Chapter 5

The Ghrelin Receptor Regulates Dendritic Spines and the NMDA Receptor–Mediated Synaptic Transmission in the Hippocampus

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Additional information is available at the end of the chapter

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Abstract

Increasing evidence suggests the involvement of ghrelin (an orexigenic hormone) and its cognate receptor growth hormone secretagogue receptor (GHSR1a, also known as the ghrelin receptor) in extra-hypothalamic functions such as hippocampal learning and memory. However, cellular and molecular mechanisms underlying the ghrelin-regulated hippocampal neuron activity are poorly understood. In this chapter, we show the following: (1) ghrelin promoted phosphorylation of the *N*-methyl-D-aspartate receptor (NMDAR) subunit 1 (GluN1) in a PKC/PKA-dependent manner and amplified NMDAR-mediated excitatory postsynaptic currents, (2) ghrelin stimulated phosphorylation of CREB (cAMP response-element-binding protein), and (3) ghrelin increased phalloidin binding to F-actin, suggesting possible reorganization of dendritic spines; all occurred through the activation of GHSR1a in the CA1 pyramidal cell of the hippocampus in cultured slice preparations. Interestingly, the ghrelin's effects on GluN1 and CREB phosphorylation were negatively modulated by exogenous application of endocannabinoids, 2-arachidonoylglycerol (2-AG), and anandamide (ANE), in type 1 cannabinoid receptor (CB1R)-dependent and -independent manners, respectively. It is suggested that ghrelin and the ghrelin receptor regulate synaptic transmission and plasticity in the hippocampus, interacting with the endogenous cannabinoid system, which may be essential and necessary for successful acquisition of metabolic state–dependent learning and adaptive appetitive behavior.

Keywords: GluN1 phosphorylation, NMDAR-EPSC, ghrelin binding, GHSR1a KO mice, Phalloidin, CREB, endocannabinoid, CB1R

1. Introduction

We empirically know that we can learn things more easily, accurately, and quickly when we are interested, motivated, and reward-driven. Numerous animal models of learning have placed experimental subjects under fasted conditions in order for successful acquisition of specific tasks by using food as a reward [1]. This suggests the possibility that a molecule, which plays a critical role in appetitive behavior and/or reward-related feeling, may also be involved in the acquisition of learning.

1.1. Hippocampus in reward-related learning

Although the mesolimbic dopaminergic system is central to the study of motivational and reward-related learning, the neurobiological basis of reward-related learning cannot be explained completely without the participation of the hippocampus. The hippocampus is a primary site of activity-dependent plasticity and neuromodulation that have been hypothesized to be the neuronal substrate for learning and memory. The hippocampus lies upstream of the dopaminergic reward circuit and sends a major output to the reward system. More specifically, the hippocampal glutamatergic outputs regulate reward responses in the nucleus accumbens [2]. Therefore, cellular and synaptic plasticity within the hippocampus alters the transfer of information throughout the brain's reward system.

1.2. Ghrelin in reward-related learning

Ghrelin is a unique acylated 28 amino acid peptide hormone that is released from the stomach when it is empty. Ghrelin was originally identified as an endogenous ligand for the growth hormone secretagogue receptor (GHSR1a, now known as the ghrelin receptor) [3]. Activation of GHSR1a initiates a release of growth hormone from pituitary glands. Activation of GHSR1a also stimulates feeding center in the hypothalamus [4]. Indeed, the hypothalamus shows the highest localization of GHSR1a [4]. However, increasing evidence suggests that ghrelin may have numerous physiological functions in the brain outside the hypothalamus. For example, ghrelin stimulates the brain's reward center [5]. Ghrelin improves memory retention [6]. Thus, the accelerated acquisition of learning under fasted conditions suggests the potential importance of ghrelin as a key molecule for cellular and molecular mechanisms of reward-related learning and memory [7–9].

1.3. Source of ghrelin: brain or stomach?

Systemic ghrelin can cross the blood-brain barrier and enter the hippocampus [10]. Thus, peripheral ghrelin could be a source to be utilized by hippocampal neurons. On the other hand, in the mouse model, acylated ghrelin was readily transported across the blood-brain barrier in the brain-to-blood direction, but the quantity of its transport in the blood-to-brain direction appeared negligible [11]. Furthermore, vagotomy prevented peripheral ghrelin's effect on the hypothalamus [12], suggesting that ghrelin's direct effect on the brain may be of intrinsic origin [13, 14]. Neurons in the hypothalamus and septum are reported to be immunopositive to ghrelin [4] and likely to release ghrelin [15]. The septal neurons project

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directly to the hippocampus making monosynaptic connections [16]. Thus, centrally produced ghrelin in the septal neuron could be a source of ghrelin for hippocampal synapses, independently of systemic ghrelin, and contributes to neuron plasticity leading to contextual learning.

1.4. Investigating hippocampal GHSR1a in isolation

The hippocampus receives hypothalamic and arcuate projections directly from the fornix, while being situated centrally for functional interactions with other limbic cortexes by exchanging reciprocal synaptic connections. Thus, GHSR1a in the hippocampus is likely a direct target of hypothalamic and limbic cortical inputs including those from the septum. Each of these inputs may provide ghrelin independently or collectively to the hippocampal GHSR1a. Identifying the source(s) of ghrelin that affects hippocampal neuron function is an important scientific issue. Unfortunately, it is beyond the scope of this chapter because our goal is to first identify cellular and molecular mechanisms underlying the GHSR1a-involved process of hippocampal learning. Here, we review our progress in determining direct effects of ghrelin and GHSR1a-mediated cellular signaling in the hippocampal neuron synapse transmission and plasticity in isolation, without secondary modulation originating in the extra-hippocampal network activity, by using the hippocampal slice culture model and exogenous application of ghrelin.

2. Localization of GHSR1a in the hippocampus of cultured slices

2.1. Fluorescent ghrelin binding

We demonstrate the localization of GHSR1a in the cultured hippocampal slices with the use of three different methods. First of all, an octanoylated form of FITC-conjugated ghrelin (1 μM) was used in the rat hippocampal slice culture. Ghrelin can bind to GHSR1a only when it is octanoylated [17]. Thus, FITC-conjugated octanoylated ghrelin is a useful and reliable molecule that specifically binds to the receptor [18]. Non-octanoylated form of FITC-conjugated ghrelin was used as a control in order to confirm the specificity of octanoylated ghrelin binding. After 1 h of incubation followed by fixation (with 4% paraformaldehyde), intense binding was detected in the pyramidal cell layer of all CA fields with the given focal plane shown in **Figure 1a** [19]. However, by imaging FITC signals in many different focal planes in the same specimen using a confocal microscope (Fluoview 1000, Olympus), we learned that the actual binding ranged from dentate gyrus granule cell layer to CA1, CA2, and CA3 pyramidal cell layers, suggesting that GHSR1a has ample expression in the cultured hippocampus and is widely distributed throughout the dentate gyrus and Ammons horn.

2.2. eGFP-tagged GHSR1a expression in transgenic mouse hippocampus in slice culture

According to the eGFP-tagged reporter gene mapping of the whole mouse brain, GHSR1a is reported to be highly localized in the hippocampus [20]. However, it is not determined whether transgenic mouse hippocampus retains the expression of functional GHSR1a in the

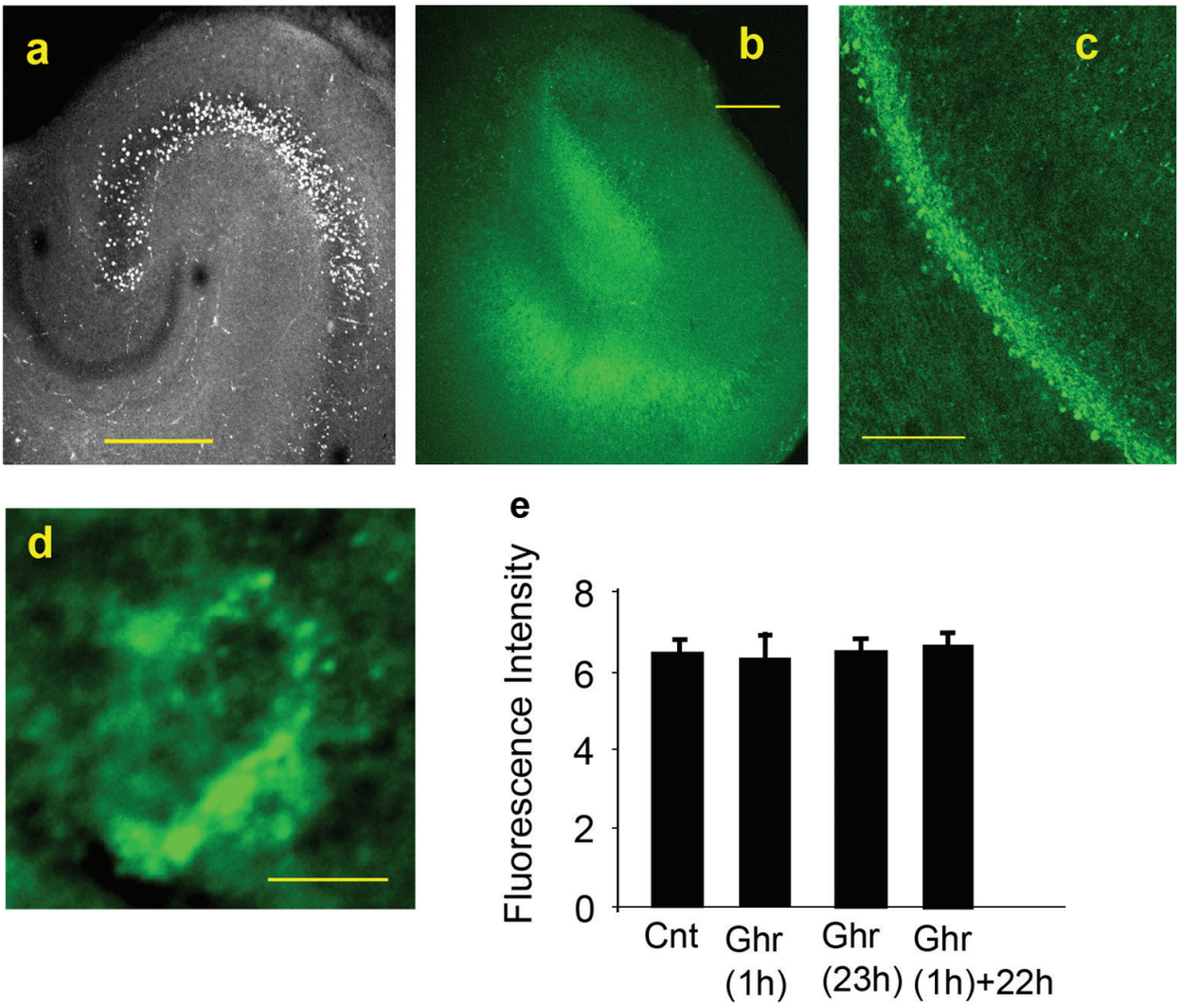

Figure 1. Localization of GHSR1a in cultured hippocampus in slices. **a**. Binding of the octanoylated form of FITC-conjugated ghrelin. **b**. eGFP signals from GHSR1a-expressing cells in mouse hippocampal slices. **c**. eGFP signals from CA1 pyramidal cell layer. **d**. GHSR1a immunoreactivity. **e**. GHSR1a expression was quantified in an arbitrary scale in control, 1-h incubation in ghrelin, 23-h incubation in ghrelin, and 1-h incubation in ghrelin followed by 22-h incubation in control media without ghrelin. Calibrations: (a) and (b) 500 μm, (c) 200 μm, and (d) 5 μm. (Modified with permission from Muniz and Isokawa [19]).

in vitro culture. Hippocampal slices were prepared from eGFP-tagged GHSR1a-expressing transgenic mouse brains, where the eGFP gene was inserted downstream of the GHSR1a promoter (Jackson Lab, B6;129S7-Ghsr<tm2Rgs</J/Stock# 019908). Up to 3 weeks in culture, the localization of GHSR1a was identified by live-imaging eGFP fluorescence. **Figure 1b** shows eGFP signals that are visible in the infra-pyramidal blade of the dentate gyrus and the pyramidal cell layer of the CA1 region. With higher magnification, the CA1 pyramidal cell layer became clearly detectable with resolution that is sufficient to identify individual neurons (**Figure 1c**). Under different focal planes, eGFP signals were detected in other CA fields (i.e., CA2 and CA3) in the pyramidal cell layer and from the supra-pyramidal blade of the dentate gyrus granule cell layer.

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2.3. Immunohistochemistry of GHSR1a

In addition to receptor binding and eGFP tagging, we investigated the localization of GHSR1a immunohistochemically in the cultured rat hippocampal slices using two different antibodies raised against GHSR1a (rabbit polyclonal anti-GHSR1a from Phoenix Pharmaceutical, Burlingame, CA, and from Santa Cruz Biotechnology Lab, Santa Cruz, CA). With both antibodies, GHSR1a immunoreactivity was successfully detected in the dentate gyrus and the Ammons horn of our rat hippocampal slice culture. More specifically, fluorescent signals collected from secondary antibody (Alexa 488, Life Technologies, Grand Island, NY) by confocal imaging revealed that GHSR1a was localized primarily as numerous aggregates surrounding the soma (**Figure 1d**). Together, these observations provide cellular and molecular evidence that the ghrelin receptor, GHSR1a, is expressed and localized in the cultured rat and mouse hippocampus in slices in a similar manner to what was reported in the in vivo whole brain specimen.

2.4. Receptor internalization in the hippocampal slice culture

GHSR1a exhibits an unusually high constitutive activity, which is the ability to propagate the intracellular signal in the absence of agonist [21, 22]. Thus, it may be possible that the downstream-signaling level, determined by constitutive activity, could reflect membrane expression of the receptor. In addition, agonist-induced internalization is a widely acknowledged process among all G-protein–coupled receptors including GHSR1a [23]. Thus, we were concerned whether GHSR1a in our hippocampal slice culture might have undergone such a process during the application of ghrelin in our experiments. Live slice cultures were incubated in ghrelin (100 nM) for either 1 or 23 h. At the end of the incubation, the specimens were fixed and processed for immunohistochemistry. GHSR1a immunoreactivity was imaged with a confocal microscope and quantified using image analysis software (IPLab, BD Bioscience, San Jose, CA). We did not find any difference in the magnitude of GHSR1a immunoreactivity between 1-h incubation and 23-h incubation, when compared with control (**Figure 1e**) [24]. We also conducted a "second" control, which was to incubate the slices in ghrelin for 1 h and the additional 22 h in control media (without ghrelin). At the end of the experiment, slices were fixed and processed for immunohistochemistry. There were no differences in the GHSR1a immunoreactivity between control and the "second" control. This finding suggested that GHSR1a did not appear to be internalized by the exogenous application of agonist to a significant extent even if a long-term incubation of 23 h was employed. There was a report that GHSR1a hardly desensitized with the nM range of concentrations of ghrelin [25], which is in agreement with our observation and supports the present finding.

3. Phosphorylation of GluN1 by ghrelin

Subunit phosphorylation is a critical step to facilitate the NMDAR channel function and to induce plasticity in excitatory synapses [26]. Among various phosphorylation sites in the NMDAR , we focused on the phosphorylation of Ser 896 and Ser 897 in the GluN1 subunit. The phosphorylation of Ser 896 is dependent on protein kinase C, and the phosphorylation

of Ser 897 is dependent on protein kinase A [27]. The reason for investigating these two phosphorylation sites are as follows: (1) GHSR1a is a Gq-coupled receptor, so that the activation of GHSR1a initiates inositol trisphosphate-mediated-signaling pathways leading to the activation of protein kinase C [28]; and (2) GHSR1a can cause a robust activation of cAMP/PKA-signaling cascade, which has been reported in the process of procuring sufficient energy [29].

3.1. Dose-dependent increase of pGluN1

Phosphorylation of GluN1 was studied using a goat polyclonal anti-pGluN1 at Ser 896/897 (Santa Cruz Biotechnology, Santa Cruz, CA) and Alexa 488 secondary antibody. Phosphorylated GluN1 (pGluN1) was quantified based on the fluorescent signal, captured by a confocal microscope. Representative pGluN1 signals were manually selected as a strongly fluorescing small puncta (examples are shown by yellow arrows in **Figure 2a**). A total of 236 representative puncta were manually selected from 30 confocal images, and the area and intensities of these puncta were measured using IPLab (BD Bioscience, San Jose, CA). Based on the measurement, selection criteria were established and applied to 2024 confocal images taken from 157 hippocampal slices. Among them, 35513 pGluN1 immunoreactive puncta, taken from 1714 confocal images, satisfied the selection criteria, and were used for data analysis.

Exogenous application of ghrelin (1–1000 nM) increased the magnitude of phosphorylation in GluN1 in a dose-dependent manner. The ghrelin-induced increase in pGluN1 immunoreactivity peaked at a concentration of 10 nM ($r^2 = 0.899$, $p < 0.0001$, $n = 56$; analysis of variance (ANOVA)) (**Figure 2b**). The ghrelin-induced changes in pGluN1 immunoreactivity were sensitive to and inhibited by the GHSR1a antagonist, D-Lys3-GHRP6 (1 μM), and an inverse agonist, substance *P*-analog (10 μM), when they were applied with ghrelin (100 nM). However, the application of D-Lys3-GHRP6 or substance *P*-analog alone (without ghrelin) did not have any effect as compared with control ($p < 0.001$, $n = 47$, unpaired *t*-test) (**Figure 2c** and **d**). This suggested the possibility that (1) there were few endogenous ghrelin molecules present in our cultured hippocampal slices, or (2) constitutive activation of GHSR1a (if any) did not appear to affect the phosphorylation state of GluN1 in cultured hippocampus in slices.

3.2. Effect of ghrelin on pGluN1 in GHSR1a knockout mouse

In cultured hippocampal slices prepared from homozygous GHSR1a knockout (-/-) mice, exogenous application of ghrelin (100 nM) failed to cause any change in pGluN1 immunoreactivity (**Figure 2e** and **f**) when compared with the wild-type GHSR1a (+/+) mouse hippocampus ($p < 0.005$, $n = 31$, unpaired *t*-test) (**Figure 2e** and **f**). Slices prepared from heterozygous GHSR1a knockout (+/-) mouse hippocampus also failed to show any change in pGluN1 immunoreactivity in response to ghrelin (100 nM) (**Figure 2e** and **f**). This suggested that a small reduction in the expression of GHSR1a could negate the ghrelin's effect on the GluN1 subunit phosphorylation at Ser 896/897. Furthermore, we found that the baseline pGluN1 was elevated in homozygous GHSR1a knockout (-/-) mice as compared with wild-type GHSR1a (+/+) mice in both control and ghrelin. This suggested that the NMDAR channel may be hyperactive in the hippocampus of homozygous GHSR1a knockout (-/-) mouse. It might be

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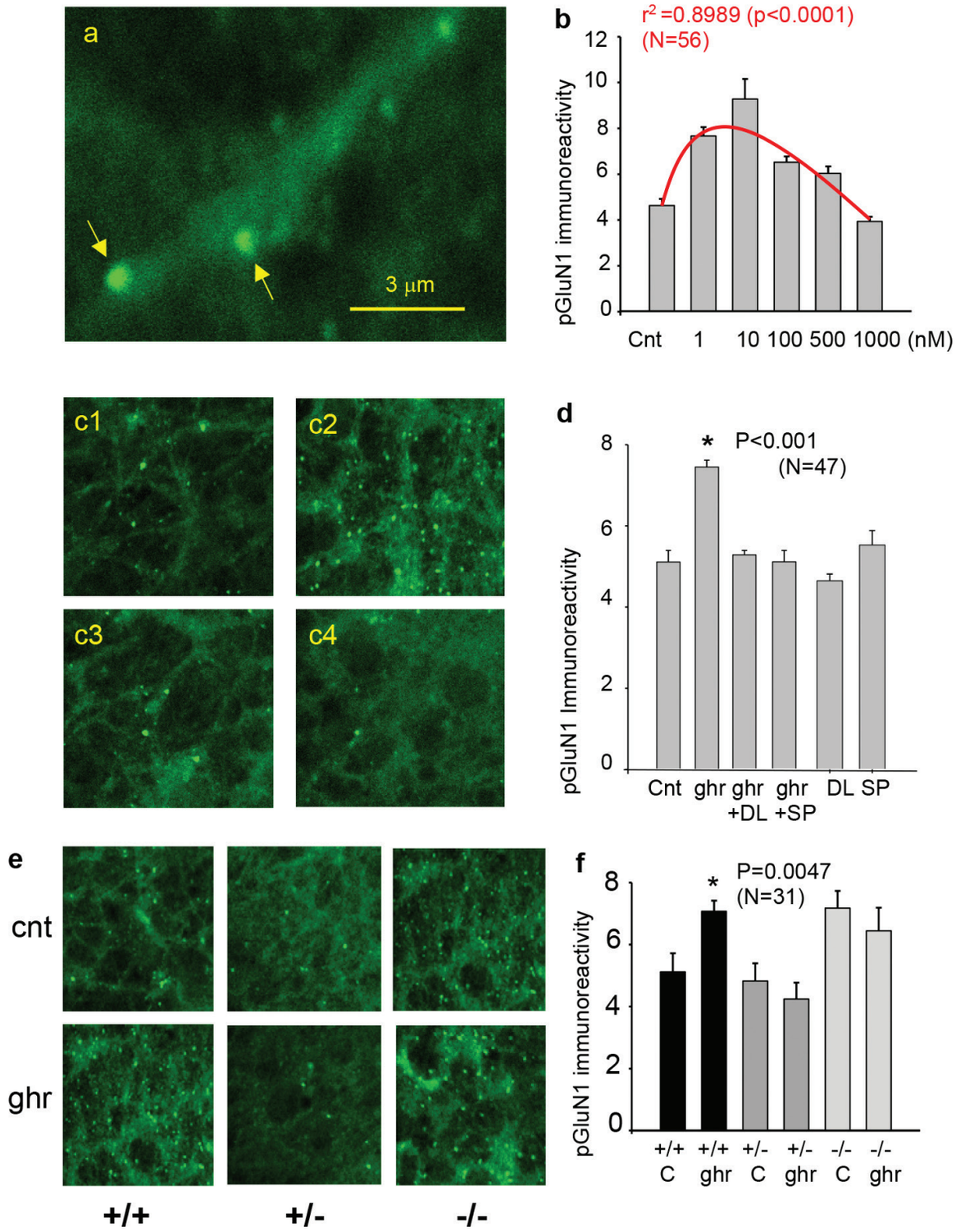

Figure 2. pGluN1 immunoreactivity in cultured rat hippocampal slices. **a**. Representative pGluN1 signals are indicated by yellow arrows. **b**. Dose-dependent changes in pGluN1. **c1–c4**. pGluN1 in control (c1), ghrelin (c2), ghrelin and D-Lys3-GHRP6 (c3), and ghrelin and substance *P*-analogue (c4). **d**. Quantification of pGluN1 in control (c), ghrelin (ghr), ghrelin and D-Lys3-GHRP6 (ghr + DL), ghrelin and substance *P*-analogue (ghr+SP), D-Lys3-GHRP6 alone (DL), and substance *P*-analogue alone (SP). **e**. pGluN1 in control (cnt) and ghrelin (ghr) in wild type (+/+), heterozygous GHSR1a knockout mice (+/-), and homozygous GHSR1a knockout mice (-/-). **f**. Quantification of pGluN1 in wild-type and GHSR1a knockout mice. (Modified with permission from Muniz and Isokawa [19]).

possible that some compensatory mechanisms had switched on under the total absence of GHSR1a, in order to readjust homeostatic balance in the NMDAR channel activity.

3.3. Endocannabinoids negatively modulate ghrelin's effect on pGluN1

Endocannabinoids (eCB) and the type 1 cannabinoid receptor (CB1R) have been implicated essentially in regulating a feeding behavior. They stimulate hypothalamic orexigenic neurons, enhance appetite, and initiate food consumption [30]. Interestingly, there is a report to suggest that ghrelin may exert its orexigenic effect through the endogenous cannabinoid system by producing eCBs in the hypothalamus [31]. However, to date, there is no evidence in the hippocampus that a similar interaction might occur between ghrelin and the endocannabinoid system. We tested if eCBs such as 2-AG (2-arachidonoylglycerol) and anandamide might modulate the effect of ghrelin on the phosphorylation of GluN1 [32].

Hippocampal slices were incubated in 20 nM of R(+)-methanandamide (non-hydrolyzing form of anandamide) together with 100 nM of ghrelin. The magnitude of pGluN1 immunoreactivity remained unchanged when compared to the control ($p = 0.939$) (**Figure 3a**), suggesting that the effect of ghrelin on pGluN1 was negated by R(+)-methanandamide. Interestingly, the inhibitory effect of R(+)-methanandamide was not blocked by the CB1R antagonist, AM251 (5 μM), which suggested that R(+)-methanandamide exerted its effect independently of CB1R. We then tested the antagonist of TRPV1 (transient receptor potential vanilloid type 1), since anandamide is also an agonist of this receptor [33]. Neither capsazepine (caps, 5 μM) nor iodoresiniferatoxin (IRTX, 10 nM), antagonists of TRPV1, was effective of blocking the inhibitory action of R(+)-methanandamide (**Figure 3a**). It may be possible that R(+)-methanandamide directly inhibited the NMDAR channel, since anandamide was reported to directly interact and inhibit nonspecific cation-permeable receptor channels [34].

Next, we tested the effect of 2-AG. Similar to the result of R(+)-anandamide, the application of 2-AG (10 μM) negated the stimulatory effect of ghrelin on the phosphorylation of GluN1 (**Figure 3b**). A negative effect of 2-AG was blocked by the CB1R antagonist, AM251 (5 μM), suggesting that the effect of 2-AG was exerted through the activation of CB1R. A synthetic agonist of CB1R, WIN 55,212 (4 μM), also blocked the ghrelin's stimulatory effect on pGluN1 in the CB1R-dependent manner. We then applied 150 mM KCl in the attempt of depolarizing neurons and mobilizing endogenous 2-AG (instead of exogenously applying 2-AG). The application of KCl mimicked the inhibitory effect of 2-AG on the ghrelin-mediated enhancement of GluN1 phosphorylation. The magnitude of pGluN1 remained unchanged in the presence of ghrelin during KCl application and was comparable to control. KCl-mediated inhibition of the ghrelin's stimulatory effect was blocked by the CB1R antagonist, AM251 (5 μM), suggesting that the application of KCl successfully mobilized endogenous 2-AG. Finally, we used an inhibitor of MAGL (monoacylglycerol lipase), JZL184 (100 nM). MAGL is the degradation enzyme for 2-AG. Thus, JZL184 slows down the rate of 2-AG degradation while maintaining an elevated concentration of ambient 2-AG and making the effect of endogenous 2-AG longer and more intense. As shown in **Figure 3b**, JZL184 was effective of negating the ghrelin's action on the phosphorylation of pGluN1 ($p < 0.0001$), and this effect was reversed by the CB1R antagonist, AM251 ($p < 0.05$). Although we cannot estimate the

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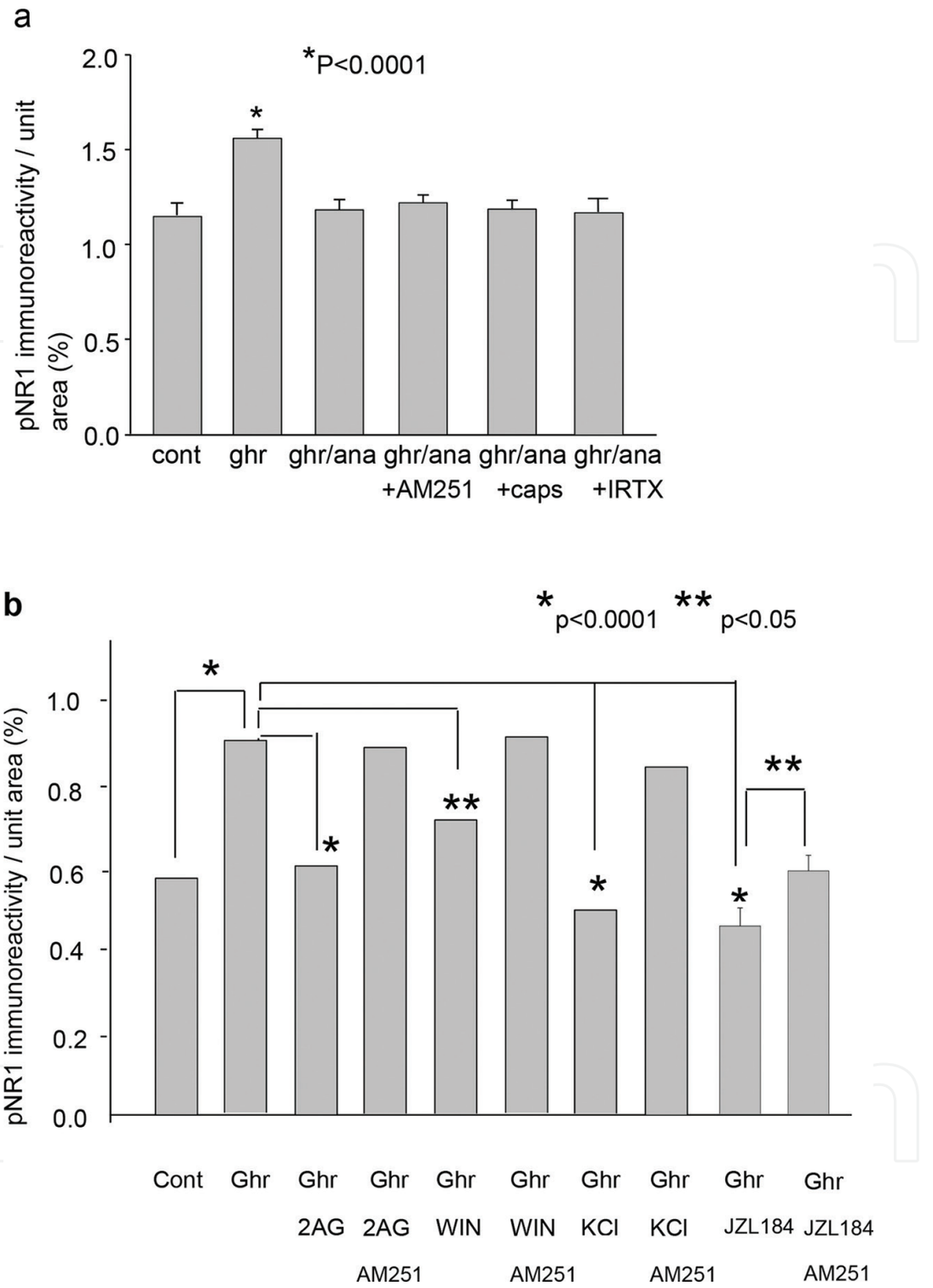

Figure 3. Effects of endocannabinoids on ghrelin-induced increase in pGluN1. **a**. Co-application of ghrelin (200 nM) and R(+)-methanandamide (20 nM) blocked the stimulatory effect of ghrelin on the phosphorylation of GluN1. This inhibitory effect of R(+)-methanandamide was independent of CB1R and TRPV1. **b**. Co-application of ghrelin (200 nM) with 2-AG (10 μM), WIN55,212 (4 μM), KCl (150 mM), and JZL 184 (100 nM), all negated the stimulatory effect of ghrelin on the phosphorylation of GluN1. The CB1R antagonist, AM251 (5 μM), blocked the inhibitory effect of 2-AG, WIN55212, KCl, and JZL 184 on the phosphorylation of GluN1, indicating that these compounds exerted their effects through the activation of CB1R. (Modified with permission from Cuellar and Isokawa [32]).

intrinsic concentration of 2-AG and the rate of increase by JZL184 in our hippocampal slice culture, a physiological range of fluctuation in the concentration of eCBs appears to be sufficient to interact and modulate the ghrelin-signaling cascade on the GluN1 subunit.

4. Ghrelin amplifies NMDAR-mediated synaptic currents

4.1. Ghrelin on evoked NMDAR-EPSCs

NMDAR-EPSCs (*N*-methyl-ᴅ-aspartate receptor–mediated excitatory postsynaptic currents) were isolated with a patch electrode in the whole-cell voltage-clamp configuration in single CA1 pyramidal cells at a holding potential of +40 mV (Axopatch 200A and pClamp 10, Molecular Devices), while stimulating the stratum radiatum every 20 s. Extracellular solution (ACSF: artificial cerebrospinal fluid) contained 50 μM picrotoxin and 10 μM NBQX (2,3-dihydroxy-6-nitro-7-sulfamoyl-benzo[F] quinoxaline) in order to block GABAergic induced pluripotent stem cells (IPSCs) and AMPA (α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid) receptor–mediated EPSC component. A brief local application of ghrelin (100 nM) enhanced NMDAR-EPSCs (**F = 29.12, p < 0.001, **F = 28.39, p < 0.005, n = 30, one-way repeated measure ANOVA*) (**Figure 4a**). This increase was sensitive to D-Lys3-GHRP-6 (GHSR1a antagonist, 100 μM). Subsequent bath application of ᴅ-Lys3-GHRP6 reduced the amplitude of NMDAR-EPSCs (**t = 10.18, p < 0.0006, **t = 14.96, p < 0.0002, n = 41, unpaired t-test*). Complete blockade of EPSCs by APV (100 μM, the antagonist of NMDAR) supported our interpretation that the recorded EPSCs were indeed generated solely by the activation of the NMDAR.

Interestingly, in the absence of exogenous ghrelin, NMDAR-EPSCs were reduced in the peak amplitude in response to D-Lys3-GHRP6 (**Figure 4c**) (**F = 63.58, p < 0.001, n = 37, one-way repeated measure ANOVA*). This reduction was reversible. Upon washout of the antagonist of GHSR1a, the amplitude of NMDAR-EPSCs recovered. We suggest that, in cultured hippocampal slices, (1) GHSR1a is likely to be constitutively active and (2) endogenous ghrelin might be present and available for the NMDAR. Finally, none of the responses presented above with ghrelin and the GHSR1a antagonist, D-Lys3-GHRP6, were generated in slices prepared from GHSR1a knockout mice (*F = 0.772, p > 0.77, n = 18, one-way repeated measure ANOVA*).

4.2. Ghrelin on spontaneous NMDAR-EPSCs

Spontaneously occurring NMDAR-EPSCs (sEPSCs) responded to exogenous application of ghrelin and the GHSR1a antagonist similarly to evoked NMDAR-EPSCs. The amplitude of sEPSCs was 100 pA in average in control ACSF (**Figure 4d1**). However, it was increased by twofolds in response to exogenous application of ghrelin (**Figure 4d2**). The increase was recovered to the control level following the bath application of D-Lys3-GHRP6 (100 μM) (**Figure 4d3**). Although the amplitude of sEPSCs changed in response to the application of agonist and antagonist of GHSR1a, the frequency of sEPSCs did not change significantly, suggesting that the effect of ghrelin and GHSR1a signaling was likely postsynaptic.

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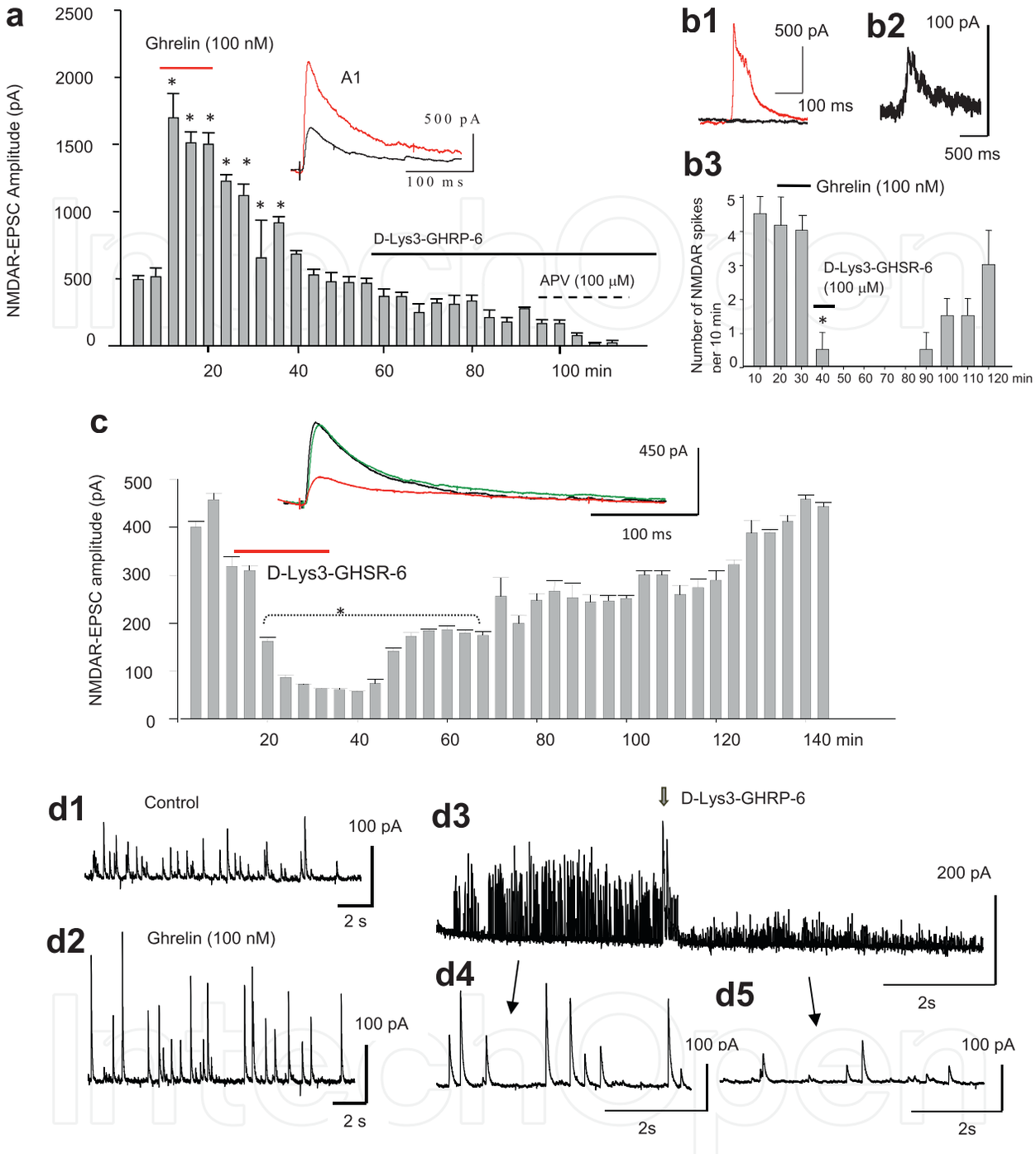

Figure 4. Effect of ghrelin on the NMDA receptor–mediated EPSCs. **a**. Single pyramidal neurons in the CA1 of cultured rat hippocampal slices were voltage clamped at +40 mV in the presence of picrotoxin and NBQX. Stratum radiatum was stimulated every 20 s in order to avoid a rundown. Recording pipette contained cesium-based solution with QX-314 and MgATP. Local brief puff application of ghrelin (100 nM) increased the EPSC amplitude transiently, which was blocked by the GHSR1a antagonist, D-Lys3-GHRP6 (100 μM), and was totally eliminated by the NMDAR antagonist, APV (100 μM). **b**. NMDA spike currents in control (red trace in **b1**), in the presence of D-Lys3-GHRP6 (black trace in **b1**), and in wash (**b2**). Bar graph in b3 represents the occurrence of NMDA spikes per 10 min for a total of 120 min of gap-free recording. **c**. Reversible inhibition of NMDAR-EPSCs by D-Lys3-GHRP6 in the absence of ghrelin. **d1–4**. Spontaneous NMDAR-EPSCs. (Modified with permission from Muniz and Isokawa [19]).

4.3. Ghrelin on NMDA spike currents

NMDA spikes are spontaneously generated local electrical signals at dendritic branches [35] where NMDARs are highly localized [36]. The generation of NMDA spikes is promoted by glutamate spillover at any single point in the entire dendritic tree [37] that may involve extra-synaptic receptors [38]. In our rat hippocampal slice culture, NMDA spike currents were insensitive to exogenous application of ghrelin. However, the generation of NMDA spike currents was blocked by the bath application of GHSR1a antagonist, D-Lys3-GHRP6, in the absence of ghrelin (**Figure 4d**) (*chi-square = 20.135, $p < 0.045$, $n = 12$, Friedman one-way repeated measure ANOVA*). The blockade of NMDA spike currents by the GHSR1a antagonist was reversible, and upon washout of D-Lys3-GHRP6, NMDA spike currents recovered. Although we cannot rule out the possibility that D-Lys3-GHRP6 interacted with the NMDAR directly, inhibiting its function independently of GHSR1a [39], our finding of the inhibitory effect of D-Lys3-GHRP6 on the NMDA spike suggests that GHSR1a-mediated intracellular signaling modulates the activity of extra-synaptic NMDARs and supports our interpretation that GHSR1a is likely present on pyramidal cell dendrites with physical proximity to the NMDA receptor.

5. Ghrelin-induced phosphorylation of CREB

The family of CREB (cAMP response element-binding protein) transcription factors is involved in a variety of biological processes including the plasticity of the nervous system [40]. In order for CREB to be active, it needs to be phosphorylated before being translocated to the nucleus. Thus, the identification of a phosphorylated CREB is a reliable assay for predicting the occurrence of plasticity, learning, and memory in neurons. We previously reported in the in vivo-fasting model in rats that metabolic demand stimulated and upregulated the phosphorylation of CREB by twofolds in the hippocampus together with other limbic cortexes such as piriform cortex, the entorhinal cortex, and the cortico-amygdala transitional zone [41]. Here, we discuss the NMDA receptor–mediated and ghrelin-enhanced phosphorylation of CREB in our cultured hippocampal slices.

5.1. Ghrelin-stimulated phosphorylation of CREB

CREB activity was assayed immunohistochemically using a rabbit polyclonal antibody against phosphorylated CREB (pCREB at Ser 133) (Cell Signaling, Danvers, MA) (**Figure 5a–c**). pCREB immunoreactivity was quantified using an auto-segmentation tool provided by IPLab imaging software. Low concentrations of ghrelin in 50 and 100 nM did not have any effect on pCREB expression. However, 200 nM and above concentrations of ghrelin increased the expression of pCREB by fourfolds compared to control ($p < 0.01$) (**Figure 5d**). The magnitude of pCREB expression was not different among 200, 500, and 1000 nM. A steep change in response to differing concentrations of ghrelin may be explained by the unique process of ghrelin des-octanoylation [42]. Finally, the effect of ghrelin was mediated by GHSR1a, since the antagonist of GHSR1a, D-Lys3-GHRP6 (100 μM), blocked the ghrelin-mediated increase in the expression of pCREB (**Figure 5e**).

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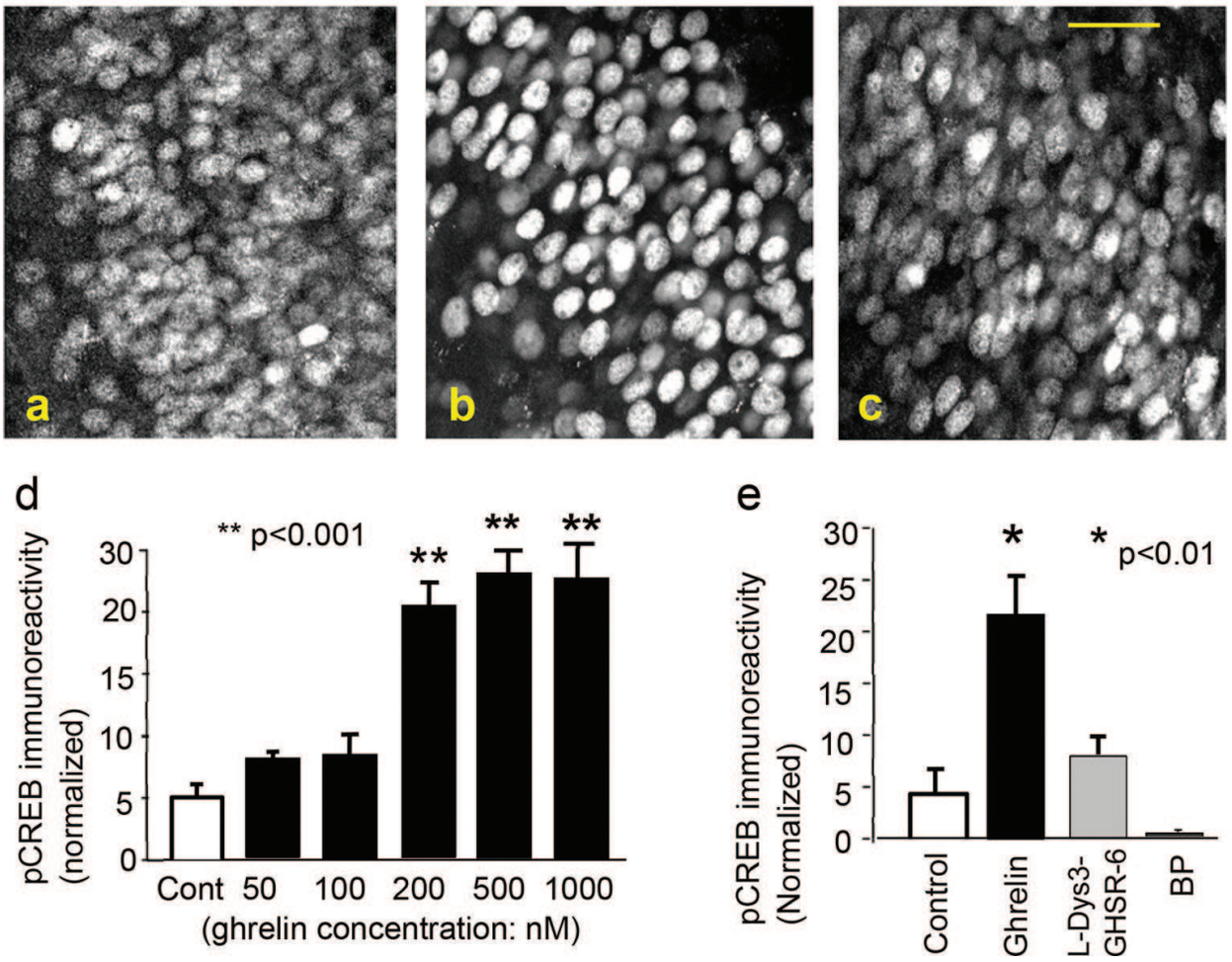

Figure 5. Effects of ghrelin on pCREB. **a–c**. pCREB immunoreactivity in control (**a**), ghrelin (**b**, 200 nM), and in D-Lys3-GHRP6 (**c**, 100 μM). **d**. pCREB in response to ghrelin doses. **e.** Summary graph of pCREB in control, ghrelin, and D-Lys3-GHRP6. Calibration: 30 μm. (Modified with permission from Cuellar and Isokawa [32]).

5.2. Effect of endocannabinoids on ghrelin-mediated upregulation of pCREB

Synergistic involvement of the endogenous cannabinoid system is suggested in the ghrelin-mediated CREB phosphorylation in the hypothalamus [31]. However, in the hippocampus, the contribution of endocannabinoids and the cannabinoid receptor in short- and long-term plasticity has been explained independently of ghrelin and GHSR1a. Furthermore, in Section 3.3, we discussed that ghrelin-mediated enhancement of GluN1 subunit phosphorylation appeared to be negatively modulated, instead of synergistically amplified, by eCBs. Here, we examined potential interactions of the endogenous cannabinoid system to ghrelin-induced hippocampal plasticity at the level of CREB phosphorylation.

A low concentration (20 nM) of R(+)-methanandamide, a nonhydrolyzing form of anandamide, inhibited ghrelin-induced increase of pCREB (**Figure 6**). This inhibitory effect of R(+)-methanandamide was not blocked by the CB1R antagonist AM251 (5 μM) or the TRPV1 antagonist capsazepine (5 μM), suggesting that the action of R(+)-methanandamide on the ghrelin-mediated phosphorylation of CREB may be independent of the CB1R or TRPV1.

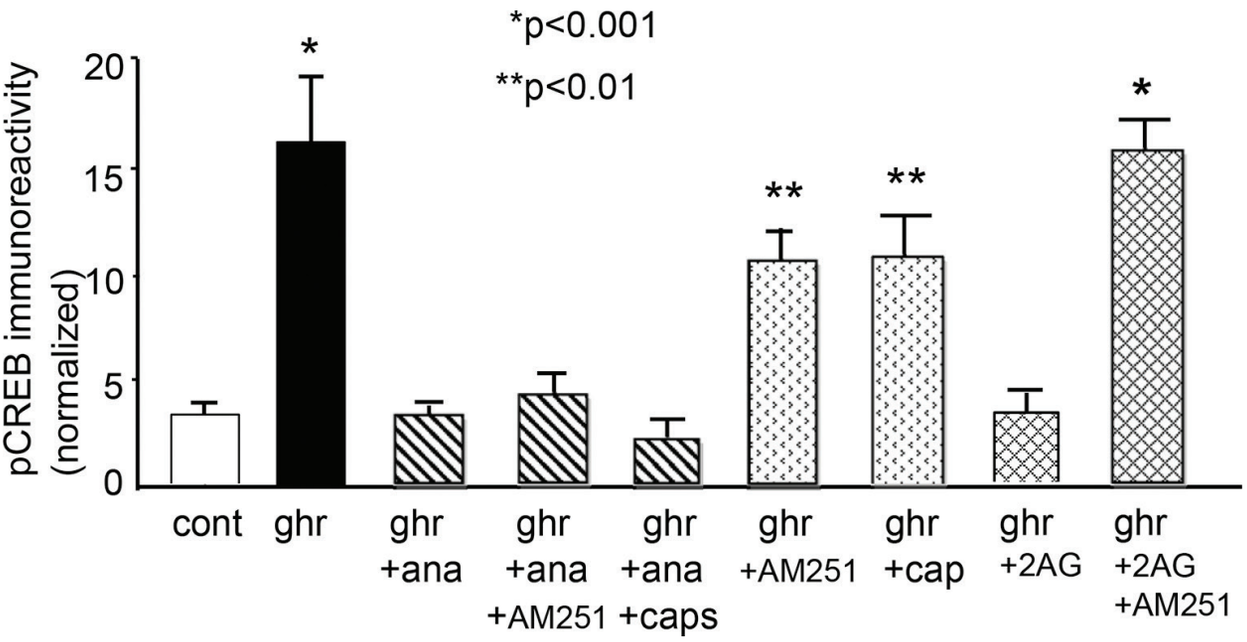

Figure 6. Effects of eCBs on ghrelin-mediated phosphorylation of CREB in the hippocampus. (Modified with permission from Cuellar and Isokawa [32]).

Furthermore, incubation of slices in AM251 alone (without anandamide) or in capsazepine alone (without anandamide) did not block a ghrelin-induced increase in CREB phosphorylation. These results suggested that neither CB1R nor TRPV1 appeared to be involved in the negative effect of R(+)-methanandamide on the ghrelin-induced phosphorylation of CREB.

We next examined the effect of 2-AG on the ghrelin-induced upregulation of pCREB. Similar to R(+)-methanandamide, 2-AG (10 μM) inhibited ghrelin-induced increase in pCREB (**Figure 6**). However, in contrast to R(+)-methanandamide, the inhibitory effect of 2-AG was blocked by CB1R antagonist AM251, suggesting that the action of 2-AG was mediated through the activation of CB1R.

Although we cannot rule out the possibility that eCBs negatively modulated the ghrelin's stimulatory effect on CREB phosphorylation independently of the phosphorylation of the NMDAR GluN1 subunit, our interpretation is that the target of the negative effect of eCBs is the NMDAR, because (1) GluN1 phosphorylation by ghrelin was negated by both 2-AG and anandamide in the identical manner to CREB phosphorylation and (2) the NMDAR is situated upstream of the signaling cascade of CREB activation, having the NMDAR as a necessary molecule in the induction of hippocampal synaptic plasticity.

6. Ghrelin and dendritic spines

CREB-induced gene expression includes reorganization of cytoskeletal proteins. Diano et al. [10] reported that ghrelin upregulated the number of spine synapses in the hippocampus. However, it is elusive whether the increase in synapse occurred on existing spines or on newly generated spines. We examined changes in the number of dendritic spines with a

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hypothesis that ghrelin might stimulate the generation of dendritic spines. Polymerized actin (F-actin) is highly localized in dendritic spines. Thus, we used phalloidin, a mushroom toxin that has a high affinity to F-actin, as a marker for the identification of dendritic spines. Alexa 488-conjugated phalloidin was visualized and relative changes in fluorescence puncta were quantified using confocal microscope and imaging software (IPLab) (**Figure 7a**).

6.1. Short-term effect of ghrelin on dendritic spines

Ghrelin was applied for 60 min with a concentration of 200 nM to cultured rat hippocampal slices. At the end of the incubation, the slices were fixed with 4% paraformaldehyde and treated with fluorescent phalloidin for confocal visualization of dendritic spines. In control, the average spine density, measured as phalloidin fluorescence was 0.302/unit area ± 0.039 standard error of mean (SEM) ($n = 30$ images taken from 10 slices). Ghrelin increased the average spine density to 0.499/unit area ± 0.058 SEM ($n = 30$, $p < 0.001$) (**Figure 7b**). The antagonist of GHSR1a, D-Lys3-GHRP6 (100 μM), blocked the ghrelin's stimulatory effect on spine generation, and the average spine density returned to the control level (0.333/unit area + 0.041 SEM, $n = 30$). These results suggested that ghrelin may increase the number (or the size) of spines by activating GHSR1a and the downstream-signaling molecules, and that 60 min of incubation in ghrelin is sufficient to induce the generation of "new" spines.

6.2. Long-term effect of ghrelin on dendritic spines

Ghrelin was applied for 23 h at a concentration of 200 nM to cultured rat hippocampal slices. At the end of the incubation, the slices were fixed and treated with fluorescent phalloidin for confocal visualization. Similar to the 60-min application, ghrelin-treated slices expressed a higher density of dendritic spines compared with the control (0.618/unit area ± 0.043 SEM, 30 images from 10 slices, $p < 0.001$) (**Figure 7c**). Spine density remained elevated at the end of the 23-h application. Pre-application of slices with D-Lys3-GHRP6 for 2 h before the application of ghrelin blocked the increase of phalloidin fluorescence, and the spine density stayed at a control level (0.322/unit area ± 0.024 SEM, $n = 30$).

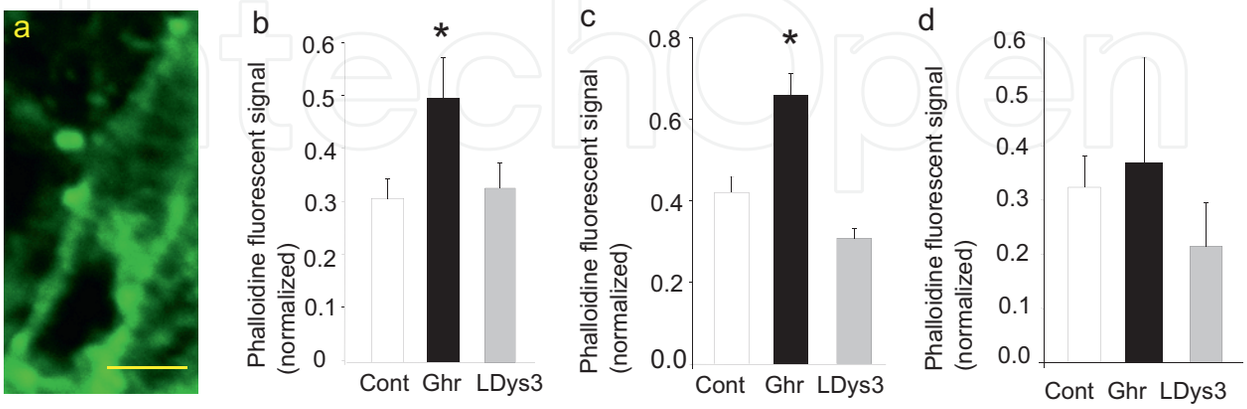

Figure 7. Dendritic spines visualized with Alexa 488-conjugated phalloidin. **a**. Representative phalloidin image. **b**. Phalloidin signals in response to 60-min incubation in ghrelin. **c.** Phalloidin signals in response to 23-h incubation in ghrelin. **d**. Phalloidin signals in response to 22-h incubation in control media after 60-min application of ghrelin. (Modified with permission from Berrout and Isokawa [24]).

6.3. Ghrelin is required to maintain "newly added" spines

Our results indicated that a 60-min application of ghrelin was sufficient to increase spine density. Our results also showed that spine density remained elevated after 23-h application of ghrelin. A question raised from this result is whether the maintenance of elevated spine density in 23 h of incubation with ghrelin really required 23 h of continual availability of ghrelin (since 60-min application was sufficient to increase spine density). In order to answer the question, we incubated hippocampal slices in ghrelin-containing culture media for 60 min, then removed the slices from ghrelin-containing media and incubated in control media for additional 22 h without ghrelin. At the end of the incubation period (of 1 h with ghrelin and the subsequent 22 h without ghrelin), the slices were fixed and treated with fluorescent phalloidin for confocal visualization of dendritic spines. At the end of this combined treatment, spine density was 0.370/unit area ± 0.193 SEM, which was comparable to a control level (0.314/unit area ± 0.057 SEM) (**Figure 7d**). It appears that spine density can increase in response to ghrelin within 60 min and remain elevated for up to 23 h as long as ghrelin is present. However, once ghrelin is removed and no longer available to activate GHSR1a, "newly added" spines retract and the spine density recovers to a control level. In conclusion, ghrelin can add "new" spines to hippocampal neuron dendrites, and that continual availability of ghrelin is a prerequisite together with non-desensitizing activity of GHSR1a for this form of spine plasticity.

7. Concluding remarks

The hippocampus plays a critical role in employing food-searching strategies. Ghrelin is thought to be essential in order to retain memories regarding the spatial localization of food sources [43]. Food search is typically initiated when metabolic demand increases, and the search typically does not end until the metabolic demand is fulfilled. During fasting, a serum ghrelin level increases and stays increased until fasting ends. The rate of ghrelin crossing the blood–brain barrier also increases in a ghrelin concentration-dependent manner [10]. Although it is not known whether the concentration of intrinsic ghrelin in the hippocampus (if any) may fluctuate with metabolic demand, ghrelin can be a key molecule for metabolic demand–induced neuron plasticity in the hippocampus, which serves as a cellular and molecular substratum for food-related memories and learning. Ghrelin-dependent maintenance of plasticity and the loss of plasticity in the absence of ghrelin may nicely explain when and how long such plasticity is required in order for organisms to successfully exercise adaptive appetitive behavior for survival.

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