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Ghrelin upregulates the phosphorylation of the GluN2B subunit of the NMDA receptor by activating GHSR1a and Fyn in the rat hippocampus

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Abstract

Ghrelin and its receptor GHSR1a have been shown to exert numerous physiological functions in the brain, in addition to the well-established orexigenic role in the hypothalamus. Earlier work indicated that ghrelin stimulated the phosphorylation of the GluN1 subunit of the NMDA receptor (NMDAR) and enhanced synaptic transmission in the hippocampus. In the present study, we report that the exogenous application of ghrelin increased GluN2B phosphorylation. This increase was independent of GluN2B subunit activity or NMDAR channel activity. However, it depended on the activation of GHSR1a and Fyn as it was blocked by D-Lys3-GHRP-6 and PP2, respectively. Inhibitors for G-protein-regulated second messengers, such as Rp-cAMP, H89, TBB, ryanodine, and thapsigargin, unexpectedly enhanced GluN2B phosphorylation, suggesting that cAMP, PKA, casein kinase II, and cytosolic calcium signaling may oppose to the effect of ghrelin on the phosphorylation of GluN2B. Our findings suggest that 1) GluN2B is likely a molecular target of ghrelin and GHSR1a-driven signaling cascades, and 2) the ghrelin-mediated phosphorylation of GluN2B depends on Fyn activation under complex negative regulation by other second messengers.

Keywords

hippocampal CA1; GHSR1a knockout mice; slice culture; GluN2B

1. Introduction

The composition of subunits is an important source of diversity for the functional regulation of the NMDAR (Cull-Candy et al., 2004). GluN1 is a channel-forming obligatory subunit,

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and GluN2A and GluN2B are the primary regulatory subunits. Although a trend for the decrease in the expression of GluN2B during development cannot be underestimated, the GluN2B subunit is essential for both neonatal and mature NMDARs and is highly expressed in the entire embryonic brain and the adult forebrain (Watanabe et al., 1992). GluN2B is a necessary subunit for NMDAR to be localized at synapses (Kutsuwada et al., 1996) and to generate synaptic responses, enhance LTP, and promote plasticity-associated spine growth (Ster et al., 2014). The majority of native receptors in the adult hippocampus are triheteromers that contain two GluN1 and two different GluN2 subunits, such as GluN1/GluN2A/GluN2B (Hansen, et al., 2014).

There is ample evidence that NMDAR subunits are phosphorylated by a variety of synapse-enriched protein kinases (Roche et al., 1994), including protein kinase A (PKA), protein kinase C (PKC), and calcium/calmodulin-dependent protein kinase II (CaMKII) (Wang et al., 2014). When subunits are phosphorylated, they cause changes in channel properties and synaptic strength that underlie many forms of synaptic plasticity (Lee et al., 2006). Phosphorylation of the GluN1 subunit at Ser896 by PKC and Ser897 by PKA was shown to facilitate receptor trafficking and surface expression by disrupting GluN1 clustering in the endoplasmic reticulum and Golgi apparatus (Scott et al., 2001). We previously reported that the exogenous application of ghrelin facilitated the phosphorylation of GluN1 at Ser896 and Ser897 and increased NMDAR-mediated synaptic currents in hippocampal slices (Muniz and Isokawa, 2015). This finding led us to investigate whether ghrelin is also involved in the phosphorylation of GluN2B, which could lead to the synergistic or cooperative upregulation of NMDAR function and receptor-mediated synaptic activity. In the present study, we focused on GluN2B phosphorylation on Tyr1336 because 1) ghrelin-induced activation of GHSR1a increased Fyn kinase activity in macrophages (Demers et al., 2009), and 2) Fyn kinase was reported to phosphorylate GluN2B at Tyr1336 in cultured hippocampal neurons (Wu et al., 2007). Our goal is to fill a gap in the knowledge by testing the hypotheses that 1) GHSR1a activation by ghrelin leads to the phosphorylation of GluN2B at Tyr1336 in the hippocampus, and 2) this process involves the activity of Fyn as a downstream kinase.

2. Results

2.1. Localization of GHSR1a

GHSR1a immunoreactivity (GHSR1a-IR) was detected in the stratum radiatum (S.R.), stratum pyramidale (S.P.), and stratum oriens (S.O.) of CA1 (Fig. 1a). GHSR1a-IR was selected as a red ROI (region of interest) (Fig. 1b1 and b2) in the images that were taken at high magnification (40x and 5 zooms), and quantified as described in the Methods section. We found that S.P. showed a higher density of GHSR1a-IR when compared with S.R. and S.O. (Fig. 1b3, $p<0.001$). The antibodies were validated for specificity in the GHSR1a Knockout mouse hippocampus in which no GHSR1a-IR was detected (Fig. 1c2), while GHSR1a-IR was clearly observed in wild type mouse (Fig. 1c1).

Our immunohistochemical results were complemented by our ghrelin-binding experiment using FITC-conjugated octanoylated and non-octanoylated ghrelin (F302 and F203, respectively). F302 octanoylated-ghrelin binding was identified in sparsely distributed punctate structures over the soma and dendritic membrane (Fig. 1d1). In contrast, we did not

detect any signal with F203 non-octanoylated-ghrelin (Fig. 1d2). As the octanoylated form of ghrelin is the only ligand that binds to GHSR1a, this observation supported our finding that GHSR1a was expressed on the somatic and dendritic layers of the pyramidal cell.

2.2. Ghrelin enhanced the phosphorylation of GluN2B by activating GHSR1a and Fyn kinase

Phosphorylated GluN2B (pGluN2B) on Tyr1336 was detected as immunopositive fluorescent signals (Fig. 2a). These fluorescent signals were selected as red ROIs (Fig. 2b). In some cases, the pattern of pGluN2B immunoreactivity (pGluN2B-IR) appeared circular. However, as shown by DAPI-staining, pGluN2B-IR was independent of the location of presumable inhibitory neuron somata in the stratum radiatum (Fig. 2c and d).

The exogenous application of ghrelin (100 nM for 2 hours) increased pGluN2B-IR in the stratum radiatum of CA1 (a dendritic zone of the pyramidal cell, Fig. 2f), when compared with controls (Fig. 2e) ($p<0.001$ in Fig. 2i). The ghrelin-induced increase in pGluN2B was mediated by the activation of GSHR1a; as the antagonist of GHSR1a, D-Lys3-GHRP-6 (1 μM) completely negated the effect of ghrelin (Fig. 2g) ($p=0.991$ in cnt vs DLys in Fig. 2i). This finding was supported by the lack of an increase in pGluN2B-IR in GHSR1a KO mice in response to the exogenous application of ghrelin (KO vs. WT in Fig. 2i) ($p=0.999$).

We tested the involvement of Fyn. Bath-application of PP2 in 10 μM, the inhibitor of Fyn, blocked ghrelin's stimulatory effect on GluN2B phosphorylation (Fig. 2h) ($p<0.001$ in Fig. 2i). The magnitude of the inhibition exerted by PP2 was similar to that of D-Lys3-GHRP-6 ($p=0.942$ in PP2 vs DLys in Fig. 2i), suggesting that Fyn is likely the primary kinase that is responsible for the ghrelin-induced and GHSR1a-mediated phosphorylation of GluN2B.

2.3. Negative regulation of ghrelin-induced phosphorylation of GluN2B by intracellular calcium

The ghrelin-induced increase in the magnitude of pGluN2B-IR was insensitive to the NMDAR channel blocker, MK801 (100 μM) and the specific GluN2B inhibitor, ifenprodil (5 μM) ($P=0.997$ in ghr vs. ghr plus MK801; $p=0.999$ in ghr vs. ghr plus ifenprodil) (Fig. 3). This finding suggested that the phosphorylation of GluN2B at Tyr1336 by ghrelin (and the subsequent activation of GHSR1a) does not require NMDA receptor channel activity or GluN2B subunit activity, although others reported that the channel activity might be required under some instances as a co-regulator for the maintenance of phosphorylation state (Plattner et al., 2014).

GHSR1a is primarily coupled to the Gq protein (Kojima, 1999). Thus, we tested the contribution of calcium-release from internal stores upon Gq activation in the ghrelin-induced phosphorylation of GluN2B. The chelation of extracellular calcium ions by the bath-application of BAPTA (10 mM) did not have any effect ($p=1.000$; ghr vs. ghr plus BAPTA) (Fig. 3). By contrast, a blockade of calcium-release from internal stores by ryanodine ($p<0.001$ when compared with ghrelin) and thapsigargin ($p<0.001$ when compared with ghrelin) amplified the effect of ghrelin on the phosphorylation of GluN2B by increasing the magnitude of pGluN2B-IR (Fig. 3). The application of ryanodine or thapsigargin alone (without ghrelin) increased pGluN2B-IR similar to the level that ghrelin

alone had achieved (p=0.005 in cnt vs. Ryn only; p=0.01 in cnt vs TG only). This suggested that GluN2B phosphorylation may be homeostatically regulated by the ambient fluctuation of cytosolic calcium-concentration caused by the internal release of calcium from the ryanodine receptor.

2.4. Negative regulation of ghrelin-induced phosphorylation of GluN2B by protein kinase A

Ligand-activated GHSR1a was reported to initiate cAMP signal transduction (Cuellar and Isokawa, 2011). Thus, we tested the involvement of protein kinase A (PKA) in the ghrelin-induced phosphorylation of GluN2B. Forskolin (30 μM), when applied alone without ghrelin, doubled the amount of ghrelin-induced pGluN2B-IR (p<0.001) (frs in Fig. 4). When forskolin was applied with ghrelin, the magnitude of the combined effect on pGluN2B-IR was not different from the effect of forskolin alone (p=0.997 in forskolin alone vs. ghr plus forskolin) (Fig. 4). This result can be interpreted as follows: 1) the cAMP-mediated signaling pathway was involved in the ghrelin-induced phosphorylation of GluN2B, or 2) forskolin phosphorylated GluN2B by activating cAMP signaling cascades, independently of ghrelin and GHSR1a. We tested the possibility that cAMP/PKA pathways were activated in the process of GluN2B phosphorylation by ghrelin. Increasing doses of Rp-cAMP (the inhibitor of PKA) amplified ghrelin's effect on the magnitude of pGluN2B-IR in a dose-dependent manner (p=0.065 in ghr vs. Rp-cAMP 50; p<0.001 in ghr vs. Rp-cAMP 100; p<0.001 in Rp-cAMP 50 vs. 100; p<0.001 in Rp-cAMP 50 vs. 700) (Fig. 4). The effect reached to a plateau at 100 μM (p=0.966 in Rp-cAMP 100 vs 700). H-89 (20 μM), another inhibitor of PKA, mimicked the effect of Rp-cAMP (p<0.001 when compared with ghrelin). This outcome was the opposite of what we expected based on the result obtained from forskolin administration. When Rp-cAMP and H-89 were applied alone without ghrelin, they did not have any effect on pGluN2B-IR (p=0.999 in cnt vs. Rp-cAMP50; cnt vs. Rp-cAMP 100; cnt vs. Rp-cAMP 700; and cnt vs. H89) (Fig. 4). PKA and its downstream signaling molecules were therefore activated by ghrelin. However, once activated, they exerted an effect that opposed ghrelin-induced and GHSR1a-mediated phosphorylation of GluN2B at Tyr1336.

Lastly, the involvement of calcium/calmodulin kinase II (CaMKII) and casein kinase II (CKII) was tested. An inhibitor of CaMKII, KN93 (10 μM) did not have any effect on ghrelin-induced increase in pGluN2B-IR (p=0.891 in ghr vs. ghr plus KN93) (Fig. 4), suggesting that CaMKII was not involved. On the other hand, the co-application of ghrelin and an inhibitor of CKII, TBB (25 μM), amplified the magnitude of pGluN2B-IR over 2-folds (p<0.001) (Fig. 4). The application of TBB alone without ghrelin did not have any effect (p=0.999 in cnt vs. TBB). This result suggested that, in the hippocampus, CKII was a downstream molecule that was activated by the GHSR1a signaling pathway and inhibited GluN2B phosphorylation in the presence of ghrelin.

3. Discussion

The present study demonstrates that ghrelin stimulated the phosphorylation of GluN2B subunit on Tyr1336 by activating GHSR1a and a non-receptor Src family tyrosine kinase Fyn in cultured hippocampal slices. Although there is no evidence available to date that

demonstrates co-localization of GHSR1a and GluN2B in the stratum radiatum of the CA1, the localization of GHSR1a in this region of the hippocampus is demonstrated in the present study and the localization of GluN2B was reported by Watanabe et al (1998). The functional interaction between GHSR1a and the NMDAR subunit GluN2B which we report in the present study suggests a likelihood of physical proximity of these two receptors and the possibility that dendritic co-localization exists between GHSR1a and triheteromeric NMDARs that express GluN2B subunits.

The present observation of the localization of GHSR1a in CA1 hippocampal neurons is in agreement with reports by Cabral et al. (2013), Mani et al (2014), and Muniz and Isokawa (2015); all identified GHSR1a in the hippocampus using either a fluorescent ghrelin or a transgenic mouse model carrying eGFP-tagged GHSR1a. The present study also revealed that GHSR1a was expressed and localized in small punctate structures on the somatic and dendritic membranes of pyramidal neurons, or possibly on axon terminals that synapsed on the pyramidal cell soma and dendrites. In the hypothalamus, GHSR1a was reported to be localized at GABAergic axon terminals (Soto et al., 2015). However, in the hippocampus, there is no report available to date to determine the subcellular localization of GHSR1a in any neurochemically or anatomically identified neuron subtypes.

There are a couple of concerns regarding our results. First, we identified the ghrelin-induced and GHSR1a-mediated upregulation of phosphorylated GluN2B subunit immunocytochemically, using two negative controls: 1) GHSR1a knockout mice did not show any increase in pGluN2B-IR in response to ghrelin, and 2) the pharmacological block of GHSR1a by the receptor antagonist (D-Lys3-GHRP-6) negated ghrelin's stimulatory effect on the phosphorylation of GluN2B. Our immunohistochemical conclusion could be further substantiated if secondary evidence such as western blots and/or PCR with single-cell resolution was available. Second, our hippocampal slice specimens were prepared from P6 (postnatal day 6) pups of rats and GHSR1a KO and WT mice. Since there is a report that mRNA expression for GHSR1a was weak in the adult rat CA1 region (Zigman et al., 2006), our present findings may be age-dependent and thus should be interpreted accordingly. It is important to study the effect of ghrelin on the phosphorylation of the GluN2B subunit in adult rat hippocampus.

GluN2B is the predominant tyrosine-phosphorylated protein in the PSD (Moon et al., 1994). GluN2B contains three tyrosine phosphorylation sites (Tyr1252, Tyr1336, and Tyr1472), which are all phosphorylated by Fyn (Nakazato et al., 2001). However, in the hippocampus, Tyr1336 was the only target reported to interact with Fyn (Wu et al., 2007). Thus, we focused on the Tyr1336 phosphorylation site to test for its interaction with GHSR1a signaling. It has been reported that the up-regulation of activities of Src family kinases including Fyn potentiated NMDA receptor currents, and the down-regulation of these kinase activities reduced NMDA receptor currents (Wang and Salter, 1994). Thus, it may be of interest to determine whether the other two target sites for Fyn also respond to ghrelin in the hippocampus.

It has been demonstrated that GluN2B-containing NMDA receptors undergo more robust endocytosis (Roche et al., 2001) and have higher surface mobility (Groc et al., 2006) than

GluN2A-containing receptors. Phosphorylation of GluN1 at Ser 896/897 was also reported to facilitate NMDAR surface expression (Scott et al., 2001), and ghrelin was reported to facilitate GluN1 phosphorylation at Ser896/897 (Muniz and Isokawa, 2015). Together this evidence suggests that ghrelin and GHSR1a receptor-mediated signaling may preferentially target phosphorylation sites that regulate receptor trafficking and surface expression on both subunits (GluN1 and GluN2B).

We found that ghrelin-induced and GHSR1a-mediated phosphorylation of GluN2B on Tyr1336 might be under the negative regulation by PKA, CKII, and cytosolic calcium (as demonstrated by the inhibitors of these signaling molecules in Fig. 3 and Fig. 4). GluN2B has phosphorylation sites that are substrates for kinases such as cAMP-dependent kinase A (PKA), protein kinase C (PKC), CaMKII, and casein kinase II (CKII) (Lee et al., 2006), indicating complex regulation of GluN2B activities (Chen and Roche, 2007). Moreover, GHSR1a was reported to couple to more than one type of G-protein (Yin et al., 2014). Through the activation of multiple G-proteins and phosphorylation sites, the second messenger kinases may limit or indirectly restrain Fyn activities on Tyr1336. On the other hand, increased evidence indicates that G-protein coupled receptors may signal through G-protein-independent pathways, i.e., JAK/STATs, Src-family tyrosine kinases, GRK/β-arrestins, and PDZ domain-containing proteins (Sun et al., 2007). We cannot rule out the possibility that Fyn, a non-receptor Src-family tyrosine kinase, might have directly interacted with GHSR1a in a G-protein independent manner in the present study.

We observed that the inhibition of CKII and PKA caused an additional enhancement in the ghrelin-induced phosphorylation of GluN2B at Tyr1336. This finding suggests that CKII and PKA were activated as a result of ghrelin binding to and activating GHSR1a. However, the consequence of their activation was the negative regulation of GluN2B phosphorylation on Tyr1336. CKII was reported to phosphorylate GluN2B on Ser1480 and to disrupt the interaction between GluN2B and PSD-95 (Chung et al., 2004). PKA was reported to phosphorylate GluN2B on Ser1166 and regulate Ca^{2+}-signaling in dendritic spines (Murphy et al., 2014). Taken together, the phosphorylation of Ser1480 by CKII and phosphorylation of Ser1166 by PKA may both oppose the Fyn-mediated phosphorylation of Tyr1336, even if all kinases (including Fyn) were activated by ghrelin and GHSR1a.

GHSR1a has been suggested to have intrinsically-high constitutive activity (Damian et al., 2012). However, we did not find any difference in the level of basal phosphorylation of GluN2B among GHSR1a KO mice, WT mice, and naïve rats. Although it may be premature to conclude that constitutive activity of GHSR1a was not involved in the present study, Soto and colleagues (2015) reported that intracellular signaling pathways involved in the ligand-induced activation of GHSR1a and constitutively active GHSR1a were different. There is also a report to suggest that the constitutive activity of this receptor may be significant in artificially expressed cell lines and re-constituted systems, but may not be a contributing factor in vivo (Muller et al., 2015).

In conclusion, NMDAR subunits have potential sites of phosphorylation by various protein kinases. Phosphorylation at these sites regulates NMDAR channel activity through a variety of means, including changes in channel conductance, surface expression, and receptor

trafficking. Fyn has been indicated to act as a point of convergence for many signaling pathways that upregulate GluN2B-containing NMDAR (Trepanier et al., 2012). Fyn lowers the threshold for LTP induction at CA1 synapses by reducing GABAergic inhibition (Lu et al., 1999). Although this facilitates the development of synaptic plasticity, it also makes the system vulnerable to excitotoxicity (Wu et al., 2007). In addition to the activation of Fyn, GHSR1a activates multiple signaling pathways that are involved in neuroprotection (Bayliss et al., 2016), the elevation of seizure thresholds (Portelli et al., 2015), and nurturing dendritic spines (Berrout and Isokawa, 2012). Further investigation is needed to understand possible consequences of our present findings in hippocampal neuron physiology and pathophysiology.

4. Experimental Procedure

4.1. Animals

Sprague-Dawley rats, GHSR1a knock-out mice (GHSR1a −/−) (B6N(Cg)-*Ghsr*$^{tm1.1(KOMP)Vlcg}$/J/Stock# 018595), and the same strain of wild type mice were used. The rats were purchased from Charles River (Wilmington, MA), and the genetically modified mouse model was purchased from The Jackson Laboratory (Bar Harbor, ME). Experiments were carried out in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals (NIH Publications No. 80-23). All protocols were approved by the University of Texas Rio Grande Valley Institutional Animal Care and Use Committee.

4.2. Hippocampal slice culture

Hippocampi from both hemispheres were dissected from P6 pups of both sexes of rats and mice. They were cut into 400 μm thick slices with a chopper (Stoelting, Wood Dale, IL). Slices were placed in the Transwell Culture Insert (Costar, Corning, NY) and incubated in culture media that consisted of: 50% MEM, 25% HBSS, 24% horse serum, 0.5% penicillin/streptomycin solution, 0.5% glucose solution, and 25 mM HEPES with 5% CO_2 at 35°C for up to 3 weeks (Stoppini et al., 1991). Slice culture was used because 1) chemical effect of agonists and antagonists of GHSR1a can be assessed directly on the hippocampal synapses in isolation while eliminating multi-synaptic circuit activities projected from extra-hippocampal regions, and 2) acutely prepared slices may reflect a fluctuating level of systemic ghrelin at the time of decapitation among animals, as systemic ghrelin may cross the blood-brain barrier (Muller et al., 2015). However, slice culture can provide a stable control level in order to test the effect of exogenously applied ghrelin on hippocampal neurons, independent of systemic ghrelin.

In the present study, the following compounds were used: ghrelin (100 nM, Phoenix Pharmaceuticals, Burlingame, CA), D-Lys3-GHSR6 (10 μM; Bachem, Torrance, CA), ifenprodil (5 μM), Rp-cAMP (50 μM), and ryanodine (50 μM); all from Sigma Chemical, St. Louis, MO. Thapsigargin (4 μM), Bapta (10 mM), MK801 (100 μM), forskolin (30 μM), H-89 (20 μM), TBB (25 μM), KN-93 (10 μM), and PP2 (10 μM); all from Tocris/R&D Systems, Minneapolis, MN.

4.3. Immunohistochemistry

Slices were immersion-fixed with 4% paraformaldehyde in 0.1 M phosphate buffered saline (PBS). At the end of the fixation, slices were rinsed and treated with 0.2% Triton-X-100 and 10% BSA for 1 hour, and incubated in a rabbit polyclonal anti-pGluN2B on Tyr1336 (1:500, Novus, cat# NB300-295, Littleton, CO; 1:400 Phosphosolution, cat# P1516-1336, Aurora, CO), or polyclonal anti-GHSR1a (1:200, Phoenix Pharmaceuticals, cat# H001-62 in rabbit, Burlingame, CA; 1:50 Santa Cruz Biotechnology, cat# SC-10359/374515 in goat, Santa Cruz, CA) for 48 hours at 4° C with gentle agitation. We did not find a difference in the immunostaining outcomes between the two different suppliers for the GHSR1a antibody or pGluN2B antibody (so the data were pooled for analysis). Slices were then incubated in Alexa 488-conjugated secondary antibody (1:200, Life Technologies, Grand Island, NY) for 1 hour at room temperature. For control conditions, we used a blocking peptide, omitted a primary antibody, or simultaneously applied primary and secondary antibodies. Slices were glass-mounted, cover-slipped, and imaged at a single cell resolution with a confocal microscope (Olympus Fluoview 1000). No dual labeling was attempted. Antibody information and control/references for specificity are summarized in Table 1.

4.4. Fluorescent ghrelin binding

The octanoylated (F302) and non-octanoylated (F203) forms of fluorescein isothiocyanate (FITC)-conjugated ghrelin were provided by L Luyt at the University of Western Ontario in Canada (McGirr et al., 2011). After fixation with 4% paraformaldehyde, hippocampal slices were incubated in F302 or F203 (1 μM)-containing phosphate-buffered saline (PBS: 0.1 M) for 1 h. At the end of incubation, slices were rinsed and glass-mounted, and examined with a confocal microscope (Fluoview 1000, Olympus, Center Valley, PA, USA).

4.5. Quantification of GHSR1a and pGluN2B immunoreactivity

Confocal images were acquired with 10x and 40x objectives, starting at low magnification. In some cases, a 40x objective was combined with a zoom function (x5). Single scanning was used. No repeated exposures, such as Kalman filtering in order to increase the signal-to-background contrast, were attempted. Laser power was kept constant under a given magnification among different experiments untill all specimens were scanned completely. Acquired images were stored in the Olympus Fluoview format for off-line analysis.

GHSR1a and pGluN2B IRs were analyzed with the IPLab imaging software (BD Bioscience, San Jose, CA). In each slice, 8–12 defined regions (65 x 70 μm^2/region) were sampled across CA1. In each region, immunofluorescence signals were measured using an auto-segmentation tool with the Triangle logic, and the magnitude of immunoreactivity was quantified by summing the “area” of individual immunofluorescence signals.

4.6. Statistical analysis

We used Sigma Stat 4.0 to test statistical significance. The results were presented as the mean ± SEM (standard error of the mean). Differences observed between experimental groups and control group were tested with One Way Analysis of Variance and Kruskal-Wallis One Way Analysis of Variance on Ranks. Normality was checked with the Kolmogorov-Smirnov test. All Pairwise Multiple Comparison Procedures were done with

Tukey Test. $P < 0.05$ was considered significant. Sample size: Six rats (12 regions/slice, 10 slices/rat) were used in the experiments reported in Figure 1. Seven rats (12 regions/slice, 10 slices/rat) and 19 KO and 20 WT mice (5 regions/slice, 5 slices/mouse) were used in each experimental condition reported in Figure 2. Six rats (8 regions/slice, 9 slices/rat) were used in each experimental condition reported in Figure 3. Five rats (8 regions/slice, 8 slices/rat) were used in each experimental condition reported in Figure 4.

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Highlights

1. Exogenous application of ghrelin increased the phosphorylation of GluN2B.
2. GHSR1a-induced pGluN2B depended on the activation of Fyn.
3. Ghrelin-mediated phosphorylation of GluN2B may be negatively regulated by some second messengers.
4. GluN2B is likely a molecular target of ghrelin and GHSR1a signaling.

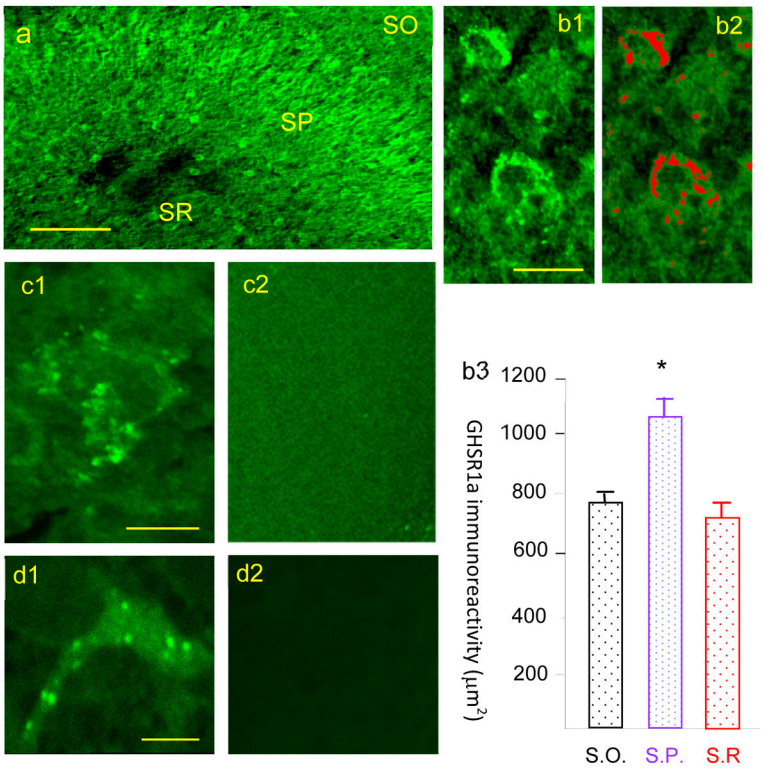

Figure 1.

Localization of GHSR1a. (a). GHSR1a immunoreactivity (IR) in the CA1 of the rat hippocampus. (b). GHSR1a IR in b1 was selected in red ROIs shown in b2, and quantified in the Stratum Oriens (SO), Stratum Pyramidale (SP), and Stratum Radiatum (SR) in the rat hippocampus in b3. Phoenix antibodies were used in a and b. (c) GHSR1a IR in wild type mouse in c1 and GHSR1a KO mouse in c2. Santa Cruz antibodies were used in c. (d). FITC-conjugated octanoylated ghrelin binding (d1) and FITC-conjugated non-octanoylated ghrelin binding (d2) in the rat hippocampus. Calibrations: 200 µm (a), 10 µm (b1, shared with b2), 5 µm (c1, shared with c2), 5 µm (d1, shared with d2). *$p<0.001$.

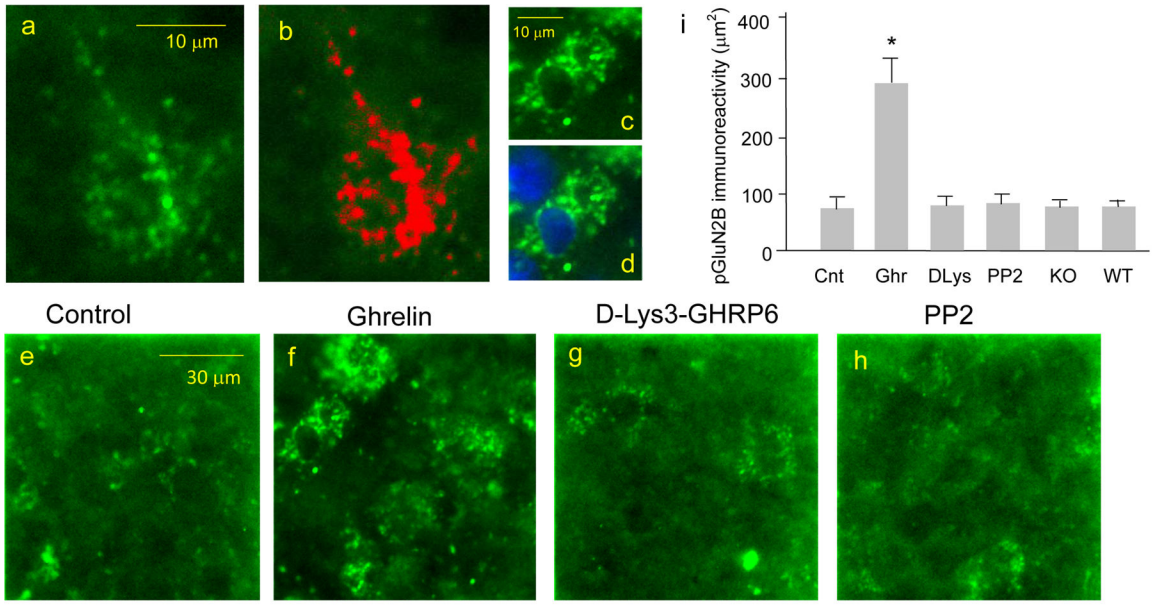

Figure 2.

GluN2B phosphorylation mediated by the activation of GHSR1a and Fyn. (a) pGluN2B immunoreactivity. (b) Immunofluorescence signals were selected in red ROIs. (c) pGluN2B immunoreactivity was dual stained with DAPI in d. (e) pGluN2B in control. (f) pGluN2B in ghrelin. (g) pGluN2B in D-Lys3-GHRP6. (h) pGluN2B in PP2. (i) A summary graph of pGluN2B expression in control, ghrelin, D-Lys3-GHRP6, PPT, and in GHSR1a knockout (KO) mice. *p<0.001.

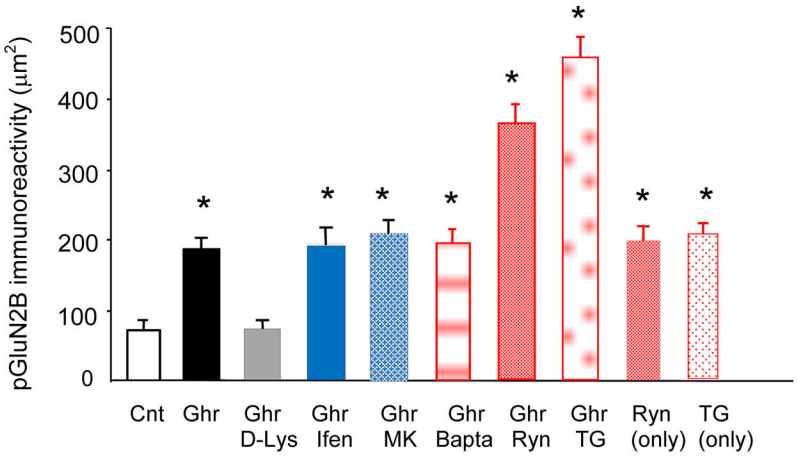

Figure 3.

Effect of the NMDA receptor channel blocker MK801 (MK), the GluN2B inhibitor ifenprodil (Ifen), BAPTA, ryanodine (Ryn), and thapsigargin (TG) on the ghrelin-mediated phosphorylation of GluN2B. * $p<0.001$.

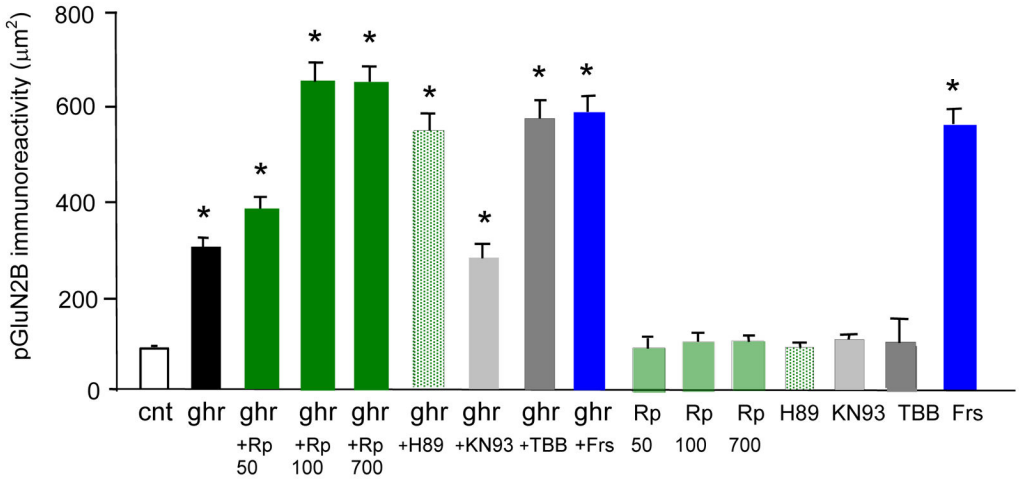

Figure 4.

Effects of kinase inhibitors, Rp-cAMP (PKA inhibitor), H89 (PKA inhibitor), KN-93 (CaMKII inhibitor), and TBB (casein kinase II inhibitor) on the ghrelin-mediated phosphorylation of GluN2B. *p<0.001.

Table 1

Antibodies used in the present study.

Antibody	Host	Supplier	Catalogue #	Control	Sources/References
Anti-GHSR1a	Rabbit	Phoenix Pharmaceutical	H-001-62	Sheep stomach Sheep brain (IHC)	Miller et al., 2005.
Anti-GHSR1a	Goat	Santa Cruz Biotechnology	SC-10359 (SC-374515)	Rat spinal cord (WB, IHC)	Lee et al., 2010.
Anti-pGluN2B		PhosphoSolution	P1516-1336	Rat hippocampus (WB) Rat frontal cortex & hippocampus (WB) Mouse cortex (WB)	Hicklin et al., 2011. Zamzow et al., 2016 Chernova et al., 2007
Anti-pGluN2B		Novus	NB300-295	Rat hippocampus (IHC, WB)	Novus data sheet

IHC: immunohistochemistry, WB: western blot