The time mice spent interacting with objects during the test session (C), expressed as a percentage of total duration of interactions with both objects (means $\pm$ SD) and assessed by paired t tests: old versus novel object in Veh group ($t = 2.968$, $df = 12$, and $*P = 0.0117$) and AZ group ($t = 2.04$, $df = 10$, and $P = 0.0686$). (D to F) As described for (A to C), respectively, in an object displacement recognition test ($n = 8$ to 9). (E) Wilcoxon matched-pairs signed-rank test, $*P < 0.05$; (F) paired t test (Veh: $t = 2.325$, $df = 7$; AZ: $t = 0.1798$, $df = 8$), $P = 0.053$ and 0.8618 , respectively. (G) Animals treated as in (A) and assessed on the elevated plus maze test (schematic), wherein distance traveled in the maze (centimeters, means $\pm$ SD, $n = 5$ each group) was compared by unpaired t test ($t = 1.085$, $df = 8$), $P > 0.3$. Heatmaps represent cumulative time spent in each part of the maze. See also fig. S3. ns, not significant.

GHS-R1a in the GHS-R1a:DRD1 complex (53). This suggests that the role of the ligand-independent activity of GHS-R1a on memory described here runs parallel to the effects of dopamine on memory through the GHS-R1a:DRD1 complex.

The ligand-independent GHS-R1a activity has also been shown to reduce presynaptic Ca_v2 currents and γ -aminobutyric acid (GABA) release in hypothalamic and hippocampal neurons (54, 55) by reducing the cell surface expression of Ca_v2 channels (56). Our results complement this observation, but further work should be done to explore how the effects of the ligand-independent activity of GHS-R1a on the inhibitory and excitatory systems contribute to memory formation.

It was recently reported that the melanocortin receptor accessory protein 2 (MRAP2) controls GHS-R1a signaling by inhibiting its ligand-independent activity, as well as by increasing its G protein-mediated signaling and blocking the recruitment and signaling of β -arrestin elicited by ghrelin binding (57). Disruption of the gene for *MRAP2* has been associated with obesity in animal models and humans (58). *MRAP2* mRNA has low expression in the hippocampus of both animals (58, 59) and humans (60), which suggests that ligand-independent activity of GHS-R1a is unimpeded in this region and is thus more likely to have an influence in hippocampal excitatory synapse protein dynamics and hippocampal-dependent behavior. We observed PIP_2 membrane accumulation in hippocampal neurons treated with GHS-R1a inverse agonists, in direct support of basal ligand-independent activity of GHS-R1a in hippocampal neurons.

Recent evidence suggests that the ligand-independent activity of the receptor is endogenously regulated by plasma levels of LEAP2 (34), which are proportional to the levels of adiposity and blood glucose (33). The levels of ligand-independent activity of GHS-R1a are highly dependent on the expression levels of the receptor (20), which, in turn, change according to the animal's feeding status (19, 20). Therefore, our observations support a physiological mechanism in

which the internal metabolic state of animals exerts control over cognitive processes. The ligand-independent activity of GHS-R1a may be particularly important in the hippocampus, given that, in contrast to the hypothalamus, which is in close proximity to fenestrated capillaries, access of plasma ghrelin to the hippocampal structure may be more limited [reviewed in (61)], and it is still a matter of debate whether ghrelin can be produced in the brain (62). Certainly, thus, the role of the ligand-independent GHS-R1a activity reported in this work should be taken into account when considering GHS-R1a inverse agonists as treatments for obesity (63) or alcohol use disorders (38, 39).

MATERIALS AND METHODS

Reagents

The GHS-R1a inverse agonist [D-Arg¹, D-Phe⁵, D-Trp^{7,9}, Leu¹¹]-substance P (SP-A) was purchased from Bachem (Bubendorf, Switzerland), and the AZ12861903 (AZ) GHS-R1a inverse agonist was provided by AstraZeneca. The GHS-R1a agonist MK-0677 was purchased from Axon Medchem (Groningen, The Netherlands). The GHS-R1a antagonist [D-Lys³]-GHRP-6, tetrodotoxin (TTX), and picrotoxin were purchased from Tocris Bioscience (Bristol, UK), and the GHS-R1a antagonist JMV2959 was obtained from Calbiochem (Merck Millipore, Portugal). Antibody to tubulin was purchased from Sigma-Aldrich (Sintra, Portugal); antibodies to Akt, phospho-Ser⁴⁷³ Akt, PSD95 (rabbit), and CaMKIV were obtained from Cell Signaling Technology; antibodies to GluA1, GluA2, phospho-Ser⁸⁴⁵ GluA1, phospho-Ser^{239/240} stargazin, and VGlut1 were from Millipore; the antibody to MAP2 was from Abcam; the antibody to phospho-Ser⁸³¹ GluA1 was from Tocris Bioscience; the antibody to PSD95 (mouse) was from Affinity BioReagents; antibody to phospho-Thr¹⁹⁶ CaMKIV was from Santa Cruz Biotechnology; and antibodies to GFP, rabbit and mouse, were from MBL International and Roche, respectively. QD655 goat F(ab')₂ anti-mouse immunoglobulin G (IgG) conjugate (H⁺L) was purchased from Invitrogen. The antibody for the N terminus of GluA1 was a gift from A. Irving (University College Dublin). All other reagents were purchased from Sigma-Aldrich, Fisher Chemicals, or Merck.

DNA constructs

For the generation of the short hairpin interfering RNA construct targeting GHS-R1a, a previously described and validated sequence (42) was used. Complementary oligonucleotides, each containing a unique 19-nucleotide sequence derived from within the target mRNA transcripts of *ghsr1a* gene (NM_032075) targeting nucleotides 79 to 96 (GACTCACTGCCTGACGAAC) (42), were annealed and subcloned into the Hpa I/Xho I sites of the U6 promoter-driven shRNA expression vector pLentiLox3.7(CMV)EGFP, which coexpresses EGFP under the CMV (cytomegalovirus) promoter. The control shRNA that targets firefly luciferase was described previously (64). Homer1C-DsRed and Homer1C-GFP were previously described (65). PLCδPH-GFP was a gift from T. Balla (Addgene plasmid #51407) (41).

Neuronal and slice cultures

Primary cultures of rat hippocampal neurons were prepared as previously described (66). Hippocampal slices were prepared from young Wistar rats of either sex (postnatal days 5 to 6) as previously described (67).

Neuron transfection

DNA constructs expressing [Luciferase shRNA-GFP, GHS-R1a shRNA-GFP (knockdown), hGHS-R1a (rescue), SEP-GluA1, Homer1C-DsRed, and Homer1C-GFP] or [PLCδPH-GFP and mCherry] were expressed in primary cultures of hippocampal neurons either at 9 or 13 to 14 DIV, respectively, using an adapted calcium phosphate transfection protocol (68), as previously described (66).

Application of GHS-R1a inverse agonists, antagonists, and agonist

Hippocampal organotypic slices (6 DIV) were treated with the GHS-R1a inverse agonist [D-Arg¹, D-Phe⁵, D-Trp^{7,9}, Leu¹¹]-substance P (SP-A; 1 μM) for 20 hours or chronically treated with the GHS-R1a antagonist [D-Lys³]-GHRP-6 (100 μM) from 3 DIV up to 7 DIV. Hippocampal neurons in culture were incubated with the GHS-R1a inverse agonists SP-A and AZ1286190 (AZ; 50 nM), antagonist JMV2959 (100 μM), and agonist MK-0677 (1 μM). The compounds were added directly to the culture medium. AZ for injection in vivo was dissolved in 95% β-hydroxypropylcyclodextrin (β-hpC)/5% (v/v) dimethyl sulfoxide. β-hpC was prepared at 25% (w/v) in Sorenson's buffer (pH 5.5). All the injected solutions were prepared under sterile conditions. The drug and vehicle were injected intraperitoneally at volumes of 100 to 150 μl. The dose of 100 mg/kg was based on previously described doses by McCoull and colleagues (25).

Imaging and analysis of PLCδPH-GFP translocation

For live-cell imaging experiments, hippocampal neurons (15 to 16 DIV) transfected with the PLCδPH-GFP construct were imaged with a spinning-disk confocal microscope using an LCI Plan-Neofluar 63×/1.3 objective. Cultured hippocampal neurons were kept at 37°C and perfused with Sham medium [10 mM Hepes, 0.116 M NaCl, 5.4 mM KCl, 0.8 mM MgSO₄, 1 mM sodium phosphate buffer, 25 mM glucose, 1.8 mM CaCl₂, and 25 mM NaHCO₃ (pH 7.3)] while imaging. To analyze PLCδPH-GFP translocation, regions of interest (ROIs) were defined in the cytosol or membrane regions of dendrites. The fluorescence intensity inside ROIs (*F*) was normalized to baseline values (*F*₀), before the application of MK-0677 or SP-A. Three ROIs (each for cytoplasm and membrane regions) were analyzed and averaged per neuron. All fluorescence measurements were performed using ImageJ software. Images obtained from experiments in fixed cells were captured on a Leica SP8 laser scanning confocal microscope. To quantify the spine/shaft ratio of PLCδPH-GFP fluorescence intensity in fixed neurons, line profiles were traced along the dendritic spine heads, the plasma membrane of the dendritic shaft, and the cytosol of the dendritic shaft, and the mean fluorescence intensity of PLCδPH-GFP from the spine and respective dendritic shaft was determined by using the plot profile tool from ImageJ software. An average of 5 to 10 spine/shaft ratios were used per neuron.

Immunocytochemistry

For labeling surface GluA1-containing AMPARs, live neurons were incubated for 10 min at room temperature using an antibody against an extracellular epitope in the GluA1 N terminus diluted in conditioned neuronal culture medium or extracellular solution (ECS) (used for cLTP). Neurons were then fixed and stained as previously described (66). For labeling GluA2-containing AMPARs, neurons were fixed and then incubated overnight with an anti-GluA2 antibody diluted at 1:100 in 3% bovine serum albumin (BSA)/phosphate-buffered

saline (PBS), at 4°C. Neurons were then stained as previously described (66).

Quantitative imaging analysis

Imaging was performed on a Zeiss Axio Observer Z1 microscope using a Plan Apochromat 63×/1.4 numerical aperture (NA) oil objective and an AxioCam HRm charge-coupled device camera. Images were quantified using image analysis software ImageJ. For quantification of total fluorescence intensity of GluA1 cell surface puncta and GluA1 synaptic clusters (VGluT1/PSD95-colocalized or VGluT1-colocalized), sets of cells were cultured and stained simultaneously and imaged using identical settings. The ROI was randomly selected avoiding primary dendrites, and dendritic length was measured using MAP2 staining. Measurements were performed in two to five independent preparations, and at least seven cells per condition were analyzed for each preparation. Quantitative imaging analysis was performed as previously described (66).

Electrophysiology

Voltage-clamp whole-cell recordings were performed stimulating Schaffer collateral fibers and recording evoked synaptic responses from CA1 pyramidal neurons at different holding potentials. The AMPA/NMDA ratios were calculated by acquiring AMPAR responses at −60 mV and NMDA receptor responses at +40 mV at a latency at which AMPAR responses were fully decayed (60 ms after stimulation). Picrotoxin (100 μM) was present in the external solution to block the GABA_A receptor responses. The recording chamber was perfused with external solution [119 mM NaCl, 2.5 mM KCl, 1 mM sodium phosphate buffer, 11 mM glucose, 26 mM NaHCO₃, 4 mM MgCl₂, 4 mM CaCl₂, and 0.004 mM 2-chloroadenosine (at pH 7.4)] and was gassed with 5% CO₂/95% O₂. Patch recording pipettes (3 to 6 megohms) were filled with internal solution [115 mM CsMeSO₃, 20 mM CsCl, 10 mM Hepes, 2.5 mM MgCl₂, 4 mM Na₂ATP, 0.4 mM Na₃GTP, 10 mM sodium phosphocreatine, and 0.6 mM EGTA (at pH 7.25)]. Synaptic responses were evoked with bipolar electrodes using single-voltage pulses (200 μs, up to 20 V). The stimulating electrodes were placed over Schaffer collateral fibers between 300 and 500 μm from the CA1 recorded cells. Synaptic responses were averaged over 50 trials. Whole-cell recordings were carried out with a Multiclamp 700A amplifier (Molecular Devices, Sunnyvale, USA).

cLTP protocol

cLTP was induced as previously described (45). Hippocampal cultures (19 to 20 DIV) were washed with ECS containing 150 mM NaCl, 2 mM CaCl₂, 5 mM KCl, 10 mM Hepes, 30 mM glucose, 0.001 mM TTX, 0.01 mM strychnine, and 0.03 mM picrotoxin (pH 7.4). After washing, neurons were stimulated with glycine (300 μM) at room temperature for 3 min in ECS and then incubated for 20 to 25 min in ECS at a 37°C, 5% CO₂/95% air incubator.

QD labeling and imaging

Endogenous GluA2 and GluA1-SEP labeling was performed in two steps: First, neurons were incubated for 10 min at 37°C with anti-GFP antibody (1/300,000) or anti-GluA2 antibody (1/1000), diluted in conditioned medium. After one washing step, anti-mouse IgG-conjugated QD655 (diluted at 1:10 in PBS) was diluted in conditioned medium with BSA 2% (1/2000) and was incubated on cells for 5 min at 37°C. Synapses were labeled using transfection with Homer1C-DsRed or Homer1C-GFP. All washes were performed in

ECS containing 145 mM NaCl, 5 mM KCl, 10 mM glucose, 10 mM Hepes, 2 mM CaCl₂, and 2 mM MgCl₂, supplemented with BSA 2% at 37°C. After washing, neurons were mounted in an open chamber (K.F. Technology SRL) and imaged in ECS. Single-particle tracking was performed as in (69). Cells were imaged at 37°C on an inverted microscope (Axio Observer Z1, Carl Zeiss) equipped with a Plan Apochromat 63× oil objective (NA = 1.4). QD, Homer1C-DsRed, and Homer1C-GFP signals were detected by using an HXP fluorescence lamp (for QDs: excitation filter, 425/50 and emission filters, 655/30, Chroma). Fluorescent images from QDs were obtained with an integration time of 50 ms with up to 600 consecutive frames. Signals were recorded with a digital complementary metal-oxide semiconductor camera (ORCA Flash 4.0, Hamamatsu). QD-labeled GluAs were imaged on randomly selected dendritic regions over up to 30 min of total experimental time. QDs fixed on the coverslip allowed us to compensate mechanical drifts of the stage.

QD tracking and analysis

The tracking of single QDs was performed with homemade software based on MATLAB (MathWorks Inc., Natick, USA). Single QDs were identified by their diffraction limited signals and their blinking fluorescent emission. The trajectory of a QD-tagged receptor could not be continuously tracked because of the random blinking events of the QDs. When the positions before and after the dark period were compatible with borders set for maximal position changes between consecutive frames and blinking rates, the subtrajectories of the same receptor were reconnected. The values were determined empirically: 2 to 3 pixels (0.32 to 0.48 μm) for maximal position change between two frames and maximal dark periods of 25 frames (1.25 s). MSD curves were calculated for reconnected trajectories of at least 20 frames. The QDs were considered synaptic if colocalized with Homer dendritic clusters for at least five frames. Diffusion coefficients were calculated by a linear fit of the first four to eight points of the MSD plots versus time depending on the length of the trajectory within a certain compartment. The resolution limit for diffusion was 0.0075 μm²/s as determined in (70), whereas the resolution precision was ~40 nm.

Biochemistry

Protein extracts were prepared in lysis buffer [10 mM Hepes (pH 7.4), 150 mM NaCl, 10 mM EDTA, 1% (v/v) Triton X-100 supplemented with 1 mM dithiothreitol, 0.1 mM phenylmethylsulfonyl fluoride, chymostatin (1 μg/ml), leupeptin (1 μg/ml), antipain (1 μg/ml), pepstatin (1 μg/ml), and a cocktail of phosphatase inhibitors (1×; Roche, Carnaxide, Portugal)]. After centrifugation at 16,100g for 10 min at 4°C, protein in the supernatant was quantified using the bicinchoninic acid assay kit (Pierce, Thermo Fisher Scientific, Rockford, USA), and the samples were denatured with 5× concentrated denaturing buffer [62.5 mM tris-HCl (pH 6.8), 10% (v/v) glycerol, 2% (v/v) SDS, 0.01% (w/v) bromophenol blue, and 5% (v/v) β-mercaptoethanol (added fresh)] and boiled for 5 min. Protein extracts were resolved by SDS-polyacrylamide gel electrophoresis in 7.5 or 12% polyacrylamide gels. For Western blot analysis, proteins were transferred onto a polyvinylidene difluoride membrane (Millipore, Madrid, Spain) by electroblotting (40 V, overnight at 4°C). The membranes were blocked for 1 hour at room temperature in tris-buffered saline [137 mM NaCl and 20 mM tris-HCl (pH 7.6)] containing 0.1% (v/v) Tween 20 (TBS-T) and 5% (w/v) low-fat milk or BSA. Membranes were probed for 1 hour, at room temperature, or overnight, at 4°C,

with the primary antibodies diluted in TBS-T containing 5 or 0.5% (w/v) low-fat milk or 5% (w/v) BSA. After several washes, membranes were incubated for 1 hour with alkaline phosphatase-conjugated secondary antibodies (anti-mouse or anti-rabbit, depending on the primary antibody host species) at room temperature, washed again, and incubated with chemifluorescent substrate (ECF) (GE Healthcare, Carnaxide, Portugal) for 5 min at room temperature. Membranes were scanned with the Storm 860 scanner (GE Healthcare, Carnaxide, Portugal) and quantified using the ImageQuant software under linear exposure conditions. When necessary, the membranes were stripped (0.2 M NaOH for 5 min) and reprobed.

Animals and behavior

For the behavior experiments, 8- to 15-week-old male C56BL/6 mice were housed in the Animal Facility of the CNC/Faculty of Medicine of the University of Coimbra with access to food and water *ad libitum*. The environment was kept under controlled temperature and humidity conditions under a 12-hour dark-light cycle (light period, 0600 to 1800). Behavioral testing was reviewed and approved by the animal use and ethics committee (Orgão Responsável pelo Bem-Estar dos Animais) of the CNC/Faculty of Medicine, University of Coimbra, and by the Portuguese National Authority for animal experimentation (Direção Geral de Alimentação e Veterinária), and all procedures were performed according to the guidelines of the DGAV and Directive 2010/63/EU of the European Parliament.

The novel object recognition task was adapted from Leger and colleagues (48). This task consisted of three phases: In the first phase, the animals freely explored the empty open-field arena for 10 min (habituation phase). Twenty-four hours after, the animals were allowed to explore two similar, symmetrically disposed objects for 10 min. Ten minutes before this phase, the animals were submitted to intraperitoneal injection of either the drug or the vehicle and stayed in an empty transport cage before entering the training phase. Six hours after the training phase, the animals were exposed to two objects located in the same positions as previously, but this time, one of the objects was substituted by a new object that the animal had not contacted previously (test phase).

The object displacement recognition test was adapted from Oliveira and colleagues (49). The test took place for 2 days. During the first day, the animals were allowed to explore an empty open field for 6 min (habituation phase). Immediately after, the animals were intraperitoneally injected with the inverse agonist of GHS-R1a or its respective vehicle and placed in their homecage. After 10 min, the animals explored two different objects placed in a specific location of the open field for 6 min (training phase). The animals were then returned to their homecage and waited for 3 min. Two more similar training phases were conducted, with a 3-min waiting period in between. On the next day, the animals returned to the open field, where one of the objects was placed in a new location, and were allowed to explore the objects for 6 min (test phase).

The objects and their positions were randomized for both tests. The used objects correspond to those described by Leger and colleagues (48). The arena and the objects were carefully cleaned before running each animal and in between phases. The test was conducted at a room temperature of 23°C and 15 lux at the center of the arena (homogenously distributed light). Videos of the test were acquired using Noldus EthoVision software, and scoring was performed blinded to the treatment of the animals, using Noldus Observer.

The elevated plus maze was performed according to (71), using a maze made in-house according to previously described specifications (72). Animals were weighted and injected with the corresponding dose of inverse agonist or vehicle 10 min before starting the test and stayed in an empty transport cage. The test started by putting the animals in the central part of the maze with the nose aligned with the closed arms and run for 10 min. The test was conducted under 100 lux at the center of the arena. The arena was carefully cleaned before and after each run. Videos of the test were acquired and automatically quantified using Noldus EthoVision.

Statistical analysis

We first evaluated the adjustment of quantitative sample distributions to a theoretical normal one using the Shapiro-Wilk test. Even when quantitative sample distributions were considered to fit a Gaussian, if more than two nonbalanced groups were in analysis, then Bartlett's test for homoscedasticity was considered to decide whenever to apply parametric or nonparametric tests. Mann-Whitney test, unpaired *t* test, or paired *t* test was used to compare statistical differences between any two groups. Comparisons between multiple groups were performed with the Kruskal-Wallis analysis of variance (ANOVA) followed by Dunn's multiple comparison test or with one-way ANOVA followed by Dunnett's multiple comparison test. In addition, data of behavioral tests were analyzed using Wilcoxon matched-pairs signed-rank test or two-way ANOVA with Bonferroni adjustment for correction of multiple comparisons. Data were analyzed using GraphPad Prism 7.04, and results were evaluated at a 5% significance level.

SUPPLEMENTARY MATERIALS

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Fig. S1. Blockade of the ligand-independent activity of GHS-R1a decreases the synapse density in mature primary cultured hippocampal neurons.

Fig. S2. Blockade of the ligand-independent activity of GHS-R1a increases the cell surface diffusion of GluA2-AMPA.

Fig. S3. Administration of an inverse agonist of GHS-R1a does not impair overall movement.

Movie S1. Application of the GHS-R1a agonist MK-0677 induces the translocation of PLC β H-GFP from the plasma membrane into the cytosol.

Movie S2. Application of the GHS-R1a inverse agonist SP-A increases the plasma membrane levels of PLC β H-GFP.

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REFERENCES AND NOTES

1. M. Kojima, H. Hosoda, Y. Date, M. Nakazato, H. Matsuo, K. Kangawa, Ghrelin is a growth-hormone-releasing acylated peptide from stomach. *Nature* **402**, 656–660 (1999).
2. M. Nakazato, N. Murakami, Y. Date, M. Kojima, H. Matsuo, K. Kangawa, S. Matsukura, A role for ghrelin in the central regulation of feeding. *Nature* **409**, 194–198 (2001).
3. D. E. Cummings, J. Q. Purnell, R. S. Frayo, K. Schmidova, B. E. Wisse, D. S. Weigle, A preprandial rise in plasma ghrelin levels suggests a role in meal initiation in humans. *Diabetes* **50**, 1714–1719 (2001).
4. Y. Date, M. Kojima, H. Hosoda, A. Sawaguchi, M. S. Mondal, T. Suganuma, S. Matsukura, K. Kangawa, M. Nakazato, Ghrelin, a novel growth hormone-releasing acylated peptide, is synthesized in a distinct endocrine cell type in the gastrointestinal tracts of rats and humans. *Endocrinology* **141**, 4255–4261 (2000).
5. B. L. Mason, Q. Wang, J. M. Zigman, The central nervous system sites mediating the orexigenic actions of ghrelin. *Annu. Rev. Physiol.* **76**, 519–533 (2014).
6. T. M. Hsu, J. D. Hahn, V. R. Konanur, E. E. Noble, A. N. Suarez, J. Thai, E. M. Nakamoto, S. E. Kanoski, Hippocampus ghrelin signaling mediates appetite through lateral hypothalamic orexin pathways. *eLife* **4**, e11190 (2015).
7. S. E. Kanoski, S. M. Fortin, K. M. Ricks, H. J. Grill, Ghrelin signaling in the ventral hippocampus stimulates learned and motivational aspects of feeding via PI3K-Akt signaling. *Biol. Psychiatry* **73**, 915–923 (2013).
8. D. Serrenho, S. D. Santos, A. L. Carvalho, The role of ghrelin in regulating synaptic function and plasticity of feeding-associated circuits. *Front. Cell. Neurosci.* **13**, 205 (2019).

9. V. P. Carlini, M. E. Monzón, M. M. Varas, A. B. Cragnolini, H. B. Schiöth, T. N. Scimonelli, S. R. de Bariooglio, Ghrelin increases anxiety-like behavior and memory retention in rats. *Biochem. Biophys. Res. Commun.* **299**, 739–743 (2002).
10. S. Diano, S. A. Farr, S. C. Benoit, E. C. McNay, I. da Silva, B. Horvath, F. S. Gaskin, N. Nonaka, L. B. Jaeger, W. A. Banks, J. E. Morley, S. Pinto, R. S. Sherwin, L. Xu, K. A. Yamada, M. W. Sleeman, M. H. Tschop, T. L. Horvath, Ghrelin controls hippocampal spine synapse density and memory performance. *Nat. Neurosci.* **9**, 381–388 (2006).
11. A. Abizaid, Z. W. Liu, Z. B. Andrews, M. Shanabrough, E. Borok, J. D. Elsworth, R. H. Roth, M. W. Sleeman, M. R. Picciotto, M. H. Tschop, X. B. Gao, T. L. Horvath, Ghrelin modulates the activity and synaptic input organization of midbrain dopamine neurons while promoting appetite. *J. Clin. Invest.* **116**, 3229–3239 (2006).
12. M. Lutter, I. Sakata, S. Osborne-Lawrence, S. A. Rovinsky, J. G. Anderson, S. Jung, S. Birnbaum, M. Yanagisawa, J. K. Elmquist, E. J. Nestler, J. M. Zigman, The orexigenic hormone ghrelin defends against depressive symptoms of chronic stress. *Nat. Neurosci.* **11**, 752–753 (2008).
13. R. M. Meyer, A. Burgos-Robles, E. Liu, S. S. Correia, K. A. Goosens, A ghrelin-growth hormone axis drives stress-induced vulnerability to enhanced fear. *Mol. Psychiatry* **19**, 1284–1294 (2014).
14. M. A. Cowley, R. G. Smith, S. Diano, M. Tschop, N. Pronchuk, K. L. Grove, C. J. Strasburger, M. Bidlingmaier, M. Esterman, M. L. Heiman, L. M. Garcia-Segura, E. A. Nillni, P. Mendez, M. J. Low, P. Sotonyi, J. M. Friedman, H. Liu, S. Pinto, W. F. Colmers, R. D. Cone, T. L. Horvath, The distribution and mechanism of action of ghrelin in the CNS demonstrates a novel hypothalamic circuit regulating energy homeostasis. *Neuron* **37**, 649–661 (2003).
15. L. F. Ribeiro, T. Catarino, S. D. Santos, M. Benoist, J. F. van Leeuwen, J. A. Esteban, A. L. Carvalho, Ghrelin triggers the synaptic incorporation of AMPA receptors in the hippocampus. *Proc. Natl. Acad. Sci. U.S.A.* **111**, E149–E158 (2014).
16. L. Shi, X. Bian, Z. Qu, Z. Ma, Y. Zhou, K. Wang, H. Jiang, J. Xie, Peptide hormone ghrelin enhances neuronal excitability by inhibition of Kv7/KCNQ channels. *Nat. Commun.* **4**, 1435 (2013).
17. X. M. Guan, H. Yu, O. C. Palyha, K. K. McKee, S. D. Feighner, D. J. Sirinathsinghji, R. G. Smith, L. H. Van der Ploeg, A. D. Howard, Distribution of mRNA encoding the growth hormone secretagogue receptor in brain and peripheral tissues. *Brain Res. Mol. Brain Res.* **48**, 23–29 (1997).
18. G. Fernandez, A. Cabral, M. F. Andreoli, A. Labarthe, C. M'Kadmi, J. G. Ramos, J. Marie, J. A. Fehrentz, J. Epelbaum, V. Tolle, M. Perello, Evidence supporting a role for constitutive ghrelin receptor signaling in fasting-induced hyperphagia in male mice. *Endocrinology* **159**, 1021–1034 (2018).
19. M. S. Kim, C. Y. Yoon, K. H. Park, C. S. Shin, K. S. Park, S. Y. Kim, B. Y. Cho, H. K. Lee, Changes in ghrelin and ghrelin receptor expression according to feeding status. *Neuroreport* **14**, 1317–1320 (2003).
20. P. S. Petersen, D. P. Woldbye, A. N. Madsen, K. L. Egerod, C. Jin, M. Lang, M. Rasmussen, A. G. Beck-Sickinger, B. Holst, In vivo characterization of high basal signaling from the ghrelin receptor. *Endocrinology* **150**, 4920–4930 (2009).
21. B. Holst, A. Cygankiewicz, T. H. Jensen, M. Ankersen, T. W. Schwartz, High constitutive signaling of the ghrelin receptor—Identification of a potent inverse agonist. *Mol. Endocrinol.* **17**, 2201–2210 (2003).
22. M. Damian, S. Mary, M. Maingot, C. M'Kadmi, D. Gagne, J. P. Leyris, S. Denoyelle, G. Gaibelet, L. Gava, M. Garcia de Souza Costa, D. Perahia, E. Trinquet, B. Mouillac, S. Galandrin, C. Gales, J. A. Fehrentz, N. Floquet, J. Martinez, J. Marie, J. L. Baneres, Ghrelin receptor conformational dynamics regulate the transition from a preassembled to an active receptor: Gq complex. *Proc. Natl. Acad. Sci. U.S.A.* **112**, 1601–1606 (2015).
23. M. P. Cornejo, D. Castrogiovanni, H. B. Schiöth, M. Reynaldo, J. Marie, J. A. Fehrentz, M. Perello, Growth hormone secretagogue receptor signalling affects high-fat intake independently of plasma levels of ghrelin and LEAP2, in a 4-day binge eating model. *J. Neuroendocrinol.* **31**, e12785 (2019).
24. S. Els, E. Schild, P. S. Petersen, T. M. Kilian, J. Mokrosinski, T. M. Frimurer, C. Chollet, T. W. Schwartz, B. Holst, A. G. Beck-Sickinger, An aromatic region to induce a switch between agonism and inverse agonism at the ghrelin receptor. *J. Med. Chem.* **55**, 7437–7449 (2012).
25. W. McCoull, P. Barton, A. J. Brown, S. S. Bowker, J. Cameron, D. S. Clarke, R. D. Davies, A. G. Dossetter, A. Ertan, M. Fenwick, C. Green, J. L. Holmes, N. Martin, D. Masters, J. E. Moore, N. J. Newcombe, C. Newton, H. Pointon, G. R. Robb, C. Sheldon, S. Stokes, D. Morgan, Identification, optimization, and pharmacology of acylurea GHS-R1a inverse agonists. *J. Med. Chem.* **57**, 6128–6140 (2014).
26. N. Li, G. Song, Y. Wang, Q. Zhu, F. Han, C. Zhang, Y. Zhou, Blocking constitutive activity of GHSR1a in the lateral amygdala facilitates acquisition of conditioned taste aversion. *Neuropeptides* **68**, 22–27 (2018).
27. H. Inoue, N. Kangawa, A. Kinouchi, Y. Sakamoto, C. Kimura, R. Horikawa, Y. Shigematsu, M. Itakura, T. Ogata, K. Fujieda; Japan Growth Consortium, Identification and functional analysis of novel human growth hormone secretagogue receptor (GHSR) gene mutations in Japanese subjects with short stature. *J. Clin. Endocrinol. Metab.* **96**, E373–E378 (2011).
28. J. Pantel, M. Legendre, S. Cabrol, L. Hilal, Y. Hajaji, S. Morisset, S. Nivot, M. P. Vie-Luton, D. Grouselle, M. de Kerdanet, A. Kadir, J. Epelbaum, Y. Le Bouc, S. Amselem, Loss of constitutive activity of the growth hormone secretagogue receptor in familial short stature. *J. Clin. Invest.* **116**, 760–768 (2006).
29. J. Pantel, M. Legendre, S. Nivot, S. Morisset, M. P. Vie-Luton, Y. Le Bouc, J. Epelbaum, S. Amselem, Recessive isolated growth hormone deficiency and mutations in the ghrelin receptor. *J. Clin. Endocrinol. Metab.* **94**, 4334–4341 (2009).
30. H. J. Wang, F. Geller, A. Dempfle, N. Schauble, S. Friedel, P. Lichtner, F. Fontenla-Horro, S. Wudy, S. Hagemann, L. Gortner, K. Huse, H. Remschmidt, T. Bettecken, T. Meitinger, H. Schafer, J. Hebebrand, A. Hinney, Ghrelin receptor gene: Identification of several sequence variants in extremely obese children and adolescents, healthy normal-weight and underweight students, and children with short normal stature. *J. Clin. Endocrinol. Metab.* **89**, 157–162 (2004).
31. L. J. Torz, S. Osborne-Lawrence, J. Rodriguez, Z. He, M. P. Cornejo, E. R. Mustafa, C. Jin, N. Petersen, M. A. Hedegaard, M. Nybo, V. M. Damonte, N. P. Metzger, B. K. Mani, K. W. Williams, J. Raingo, M. Perello, B. Holst, J. M. Zigman, Metabolic insights from a GHSR-A203E mutant mouse model. *Mol. Metab.* **39**, 101004 (2020).
32. X. Ge, H. Yang, M. A. Bednarek, H. Galon-Tilleman, P. Chen, M. Chen, J. S. Lichtman, Y. Wang, O. Dalmás, Y. Yin, H. Tian, L. Jermutus, J. Grimsby, C. M. Rondinone, A. Konkar, D. D. Kaplan, LEAP2 is an endogenous antagonist of the ghrelin receptor. *Cell Metab.* **27**, 461–469.e6 (2018).
33. B. K. Mani, N. PuzziFerri, Z. He, J. A. Rodriguez, S. Osborne-Lawrence, N. P. Metzger, N. China, B. Gaylinn, M. O. Thorne, E. L. Thomas, J. D. Bell, K. W. Williams, A. P. Goldstone, J. M. Zigman, LEAP2 changes with body mass and food intake in humans and mice. *J. Clin. Invest.* **129**, 3909–3923 (2019).
34. C. M'Kadmi, A. Cabral, F. Barriale, J. Giribaldi, S. Cantel, M. Damian, S. Mary, S. Denoyelle, S. Dutertre, S. Peraldi-Roux, J. Neasta, C. Oiry, J. L. Baneres, J. Marie, M. Perello, J. A. Fehrentz, N-terminal liver-expressed antimicrobial peptide 2 (LEAP2) region exhibits inverse agonist activity toward the ghrelin receptor. *J. Med. Chem.* **62**, 965–973 (2019).
35. Y. Mear, A. Enjalbert, S. Thirion, GHS-R1a constitutive activity and its physiological relevance. *Front. Neurosci.* **7**, 87 (2013).
36. R. G. Albarran-Zeckler, A. F. Brantley, R. G. Smith, Growth hormone secretagogue receptor (GHS-R1a) knockout mice exhibit improved spatial memory and deficits in contextual memory. *Behav. Brain Res.* **232**, 13–19 (2012).
37. J. F. Davis, D. L. Choi, D. J. Clegg, S. C. Benoit, Signaling through the ghrelin receptor modulates hippocampal function and meal anticipation in mice. *Physiol. Behav.* **103**, 39–43 (2011).
38. M. R. Lee, M. Farokhnia, E. Cobbina, A. Saravanakumar, X. Li, J. T. Battista, L. A. Farinelli, F. Akhlaghi, L. Leggio, Endocrine effects of the novel ghrelin receptor inverse agonist PF-5190457: Results from a placebo-controlled human laboratory alcohol co-administration study in heavy drinkers. *Neuropharmacology*, 107788 (2020).
39. M. R. Lee, J. D. Tapocik, M. Ghareeb, M. L. Schwandt, A. A. Dias, A. N. Le, E. Cobbina, L. A. Farinelli, S. Bouhail, M. Farokhnia, M. Heilig, F. Akhlaghi, L. Leggio, The novel ghrelin receptor inverse agonist PF-5190457 administered with alcohol: Preclinical safety experiments and a phase 1b human laboratory study. *Mol. Psychiatry* **25**, 461–475 (2020).
40. H. H. Kim, K. H. Lee, D. Lee, Y. E. Han, S. H. Lee, J. W. Sohn, W. K. Ho, Costimulation of AMPA and metabotropic glutamate receptors underlies phospholipase C activation by glutamate in hippocampus. *J. Neurosci.* **35**, 6401–6412 (2015).
41. P. Varnai, T. Balla, Visualization of phosphoinositides that bind pleckstrin homology domains: Calcium- and agonist-induced dynamic changes and relationship to myo-[3H] inositol-labeled phosphoinositide pools. *J. Cell Biol.* **143**, 501–510 (1998).
42. Y. B. Shrestha, K. Wickwire, S. Giraudo, Effect of reducing hypothalamic ghrelin receptor gene expression on energy balance. *Peptides* **30**, 1336–1341 (2009).
43. B. Holst, N. D. Holliday, A. Bach, C. E. Elling, H. M. Cox, T. W. Schwartz, Common structural basis for constitutive activity of the ghrelin receptor family. *J. Biol. Chem.* **279**, 53806–53817 (2004).
44. N. D. Holliday, B. Holst, E. A. Rodionova, T. W. Schwartz, H. M. Cox, Importance of constitutive activity and arrestin-independent mechanisms for intracellular trafficking of the ghrelin receptor. *Mol. Endocrinol.* **21**, 3100–3112 (2007).
45. M. Ahmad, J. S. Polepalli, D. Goswami, X. Yang, Y. J. Kaeser-Woo, T. C. Sudhof, R. C. Malenka, Postsynaptic complexin controls AMPA receptor exocytosis during LTP. *Neuron* **73**, 260–267 (2012).
46. M. C. Oh, V. A. Derkach, E. S. Guire, T. R. Soderling, Extrasynaptic membrane trafficking regulated by GluR1 serine 845 phosphorylation primes AMPA receptors for long-term potentiation. *J. Biol. Chem.* **281**, 752–758 (2006).
47. N. J. Broadbent, L. R. Squire, R. E. Clark, Spatial memory, recognition memory, and the hippocampus. *Proc. Natl. Acad. Sci. U.S.A.* **101**, 14515–14520 (2004).
48. M. Leger, A. Quiedeville, V. Bouet, B. Haelewyn, M. Boulouard, P. Schumann-Bard, T. Freret, Object recognition test in mice. *Nat. Protoc.* **8**, 2531–2537 (2013).
49. A. M. Oliveira, T. J. Hemstedt, H. Bading, Rescue of aging-associated decline in Dnmt3a2 expression restores cognitive abilities. *Nat. Neurosci.* **15**, 1111–1113 (2012).

50. H. K. Lee, K. Takamiya, J. S. Han, H. Man, C. H. Kim, G. Rumbaugh, S. Yu, L. Ding, C. He, R. S. Petralia, R. J. Wenthold, M. Gallagher, R. L. Huganir, Phosphorylation of the AMPA receptor GluR1 subunit is required for synaptic plasticity and retention of spatial memory. *Cell* **112**, 631–643 (2003).
51. M. Damian, J. Marie, J. P. Leyris, J. A. Fehrentz, P. Verdier, J. Martinez, J. L. Baneres, S. Mary, High constitutive activity is an intrinsic feature of ghrelin receptor protein: A study with a functional monomeric GHS-R1a receptor reconstituted in lipid discs. *J. Biol. Chem.* **287**, 3630–3641 (2012).
52. A. Kern, R. Albarran-Zeckler, H. E. Walsh, R. G. Smith, Apo-ghrelin receptor forms heteromers with DRD2 in hypothalamic neurons and is essential for anorexigenic effects of DRD2 agonism. *Neuron* **73**, 317–332 (2012).
53. A. Kern, M. Mavrikaki, C. Ullrich, R. Albarran-Zeckler, A. F. Brantley, R. G. Smith, Hippocampal dopamine/DRD1 signaling dependent on the ghrelin receptor. *Cell* **163**, 1176–1190 (2015).
54. E. J. Lopez Soto, F. Agosti, A. Cabral, E. R. Mustafa, V. M. Damonte, M. A. Gandini, S. Rodriguez, D. Castrogiovanni, R. Felix, M. Perello, J. Raingo, Constitutive and ghrelin-dependent GHSR1a activation impairs CaV2.1 and CaV2.2 currents in hypothalamic neurons. *J. Gen. Physiol.* **146**, 205–219 (2015).
55. V. Martinez Damonte, S. S. Rodriguez, J. Raingo, GHSR constitutive activity impairs voltage-gated calcium channel (CaV)-dependent inhibitory neurotransmission in hippocampal neurons. *J. Physiol.* (2018).
56. E. R. Mustafa, E. J. Lopez Soto, V. Martinez Damonte, S. S. Rodriguez, D. Lipscombe, J. Raingo, Constitutive activity of the Ghrelin receptor reduces surface expression of voltage-gated Ca²⁺ channels in a CaV β -dependent manner. *J. Cell Sci.* **130**, 3907–3917 (2017).
57. A. A. J. Rouault, L. K. Rosselli-Murai, C. C. Hernandez, L. E. Gimenez, G. G. Tall, J. A. Sebag, The GPCR accessory protein MRAP2 regulates both biased signaling and constitutive activity of the ghrelin receptor GHSR1a. *Sci. Signal.* **13**, eaax4569 (2020).
58. M. Asai, S. Ramachandrapa, M. Joachim, Y. Shen, R. Zhang, N. Nuthalapati, V. Ramanathan, D. E. Strohlic, P. Ferket, K. Linhart, C. Ho, T. V. Novoselova, S. Garg, M. Ridderstrale, C. Marcus, J. N. Hirschhorn, J. M. Keogh, S. O'Rahilly, L. F. Chan, A. J. Clark, I. S. Farooqi, J. A. Majzoub, Loss of function of the melanocortin 2 receptor accessory protein 2 is associated with mammalian obesity. *Science* **341**, 275–278 (2013).
59. Genome-wide atlas of gene expression in the adult mouse brain (2021); <http://mouse.brain-map.org/gene/show/89308>.
60. Tissue-based map of the human proteome; www.proteinatlas.org/ENSG00000135324-MRAP2/brain.
61. A. Edwards, A. Abizaid, Clarifying the ghrelin system's ability to regulate feeding behaviours despite enigmatic spatial separation of the ghrelin and its endogenous ligand. *Int. J. Mol. Sci.* **18**, 859 (2017).
62. A. Cabral, E. J. Lopez Soto, J. Epelbaum, M. Perello, Is ghrelin synthesized in the central nervous system? *Int. J. Mol. Sci.* **18**, 638 (2017).
63. S. Els, A. G. Beck-Sickinger, C. Chollet, Ghrelin receptor: High constitutive activity and methods for developing inverse agonists. *Methods Enzymol.* **485**, 103–121 (2010).
64. S. W. Flavell, C. W. Cowan, T. K. Kim, P. L. Greer, Y. Lin, S. Paradis, E. C. Griffith, L. S. Hu, C. Chen, M. E. Greenberg, Activity-dependent regulation of MEK2 transcription factors suppresses excitatory synapse number. *Science* **311**, 1008–1012 (2006).
65. M. Renner, D. Choquet, A. Triller, Control of the postsynaptic membrane viscosity. *J. Neurosci.* **29**, 2926–2937 (2009).
66. S. D. Santos, O. Iuliano, L. Ribeiro, J. Veran, J. S. Ferreira, P. Rio, C. Mulle, C. B. Duarte, A. L. Carvalho, Contactin-associated protein 1 (Caspr1) regulates the traffic and synaptic content of α -amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA)-type glutamate receptors. *J. Biol. Chem.* **287**, 6868–6877 (2012).
67. B. H. Gähwiler, M. Capogna, D. Debanne, R. A. McKinney, S. M. Thompson, Organotypic slice cultures: A technique has come of age. *Trends Neurosci.* **20**, 471–477 (1997).
68. M. Jiang, L. Deng, G. Chen, High Ca²⁺-phosphate transfection efficiency enables single neuron gene analysis. *Gene Ther.* **11**, 1303–1311 (2004).
69. P. Opazo, S. Labrecque, C. M. Tigaret, A. Frouin, P. W. Wiseman, P. De Koninck, D. Choquet, CaMKII triggers the diffusional trapping of surface AMPARs through phosphorylation of stargazin. *Neuron* **67**, 239–252 (2010).
70. L. Groc, M. Heine, L. Cognet, K. Brickley, F. A. Stephenson, B. Lounis, D. Choquet, Differential activity-dependent regulation of the lateral mobilities of AMPA and NMDA receptors. *Nat. Neurosci.* **7**, 695–696 (2004).
71. C. R. Gerfen, M. A. Rogawski, D. R. Sibley, P. Skolnick, S. Wray, *Short Protocols in Neuroscience: Systems and Behavioral Methods*, C. R. Gerfen, D. R. Sibley, P. Skolnick, S. Wray, Eds. (John Wiley & Sons, 2006).
72. N. Matsuo, K. Takao, K. Nakanishi, N. Yamasaki, K. Tanda, T. Miyakawa, Behavioral profiles of three C57BL/6 substrains. *Front. Behav. Neurosci.* **4**, 29 (2010).

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Ligand-independent activity of the ghrelin receptor modulates AMPA receptor trafficking and supports memory formation

Luís F. Ribeiro, Tatiana Catarino, Mário Carvalho, Luísa Cortes, Sandra D. Santos, Patricio O. Opazo, Lyn Rosenbrier Ribeiro, Bárbara Oliveiros, Daniel Choquet, José A. Esteban, João Peça and Ana Luísa Carvalho

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No ligand needed for learning

In addition to its role in stimulating appetite, the hormone ghrelin and its receptor GHS-R1a are implicated in cognition. Ribeiro *et al.* found a role for ghrelin-independent GHS-R1a signaling in learning in mice. The use of inverse agonists and mutants revealed that ligand-independent activity of GHS-R1a maintained the synaptic abundance of AMPA-type glutamate receptors in hippocampal neurons through a phosphorylation-dependent trafficking mechanism in cultured neurons and brain slices, thereby ensuring tonic control of synaptic plasticity. Treating mice with a GHS-R1a inverse agonist impaired spatial and contextual memory formation. Thus, the use of ghrelin receptor–blocking therapies—which have been proposed for treating metabolic disorders, acromegaly, cancer, and alcoholism—may also have cognitive side effects.

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