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Effects of ghrelin on neuronal activity in the ventromedial nucleus of the hypothalamus in infantile rats: An in vitro study

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ABSTRACT

Ghrelin is an endogenous ligand for the growth hormone (GH) secretagogue (GHS) receptor (GHS-R) and a potent stimulant for GH secretion even in infantile rats before puberty. Although the ventromedial nucleus of the hypothalamus (VMH) might be a site of action for ghrelin to induce GH release, the electrophysiological effect of ghrelin on VMH neurons in infantile rats remains to be elucidated. Thus, the purpose of the present study was to investigate the effect of ghrelin on VMH neurons using hypothalamic slices of infantile rats. Ghrelin excited a majority of VMH neurons in a concentration-dependent manner. VMH neurons that were excited by GH releasing peptide-6 (GHRP-6), a synthetic GHS, were also excited by ghrelin and vice versa. Repeated application of ghrelin to the same VMH neuron decreased progressively the excitatory responses depending on the number of times it was administered. The excitatory effect of ghrelin on VMH neurons in normal artificial cerebrospinal fluid (ACSF) persisted in low Ca^{2+}-high Mg^{2+} ACSF. The present results indicate that (1) ghrelin excites a majority of VMH neurons dose-dependently and postsynaptically and (2) the excitatory effects of ghrelin are mimicked by GHRP-6 and desensitized by repeated applications of ghrelin.

1. Introduction

Growth hormone secretagogues (GHSs) are a family of small synthetic peptidyl and nonpeptidyl molecules that stimulate growth hormone (GH) secretion in animals and humans [3,37,38]. The first peptidyl GHS discovered is a growth hormone-releasing peptide-6 (GHRP-6) that was derived from the opioid peptide met-enkephalin [4]. A series of nonpeptidyl GHSs such as L-692,429 and MK-0677 were also discovered using GHRP-6 as a template [3,7]. Actions of GHSs are mediated through a specific G-protein coupled receptor GHS receptor (GHS-R). The GHS-R is distinct from the GH-releasing hormone (GHRH) receptor (GHRH-R) and consists of 366 amino acids with a molecular mass of approximately 41 kDa [22,43]. Recently a natural endogenous ligand for the GHS-R has been identified and named ghrelin. Ghrelin is a 28-amino-acid gastric peptide that is produced in peripheral and brain tissues such as the stomach and hypothalamus and displays strong GH-releasing activity [24].

GHS-Rs are mainly distributed in the pituitary and hypothalamus such as the ventromedial nucleus (VMH) and arcuate nucleus (ARC) of the hypothalamus [17,22,28,36,47,48].

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In the VMH and ARC a subpopulation of GHRH mRNA-containing neurons expresses the GHS-R gene [39,40] indicating that ghrelin and GHSs act both at the pituitary and hypothalamic levels to release GH. However, several lines of evidence suggest that the most important action of ghrelin and GHSs for GH secretion takes place at the hypothalamic level through the mediation of GHRH-secreting neurons [43]. For example, the GH-releasing activity of GHSs was significantly attenuated in rats administered GHRH antiserum or a GHRH receptor antagonist [2,5,10]. The GH secretion following ghrelin or GHSs administration was also dramatically reduced in animals by transecting the hypophyseal stalk [15,21] and in humans with hypothalamopituitary disconnection [31,32].

Ghrelin mRNA and peptide in the stomach of rats were first detectable at 1 and 7 days of age, respectively [27,41]. Stomach ghrelin mRNA and peptide increased progressively during the second and third weeks of age and attained a plateau level [27]. Another study also showed that the amount of ghrelin in the rat stomach also increased, in an age-dependent fashion, from the neonatal stage to adult [18]. In parallel with stomach ghrelin levels, plasma ghrelin levels also increased postnatally [27]. The expression of GHS-R mRNA in the brain is detected at embryonic day 19 [23] and the GHS-R mRNA levels increase with age in the pituitary while they remain constant in the hypothalamus during development [6,28]. Peripheral injection of ghrelin caused a significant increase in plasma or serum GH concentrations in rats with 1–3 weeks of age [18,30]. Intracerebroventricular administrations of ghrelin also significantly increased serum GH levels in these rats, but in vitro ghrelin challenge to pituitary tissues failed to enhance GH release into the incubation medium [30]. These data suggest that the stimulatory effect of ghrelin on GH secretion in rats is established in an early stage of the infantile period before puberty, and like in adult rats ghrelin functions at the hypothalamic level for GH release, possibly via the mediation of GHRH-secreting neurons [30]. Therefore, we hypothesized that ghrelin as well as GHRP-6 will have an excitatory effect on VMH neurons in infantile rats. Also there does not appear to be any electrophysiological evidence showing that ghrelin acts on hypothalamic neurons in infantile rats. Thus, the purpose of the present study was to determine the effect of ghrelin on the electrical activity of VMH neurons in vitro using hypothalamic slice preparations of infantile rats and to determine whether or not the effect of ghrelin is mimicked by GHRP-6 in the VMH.

2. Materials and methods

2.1. Animals

Seventy-six male Wistar rats (Sankyo Lab., Shizuoka, Japan) 2–4 weeks of age were used. They were housed with their mothers in a light-controlled room, light on 06:00–18:00, at a temperature of $23 \pm 1\,^{\circ}C$ for several days before the experiments. Food and water were available ad libitum. The animals and experimental procedures used were approved by the Institutional Animal Care and Use Committee of the University of Toyama.

2.2. Slice preparation

After ether anesthesia, rats were decapitated, and the brain was rapidly removed from the skull. The brain was then submerged in ice cold, oxygenated (95% O_2 and 5% CO_2) artificial cerebrospinal fluid (ACSF), composition in mM: NaCl 126, KCl 3, $CaCl_2$ 2.4, $MgSO_4$ 1.3, KH_2PO_4 1.25, $NaHCO_3$ 26 and glucose 10 with a pH of 7.4. Frontal hypothalamic slices 400 μm thick were cut by a microslicer (ZERO 1, Dosaka EM, Kyoto, Japan). Slices including the VMH were selected and cut with a scalpel along the third ventricle so that two VMH slices could be obtained from each frontal slice. The slices containing the VMH were then preincubated in a chamber with oxygenated ACSF for about 1 h at room temperature.

2.3. Extracellular recording

After preincubation, slices were transferred into a recording chamber, and perfused with oxygenated ACSF containing in mM: NaCl 124, KCl 5, $CaCl_2$ 2.4, $MgSO_4$ 1.3, KH_2PO_4 1.25, $NaHCO_3$ 26 and glucose 10, at 1 ml/min and at 34 °C. ACSF containing 5 mM in place of 3 mM KCl was used to depolarize neurons slightly and to make it easier to obtain spontaneous firing of action potentials. Extracellular electrical activity was recorded from the dorsomedial part of the VMH via a glass microelectrode, resistance 5–15 MΩ, filled with ACSF, and then fed into a main amplifier via a preamplifier. The dorsomedial part was selected because of the abundant expression of GHS-Rs [17,48]. The output of the main amplifier was monitored on an oscilloscope and recorded on a magnetic tape. The output was also passed through a pulse former and then into a computer through an A/D converter. The computer calculated the firing rate of a neuron in 1 s intervals in spikes/s and recorded and displayed them on a computer screen as a function of time. The basal firing rate in each neuron was then calculated as an average for 3 min immediately before the application of ghrelin or GHRP-6 and this value was subtracted from subsequent changes in firing rate after the application of ghrelin or GHRP-6. The response to ghrelin and GHRP-6 was evaluated as the change of mean firing rate averaged during the entire response time after the drug application. If the change in mean firing rate after the application of ghrelin and GHRP-6 was greater than ±20% compared with the basal firing rate, the drug was considered to be effective. In the present study a recording was made from only one neuron in each slice. When a repeated application of a drug was tested it was tested only after the firing rates of the neurons returned to baseline levels of activity usually 10–20 min.

2.4. Drugs

Ghrelin (Peptide Institute, Osaka, Japan) and GHRP-6 (Sigma, Tokyo, Japan) were dissolved in saline and stored at −40 °C in small aliquots. They were then diluted with ACSF to desired concentrations and were added to the perfusate.

2.5. Statistics

All data are expressed as means ± SEMs. A dose–response curve for ghrelin was analyzed by the Kruskal–Wallis test,

whereas responses to repeated applications of ghrelin were analyzed by a one-way analysis of variance (ANOVA) with repeated measures followed by a Scheffe test. Paired *t*- and χ^2-tests were also used, $p < 0.05$.

3. Results

3.1. Excitatory effects of ghrelin on VMH neurons

Based upon previous studies of the effects of ghrelin in ARC neurons [33,34,42], ghrelin at 10^{-7} M was applied to 37 VMH neurons with a mean spontaneous firing rate of 1.10 ± 0.15 spikes/s. Of the 37 VMH neurons, 24 (64.9%) were excited by ghrelin, and the remaining 13 (35.1%) were unchanged. None of the neurons were suppressed by ghrelin. Fig. 1A shows a sample record of a VMH neuron tested with 10^{-7} M ghrelin. The neuron increased its firing rate of responding after ghrelin was administered. The mean latency, duration and firing rate for ghrelin-induced excitations obtained from 24 neurons were 143.6 ± 11.8 s, 581.8 ± 77.5 s and 1.10 ± 0.10 spikes/s, respectively. These results indicate that VMH neurons are predominantly excited but not inhibited by ghrelin. To obtain a dose–response relationship, responses to ghrelin were further investigated in another 21 VMH neurons. Each neuron was tested with one of four different concentrations of ghrelin and the four ghrelin concentrations used were 10^{-10} M ($n = 3$), 10^{-9} M ($n = 4$), 10^{-8} M ($n = 8$), and 10^{-6} M ($n = 6$). The neurons that were tested with low doses of ghrelin such as 10^{-10} M and 10^{-9} M were further tested with 10^{-7} M ghrelin to confirm a definite excitatory response to ghrelin. A dose–response curve obtained from the 45 neurons is displayed in Fig. 1B; E_{max} was 1.17 ± 0.08 spikes/s at 10^{-6} M and the EC_{50} value was 0.59×10^{-8} M. A Kruskal–Wallis analysis test indicates a significant increase in mean excitatory responses, $H_4 = 22.8$, $p < 0.001$.

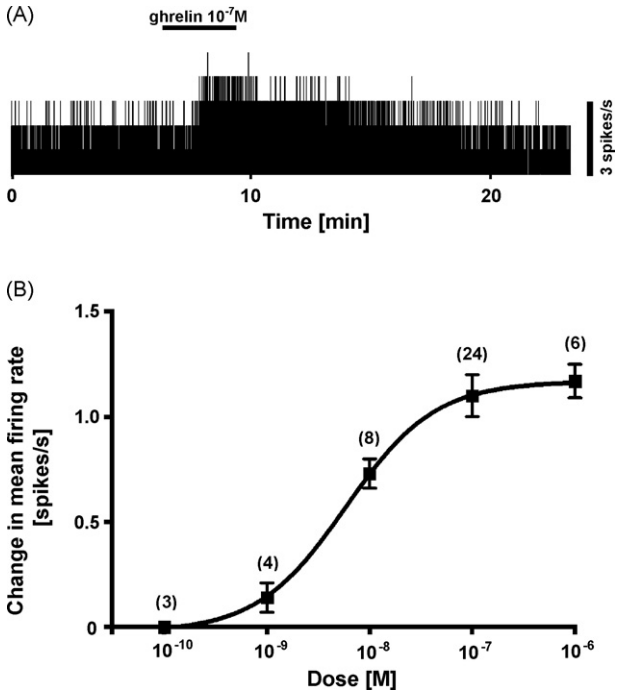

Fig. 1 – Excitatory effects of ghrelin on VMH neurons. (A) A sample record of a VMH neuron tested with 10^{-7} M ghrelin. Ghrelin was applied for the period indicated by a solid horizontal bar. Neuronal activity was increased after ghrelin application. Firing rates in spikes/s (ordinate) are shown as a function of time in min (abscissa). (B) Dose–response curve of VMH neurons to ghrelin; means ± SEMs. Each VMH neuron was challenged with one of five ghrelin concentrations at 10^{-10} M, 10^{-9} M, 10^{-8} M, 10^{-7} M and 10^{-6} M, and neurons that were challenged with low doses of ghrelin such as 10^{-10} M and 10^{-9} M were further tested by 10^{-7} M ghrelin to confirm a definite excitatory response to ghrelin. Numerals enclosed within parentheses are the number of neurons tested for each concentration.

3.2. Effect of repeated applications of ghrelin on VMH neurons

The regulation of GHS-R signaling has been characterized by a rapid desensitization process that is induced after ghrelin binding [8]. Therefore, the excitatory responses as indicated in Fig. 1. A might decrease during repeated applications of ghrelin. To investigate such a possibility, application of ghrelin at 10^{-7} M was repeated three times in seven VMH neurons. A sample record is shown in Fig. 2A. Neurons were excited strongly by the first application of ghrelin and the subsequent increases in firing rates for the second and third applications decreased considerably. As shown in Fig. 2B, the mean excitatory responses obtained in seven VMH neurons were progressively reduced depending on the number of times ghrelin administration was repeated. A one-way ANOVA analysis for repeated measures resulted in an $F_{2,10} = 15.9$ and indicated that the reduction is statistically significant, $p < 0.001$. A Scheffe test further revealed that the mean excitatory response for the third application was significantly lower than that for the first application, $p < 0.01$. These results suggest that the GHS-Rs in the VMH are easily desensitized by repeated applications of ghrelin thereby leading to decreases of firing rate and possibly GH release.

3.3. Effects of ghrelin and GHRP-6 on VMH neurons

Whether or not ghrelin, a natural endogenous ligand for the GHS-Rs, had similar effects with GHRP-6 on the same neuron was examined in 12 VMH neurons. Four neurons that were excited by GHRP-6 were also excited by ghrelin, and five neurons that were exited by ghrelin were also exited by GHRP-6, as shown in sample records of Fig. 3A and B, respectively. However, the remaining three were not affected by both agents as shown in Fig. 3C. In nine neurons that were excited by both ghrelin and GHRP-6 the mean latency, duration and firing rate to ghrelin-induced excitation were 126.3 ± 10.0 s, 706.7 ± 114.7 s and 0.66 ± 0.13 spikes/s, respectively whereas the corresponding values for GHRP-6-induced excitation were 144.1 ± 14.9 s, 620.2 ± 50.3 s and 0.61 ± 0.15 spikes/s. There were no statistical significances in these three parameters between ghrelin- and GHRP-6-induced excitatory responses, paired *t*-test, $p > 0.05$. These results suggest that both ghrelin

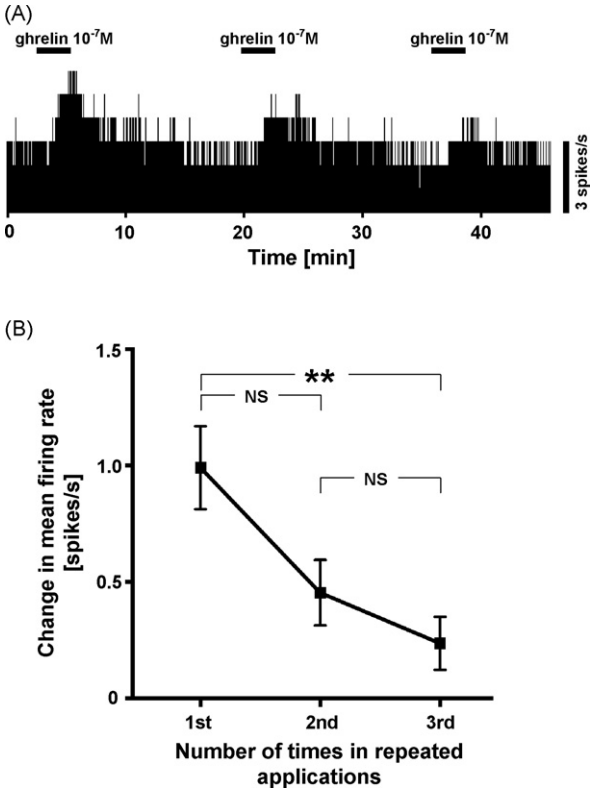

Fig. 2 – Effect of repeated application of ghrelin on the same VMH neuron. (A) A sample record of a VMH neuron tested with three applications of 10^{-7} M ghrelin. First application of 10^{-7} M ghrelin indicated by the first solid horizontal bar produced a strong excitatory response, but the magnitude of excitatory responses was progressively decreased to the subsequent second (second horizontal bar) and third (third horizontal bar) applications of ghrelin. Firing rates in spikes/s (ordinate) are shown as a function of time in min (abscissa). (B) Decrease of the mean excitatory responses to repeated applications of 10^{-7} M ghrelin. The mean excitatory response for the third application was significantly lower than that for the first application. The number of neurons tested for three administrations of ghrelin from the first to third is 7. $^{**}p < 0.01$; NS, not significant.

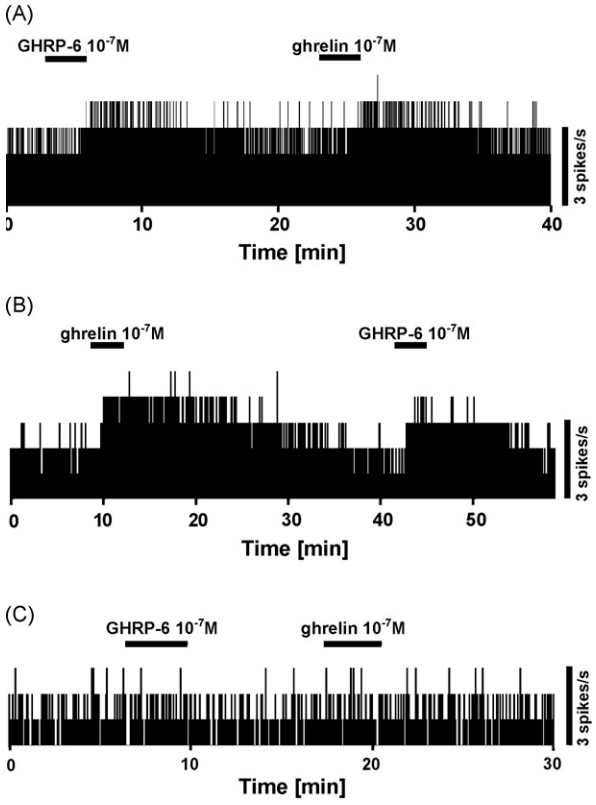

Fig. 3 – Excitatory effect of ghrelin and GHRP-6 on the same VMH neuron. (A) A neuron that was excited by GHRP-6 was also excited by ghrelin. First and second solid horizontal bars, GHRP-6 and ghrelin at 10^{-7} M, respectively. Firing rates in spikes/s (ordinate) are shown as a function of time in min (abscissa). (B) A neuron that was excited by ghrelin was also excited by GHRP-6. First and second solid horizontal bars, ghrelin and GHRP-6 at 10^{-7} M, respectively. (C) A neuron that was unresponsive to GHRP-6 was also unresponsive to ghrelin. First and second solid horizontal bars, GHRP-6 and ghrelin at 10^{-7} M, respectively.

and GHRP-6 share the GHS-R and when activated induce an excitatory response in the same VMH neuron.

3.4. *Effects of ghrelin on VMH neurons in low-Ca^{2+} and high-Mg^{2+} ACSF*

To determine whether the excitatory effect of ghrelin on VMH neurons was presynaptic or postsynaptic, neuronal activity was recorded in 10 VMH neurons in normal as well as low-Ca^{2+} and high-Mg^{2+} ACSF. As shown in Fig. 4, a neuron excited by 10^{-7} M ghrelin in normal ACSF was also excited by 10^{-7} M ghrelin in low-Ca^{2+} and high-Mg^{2+} ACSF, which blocks synaptic transmission. Similar results were also obtained in the other nine neurons. These results suggest that the excitatory effect of ghrelin on VMH neurons is due to a direct postsynaptic action of ghrelin on GHS-Rs.

4. Discussion

In the present study, we demonstrated that ghrelin at 10^{-7} M excites 64.9% of VMH neurons dose-dependently in infantile rats; that the VMH neurons excited by GHRP-6 are also excited by ghrelin and vice versa; and that excitatory responses of VMH neurons evoked by ghrelin are persisted during synaptic blockade due to low-Ca^{2+} and high-Mg^{2+} ACSF. These results suggest that the major effect of ghrelin on VMH neurons in infantile rats was excitation and ghrelin and GHRP-6 caused excitatory responses postsynaptically on the same VMH neuron through the mediation of common GHS-Rs. In the previous in vitro studies using adult rat hypothalamic slices neurons in the VMH were excited predominantly by bath applications of GHRP-6 [20,25], and the percentage of VMH neurons excited by GHRP-6 in adult rats was comparable with those of VMH neurons excited by ghrelin in infantile rats. For example, Kumarnsit et al. [25] reported that in adult rats 10^{-7} M GHRP-6 excited 33 (73.3%) of 45 VMH neurons tested,

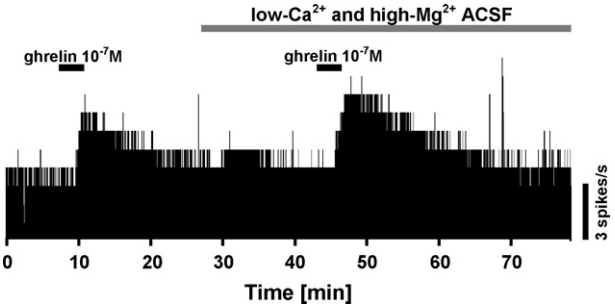

Fig. 4 – Persistence of ghrelin-induced excitatory response in a VMH neuron during synaptic blockade. The excitatory effect of ghrelin in normal ACSF persisted in the low-Ca^{2+} and high-Mg^{2+} ACSF, which blocks synaptic transmission. Lower solid horizontal bars, 10^{-7} M ghrelin. Upper dotted horizontal bar, low-Ca^{2+} and high-Mg^{2+} ACSF perfusate. Firing rates in spikes/s (ordinate) are shown as a function of time in min (abscissa).

inhibited 2 (4.4%) and did not affect the remaining 10 (22.2%). Using a χ^2 analysis a comparison of the ratios of neurons excited in the previous study [25] with those in this study revealed that there was no significant difference between adult and infantile rats, $p > 0.05$. Expression of GHS-R mRNA in the hypothalamus occurs at embryonic day 19 [23] and the GHS-R mRNA levels remain constant in the hypothalamus during development [6,23]. Peripheral and central administrations of ghrelin induced plasma GH responses in infantile rats without a direct pituitary effect [18,30]. In addition, it has been reported that a large number of GHRH mRNA-containing neurons exist in the VMH and ARC and more than 20% of them express the GHR-R gene [39,40]. Furthermore recent in vitro studies demonstrated that 61–80% of ARC neurons in rats are excited by ghrelin [33,34,42]. Taking these previous findings into consideration, the present study supports the notion that in infantile rats as well as in adult rats ghrelin and GHSs directly excite GHRH-containing neurons with GHS-Rs in the VMH as well as in the ARC thereby secreting GHRH into pituitary portal blood and thereby inducing GH release [43].

Recently, Chen et al. [9] reported that 39.5% of VMH neurons in anesthetized adult rats are inhibited by micropressure application of ghrelin, 25.9% are excited, and the remaining 34.6% are unresponsive; indicating that VMH neurons are predominantly inhibited by ghrelin. The results obtained in this earlier experiment are not consistent with those obtained in the present study. While the exact cause of the discrepancy remains to be elucidated, the difference might be related to different experimental conditions. In the earlier experiment, the animals were anesthetized with urethane; whereas, in our brain slice experiments using ether, a volatile and short-acting anesthetic, anesthesia was not a confounding factor. The methodological difference in ghrelin application; that is, micropressure application in the earlier study and bath application in the present study might also contribute to the discrepancy. Furthermore the fact that the recording site of neuronal activity in the present study is limited to the dorsomedial part of the VMH but not the whole VMH is another possible explanation for the discrepancy. Although it is conceivable that the difference in age of the animals used is a possible factor in the discrepancy, it should be noted as discussed above that VMH neurons are predominantly excited by both ghrelin and GHRP-6 in infantile as well as adult rats.

It has been demonstrated in both in vitro and in vivo experiments that the effects of GHSs on GH secretion are reproducible but undergo desensitization during intermittent administration and during continuous infusion [16,43]. A decrease of GH responses to intermittent administration of ghrelin also occurred when using a perifusion system of isolated rat anterior pituitary cells [46]. In the present study, when 10^{-7} M ghrelin was applied to the same VMH neuron three times with an interval of about 10–20 min the magnitude of excitatory responses to the third application was significantly lower than that to the first application. It seems likely that the decrease of the excitatory responses evoked by repeated applications of ghrelin is due to the desensitization of GHS-Rs. Desensitization is induced by the uncoupling of the receptor from G proteins and the internalization of the cell surface receptors to intracellular domains a mechanism that protects the cell from receptor overstimulation [8]. Taking the results of previous studies [16,43,46] into consideration, the present data suggest that such a desensitization of GHS-Rs occurs at the hypothalamic level as well as at the pituitary level and leads to a decrease in the GH response.

In addition to electrophysiological studies, brain areas activated by ghrelin and GHSs have been examined in rats and mice by assessing the expression of c-Fos proteins a marker of neuronal activation and the product of the immediate-early-gene c-*fos*. Systemic and central injections of ghrelin and other GHSs, such as GHRP-6, consistently induce c-Fos expression in the ARC [1,11–13,19,26,29,35,44]. However, the c-Fos expression in the VMH was almost absent following systemic administration of ghrelin and GHRP-6 [12,44]. Central administration of ghrelin and GHRP-6 also induce no detectable change or only a small increase of c-Fos expression in the VMH [13,26,29]. Despite the abundant distribution of GHS-Rs in the VMH [17,22,28,36,47,48] and the excitation of VMH neurons by ghrelin and GHRP-6, as demonstrated electrophysiologically in the present study, the reason why an absence or a small increase of c-Fos expression is induced in the VMH following ghrelin and GHSs administration is unclear. GHS-R mRNA is expressed in 94% of the ARC neurons expressing NPY mRNA [45] and about 90% of the ARC neurons that expressed c-Fos after intraperitoneal injection of ghrelin also expressed NPY mRNA [44]. In addition, NPY neurons in the ARC project to the VMH [14] and NPY is a powerful inhibitor of VMH neurons [25]. Hence, it is suggested that the activation of NPY neurons in the ARC by ghrelin and GHSs might inhibit neuronal activity in the VMH and thereby counteract the excitatory effects of ghrelin and GHSs in the VMH [25]. Alternatively, when VMH neurons are predominantly inhibited by ghrelin as shown in the earlier experiment [9] the discrepancy between the level of c-Fos expression and the presence of GHS-Rs may be explained by the inhibition [26].

In summary, we demonstrated that VMH neurons in infantile rats are predominantly excited by ghrelin and GHRP-6 through the postsynaptic action of GHS-Rs and that GHS-Rs in the VMH are easily desensitized by repeated application of ghrelin. The present study supports the notion

that the excitation of GHRH-secreting cells with GHS-Rs in the VMH mediates partly the ghrelin- and GHS-induced GH secretion in infantile rats as well as in adult rats.

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