



Gamma-aminobutyric acid_A receptor agonist, muscimol, increases KiSS-1 gene expression in hypothalamic cell models

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Abstract

Purpose: Accumulating evidence indicates that hypothalamic kisspeptin plays a pivotal role in the regulation of the hypothalamic–pituitary–gonadal (HPG) axis. In this study, the direct action of the gamma-aminobutyric acid (GABA)_A receptor agonist on kisspeptin-expressing neuronal cells was examined.

Methods: A hypothalamic cell model of rat hypothalamic cell line R8 (rHypoE8) cells and primary cultures of neuronal cells from fetal rat brains were stimulated with a potent and selective GABA_A receptor agonist, muscimol, to determine the expression of the KiSS-1 gene.

Results: Stimulation of the rHypoE8 cells with muscimol significantly increased the level of KiSS-1 messenger (m)RNA expression. The ability of muscimol to increase the level of KiSS-1 mRNA also was observed in the primary cultures of the neuronal cells from the fetal rat brains. The muscimol-induced increase in KiSS-1 mRNA expression was completely inhibited in the presence of the GABA_A receptor antagonist. Although muscimol increased the expression of KiSS-1, the natural compound, GABA, failed to induce the expression of KiSS-1 in the rHypoE8 cells. Muscimol did not modulate gonadotropin-releasing hormone expression in either the rHypoE8 cells or the primary cultures of the fetal rat brains.

Conclusions: This study's observations suggest that the activation of the GABA_A receptor modulates the HPG axis by increasing kisspeptin expression in the hypothalamic neurons.

KEYWORDS

gamma-aminobutyric acid_A receptor, gonadotropin-releasing hormone, hypothalamus, kisspeptin, muscimol

1 | INTRODUCTION

Kisspeptin, a product of the KiSS-1 gene, is a neuropeptide that is closely linked to the reproductive function of multiple species. Although gonadotropin-releasing hormone (GnRH) is the most potent activator of the hypothalamic–pituitary–gonadal (HPG) axis,

mounting evidence indicates a role for hypothalamic kisspeptin in the regulation of GnRH release.^{1,2} In rodents, kisspeptin neurons in the arcuate nucleus (ARC) of the hypothalamus are implicated in the estrogen-mediated pulsatile release of GnRH and the subsequent release of pituitary gonadotropins, luteinizing hormone (LH), and follicle-stimulating hormone.^{3,4} However, kisspeptin neurons in

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the anteroventral periventricular nucleus (AVPV) generally are accepted as mediators of the GnRH and LH surge. Indeed, previous *in vivo* work has shown that ovarian estradiol (E2) upregulated kisspeptin in AVPV kisspeptin neurons, whereas E2 suppressed kisspeptin in ARC kisspeptin neurons.^{5,6} Therefore, now it is generally accepted that kisspeptin neurons in the AVPV region are a center of the E2-induced positive feedback mechanism and that those in the ARC region play a pivotal role in the E2-induced negative feedback mechanism.

Kisspeptin neurons are the final component of the HPG axis and control GnRH. In addition, substantial evidence indicates that γ -aminobutyric acid (GABA) neurons also influence GnRH neurons. Hypothalamic GnRH neurons receive abundant synaptic input from GABA neurons within the hypothalamus⁷ and functional receptors for GABA are expressed in GnRH neurons.⁸ The GABA has a direct effect on GnRH neurons and exerts both stimulatory and inhibitory influences on the reproductive axis.⁹ In the central nervous system, GABA acts through three pharmacologically different receptors: GABA_A, GABA_B, and GABA_C. The GABA_A and GABA_C receptors are ligand-gated Cl⁻ channel-type receptors, whereas the GABA_B receptors are coupled with G proteins.¹⁰

GABA_A receptors are heteromeric pentamers and most receptors in the central nervous system contain α , β , and γ subunits.¹¹ The highest concentrations of GABA_A sites have been detected in the frontal cortex, the granular cell layer of the cerebellum, the olfactory bulb, the thalamic medial geniculate, and the hypothalamus.¹²⁻¹⁴ Previous work has shown that the activation of GABA_A receptors results in different responses, depending on the species or developmental stage of the animal. *In vivo* studies using rodents and sheep showed that the inhibition of the GABA_A receptor by its specific antagonist increases LH release^{15,16} and that the GABA_A receptor antagonist advances the onset of puberty in female monkeys.¹⁷ These observations show the inhibitory effect of GABA_A receptor activation on the HPG axis. In contrast, GABA_A receptor activation excites the GnRH neurons of adult mice¹⁸ and increases the firing activity in adult mouse brain slices.¹⁹ It also exerts an excitatory effect on GnRH neurons from embryonic mice.²⁰

It is still unknown whether hypothalamic kisspeptin neurons are a direct target of GABA. In this study, using a hypothalamic kisspeptin-producing cell model, rHypoE8 cells, it was found that muscimol, a specific agonist of the GABA_A receptor,¹¹ can stimulate KiSS-1 gene expression.

2 | MATERIALS AND METHODS

2.1 | Materials

The following chemicals and reagents were obtained from the indicated sources: fetal bovine serum (Gibco FBS; Invitrogen, Carlsbad, CA, USA); Dulbecco's modified Eagle's medium (DMEM), penicillin-streptomycin, and GABA (Sigma-Aldrich Company, St. Louis, MO, USA); muscimol and GABA_A antagonist (SR95531; Abcam Inc., Cambridge, MA, USA).

2.2 | Cell culture

The embryonic rat hypothalamic cell line R8 (rHypoE-8) was purchased from Cosmo Bio Company, Ltd., Tokyo, Japan. The cells were plated in 35 mm tissue culture dishes and incubated with high-glucose DMEM containing 10% heat-inactivated FBS and 1% penicillin-streptomycin at 37°C under a humidified atmosphere of 5% CO₂ in air. After 24 hours, the culture medium was changed to high-glucose DMEM containing 1% heat-inactivated FBS and 1% penicillin-streptomycin and incubated without (control) or with the test reagents for the indicated periods.

2.3 | Primary culture of the neuronal cells of fetal rat brains

Six-to-eight fetal rat brains were dissected from fetuses at 16-18 days of gestation from a female rat under deep sodium pentobarbital anesthesia. Whole fetal brains were excised and minced before being incubated in Ca⁺⁺/Mg⁺⁺-free Hank's balanced salt solution (CMF-HBSS) containing 10 mg/mL trypsin and 2 mg/mL collagenase (Nitta Gelatin, Osaka, Japan) for 15 minute at 37°C. The samples then were incubated in an identical solution containing 0.5 μ g/mL DNase I (Boehringer-Mannheim, Mannheim, Germany) for 5 minute at 37°C. After incubation in CMF-HBSS that contained 5 mol L⁻¹ ethylenediaminetetraacetic acid (Wako Pure Chemicals, Osaka, Japan) for 5 minute at 37°C, the samples were washed with CMF-HBSS. The dispersed cells then were suspended in CMF-HBSS with a pipette, passed through a 70 μ m nylon mesh (Becton Dickinson Labware, Franklin Lakes, NJ, USA), and then collected by centrifugation. The pellet was suspended and 2-3 \times 10⁵ cells were cultured on 35 mm Petri dishes in DMEM with 10% FBS and 1% penicillin-streptomycin until use. This protocol was approved by the committee of the Experimental Animal Center for Integrated Research at Shimane University, Izumo, Japan.

2.4 | RNA preparation, reverse transcription, and quantitative real-time polymerase chain reaction

The cells were stimulated with muscimol or GABA in 1% FBS-containing medium and cultured for 24 hours. When the GABA_A antagonist was applied, the cells were incubated for 1 hour with GABA_A antagonist prior to stimulation. After stimulation, the total RNA from these cells was extracted by using TRIzol-LS (Invitrogen) according to the manufacturer's instructions. In order to obtain the cDNA, 1.0 μ g total RNA was reverse-transcribed by using an oligo-dT primer (Promega Corporation, Madison, WI, USA) and prepared by using a First-Strand cDNA Synthesis Kit (Invitrogen) in a reverse-transcription buffer. The preparation was supplemented with 10 mol L⁻¹ dithiothreitol, 1 mM of each dNTP, and 200 U RNase inhibitor/human placenta ribonuclease inhibitor (Code No. 2310; Takara, Tokyo, Japan) in a final volume of 10 μ L. The reaction was incubated at 37°C for 60 minute. The quantification of the KiSS-1 and GnRH mRNA was performed by using quantitative real-time polymerase chain reaction (PCR; ABI Prism 7000;

Perkin-Elmer Applied Biosystems, Foster City, CA, USA), following the manufacturer's protocol (User Bulletin No. 2) and with a Universal Probe Library Probe and Fast Start Master Mix (Roche Diagnostics, Mannheim, Germany). Using specific primers for rat *KISS-1*²¹ (forward: 5'-ATGATCTCGC TGGCTTCTTG GCA-3'; reverse: 5'-AGTTCCAGTT GTAGGTGGAC AGGT-3') and mouse *GnRH*²² (forward: 5'-ACTGTGTGTT TGGAAAGGCTG C-3'; reverse: 5'-TTCCAGAGCT CCTCGCAGAT C-3'), the simultaneous measurement of mRNA and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) permitted the normalization of the amount of cDNA that was added per sample. For each set of primers, a no-template control was included. The thermal cycling conditions were as follows: 10 minute denaturation at 95°C, followed by 40 cycles of 95°C for 15 seconds and 60°C for 1 minute. The reactions were followed by a melting curve analysis (55–95°C). In order to determine the PCR's efficiency, a 10-fold serial dilution of cDNA was performed, as previously described.²³ The PCR conditions were optimized to generate >95% PCR efficiency and only those reactions with between 95% and 105% efficiency were included in the subsequent analyses. Relative differences in the cDNA concentration between the baseline and experimental conditions then were calculated by using the comparative threshold cycle (Ct) method.²⁴ Briefly, for each sample, the ΔC_t was calculated to normalize for the internal control by using the equation: $\Delta C_t(\text{gene}) - C_t(\text{GAPDH})$. In order to obtain the difference between the experimental and the control conditions, the $\Delta\Delta C_t$ was calculated: $\Delta C_t(\text{sample}) - \Delta C_t(\text{control})$. The relative mRNA levels then were calculated by using the following equation: $\text{fold difference} = 2^{\Delta\Delta C_t}$.

2.5 | Statistical analysis

All the experiments were repeated independently at least three times. Each experiment in each experimental group was performed by using duplicate samples. When the mRNA expression was determined, two samples were assayed in duplicate. Six averages from three independent experiments were statistically analyzed. The data are expressed as the mean \pm standard error of the mean values. The statistical analysis was performed by using a one-way ANOVA, followed by Duncan's multiple range test or the Student's *t* test. $P < .05$ was considered to be statistically significant.

3 | RESULTS

3.1 | Effect of muscimol on *KISS-1* messenger RNA expression

The GABA_A receptor-specific agonist, muscimol, was able to stimulate *KISS-1* mRNA expression in the hypothalamic rHypoE8 cells, with *KISS-1* mRNA expression significantly increased with 100 mol L⁻¹ muscimol and increased by $\leq 4.10 \pm .72$ -fold with 1 μM muscimol (Fig. 1a). In order to examine the response of the kisspeptin-expressing neuronal cells in a more natural state, the primary cultures of the brain cells from the fetal rats were examined. The ability of muscimol to

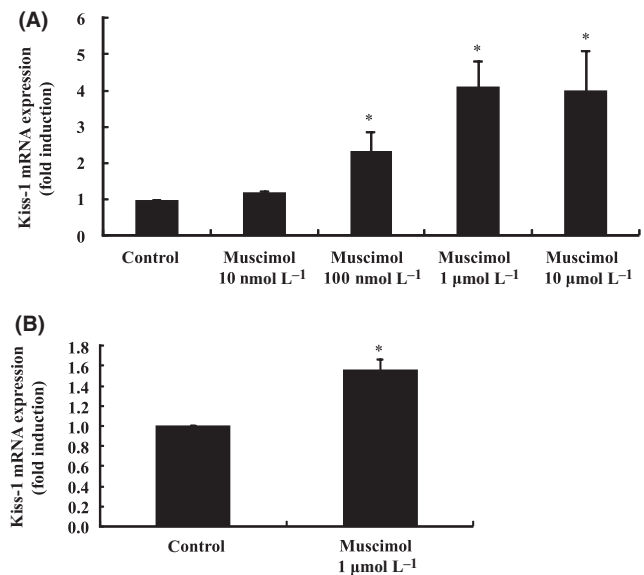


FIGURE 1 Effect of muscimol on *KISS-1* messenger (m)RNA expression. (A) rHypoE-8 cells were stimulated with the indicated concentrations of muscimol in 1% fetal bovine serum (FBS)-containing medium and cultured for 24 hours. (B) Primary cultures of fetal rat neuronal cells were stimulated with 1 mol L⁻¹ muscimol in 1% FBS-containing medium and cultured for 24 hours. Subsequently, the mRNAs were extracted and reverse-transcribed and the *KISS-1* mRNA levels were measured by quantitative real-time polymerase chain reaction. The results are expressed as the fold stimulation over the unstimulated cells and presented as the mean \pm standard error of the mean values of three independent experiments, each performed with duplicate samples. * $P < .05$, compared to the control

increase *KISS-1* gene expression also was observed in these primary cultures, with 1 μM muscimol significantly increasing *KISS-1* mRNA expression by $\leq 1.57 \pm .10$ -fold (Fig. 1b).

3.2 | Effect of the GABA_A receptor antagonist on muscimol-induced *KISS-1* messenger RNA expression

Muscimol-increased *KISS-1* mRNA expression was inhibited completely in the presence of the GABA_A receptor antagonist in the rHypoE8 cells (Fig. 2a). In contrast, the natural GABA compound failed to stimulate *KISS-1* mRNA expression (Fig. 2b).

3.3 | Effect of muscimol on gonadotropin-releasing hormone messenger RNA expression

Next, the effect of muscimol on *GnRH* mRNA expression was examined. Muscimol did not increase the expression of *GnRH* in either the rHypoE8 cells (Fig. 3a) or the primary cultures of the fetal rat brain (Fig. 3b).

4 | DISCUSSION

As the investigation of the molecular mechanisms occurring in kisspeptin neurons is difficult due to the heterogeneous nature

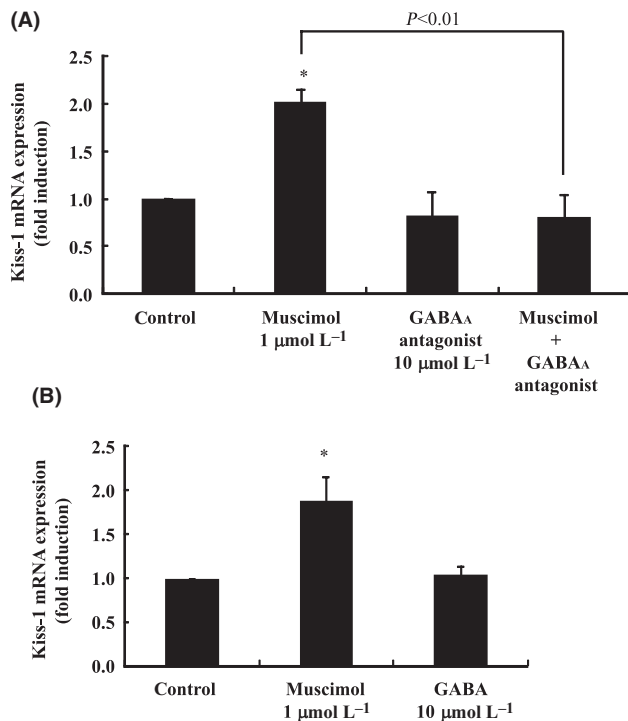


FIGURE 2 Effect of the GABA_A receptor antagonist and natural compound, GABA, on KiSS-1 messenger (m)RNA expression. (a) rHypoE-8 cells were stimulated with 1 mol L⁻¹ muscimol in 1% fetal bovine serum (FBS)-containing medium in the presence or absence of 10 μM GABA_A antagonist for 24 hours, after which the mRNA was extracted and reverse-transcribed. The GABA_A antagonist was added to the culture dishes 60 minute prior to muscimol stimulation. (B) The rHypoE-8 cells were stimulated with 1 μM muscimol and 10 μM of GABA in 1% FBS-containing medium and cultured for 24 hours, after which the mRNA was extracted and reverse-transcribed. The KiSS-1 mRNA levels were measured by quantitative real-time polymerase chain reaction. The results are expressed as the mean ± standard error of the mean values of three independent experiments, each performed with duplicate samples. *P < .05, compared to the control

and complexity of the hypothalamus, a kisspeptin-expressing hypothalamic cell model, rHypoE-8 cells, was used. Also used were neuronal cell cultures from fetal rat brain containing kisspeptin-expressing cells. Using these cell models, it was shown that the GABA_A receptor-specific agonist, muscimol, stimulated KiSS-1 mRNA expression.

Muscimol was isolated from the mushroom, *Amanita muscaria*, in the early 1960s. This mushroom is psychoactive and has a rich history, with muscimol considered to contribute significantly to its behavioral effects.²⁵ This compound is a selective conformationally restricted GABA agonist that has been used to define the structure-activity relationship at the GABA_A receptor. Accordingly, muscimol-induced KiSS-1 mRNA expression was prevented completely in the presence of the GABA_A receptor antagonist in the experiments in this study, suggesting that muscimol induces KiSS-1 gene expression through the GABA_A receptors. However, it is also

true that muscimol has potent action as a partial agonist of the GABA_C receptor.²⁶

A previous study showed that GABA signaling can directly regulate GnRH secretion.²⁷ In addition, GABA could indirectly modulate GnRH secretion by inhibiting the upstream neuronal afferents of the GnRH neurons, such as opiate and noradrenergic neurons.^{28,29} Furthermore, although GABA_A receptors are widely distributed in many brain regions of rats, another previous study revealed that they are also broadly expressed in the hypothalamus region, which includes the preoptic area, ARC, and AVPV.¹⁴ The interaction between GABA and the kisspeptin neurons is not fully characterized, but several studies have suggested the possible effect of kisspeptin neurons on modulating GABA signaling toward GnRH neurons.^{30,31} In contrast, another study suggested that kisspeptin neurons were under the control of GABA neurons because the GABA_A receptor antagonist stimulated the release of kisspeptin in the medial basal hypothalamus of prepubertal monkeys.³² There might be a complicated relationship between GABA and kisspeptin neurons, but the current study clearly showed that GABA had a direct effect on the kisspeptin neurons and increased KiSS-1 gene expression via the GABA_A receptor. This observation contradicts that of the latter study,³² which showed the inhibitory influence of GABA neurons on kisspeptin neurons. The inhibitory influence of GABA on the kisspeptin neurons was evaluated by measuring the release of kisspeptin and GnRH by using in vivo models, whereas this study only determined the expression level of KiSS-1 mRNAs after muscimol stimulation. The difference could be explained by a discrepancy between the secretory processes and gene expression or by a lack of a relationship between the secretory processes and gene expression in the kisspeptin neurons. Controversial observations were noted in previous reports regarding the action of GABA on the HPG axis. The present results cannot explain these conflicting results, but the action of GABA_A might be influenced by the different types of cell models, the different hormonal milieu, or input from different neurons. However, it is true that this study's in vitro model using kisspeptin-expressing neurons could simply reflect the effect of muscimol on kisspeptin neurons without the neuron-modifying effects of other neuropeptides within the hypothalamus. These observations could be taken into account when further evaluating the function of GABA neurons on the HPG axis.

Surprisingly, the natural GABA compound had no effect on KiSS-1 gene expression, in contrast to muscimol. This observation implies that more intense activation of the GABA_A receptor is necessary to induce KiSS-1 gene expression. Artificially generated receptor agonists are usually applied to experiments exploring receptor function. As universal GABA compounds show weak stimulation of GABA_A receptors, they might fail to stimulate the KiSS-1 gene. Otherwise, muscimol might have a specific effect on KiSS-1 gene expression beyond its characterization as a GABA_A receptor agonist.

A direct effect of muscimol on GnRH mRNA expression was not observed, in contrast to a previous study.²⁷ This might mean that GABA does not act directly on GnRH neurons. However, because

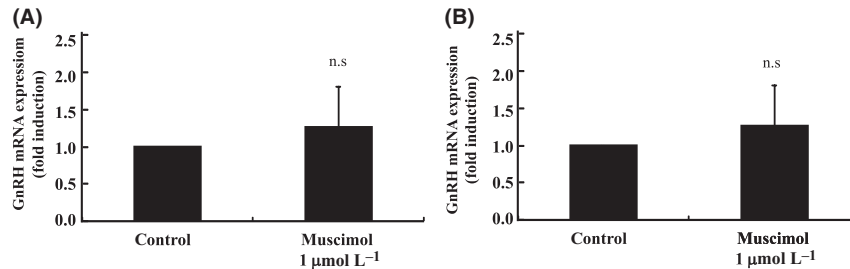


FIGURE 3 Effect of muscimol on gonadotropin-releasing hormone (GnRH) messenger (m)RNA expression. (A) rHypoE-8 cells and (B) primary cultures of the fetal rat brain were stimulated with 1 mol L⁻¹ muscimol in 1% fetal bovine serum (FBS)-containing medium and cultured for 24 hours, after which the mRNA was extracted and reverse-transcribed. The GnRH mRNA levels were measured by quantitative real-time polymerase chain reaction. The results are expressed as the fold stimulation over the unstimulated cells and presented as the mean ± standard error of the mean values of three independent experiments, each performed with duplicate samples. NS, differences between the control and muscimol-stimulated cells were not statistically significant

the rHypoE8 cells and primary cultures of the fetal rat brains were used, both of which originate from fetal brains, immaturity might be the reason for the effectiveness of muscimol. The primary cultures of the fetal rat brain cells are heterogeneous populations.³³ In addition, rHypoE8 cells are heterogeneous neuronal cell populations because these cells express at least two neuropeptides, kisspeptin and GnRH.³⁴ As muscimol was able to stimulate KISS-1 mRNA expression, it was first expected that muscimol would increase GnRH mRNA expression in this cell population, even though the GABA_A receptor agonist has no effect on GnRH neurons. However, muscimol failed to stimulate GnRH mRNA expression. The reasons for its lack of effectiveness might include an insufficient release, an inability to trigger a release in these cell populations, or because dispersed neuronal cells do not have synaptic contact with other types of neuronal cells; such contact is required for communication or the governing of neuronal cells in normal hypothalamic tissue.

In this study, it was found that the activation of the GABA_A receptor by muscimol can increase KISS-1 gene expression in this study's kisspeptin-producing cell models. The mechanisms that are involved are still obscure, but this compound could be developed as a novel therapy for the activation of the HPG axis.

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DISCLOSURES

Conflict of interest: The authors declare no conflict of interest. **Human and Animal Rights:** This study's protocol was approved by the committee of the Experimental Animal Center for Integrated Research at Shimane University, Izumo, Japan. All the institutional and national guidelines for the care and use of laboratory animals were followed. This article does not contain any study with human participants that has been performed by any of the authors.

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