Inactivation of the Constitutively Active Ghrelin Receptor Attenuates Limbic Seizure Activity in Rodents

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Abstract Ghrelin is a pleiotropic neuropeptide that has been recently implicated in epilepsy. Animal studies performed to date indicate that ghrelin has anticonvulsant properties; however, its mechanism of anticonvulsant action is unknown. Here we show that the anticonvulsant effects of ghrelin are mediated via the growth hormone secretagogue receptor (GHSR). To our surprise, however, we found that the GHSR knockout mice had a higher seizure threshold than their wild-type littermates when treated with pilocarpine. Using both *in vivo* and *in vitro* models, we further discovered that inverse agonism and desensitization/internalization of the GHSR attenuate limbic seizures in rats and

epileptiform activity in hippocampal slices. This constitutes a novel mechanism of anticonvulsant action, whereby an endogenous agonist reduces the activity of a constitutively active receptor.

Keywords Epilepsy · Seizures · Ghrelin · GHSR · Hippocampus · Pilocarpine

Introduction

Ghrelin is a pleiotropic peptide that has gained considerable attention since its discovery in 1999 [1]. This 28 amino-acid bioactive peptide displays several important biological effects, including an influence on pituitary hormone secretion, appetite, metabolism, gastrointestinal function, and memory [2–4]. Ghrelin is best known as the endogenous ligand of the growth hormone secretagogue receptor (GHSR). Most of the known biological effects of ghrelin are mediated via the GHSR, however, it is becoming increasingly recognized that ghrelin is also capable of binding to other yet unknown receptors [5, 6].

Apart from its numerous peripheral functions, several studies have highlighted the important role of ghrelin in the brain. Centrally, ghrelin has the capability of altering neuronal activity in various brain regions. Being a gut-derived hormone, the major emphasis of ghrelin research has been directed toward its orexigenic action via neuropeptide Y/agouti-related protein activation in the hypothalamus [3]. As for the hippocampus, peripheral ghrelin was shown to enter this brain region to bind to the GHSR, where it promoted spine synapse formation in the stratum radiatum of the CA1 subfield as well as facilitated long-term

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potentiation generation, which were paralleled by enhanced spatial learning and memory [2]. Since then, various reports have implicated ghrelin in hippocampal functions, and more recently in epilepsy mechanisms [7–11].

Approximately 50 million people worldwide have active epilepsy with recurrent seizures and, in spite of the medical advances along the years, 30 % of these patients remain drug resistant [12]. Thus, the search for new antiepileptic drugs (AEDs) is ongoing, and the emphasis is being placed in the discovery of compounds that have distinct profiles of activity than the AEDs currently on the market [13]. Increasing attention is given toward neuropeptides as potential leads in preventing seizures since they are less likely to fall under the category of “me-too” AEDs [14]. Recently ghrelin has been investigated with respect to its potential role in epilepsy. Data from human studies in this area are conflicting, with some studies concluding either increased [15, 16] or decreased [17–22] plasma levels of ghrelin in epileptic patients. Rodent studies are more consistent, with the majority of studies indicating that ghrelin shows an anticonvulsant action in different models [10, 23–25], but its mechanism of action is unknown.

Therefore, we have investigated the involvement of the ghrelin receptor, GHSR, in animal models of epilepsy using *in vivo* and *in vitro* techniques. When studying GHSR-null mice, we unexpectedly found that homozygous GHSR deletion rendered the mice less seizure susceptible than their wild-type littermates. Using multiple approaches, we found that various treatments that inactivate the GHSR lead to the attenuation of limbic seizures. This highlights the GHSR as a potential site for the development of novel anticonvulsant drugs.

Methods

Animals

Experimental procedures were performed on male Wistar rats (260–320 g; Charles River Laboratories, France), and male GHSR^{+/+} and GHSR^{-/-} littermate mice (25–35 g). All experiments were carried out in accordance with the National Rules on Animal Experiments and were approved by the Ethics Committee on Animal Experiments of the Vrije Universiteit Brussel, Belgium, and the animal Scientific Procedures Act, 1986, United Kingdom.

Generation of GHSR^{-/-} Mice

GHSR^{-/-} mice were developed by Janssen Pharmaceutica (Beerse, Belgium) in collaboration with Lexicon Genetics, Inc (The Woodlands, TX) on a C57BL/6 background, as previously described [26]. Homozygous male GHSR^{-/-} mice

and their corresponding wild-type GHSR^{+/+} littermates (3–5 months old) were used in this study. Genotypes were confirmed by real-time reverse transcription polymerase chain reaction using DNA isolated from mouse tail biopsy samples. Real-time reverse transcription polymerase chain reaction analysis was used to show expression or absence of the GHSR transcript. Primers sequences and additional information on the targeted disruption of the GHSR gene can be found in the study by Verhulst et al. [26]. Mice were kept in a regulated environment (22–24 °C; 50 % relative humidity; lights on at 7:00AM and off at 9:00PM) with free access to food (standard laboratory chow) and water (filtered). All mice were held in the animal facility for at least 1 month before being transferred to the experimentation rooms.

GHSR Ligands, Chemicals, and Reagents

Neuroactive substance standards, pilocarpine, and the GHSR inverse agonist [D-Arg¹,D-Phe⁵,D-Trp^{7,9},Leu¹¹]substance P were supplied by Sigma-Aldrich (Bornem, Belgium). Ghrelin was supplied by PolyPeptide Laboratories (Strasbourg, France). The ghrelin mimetic capromorelin and the presumed GHSR antagonist A778193 (found in this study to be a GHSR inverse agonist) were generously provided by Dr. Luc Ver Donck and Dr. Dieder Moechars. The ghrelin fragments (1–5) amide, (1–7), (1–9), (1–11), (1–14), and (23–28) were supplied by Phoenix Pharmaceuticals (Karlsruhe, Germany). All other chemicals were analytical reagent grade or better and were supplied by Merck (Darmstadt, Germany). Aqueous solutions were made with purified water (Seralpur pro 90 CN; Belgolabo, Overijse, Belgium) and filtered through a 0.2-μm membrane filter.

In Vivo Microdialysis in Rats

Surgery

Microdialysis in rat hippocampus was performed as previously described [27]. Rats received intraperitoneal injections of a mixture of ketamine and diazepam (start dose of 90.5:4.5 mg/kg) until full-body anesthesia was achieved. The animal was then mounted on a stereotaxic frame for precise intracranial steel guide cannula (CMA/Microdialysis, Solna, Sweden) implantation 3 mm above the actual membrane position in the left CA1–CA3 hippocampal area, using the coordinates 4.6 mm lateral from the midline, 5.6 mm posterior to the bregma, and 4.6 mm ventral from the dura [27]. Ketoprofen (4 mg/kg) was administered intraperitoneally for postoperative analgesia at the end of the surgical procedure.

At least 2 rats from each control and GHSR inverse agonist-treated rat groups were implanted with a sterilized

radiotelemetric transmitter (F20-EET; Data Sciences International, Tilburg, The Netherlands). The measuring and reference electrodes, both attached with a stainless steel screw at the end, were subcutaneously tunneled to the skull. The measuring electrode was stereotactically positioned above the right hippocampus (4.6 mm lateral and 5.6 mm anterior to the bregma), whereas the reference electrode was positioned above the cerebellum (1 mm anterior according to the lambda).

Microdialysis

Immediately after surgery, the hippocampal guide cannula obturator was replaced by a microdialysis probe (CMA/12, 3 mm membrane length, theoretical cut-off 20 kDa; CMA/Microdialysis), which was continuously perfused with modified Ringer's solution (in mM: NaCl, 147; CaCl₂, 2.3; and KCl, 4) at 2 µl/min. All experiments started on the day after surgery. Individual sampling times were set at 20 minutes each. Each experiment started with the collection of intra-hippocampal basal samples (1-6) in which the perfusion fluid was composed of only modified Ringer's solution. Consecutively, ghrelin, capromorelin, A778193, [D-Arg¹,D-Phe⁵,D-Trp^{7,9},Leu¹¹]substance P, or ghrelin (1-5) amide were dissolved in modified Ringer's solution and perfused through the hippocampal microdialysis probe from the 7th collection period onward. During the 13th and 14th collection periods, 12 mM pilocarpine was co-perfused with the ligand. Perfusion of the GHSR ligand continued for another 5 collection periods. At the end of every experiment, rats were killed by an overdose of pentobarbital (Ceva Santé Animale, Brussels, Belgium). Probe localization and tissue damage were histologically verified and evaluated postmortem.

Seizure Severity Assessment

Typical seizure-related behavioral changes after pilocarpine perfusion were rated on a Seizure Severity Score based on Racine's Scale [28]. This scale was previously optimized and validated with electrocorticographic monitoring to take into account the typical behavioral changes associated with pilocarpine-induced motor seizures [29]. This scale consists of 6 different stages: 0) normal, nonepileptic activity; 1) mouth and facial movements, hyperactivity, excessive grooming, sniffing, scratching, wet dog shakes; 2) head nodding, staring, tremor; 3) forelimb clonus, forelimb extension; 4) rearing, salivating, tonic-clonic activity; and 5) falling. The total seizure severity score (TSSS) for each animal is calculated as the sum of the highest Seizure Severity Score attained in each of the 7 collection periods after the start of pilocarpine administration (total time, 140 minutes). The rats implanted with a radiotelemetric transmitter were placed in a video electroencephalography (EEG) monitoring unit equipped with a radiotelemetric receiver

(PhysioTel™ Receiver Model RPC-1; Data Sciences International, Tilburg, The Netherlands) coupled to the Notocord-hem Evolution acquisition software (Notocord, Croissy-sur-Seine, France). During the microdialysis experiment, the EEG was sampled with a frequency of 100 Hz, and behavioral seizure activity was correlated with EEG activity in these rats.

Neurochemical Evaluation

Isocratic microbore liquid chromatography (C8 column, 5 µm, 100×1 mm internal diameter; Bioanalytical Systems, IN, West Lafayette, USA) with amperometric detection at 750 mV (LC-4 C, Bioanalytical Systems) was used for γ-amino butyric acid (GABA) analysis in microdialysates after pre-column derivatization with o-phthalaldehyde/tert-butylthiol and iodoacetamide.

In Vivo Mice Experiments

GHSR^{+/+} and GHSR^{-/-} mice were used to determine seizure thresholds, based on the pilocarpine-induced stereotyped seizure behavior [30]. The threshold for the different phases of pilocarpine-induced seizure activity was determined by infusing a pilocarpine solution (24 mg/ml) through a 29-gauge needle, attached to polyethylene tubing (Smiths, Keene, Smiths Medical, New Hampshire, USA) and inserted into the tail vein of the animals at a constant rate of 150 µl/min [30], using a Hamilton syringe mounted to an infusion pump (CMA, Microdialysis, Solna, Sweden). The animal was allowed to move freely in a cage made of plexiglass. To prevent peripheral cholinergic symptoms, all mice were administered methylscopolamine (1 mg/kg, subcutaneously) 30 minutes prior to pilocarpine infusion. Except in the case of GHSR^{+/+} and GHSR^{-/-} mice tested for genotype differences, mice were also administered (according to body weight) ghrelin or saline via intraperitoneal administration 30 minutes prior to pilocarpine tail infusion. The following endpoints were used to determine the seizure threshold: 1) head bobbing, 2) rearing, 3) clonus with loss of righting reflexes (falling), 4) tonic hindlimb extension (tonus), and 5) death. Time was measured from the start of the pilocarpine infusion until the onset of these stages. The seizure thresholds were determined for each animal according to the following equation: dose (mg/kg)=duration of infusion (seconds)×rate of infusion (ml/min)×drug concentration (mg/ml)×1000/(60 seconds×weight of mouse [g]) [30].

Slice Electrophysiology

For a detailed overview of the protocol, see Bortolotto et al. [31]. In summary, experiments were performed in a submerged recording chamber using horizontal hippocampal slices (thickness, 400 µm) obtained from Wistar rats 7 to

9 weeks old. The slices were superfused at a rate of 2 ml/min with artificial cerebrospinal fluid (CSF) medium (in mM: NaCl, 124; KCl, 3; NaHCO₃, 26; NaH₂PO₄, 1.25; CaCl₂, 2; MgSO₄, 1; and D-glucose, 10) bubbled with O₂/CO₂ (95/5 %) at 30°C. Extracellular field excitatory postsynaptic potentials (EPSPs), population spikes, as well as spontaneous epileptiform activity were recorded from the cell body layers of the CA3 region using glass microelectrodes (8–10 MΩ) filled with 3 M NaCl. The mossy fiber/CA3 pyramidal neuron responses were evoked by the stimulation of mossy fiber axons close to the granular cell layer of the dentate gyrus using bipolar electrodes (insulated NiCr wire 0.05 mm; Advent Research Materials, Oxford, UK). The recording electrodes were placed in the CA3 cell body region and lowered to a depth of 80 to 100 μm below the slice surface. Test pulses were delivered to evoke a field excitatory postsynaptic potential (fEPSP), and the stimulus intensity was increased until a population spike was evoked to determine the maximum stimulation intensity. Then the stimulus intensity was decreased until 50 % of the response amplitude was evoked. Once the stimulus intensity was established it remained constant throughout the whole experiment. Responses were evoked by low frequency stimulation (0.016 Hz, 5 to 35 V). At least 30 minutes of stable baseline was recorded before any drug was added to the perfusion medium. For control experiments, 15 μM pilocarpine was added to the perfusing medium for 15 minutes followed by a washout period of 110 minutes. Ghrelin or A778193 were added to the perfusing medium for 20 minutes, followed by pilocarpine co-perfusion for 15 minutes, then back to the test compound for another 20 minutes, and then to the perfusion medium for a minimum washout period of 90 minutes. Data were collected online using the WinLTP software [32] and re-analyzed using AxoScope (Axon Instruments, California, USA).

In Vitro GHSR Inverse Agonism Determination

Inverse agonism was assessed by determination of constitutive inositol 1-phosphate (IP1)-production in unstimulated HEK293 cells expressing the cloned human GHSR (hGHSR). Cells were grown in Dulbecco's modified eagle medium supplemented with 1 mM sodium pyruvate, 100 units/ml penicillin, 100 μg/ml streptomycin, and 10 % fetal bovine serum in a humidified atmosphere of air/CO₂ (95/5 %) at 37 ° C. Cells were subcultured at 70 to 80 % confluency and re-suspended in 96-well plates at 60,000 cells per well. Concentrations of A778193 were added in quadruplicate to the cells and incubated for 90 minutes at 37 ° C in air/CO₂ (95/5 %); 0.5 % dimethyl sulfoxide and 10⁻⁵ M of [D-Arg¹,D-Phe⁵,D-Trp^{7,9},Leu¹¹]substance P (a

full GHSR inverse agonist: pIC₅₀=6.74±0.02 [n=3]) were used as controls. The reaction was stopped by the addition of 10 μl IP1-d2 conjugate and subsequently 10 μL anti-IP1 cryptate conjugate was added (IP-One HTplex™ assay, category number 62IP1PEC; Cis Bio International, Codolet, France). The plates were read on a Pherastar reader (BMG Labtech, Ortenberg, Germany) after incubation for 24 h. Inhibition of IP1-production was calculated relative to maximal inhibition by [D-Arg¹,D-Phe⁵,D-Trp^{7,9},Leu¹¹]substance P and baseline in dimethyl sulfoxide-treated control wells.

GHSR Activation Assay

HEK293 cells stably transfected with the hGHSR were seeded on a black 96-well plate with clear poly-L-lysine treated bottom at a density of 60,000 cells per well to reach 70 % confluency. Agonist-induced Ca²⁺ fluxes were measured 1 h after loading with the fluorescent Ca²⁺ indicator Fluo-4-acetoxymethyl ester (Molecular Probes, Leiden, The Netherlands). Cell plates were assayed in the Fluorometric Imaging Plate Reader (FLIPR; Molecular Devices, California, USA). A concentration response curve was established (10⁻⁶ - 10⁻¹⁰ M) and, after background subtraction, expressed as percentage of the maximal response obtained with 5 μM ionomycin. The natural ligand ghrelin and the ghrelin fragments (1-5) amide, (1-7), (1-9), (1-11), (1-14), and (23-28) were tested next to the GHSR agonist capromorelin. A sigmoidal dose response curve was fitted using GraphPad Prism software, GraphPad Software, (La Jolla, USA), yielding a pEC₅₀ and curve maximum. For each pretreatment condition, the maximum was expressed as a percentage of ionomycin after subtraction of the blank. The effect of pretreatment was expressed as percentage of control.

GHSR Desensitization and Resensitization Assay

Desensitization was studied by 2 consecutive Ca²⁺ measurements separated by 3 washings. As previously described for activation, cells were first loaded with Fluo-4-acetoxymethyl ester and then pre-incubated for 3 minutes with ghrelin, capromorelin, ghrelin (1-14) or ghrelin (1-5) amide to induce desensitization. Three minutes later, a second stimulation with ghrelin was performed, and it consisted of a concentration response curve for ghrelin (range, 10⁻⁶-10⁻¹⁰ M). Desensitization by the first applied GHSR ligand was described as its effect on the maximum of the concentration response curve obtained after the second stimulation with ghrelin. Results were expressed as the percentage of control response (no pre-stimulation), and the negative logarithm of the pre-incubation concentration reducing the

maximum response to ghrelin to 50 % of its control value was calculated (pDC_{50}). The protocol used to investigate the effect of different pretreatment times with ghrelin was the same, with the difference being that the preincubation time for desensitization varied between 3 minutes to 60 minutes.

Resensitization was studied using HEK293 cells stably expressing enhanced green fluorescent-tagged GHSRs (GHSR-EGFP). To determine the time required for GHSR-EGFP resensitization, after pretreatment with ghrelin at 10^{-6} M, 10^{-7} M, or 10^{-8} M for the duration of 3 minutes, cells were washed and re-stimulated with ghrelin (dose response, 10^{-6} M – 10^{-10} M) after 3 minutes, 20 minutes, 1 h, 2 h, 5 h, or 24 h, respectively.

GHSR Internalization

Internalization was observed as redistribution of fluorescence to the cytosol at 37 ° C in HEK293 cells, expressing GHSR-EGFP. Cells were seeded at 300 μ l per well in an 8-well coverslip (Chambered Coverglass, glass bottom; Lab-tek, Thermo Scientific, New York, USA). When 80 % confluence was reached, cells were mounted on a confocal microscope (objective c-Apochromat 40x/1.2 W corr, laser at 488–498 nm; LSM510, Zeiss, Jena, Germany, Belgium). An image (512×512 pixels, or $0.45 \mu\text{m} \times 0.45 \mu\text{m}$) was taken after the cells were incubated with ghrelin at 1 μ M at 37 ° C for 90 minutes. Cells were fixed (HT50-1-2 formalin; Sigma, Sigma-Aldrich, Bornem, Belgium) and washed with phosphate buffered saline. A second image was taken of the same cells.

Statistics

All mouse experiments were performed by the experimenter blinded to the genotype of the mice. All *in vivo* mouse and rat experiments were performed in a randomized fashion.

Statistical analysis was performed using GraphPad Prism 4.0 software (GraphPad Software). Data are expressed as mean $\pm$ SEM. For the mice pilocarpine tail vein infusion and rat microdialysis experiments, one-way analysis of variance (ANOVA) followed by the Newman-Keuls multiple comparison test when ANOVA showed significance ($\alpha=0.05$), was used when more than 2 experimental groups were present. The assessment of differences in hippocampal GABA neurochemistry was performed by comparison of the baseline neurotransmitter levels in time with neurotransmitter levels during ghrelin administration using one-way ANOVA for repeated measures. The two-tailed unpaired Student's *t* test was performed in experiments in which 2 groups were compared ($\alpha=0.05$). The statistical test applied in each experiment is denoted in the Results section. For the

hippocampal slice electrophysiology experiments, the number of spontaneous interictal bursts per minute was noted for each experiment, and an area under the curve was calculated for each group of experiments (using mean $\pm$ SEM). The area under the curve for the ghrelin group and A778193 group was statistically compared to that of the control group using the two-tailed unpaired Student's *t* test ($\alpha=0.05$).

Results

Ghrelin and Capromorelin are Protective against *In Vivo* Limbic Seizures in Rats

To scrutinize whether ghrelin has anticonvulsant properties in our rat pilocarpine model for limbic seizures, we locally perfused ghrelin or the ghrelin-mimetic capromorelin [33] into the hippocampus via *in vivo* microdialysis (Fig. 1a, b). Both ghrelin (0.1–10 μ M) (one-way ANOVA $F=20.33$; $P<0.0001$; Fig. 1a) and capromorelin (1–10–20 μ M) (one-way ANOVA $F=4.817$; $P=0.0051$; Fig. 1b) attenuated pilocarpine-induced seizures in rats at different concentrations.

Hippocampal Extracellular GABA Levels are not Altered after Ghrelin Administration in Rats

Since it was hypothesized that ghrelin-induced elevations of the inhibitory neurotransmitter GABA could account for the anticonvulsant effect of ghrelin [25], we determined whether ghrelin induces extracellular GABA alterations in the hippocampus via *in vivo* microdialysis. Mean pooled baseline hippocampal GABA dialysate concentration (mean $\pm$ SEM) was $0.05 \pm 0.01 \mu\text{M}$ ($n=9$) (Fig. 1c). No alterations in extracellular GABA levels in the hippocampus were present after administration of anticonvulsant doses of ghrelin when compared to baseline.

Ghrelin Requires GHSR for its Anticonvulsant Effect

Next, we investigated whether ghrelin was able to alter seizure thresholds in the pilocarpine mouse tail infusion model. Intravenous pilocarpine infusion in the tail induces limbic seizures with secondary generalization in mice. This results in an array of rapidly progressing behaviors, namely head bobbing and bilateral forelimb clonus with rearing, followed by clonic convulsions with loss of righting reflexes (falling), tonic hindlimb extension (tonus), and finally death in all mice. The anticonvulsant dose of ghrelin in GHSR^{+/+} mice was found by testing a range of doses (0–0.8–1.2–1.8 $\mu\text{g/g}$) (Fig. 2a). Significant increases in seizure thresholds by ghrelin in GHSR^{+/+} mice were clearly obtained at 1.8 $\mu\text{g/g}$ (head

Fig. 1 Ghrelin attenuates limbic seizures without affecting extracellular hippocampal γ -amino butyric acid (GABA) levels. **a, b** *In vivo* microdialysis in conscious rats. The mean total seizure severity score (TSSS) with SEM for the rat groups that underwent intrahippocampal administration of (**a**) ghrelin and (**b**) capromorelin prior to pilocarpine administration (one-way analysis of variance; * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$). **c** Effect of the intrahippocampally administered

anticonvulsant doses of ghrelin on the baseline hippocampal GABA dialysate levels in rats. The first data point (0–120) represents the mean $\pm$ SEM GABA level of the first 6 basal microdialysis collections (grey box). The following data points represent the mean $\pm$ SEM GABA level of a subsequent 20-minute collection period during the 120 minutes perfusion with 1 μ M or 10 μ M ghrelin via the microdialysis probe

bobbing: one-way ANOVA $F = 7.304$; $p = 0.0016$; rearing: one-way ANOVA $F = 4.624$; $p = 0.0130$; falling: one-way ANOVA $F = 7.852$; $p = 0.0010$; tonus: one-way ANOVA $F = 6.792$; $p = 0.0021$; death: one-way ANOVA $F = 6.901$; $p = 0.0019$).

Based on this, we selected a dose of 1.8 μ g/g ghrelin to investigate whether the anticonvulsant effects of ghrelin persisted in GHSR^{-/-} mice. We observed no differences in seizure thresholds between ghrelin and saline-treated GHSR^{-/-} mice, demonstrating for the first time that ghrelin requires GHSR for its anticonvulsant effect (Fig. 2b). Interestingly, we also noted a significant effect of genotype at the threshold dose of pilocarpine for all behavioral endpoints when we looked at the saline-injected GHSR^{+/+} mice (Fig. 2a) and GHSR^{-/-} mice (Fig. 2b). This was further explored via additional tests on separate sets of mice.

GHSR^{-/-} Mice are Less Susceptible to Limbic Seizures with Secondary Generalization

Unexpectedly, we found there was a significant increase in the seizure threshold in GHSR^{-/-} mice (Fig. 2c); the effect of the genotype was significant for all behavioral endpoints: head bobbing (GHSR^{+/+}, 320.4 ± 7.6 mg/kg; GHSR^{-/-}, 379.2 ± 12.3 mg/kg; unpaired Student's t test, $p = 0.0007$), rearing (GHSR^{+/+}, 437.3 ± 15.2 mg/kg; GHSR^{-/-}, 485.9 ± 13.2 mg/kg; unpaired Student's t test, $p = 0.0266$), falling (GHSR^{+/+}, 468.6 ± 14.3 mg/kg; GHSR^{-/-}, 523.4 ± 16.0 mg/kg; unpaired Student's t test, $p = 0.0198$), tonus (GHSR^{+/+}, 483.2 ± 16.1 mg/kg; GHSR^{-/-}, 531.8 ± 16.5 mg/kg; unpaired Student's t test, $p = 0.0495$), and death (GHSR^{+/+}, 495.6 ± 16.5 mg/kg; GHSR^{-/-}, 551.1 ± 16.4 mg/kg; unpaired Student's t test, $p = 0.0284$). We did not observe any differences in weight of the GHSR^{-/-} mice when compared to

their wild-type littermates (unpaired Student's t test, $p = 0.3670$). These results suggest that the GHSR acts to lower the seizure threshold, which is puzzling given that the anticonvulsant effects of ghrelin are mediated via this receptor.

A778193, a GHSR Inverse Agonist, Also Protects against *In Vivo* Limbic Seizures

GHSR is one of the few known GPCRs having a relatively high constitutive activity of approximately 50 % [34]. If the increase in seizure threshold is due to the loss of constitutively active GHSR receptors, rather than a developmental consequence of the absence of the receptor throughout the life of the GHSR^{-/-} mice, then it may be possible to mimic the effects by inactivating the GHSR. We started investigating the pharmacological properties of the presumed GHSR antagonist A778193, which has a nanomolar affinity for the GHSR [35]. Using HEK293 cells expressing the cloned hGHSR, we found that A778193 in fact acts as a potent inverse agonist with a pIC_{50} of 6.13 ± 0.22 ($n = 3$) (Fig. 3a). Thus, we wondered whether a reduction in the constitutive activity of the GHSR can result in the attenuation of the evoked seizures. In our *in vivo* pilocarpine rat model, intrahippocampal perfusion of A778193 (10–25–50 μ M) resulted in significant attenuation of limbic seizures (one-way ANOVA $F = 10.78$; $p = 0.0002$; Fig. 3b). Another known GHSR inverse agonist [D-Arg¹,D-Phe⁵,D-Trp^{7,9},Leu¹¹]substance P (0.1–1–10 μ M) [34] was also tested, and was found to effectively inhibit limbic seizures at a concentration of 10 μ M (unpaired Student's t test, $p = 0.0064$) (Fig. 3c). The *in vivo* EEG recordings for rats receiving 10 μ M A778193 and 10 μ M [D-Arg¹,D-Phe⁵,

Fig. 2 GHSR^{-/-} mice are unresponsive to ghrelin but have a higher seizure threshold than wild-type littermates. **a** Threshold doses of pilocarpine for the induction of seizures in growth hormone secretagogue receptor (GHSR)^{+/+} mice receiving ghrelin intraperitoneally 0-0.8-1.2-1.8 µg/g (n=4-8 per group) 30 minutes prior to pilocarpine infusion in the tail vein (one-way analysis of variance; **p*<0.05, ***p*<0.01, ****p*<0.001). **b** Threshold doses of pilocarpine for the induction of seizure-associated behaviors in GHSR^{-/-} mice after saline and 1.8 µg/g ghrelin administration (n=6 per group). **c** Threshold doses of pilocarpine for the induction of seizure-associated behaviors (n=10 for each genotype) in GHSR^{+/+} and GHSR^{-/-} mice in the pilocarpine tail vein infusion model (unpaired Student's *t* test; **p*<0.05, ****P*<0.001)

D-Trp^{7,9},Leu¹¹]substance P locally in the hippocampus show no seizure activity as compared to control rats (Fig. 4).

Fig. 3 Growth hormone secretagogue receptor (GHSR) inverse agonists reduce pilocarpine-induced seizures *in vivo*. **a** Dose-dependent inhibition of constitutive inositol 1-phosphate (IP1)-production by the GHSR inverse agonist A778193 in HEK293 cells expressing the cloned hGHSR. **b, c** The mean total seizure severity score (TSS) with SEM for the rat groups that underwent intrahippocampal administration of **(b)** A778193 (one-way analysis of variance; ***p*<0.01, ****p*<0.001) and **(c)** the GHSR inverse agonist [D-Arg¹,D-Phe⁵,D-Trp^{7,9},Leu¹¹]substance P (unpaired Student's *t* test, ***p*<0.01) prior to pilocarpine administration

GHSR Agonism and Inverse Agonism Results in the Attenuation of *In Vitro* Epileptiform Activity

To confirm the *in vivo* results, we used an *in vitro* model of epileptiform activity that allows us to test ghrelin and A778193 in an isolated hippocampal system. Using *in vitro* electrophysiology, field recordings were made from the CA3 cell body region of hippocampal slices in response to

stimulation from the axons of the mossy fibers (Fig. 5d). Perfusion with pilocarpine alone caused the appearance

of multiple population spikes in response to electrical stimulation and the appearance of spontaneous interictal

Fig. 4 The sample time courses of *in vivo* electroencephalography (EEG) recordings from growth hormone secretagogue receptor (GHSR) inverse agonists test groups. Representative EEG recordings of (a) control, (b) 10 μ M of the GHSR inverse agonist A778193, and (c) 10 μ M of the GHSR inverse agonist [D-Arg¹,D-Phe⁵,D-Trp^{7,9},Leu¹¹]substance P intrahippocampally administered microdialysis experiments. Each EEG recording represents 30 minutes of baseline, 2 h of drug perfusion or sham (i.e., Ringer's solution alone in the case of control), 40 minutes co-administration of inverse agonist/sham and pilocarpine, and 2 h 40 minutes of inverse agonist/sham perfusion until the end of the experiment. The insets of magnified windows represent EEG samples for 30 seconds in each highlighted time window. Green boxes indicate a 30-second interval, 15 minutes prior to drug administration; blue boxes indicate a 30-second interval, 50 minutes after the start of inverse agonist/sham administration; and red boxes indicate a 30-second interval, 50 minutes after initiation of intrahippocampal pilocarpine and inverse agonist/sham co-administration

bursts (Fig. 5a). Perfusion of ghrelin (1 μ M) or A778193 (1 μ M) prior to pilocarpine co-administration did not lead to any differences in basal synaptic excitatory transmission, but the hippocampal slices showed a pronounced attenuation from pilocarpine-induced epileptiform activity (Fig. 5b, c). Thus, ghrelin and A778193 significantly reduced the

number of spontaneous interictal bursts per minute when compared to the control group (ghrelin vs control: unpaired Student's *t* test, $p < 0.0001$; A778193 vs control: unpaired Student's *t* test, $p < 0.0001$) (Fig. 5e, f). Our *in vitro* and *in vivo* experiments confirm that both ghrelin and the inverse agonist A778193 attenuate the convulsant action of pilocarpine.

Ghrelin Leads to Rapid GHSR Desensitization and Internalization

In light of the results obtained, we speculated that the anticonvulsant effect of ghrelin could be related to its ability to desensitize the GHSR. Desensitization was studied in HEK293 cells expressing hGHSR by comparing dose-response curves to ghrelin after preincubation with ghrelin. A 3-minute pretreatment with ghrelin resulted in a decrease of the maximal Ca^{2+} signal, as shown in Fig. 6a (left panel). The level of inhibition was dependent of the concentration of ghrelin used for the priming (Fig. 6a, right panel). The desensitization occurred rapidly since the 3-minute

Fig. 5 Ghrelin and A778193 protect against pilocarpine-induced epileptiform activity *in vitro*. **a-c** The timeline configuration of the experiments (top), electrically evoked field recordings (middle) and continuous acquisition traces (bottom). The insets are representative traces taken according to the time indicated in the timeline: (a) baseline conditions (i), 20 minutes after pilocarpine cessation (ii), and after washout for at least 2 h (iii); (b) baseline conditions (i), after 20 minutes of 1 μ M ghrelin alone (ii), 20 minutes after pilocarpine cessation (iii),

and after 95 minutes washout period (iv). **c** baseline conditions (i), after 20 minutes of 1 μ M of the growth hormone secretagogue receptor (GHSR) inverse agonist A778193 alone (ii), 20 minutes after pilocarpine cessation (iii), and after 95 minutes washout period (iv). **d** Positioning of electrodes for recording spontaneous and electrically evoked responses in the rat hippocampal slice. **e, f** Number of spontaneous interictal bursts per minute for the control group, and (e) ghrelin, or (f) A778193 pretreated group

pretreatment with ghrelin 10^{-6} M reduced the second stimulus to the same extent (8.50 ± 3.67 %), compared to a pretreatment of 10 minutes (5.2 ± 3.7 %), 20 minutes (14.7 ± 3.2 %), and 60 minutes (6.9 ± 2.1 %) (Fig. 6b). GHSR internalization was observed as a redistribution of the fluorescence to the cytosol in HEK293 cells expressing GHSR-EGFP, recorded at 37°C (Fig. 6c).

For resensitization experiments, HEK293 cells stably expressing GHSR-EGFP were pre-incubated with ghrelin for 3 minutes prior to a second stimulation with ghrelin after different periods (3 minutes, 20 minutes, 60 minutes, 2 h, 5 h, and 24 h). As shown in Fig. 6d, full resensitization of the GHSR-EGFP was achieved only after 24 h.

GHSR Desensitization is Essential for the Anticonvulsant Effect of Ghrelin

Although it is known that C-terminal elongation of ghrelin hardly induces differences in potency [36], it is unknown whether this has an effect on desensitization. Using HEK293 cells stably transfected with the hGHSR, we also investigated different truncated forms of ghrelin. We found that ghrelin (1-5) amide was approximately 300-fold (2.49 log units) less capable of inducing desensitization than ghrelin, whereas, in regard to agonistic activity, it only functioned at approximately 10-fold lower potency (Fig. 6e) (Table 1). We also found that the ghrelin-mimetic capromorelin induced GHSR desensitization (Fig. 6e) (Table 1).

Via *in vivo* microdialysis, we tested ghrelin(1-5) amide by administering it locally in the hippocampus and as based on pEC_{50} and pDC_{50} values (Table 1), only the $500\text{ }\mu\text{M}$ concentration showed significant anticonvulsant effects (unpaired Student's *t* test, $p=0.0253$) (Fig. 6f). This strengthened our hypothesis that desensitization of GHSR is required for seizure prevention.

Discussion

In this study, we unveiled that ghrelin requires the GHSR for its anticonvulsant action. For the first time we also show that GHSR deletion, inverse agonism, or desensitization leads to the attenuation of limbic seizures *in vivo* and epileptiform activity *in vitro*, meaning that both agonists and inverse agonists for the GHSR are capable of exerting anticonvulsant effects.

Ghrelin is Anticonvulsant in *In Vivo* and *In Vitro* Models and Requires GHSR for its Anticonvulsant Action

Previous studies have shown that ghrelin has an inhibitory effect on seizures induced by pentylenetetrazole [25],

penicillin [23], and kainic acid [10], but its anticonvulsant mechanism of action remains elusive. Ghrelin was shown in an *ex vivo* study to possess neuroprotective effects associated with the promotion of phosphoinositide-3-kinase (PI3K)/Akt signaling pathway and the inhibition of the mitochondrial-dependent apoptosis pathway [11]. We started by elucidating the role of ghrelin in focal pilocarpine-induced limbic seizures. Our results show an anticonvulsant action of ghrelin at different concentrations against pilocarpine-induced seizures in both mice and rats, as well as an attenuating effect on pilocarpine-induced epileptiform activity in hippocampal slices. In addition, the ghrelin-mimetic capromorelin was tested in rats where it concentration-dependently inhibited limbic seizures.

GABA, the principal inhibitory neurotransmitter in the brain, has long been accepted to play a major role in neuroexcitation and epilepsy [37, 38]. The effect of ghrelin on GABA release [39] has been proposed as a possible mechanism of action of the anticonvulsant effects of ghrelin [10, 23, 25]. However, these ghrelin–GABA interactions were only described in the hypothalamic circuitry and not in the cortical or thalamic brain regions, which are mainly involved in the generation of pentylenetetrazole-induced seizures [40]. Here we show that in the hippocampus (a brain region that plays a crucial role in temporal lobe epilepsy [41]) ghrelin did not alter extracellular GABA levels when administered intrahippocampally indicating that the anticonvulsant action of ghrelin against limbic seizures does not involve GABA alterations in the hippocampus.

Fig. 6 Desensitization, internalization, and resensitization of growth hormone secretagogue receptors (GHSRs). **a** (left) Intracellular Ca^{2+} rises in response to different concentrations of ghrelin (X-axis). Pretreated responses are expressed as percentage of responses without pretreatment (blank). (Right) The maxima of the concentration response curves in the left graph are expressed as a function of the pretreatment concentration (X-axis). **b** The effect of different pretreatment times with ghrelin at different concentrations (X-axis) on the maximum (Y-axis) of a subsequent stimulation with ghrelin. Three minutes, 10 minutes, 20 minutes, and 60 minutes of pre-incubation with ghrelin all resulted in a similar GHSR desensitization. **c** GHSR internalization was observed as redistribution of fluorescence to the cytosol at 37°C in HEK293 cells expressing an enhanced green fluorescent protein (EGFP) tagged GHSR. **d** Resensitization of GHSR. After pretreatment with ghrelin at 10^{-6} M, 10^{-7} M, or 10^{-8} M for the duration of 3 minutes, the cells were washed and re-stimulated with ghrelin (dose response 10^{-6} M – 10^{-10} M) after 3 minutes, 20 minutes, 1 h, 2 h, 5 h, or 24 h, respectively. **e** *In vitro* desensitization concentration response curve (3 minutes) for ghrelin, the GHSR agonist capromorelin, ghrelin(1-14), and ghrelin(1-5) amide. Ghrelin(1-5) amide has a much lower capacity to desensitize the GHSR compared to ghrelin and capromorelin. **f** The mean total seizure severity score (TSSS) with SEM for the rat groups that underwent intrahippocampal administration of ghrelin(1-5) amide prior to pilocarpine administration (unpaired Student's *t* test; $*p<0.05$)

Table 1 *In Vitro* Determination pEC₅₀ and pDC₅₀ of Ghrelin, Ghrelin Fragments (1-5) Amide, (1-7), (1-9), (1-11), (1-14), (23-28), and Capromorelin

	pEC ₅₀	pDC ₅₀
Ghrelin	8.90±0.09	8.15±0.08
Ghrelin (1-14)	8.29±0.07	7.81±0.24
Ghrelin (1-11)	8.88±0.08	8.48±0.36
Ghrelin (1-9)	8.90±0.09	7.37±0.50
Ghrelin (1-7)	8.59±0.09	5.70±0.13
Ghrelin (1-5) amide	8.02±0.07	5.66±0.47
Ghrelin (23-28)	< 1 μM	< 1 μM
Capromorelin	9.33±0.07	8.43±0.16

Ghrelin and capromorelin similarly activated and desensitized the growth hormone secretagogue receptors (GHSRs). The truncated forms of ghrelin (1-14) and (1-5) amide had a similar activity (pEC₅₀), however, unexpectedly ghrelin(1-5) amide did not induce desensitization of GHSR to the same extent as ghrelin(1-14). In fact, with increasing length, ghrelin(1-7), (1-9), and (1-11) increasingly desensitized the receptor while maintaining a similar pEC₅₀ as the full-length ghrelin. The C-terminal fragment ghrelin(23-28) was not able to evoke a Ca²⁺ response and not desensitization of the GHSR occurred.

In addition to acting on the GHSR, ghrelin is also known to act on other unidentified receptors [5]. Therefore, we used GHSR-null mice to establish whether the seizure-attenuating effect of ghrelin is due to its direct action on this receptor. For the first time, we conclusively show that ghrelin requires the GHSR for its anticonvulsant effect.

GHSR^{-/-} Mice Have an Elevated Seizure Threshold

Unexpectedly, we found that GHSR^{-/-} mice had a decreased vulnerability to pilocarpine-induced seizures than their GHSR^{+/+} littermates implicating that, in fact, inactivation of the receptor is required for seizure inhibition. This observation, which would not have been predicted from the effects of ghrelin, prompted us to look further into the pharmacological modulation of the receptor.

Inverse Agonism of GHSR is Anticonvulsant in *In Vitro* and *In Vivo* Models

The GHSR signals with approximately 50 % maximal activity in the absence of its peptide ligands [34], rendering it one of the few highly constitutively active GPCRs currently known. This ligand-independent activity was recently proven to be an intrinsic feature of the GHSR protein and not merely the consequence of an influence of the cellular environment [42]. The constitutive activity of GHSR

appears to be important in humans, since a missense mutation of the GHSR, which leads in the loss of the constitutive activity of the receptor while retaining its ability to respond to ghrelin-induced signaling, resulted in familial short stature [43]. In light of this knowledge, we wondered whether the constitutive activity of GHSR needed to be reduced to attenuate seizure activity. Pharmacologically, this could be obtained via inverse agonism. We found that A778193 dose dependently inhibited constitutive IP1 production in unstimulated HEK293 cells expressing the cloned hGHSR, and thus acts as an inverse GHSR agonist.

Next we tested the effect of A778193 on pilocarpine-induced seizures *in vivo*, as well as on pilocarpine-induced epileptiform activity using hippocampal slices. A778193 significantly attenuated the convulsant actions of pilocarpine in both setups. The anticonvulsant actions mediated by inverse agonism of GHSR are fully in line with the increased seizure threshold observed in GHSR^{-/-} mice *versus* GHSR^{+/+} littermates. To further confirm this finding, we tested another known GHSR inverse agonist [D-Arg¹, D-Phe⁵, D-Trp^{7,9}, Leu¹¹] substance P [34], which also significantly inhibited limbic seizures in our *in vivo* model. In the past few years, a number of ligands have been reclassified from being antagonists to inverse agonists, thus it would not be surprising if other GHSR “antagonists” are in fact inverse agonists.

Receptor Desensitization Induced by Ghrelin

Our finding that agonism, inverse agonism, and deletion of the GHSR all result in the attenuation of *in vivo* and *in vitro* seizure activity was difficult to reconcile at first sight. This led us to speculate that the anticonvulsant effect of ghrelin may be related to its ability to desensitize the GHSR. It is known that while desensitization and internalization of the GHSR occurs rapidly, resensitization of the receptor to the cell surface is slow [44–46]. We found that a 3-minute pretreatment with ghrelin was sufficient for maximal desensitization, and that full resensitization took around 24 h. Also, capromorelin showed anticonvulsant activity combined with strong receptor desensitization potential. A way to investigate this anticonvulsant mechanism of ghrelin using an *in vivo* setting is by testing a ligand that provides a similar GHSR activation to ghrelin, while showing a decreased tendency to desensitize the receptor. In this study, we successfully discovered that the shorter the C-terminal of ghrelin is, the less capable it is able to desensitize GHSR, while maintaining a similar pEC₅₀ to ghrelin. Thus, we confirmed our hypothesis by testing the ghrelin (1-5) amide *in vivo*, where we revealed for the first time that it is desensitization and not activation of the GHSR that is necessary to attenuate limbic seizures *in vivo*.

Possible Downstream Molecular Pathways for GHSR-Mediated Anticonvulsant Effects

Holliday et al. [46] unraveled that constitutive GHSR G_q signaling ceases when the GHSR is internalized and is no longer at the plasma membrane. It is known that activity of G_q signaling can be determined in cAMP response element (CRE) reporter assays via phosphorylation of the CRE binding protein (CREB). Holst et al. [34] reported that the GHSR ligand-independently signals through the CREB pathway. CREB phosphorylation has been found to be increased in rodent epilepsy models, and a reduction in CREB levels led to the suppression of seizures [47]. Activation of the G_q pathway inherently results in inositol phosphate-3 (IP_3)-dependent Ca^{2+} release from intracellular Ca^{2+} stores, leading to increased cytosolic Ca^{2+} concentrations, which is synonymous with increased membrane excitability, as well as triggering and maintaining seizures [48–50]. A number of AEDs (such as phenytoin and levetiracetam) are inhibitors of IP_3 -dependent intracellular Ca^{2+} release [50, 51]. Xu et al. [11] found that ghrelin possesses neuroprotective properties after pilocarpine-induced seizures by promoting the PI3K/Akt signaling pathway. This claim was substantiated by another recent study [7], however, the overall involvement of this pathway in seizures is still being determined [52]. The GHSR is also known to stimulate adenylyl cyclase [5]. Stimulation of adenylyl cyclase has been implicated in pro-convulsant effects [53–57]. Thus, one can theorize that GHSR desensitization/inverse agonism should result in anticonvulsant effects through inactivation of the CREB pathway, diminished IP_3 -dependent Ca^{2+} release, and decreased adenylyl cyclase activity. It is difficult to speculate which molecular pathway is implicated in seizure suppression following GHSR internalization or inverse agonism, which consequently calls for the need of future investigations regarding signaling cascades that are implicated in GHSR-mediated seizure suppression.

Implications for the Use of Ghrelin in Epilepsy

The phenomenon of having both agonism and inactivation of a receptor resulting in the same end effect is not a pharmacological oddity. Indeed, desensitization by agonists and pharmacological blockade by antagonists of the vanilloid (capsaicin) receptors (transient receptor potential cation channel subfamily V member 1, TRPV1) are well known to both lead to analgesia [58–60]. The latter is corroborated by experiments on TRPV1-deficient mice [58]. Currently, there are a number of TRPV1 agonists and antagonists undergoing clinical trials [60]. The mechanism of action of ghrelin in epilepsy could be similar to that of (TRPV1) agonists in pain.

Researchers are now looking for potential AEDs that act in new ways and through new targets since a staggering 30 % of epilepsy patients are pharmacoresistant to the current available drugs. As seen from this study, ghrelin has a diverse mechanism of anticonvulsant action than the existing AEDs on the market. Interestingly, the GHSR agonist JMV 1843 has just completed its clinical phase III for growth hormone deficiency diagnosis in man and was found to be well-tolerated by human subjects [61]. Keeping in mind the phenomenon of ligand-directed signaling [62], in which agonists acting on a selected GPCR can induce different cellular response patterns, one cannot assume that each agonist of the GHSR will result in the same end response. Nevertheless, it would be interesting to investigate whether JMV 1843 possesses anticonvulsant properties.

Moreover, ghrelin presents a number of benefits as an anticonvulsant drug [63]. Ghrelin, as well as selective GHSR agonists, are capable of crossing the blood-brain barrier [64], which enables peripheral administration in humans. GHSR is highly expressed in hippocampal structures, however, the exact localization of these receptors in this brain region are yet to be determined. As previously mentioned, ghrelin has been shown to affect hippocampal synaptic plasticity, with the benefit of improving memory [2, 65]. It has been described that patients suffering from limbic seizures had altered hippocampal synaptic plasticity, resulting in long-term potentiation impairment [66]. Ghrelin has also been repeatedly found to have neuroprotective properties [9, 11]. Thus, one could hypothesize that ghrelin administration in epileptic subjects will have a triple function of seizure attenuation, neuroprotection, as well as prevention of memory impairment associated with recurrent seizures.

Concluding Remark

To the best of our knowledge, the constitutively active GHSR is the first neuropeptide receptor that requires inactivation in order to attenuate epileptiform activity *in vitro* and limbic seizures *in vivo*.

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