Excitatory Action of GABA in the Terminal Nerve Gonadotropin-Releasing Hormone Neurons

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Nakane R, Oka Y. Excitatory action of GABA in the terminal nerve gonadotropin-releasing hormone neurons. J Neurophysiol 103: 1375–1384, 2010. First published January 13, 2010; doi:10.1152/jn.00910.2009. The terminal nerve (TN)-gonadotropin-releasing hormone (GnRH) neurons have been suggested to function as a neuromodulatory system that regulates the motivational and arousal state of the animal and have served as a model system for the study of GnRH neuron physiology. To investigate the synaptic control of the TN-GnRH neurons, we analyzed electrophysiologically the effect of GABA on the TN-GnRH neurons. GABA generally hyperpolarizes most of the neurons in the adult brain by activating GABAA receptors while the activation of GABAA receptors depolarizes some specific neurons in the mature brain. Here we examined the GABAA receptor-mediated responses in the TN-GnRH neurons of adult teleost fish, the dwarf gourami, by means of gramicidin-perforated patch-clamp and cell-attached patch-clamp recordings. The reversal potential for the currents through GABAA receptors under the voltage clamp was depolarized relative to the resting membrane potential. GABAA receptor activation depolarized TN-GnRH neurons under the current clamp and had excitatory effect on their electrical activity, whereas the stronger GABAA receptor activation had bidirectional effect (excitatory–inhibitory). This excitatory effect is suggested to arise from high [Cl–], and was shown to be suppressed by bumetanide, the blocker of Cl–-accumulating sodium-potassium-2-chloride co-transporter (NKCC). The present results demonstrate that GABAA receptor activation induces excitation in TN-GnRH neurons, which may facilitate their neuromodulatory functions by increasing their spontaneous firing frequencies. The excitatory actions of GABA in the adult brain have recently been attracting much attention, and the easily accessible large TN-GnRH neurons should be a nice model system to analyze their physiological functions.

INTRODUCTION

The terminal nerve (TN)-gonadotropin-releasing hormone (GnRH) neurons have spontaneous pacemaker activity of action potentials and project widely in various brain regions (Oka and Matsushima 1993). This is in clear contrast to the hypothalamic hypophysiotropic GnRH neurons, which show episodic and rather irregular spontaneous activity and project specifically to the median eminence (in most vertebrates) or the pituitary (in teleosts) and facilitate gonadotropin release. The characteristics of TN-GnRH neurons have led us to suggest that the TN-GnRH neurons may function as a neuromodulatory system (Eisthen et al. 2000; Oka 1997, 2002). In our working hypothesis, the pacemaker activity is considered to reflect the physiological conditions of the animal and may be modulated by neuronal inputs consisting of various sensory modalities (Yamamoto and Ito 2000). Changes in pacemaker frequency, in turn, are suggested to alter the release of GnRH peptides from the extensively projecting axonal branches and simultaneously modulate neuronal excitability in wide brain areas. Such modulation may finally lead to long-lasting changes in the motivational and arousal state for certain animal behaviors (Wirsig and Leonard 1987; Yamamoto et al. 1997). Therefore the analysis of the neuronal inputs to TN-GnRH neurons, which could change their pacemaker frequencies and lead to subsequent modulation, is the key to understanding their neuromodulatory functions.

There are some reports on the neuronal inputs to TN-GnRH neurons. Morphological study by Yamamoto and Ito (2000) of teleosts, the dwarf gourami and tilapia, has demonstrated the multimodal sensory inputs (olfactory, visual, and somatosensory) to the TN-GnRH neurons. In agreement with this, noxious tactile stimulation inhibited the pacemaker activity of TN neurons in the goldfish (Fujita et al. 1991) although it is not certain whether the TN neurons in their study are GnRH neurons or not. As for the possible synaptic inputs, the major excitatory neurotransmitter, glutamate, has been reported to increase the pacemaker frequency of TN-GnRH neurons in the dwarf gourami (Kiya and Oka 2003), which may mediate the sensory inputs mentioned in the preceding text. On the other hand, the major inhibitory neurotransmitter, γ-aminobutyric acid (GABA), generally hyperpolarizes neurons in the adult brain by acting on GABAA receptors. However, it has been shown that activation of GABAA receptors depolarizes some neurons in the adult brain (Marty and Llano 2005). Thus far, the GABAergic inputs to TN-GnRH neurons have not been investigated electrophysiologically or morphologically.

In the present study, we performed electrophysiological experiments using TN-GnRH neurons in the brain of the adult dwarf gourami to show that 1) functional GABAA receptors are expressed, 2) reversal potential for the GABAA receptor-mediated currents is depolarized relative to the resting membrane potential, and 3) the GABAA receptor activation depolarizes TN-GnRH neurons. Experiments 2 and 3 were performed by means of gramicidin-perforated patch-clamp recordings and cell-attached patch-clamp recordings, which do not disrupt [Cl–], and maintain intact GABAA receptor responses (Akaike 1996).

METHODS

Preparation of acute brain block with exposed TN-GnRH neurons

Acute brain blocks including the TN-GnRH neurons were prepared as previously described (Haneda and Oka 2004). These procedures were performed in accordance with the guidelines of the Physiological

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Society of Japan and the University of Tokyo for the Use and Care of Experimental Animals. Briefly, adult male and female dwarf gouramis (Colisa lalia), ~4.5 cm in standard length and 4 g in body weight (n = 40 fish), were purchased from a local dealer. Each fish tank containing ~20 fish was maintained at 27°C and 14-h light, 10-h dark cycle. They were deeply anesthetized by immersing them in a 0.02% 3-amino benzoic acid ethyl ester (MS-222) solution and were quickly killed by decapitation. The brain was immersed in the standard external solution that included (in mM) 150 NaCl, 5.0 KCl, 1.3 MgSO4, 2.4 CaCl2, 10 HEPES, and 10 glucose (adjusted to pH 7.4 with NaOH). The ventral meningeal membrane of the forebrain was carefully removed. Thick brain block of ~500 μm containing TN-GnRH neurons were manually cut out with a razor blade. All the experiments were performed at room temperature (22–27°C). In the present study, the TN-GnRH neurons that were completely exposed to the surface and could be clearly identified by their size (20–43 μm diam), which is much larger compared with the surrounding cells about ≤10 μm diam) (Haneda and Oka 2004) were electrophysiologicaly analyzed. In some experiments, we obtained recordings from unidentified non-GnRH neurons (5–13 analyzed. In some experiments, we obtained recordings from unidentified non-GnRH neurons (5–13 analyzed. In some experiments, we obtained recordings from unidentified non-GnRH neurons (5–13 analyzed. In some experiments, we obtained recordings from unidentified non-GnRH neurons (5–13 analyzed. In some experiments, we obtained recordings from unidentified non-GnRH neurons (5–13 analyzed. In some experiments, we obtained recordings from unidentified non-GnRH neurons (5–13 analyzed.

Conventional whole cell recordings

Conventional whole cell recordings were performed as previously described (Haneda and Oka 2004). Recording pipettes were made of borosilicate glass (GD-1.5, Narishige, Tokyo, Japan) using a puller (P-97; Sutter Instruments, Navato, CA). In current clamp recordings, we used KCl-based pipette solution, consisting of (in mM) 110 KCl, 3 MgCl2, 40 HEPES, 0.3 EGTA, 2 Mg-ATP, and 0.2 Li3-GTP (adjusted to pH 7.2 with KOH). In voltage-clamp recordings, we used the CsCl-based pipette solution, consisting of (in mM) 110 CsCl, 3 MgCl2, 40 HEPES, and 10 EGTA (adjusted to pH 7.2 with CsOH). The pipettes had resistances of 2.4–4.6 MΩ. The osmolarity of the pipette and standard external solutions were ~270 and 315 mOsm respectively. Whole cell recordings were performed using an Axopatch 200B or MultiClamp 700A amplifiers (Molecular Devices, Foster City, CA). Electrical signals were low-pass filtered at 1 kHz and sampled at 2 kHz using the Digidata 1322A and pCLAMP9.2 software (Molecular Devices). The agar bridge was used for the reference electrode. The liquid junction potential was estimated (6.4 mV) and was corrected by adjusting the pipette offset.

Gramicidin-perforated patch-clamp recordings

The cation-selective ionophore gramicidin was used for perforated patch-clamp recordings to maintain [Cl−]pip (Akaike 1996). Gramicidin (Sigma) was dissolved in DMSO and then diluted to 10–15 μg/ml in sonicated pipette solution. CsCl-based pipette solution was used. To avoid gramicidin spillover during positive pressure approach to the target cell, the pipette tip was filled with the filtered gramicidin-free pipette solution by dipping, and then the patch pipette was backfilled with the gramicidin-containing pipette solution. Pipettes had resistances of 2.1–3.4 MΩ. Electrical signals were low-pass filtered at ~1 kHz and sampled at ~2 kHz. To ensure full efficacy of the gramicidin, the solution was renewed every 2 h. Achieving successful perforation took ~60 min, and experiments were started after getting series resistance <100 MΩ. The series resistance of the perforated patch was 10.3–100 MΩ. The series resistance was electronically compensated by 15–50%. The liquid junction potential was estimated (6.5 mV) and corrected. The chloride concentration of the pipette solution was 116 mM. Degradation of perforated patch-clamp mode by accidental rupture of the patch membrane was detected by an abrupt decrease in series resistance and the shift of the reversal potential of GABA_A receptor currents closer to −8.2 mV, the equilibrium potential for Cl− (ECl) with this high Cl− pipette solution, and the data were discarded.

Using the reversal potential of K+ current through cell-attached patches to monitor the cell membrane potential, Vm

This method has been specially developed to measure the membrane potential from cell-attached K+ currents under the conditions maintaining the intracellular ionic milieu (Fricker et al. 1999; Verheugen et al. 1995, 1999). With a 150 mM K+ internal solution, which is close to the estimated intracellular [K+] in mammalian cell (155 mM) (Hille 1992), the equilibrium potential for K+ (EK) across the patch is ~0 mV, and K+ currents will reverse when the pipette potential (Vpip) cancels out the cell membrane potential (Vm). Therefore the holding potential of the cell (−Vpip) at which the K+ current reverses direction gives a direct quantitative measure for the membrane potential (at K+ current reversal). Vpip was determined by 10.2 ± 0.3 mV, where Vpip indicates the potential across the patch membrane, Vm the membrane potential, and Vpip the pipette potential. Depolarizing voltage ramps (Fig. 4A) were applied to activate voltage-gated K+ channels. For the analysis of currents evoked by ramp stimulation, a correction was made for the leak component (~80–50 mV) by linear fit and extrapolation of the channel-closed level. KCl-based pipette solution containing 150 mM K+ consisted of (in mM) 120 KCl, 3 MgCl2, 20 HEPES, and 10 EGTA (adjusted to pH 7.2 with KOH, equivalent to 30 mM K+ in final concentration). Pipettes had resistances of 2.2–5.6 MΩ. Electrical signals were low-pass filtered at 2 kHz and sampled at 50 kHz. The liquid junction potential was estimated (5.9 mV) and corrected.

Cell-attached loose patch-clamp recordings

The cell-attached loose patch-clamp recordings were obtained by referring to the methods described in a previous literature (Nunemaker et al. 2003). In some experiments, HEPES-buffered standard external solution was replaced by bicarbonate-buffered external solution (in mM) 124 NaCl, 5 KCl, 1.2 KH2PO4, 2.4 CaCl2, 1.3 MgSO4, 26 NaHCO3, and 10 glucose, which was continuously bubbled with 95% O2–5% CO2 for ≥30 min before recording (pH ~7.4 after bubbling). Pipettes had resistances of 3.0–6.5 MΩ when filled with external solution or KCl-based pipette solution. The results were not different between the two solutions. A loose seal (22–58 MΩ) was established with mild suction to monitor spontaneous action currents in voltage-clamp mode. We adjusted the pipette offset dial to shift the baseline to 0 pA as needed to negate current due to a change in the junction potential. Electrical signals were low-pass filtered at 2 kHz and sampled at 5 kHz.

Drug applications

Both bath and puffer applications of the drugs were used. Muscimol was applied by puffer application using a custom-made computer-controlled pressure ejection system (200- to 500-ms puff, 50 kPa). GABA_A receptor antagonists and other blockers were applied by bath application.

Chemicals and reagents

MS-222, muscimol, bicuculline methiodide, picrotoxin, gabazine (SR-95531), gramicidin and bumetanide were purchased from Sigma (St Louis, MO). Tetrodotoxin (TTX) was obtained from Wako (Osaka, Japan). 6-cyano-7-nitroquinazoline-2,3-dione (CNQX) and d-(-)-2-amino-5-phosphono pentanoic acid (d-APV) were obtained from Tocris (Bristol, UK). The following stock solutions were made by dissolving each drug in DMSO: 50 mM bumetanide and 20 mM CNQX.

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Data analysis

Statistical analyses were performed with Kyplot (version 5, Ky-ence, Tokyo, Japan) and Clampfit (Molecular Devices, Foster City, CA) softwares. All data in this report are presented as means ± SE.

RESULTS

TN-GnRH neurons express functional GABA<sub>A</sub> receptors

Pharmacological characterization of the GABA current was performed in TN-GnRH neurons using the conventional whole cell recording technique (Fig. 1). All the recordings were done in the presence of TTX to block synaptic inputs. In current-clamp recordings using KCl-based pipette solution, repetitive hyperpolarizing square current pulses (amplitude: 200 pA, duration: 200 ms) were delivered in every 10 s to monitor input resistance. Bath application of GABA (50 μM) and muscimol (10 μM) depolarized the membrane potential accompanied by a decrease of input resistance, while baclofen (100 μM for 5 min, n = 5) had no effect on membrane potential and input resistance (Fig. 1A). As a positive control experiment, baclofen hyperpolarized the membrane potential of an unidentified neuron in the olfactory bulb (data not shown), showing that baclofen works in dwarf gourami. Puffer application of 5 μM muscimol induced transient inward currents recorded at a holding potential of ~60 mV. The progressive blockage of muscimol-induced current by increasing concentrations of GABA<sub>A</sub> receptor antagonist is shown in Fig. 1, B, 1 and 2, C, 1 and 2, and D. At least three cells were tested for each concentration of antagonist. The peak values of muscimol-induced GABAergic currents were normalized to the peak amplitude of the control responses and plotted as a function of the corresponding concentration of antagonist. Numbers in parentheses (near F) represent the numbers of neurons tested for each antagonist concentration. A fit to a sigmoidal logistic equation gave an IC<sub>50</sub> of 4.1 μM for bicuculline methiodide and 0.28 μM for gabazine. All the recordings were done in the presence of TTX.

**FIG. 1.** GABA agonists-induced currents and the effect of GABA<sub>A</sub> receptor antagonists recorded in the conventional whole cell patch-clamp mode. A: bath application of GABA (50 μM) and muscimol (10 μM) depolarized the membrane potential accompanied by a decrease in input resistance, while baclofen (100 μM for 5 min, n = 5) had no effect on membrane potential and input resistance. In conventional whole cell current-clamp recordings using KCl-based pipette solution, repetitive hyperpolarizing square current pulses (amplitude: 200 pA, duration: 200 ms) were delivered every 10 s to monitor input resistance. B1, C1, and D: superimposed traces of muscimol-induced currents under control conditions and in the presence of increasing concentrations of bicuculline methiodide (B1), gabazine (SR-95531; C1), or picrotoxin (D). The membrane potentials were held at ~60 mV. Muscimol was puffer-applied at 5 μM. B2 and C2: muscimol-induced current amplitudes normalized to the peak amplitude of the control responses and plotted as a function of the corresponding concentration of antagonists. Numbers in parentheses (near F) represent the numbers of neurons tested for each antagonist concentration. A fit to a sigmoidal logistic equation gave an IC<sub>50</sub> of 4.1 μM for bicuculline methiodide and 0.28 μM for gabazine. All the recordings were done in the presence of TTX.
induced currents were used for the analysis. The resulting antagonist concentration-inhibition curve was fitted to a sigmoidal logistic function, which gave a mean IC$_{50}$ of 4.1 μM for bicuculline methiodide and 0.28 μM for gabazine (Fig. 1, B2 and C2). The muscimol-induced current was completely inhibited by bath application of bicuculline methiodide (96% blockage at 100 μM), gabazine, (96% blockage at 10 μM), and picrotoxin (91% blockage at 10 μM). These pharmacological characteristics of muscimol-induced currents indicate that the ionotropic GABA$_A$ receptors are expressed in TN-GnRH neurons: currents induced by an ionotropic GABA receptor agonist muscimol were completely blocked by the GABA$_A$ specific antagonists (bicuculline methiodide and gabazine). In a preliminary experiment, we also found that TN-GnRH neurons did not show metabotropic GABA$_B$ receptor-mediated response, whereas non-GnRH neurons in the olfactory bulb did. To avoid possible small contamination of GABA$_B$ receptor response and to focus on GABA$_A$ receptor response, we used muscimol in the present experiments described in the following text.

**I-V relationship of GABA$_A$ receptor response**

The reversal potentials of GABA$_A$ receptor currents were measured in TN-GnRH neurons using the conventional whole cell recording technique in the presence of TTX. The peak values of muscimol-induced currents were used for the analysis. The reversal potential of currents evoked by puffer application of 5 μM muscimol was −5.0 ± 0.9 mV (n = 6, Fig. 2, A and B). This value is close to the calculated $E_{Cl}$ (−8.2 mV, 25°C). Thus it is suggested that the current evoked by muscimol arises from a pure Cl$^-$ flux.

**GABA$_A$ receptor activation depolarizes TN-GnRH neurons**

To measure the reversal potential for the GABA$_A$-receptor current in a near physiological [Cl$^-$], we performed perforated patch-clamp recordings using gramicidin, which does not permeate Cl$^-$ (Akaike 1996). We used an external solution containing TTX, CNQX, and APV to block synaptic inputs, especially glutamatergic synaptic inputs, which are also depolarizing under the present experimental conditions. The peak values of muscimol-induced currents were used for the analysis. The reversal potential of currents induced by muscimol (20–50 μM) in the gramicidin-perforated patch-clamp recordings was $-22.3 \pm 2.1$ mV (n = 5 cells from 5 fish; the numbers apply to all the measurements in Fig. 3) in TN-GnRH neurons, which was depolarized relative to the resting membrane potential ($-48.9 \pm 3.2$ mV in current-clamp recordings; Fig. 3A and B). We also tested the effects of muscimol (20–50 μM) on membrane potential in current-clamp recordings (Fig. 3C). Membrane potential peaked at $-25.0 \pm 3.2$ mV, which was depolarized from the resting membrane potential ($-48.9 \pm 3.2$ mV).

As an independent confirmation of the results obtained from the gramicidin-perforated patch-clamp recordings, we measured the changes in membrane potential induced by GABA$_A$ receptor activation in the cell-attached configuration, using a recently reported method that uses the reversal potential of K$^+$ current through cell-attached patches (Fig. 4) (Fricker et al. 1999; Verheugen et al. 1995, 1999). All the experiments were performed in the presence of TTX to block synaptic inputs. In the present experimental conditions, puffer application of 100 μM muscimol always depolarized TN-GnRH neurons ($-35.3 \pm 2.3$ mV, n = 17 cells from 9 fish) relative to the resting membrane potentials ($-65.2 \pm 2.4$ mV, n = 17 cells from 9 fish; Fig. 4A, B and D). In contrast, the responses were always hyperpolarizing in the unidentified non-GnRH neurons in the olfactory bulb ($-76.3 \pm 4.5$ mV, n = 6 cells from 2 fish) relative to the resting membrane potential ($-55.8 \pm 4.9$ mV, n = 6 cells from 2 fish; Fig. 4C, I and J).

**GABA$_A$ receptor activation modulates the pacemaker activity of TN-GnRH neurons**

In the gramicidin current-clamp recordings, we investigated the effects of GABA$_A$ receptor activation on the pacemaker activity of TN-GnRH neurons under the blockage of depolarizing glutamatergic synaptic inputs by CNQX and APV. Puffer application of 20 μM muscimol depolarized these cells, increased the frequency of spikes while decreasing the spike amplitude (Fig. 5A).

We also performed cell-attached recordings of action current as an independent confirmation of the results obtained with the gramicidin current-clamp recordings. To obtain high signal-to-noise ratio, we performed cell-attached loose patch-clamp recordings (Nunemaker et al. 2003). We used the same external solution as described in the preceding text. A low concentration (5 μM) of muscimol increased the frequency of pacemaker activity (action currents in this case; n = 14 cells from 6 fish, 17 cells from 9 fish; 14 cells from 6 fish, 5 cells from 5 fish; 6 cells from 2 fish).

![FIG. 2. I-V relationship of GABA$_A$ receptor currents. A: muscimol-induced currents recorded at different holding potentials ranging from −45 to +30 mV. These experiments were performed by using conventional whole cell recordings, and 5 μM muscimol was puffer-applied. All the recordings were done in the presence of TTX. B: I-V plot of the peak value of muscimol-induced currents for the traces shown in A. The current responses reversed near −8.2 mV as expected from $E_{Cl}$ in our recording solutions. $E_{rev}$: reversal potential.](http://jn.physiology.org/DownloadedFrom)
constantly bubbled with 95% O2–5% CO2. Here puffer applied clamp recordings in bicarbonate-buffered external solution al. 1993). Therefore we performed cell-attached loose patch-responses of TN-GnRH neurons induced by GABAA receptor the effect of the NKCC blocker, bumetanide, on the excitatory interaction is the activity of the sodium-potassium-2-chloride co-transporter, NKCC-1 (Russell 2000). Therefore we examined the effect of the NKCC blocker, bumetanide, on the excitatory responses of TN-GnRH neurons induced by GABA<sub>A</sub> receptor activation. In cell-attached loose patch-clamp recordings, bath application of 50 µM bumetanide suppressed the excitatory responses of TN-GnRH neurons induced by muscimol (5 µM, 3-minute interval) (n = 3 cells from 3 fish, Fig. 6). Blocker application for 25 min substantially decreased the frequency of spikes in response to muscimol applications although it did not completely stop the firing. This inhibition could be partially reversed after washout (n = 3 cells from 3 fish). These data suggest that NKCC is one of the factors that are responsible for excitatory response induced by GABA<sub>A</sub> receptor activation in TN-GnRH neurons.

**DISCUSSION**

The present study reports on the expression and properties of functional GABA<sub>A</sub> receptors in TN-GnRH neurons of vertebrate brains for the first time. More specifically, our data show that the TN-GnRH neurons in the brain of the adult dwarf gourami have pharmacologically distinctive GABA<sub>A</sub> receptors, the reversal potential for the GABA<sub>A</sub> receptor currents is depolarized relative to the resting membrane potential, (reversal potential, -22.3 ± 2.1 mV, n = 5 cells from 5 fish; resting membrane potential from current-clamp recordings, -48.9 ± 3.2 mV, n = 5 cells from 5 fish). All the recordings were done in the presence of TTX, 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX), and tetr(–)-2-amino-5-phosphonopentanoic acid (APV). E<sub>rev</sub>, reversal potential; V<sub>rest</sub>, resting membrane potential; V<sub>muscimol</sub>, peak membrane potential after puffer application of muscimol.

**NKCC blocker bumetanide blocks excitation induced by GABA<sub>A</sub> receptor activation**

One mechanism that can cause GABA-induced depolarization is the activity of the sodium-potassium-2-chloride co-transporter, NKCC-1 (Russell 2000). Therefore we examined the effect of the NKCC blocker, bumetanide, on the excitatory responses of TN-GnRH neurons induced by GABA<sub>A</sub> receptor activation. In cell-attached loose patch-clamp recordings, bath application of 50 µM bumetanide suppressed the excitatory responses of TN-GnRH neurons induced by muscimol (5 µM, 3-minute interval) (n = 3 cells from 3 fish, Fig. 6). Blocker application for 25 min substantially decreased the frequency of spikes in response to muscimol applications although it did not completely stop the firing. This inhibition could be partially reversed after washout (n = 3 cells from 3 fish). These data suggest that NKCC is one of the factors that are responsible for excitatory response induced by GABA<sub>A</sub> receptor activation in TN-GnRH neurons.

**FIG. 3.** The reversal potential of GABA<sub>A</sub> receptor currents is depolarized in the adult terminal nerve-gonadotropin-releasing hormone (TN-GnRH) neurons as shown by gramicidin-perforated patch-clamp recordings. A: muscimol-induced currents recorded at different holding potentials ranging from -50 to 0 mV. These experiments were performed by gramicidin-perforated patch-clamp recordings, and muscimol was puffer-applied at 20 – 50 µM. B: I-V plot of the peak values of muscimol-induced currents for the traces shown in A. The reversal potential of TN-GnRH neurons from adult fish was depolarized relative to the resting membrane potential (reversal potential, -22.3 ± 2.1 mV, n = 5 cells from 5 fish; resting membrane potential from current-clamp recordings, -48.9 ± 3.2 mV, n = 5 cells from 5 fish). C: membrane depolarization induced by muscimol (20 – 50 µM) in current-clamp recordings in the gramicidin-perforated patch-clamp configuration (positive peak of membrane potential, -25.0 ± 3.2 mV; resting membrane potential -48.9 ± 3.2 mV, n = 5 cells from 5 fish). All the recordings were done in the presence of TTX, 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX), and tetr(–)-2-amino-5-phosphonopentanoic acid (APV). E<sub>rev</sub>, reversal potential; V<sub>rest</sub>, resting membrane potential; V<sub>muscimol</sub>, peak membrane potential after puffer application of muscimol.
FIG. 4. Measurements of changes in membrane potential in response to GABA_A receptor activation estimated from cell-attached patch-clamp recordings. A: the command potential protocol for monitoring the reversal potential of K^+ current through cell-attached patches. The 1st ramp was delivered to estimate the resting membrane potential. After the puffer application of 100 μM muscimol, the 2nd ramp was delivered to estimate the response to muscimol. B1 and C1: I-V curve in a TN-GnRH neuron (B1) and an unidentified non-GnRH neuron in the olfactory bulb (C1) at resting membrane potential (○) and after puffer application of muscimol (●). Voltage-gated K^+ currents through the cell-attached patches activated by the depolarizing voltage ramp were initially inward, and then reversed to outward. The reversal of K^+ currents was determined from the intersection of the fit to the linear leak (at resting membrane potential, - - -; after puffer application of muscimol, —) and the K^+ currents. The membrane potential was estimated from the reversal of K^+ currents. B2 and C2: summary of measured membrane potentials at resting membrane potential vs. after puffer application of muscimol in TN-GnRH neurons (B2) and unidentified non-GnRH neuron in the olfactory bulb (C2). All of TN-GnRH neurons tested depolarized (at resting membrane potential, Rest = -65.2 ± 2.4 mV; after puffer application of muscimol, Muscimol = -35.3 ± 2.3 mV, n = 17 cells from 9 fish), and all of the unidentified non-GnRH neurons in the olfactory bulb tested hyperpolarized (Rest = -55.8 ± 4.9 mV, Muscimol = -76.3 ± 4.5 mV, n = 6 cells from 2 fish) in response to muscimol. All the recordings were done in the presence of TTX.
GABA depolarizes TN-GnRH neurons

GABA<sub>A</sub> receptor activation induces a depolarizing effect on TN-GnRH neurons in the adult dwarf gourami as shown by the gramicidin-perforated patch-clamp and cell-attached patch-clamp recordings. A: recordings obtained from TN-GnRH neurons in the gramicidin-perforated patch-clamp configuration. Puffer application of 20 μM muscimol depolarized these cells, increased the frequency of spikes, while it decreased the spike amplitude. B–D: cell-attached loose patch-clamp recordings of action currents in TN-GnRH neurons and an unidentified non-GnRH neuron in the olfactory bulb. B: 5 μM muscimol increased pacemaker frequency of TN-GnRH neurons (n = 14 cells from 6 fish). C1 and C2: in contrast, 50 μM muscimol rapidly facilitated firing and then inhibited it (n = 11 cells from 6 fish). At the enlarged time scale (the area indicated by bar is enlarged in C2), the initial increase of pacemaker frequency is evident before the inhibition of firing (C2). C3: the decrease in input resistance on application of 50 μM muscimol was shown by delivering repetitive hyperpolarizing square current pulses (amplitude: 200 pA; duration: 200 ms; interval: 1 s) in another GnRH neuron in a conventional whole cell configuration. The time course is the same as that in C1. D: 5 μM muscimol decreased (completely inhibited in this case) the firing frequency of unidentified non-GnRH neurons in the olfactory bulb (n = 7 cells from 2 fish). All the recordings were done in the presence of CNQX and APV.

FIG. 5. GABA<sub>A</sub> receptor activation modulates the pacemaker activity of TN-GnRH neurons as shown by gramicidin-perforated patch-clamp and cell-attached patch-clamp recordings. A: recordings obtained from TN-GnRH neurons in the gramicidin-perforated patch-clamp configuration. Puffer application of 20 μM muscimol depolarized these cells, increased the frequency of spikes, while it decreased the spike amplitude. B–D: cell-attached loose patch-clamp recordings of action currents in TN-GnRH neurons and an unidentified non-GnRH neuron in the olfactory bulb. B: 5 μM muscimol increased pacemaker frequency of TN-GnRH neurons (n = 14 cells from 6 fish). C1 and C2: in contrast, 50 μM muscimol rapidly facilitated firing and then inhibited it (n = 11 cells from 6 fish). At the enlarged time scale (the area indicated by bar is enlarged in C2), the initial increase of pacemaker frequency is evident before the inhibition of firing (C2). C3: the decrease in input resistance on application of 50 μM muscimol was shown by delivering repetitive hyperpolarizing square current pulses (amplitude: 200 pA; duration: 200 ms; interval: 1 s) in another GnRH neuron in a conventional whole cell configuration. The time course is the same as that in C1. D: 5 μM muscimol decreased (completely inhibited in this case) the firing frequency of unidentified non-GnRH neurons in the olfactory bulb (n = 7 cells from 2 fish). All the recordings were done in the presence of CNQX and APV.

GABA depolarizes TN-GnRH neurons

GABA<sub>A</sub> receptor activation induces a depolarizing effect on TN-GnRH neurons in the adult dwarf gourami as shown by the gramicidin-perforated patch-clamp and cell-attached patch-clamp recordings. In the present study, we considered the reversal potential of muscimol-induced current to be equivalent to \( E_{Cl} \). Therefore the I-V plots enabled us to determine that the physiological [Cl<sup>-</sup>] is 67 mM, a concentration allowing an efflux of chloride at resting membrane potential. On the other hand, the reversal potential for GABA<sub>A</sub> receptor-mediated responses could be influenced by other permeant anions. In particular, in adult cortical neurons, the GABA-mediated depolarization is, at least in part, reported to be mediated by bicarbonate passing through the GABA<sub>A</sub> receptor (Kaila et al. 1993). Because we employed HEPES-buffered external solution without CO<sub>2</sub> equilibration, possible effects of bicarbonate were excluded. Thus in the present experimental conditions, the depolarizing effect of GABA can be ascribed to the relatively high physiological [Cl<sup>-</sup>]. As an another possibility, bicarbonate is likely to have an influence on various mechanisms involved in cellular ionic homeostasis, including those responsible for the regulation of [Cl<sup>-</sup>], (Gulacsi et al. 2003; Kaila 1994). In bicarbonate-buffered external solution, muscimol (50 μM) increased the frequency of pacemaker activity in TN-GnRH neurons (cell-attached loose patch-clamp recordings, data not shown). This indicates that the influence of bicarbonate, if any, should not be so strong as to change the nature of GABA<sub>A</sub> receptor response.
whether it is excitatory or inhibitory. Thus we conclude that
the excitatory GABA<sub>A</sub> response of the TN-GnRH neurons
mainly arises from the efflux of chloride at resting mem-
brane potential.

A depolarizing response to GABA<sub>A</sub> receptor activation has
been mainly described in immature neurons (Ben-Ari 2002).
Although the response to GABA<sub>A</sub> receptor activation is pre-
dominantly hyperpolarizing in mature neurons, depolarizing
GABA<sub>A</sub> response has been reported in some neurons of adult
animals such as the dorsal root ganglion cells (Sung et al.
2000) and fast-spiking interneurons of the cortex and amygdala
(Martina et al. 2001). The hypothalamic GnRH neurons also
show depolarization in response to GABA<sub>A</sub> receptor activation
in the mouse (Constantin et al. 2009; DeFazio et al. 2002) and
rat (Yin et al. 2008). This depolarizing GABA<sub>A</sub> response is
suggested to mediate various physiological signals in the hy-
pothalamic GnRH neurons. For example, Christian and Moen-
ter (2007) demonstrated that the rate of GABAergic transmis-
sion changes in a diurnal manner but also that the response to
GABA does not change in a diurnal manner; i.e., the ability to
induce action potentials is not altered (Christian and Moenter
2008); this was also shown in the rat (Yin et al. 2008). Thus
considering the results of the present study, it is tempting to
hypothesize that depolarizing GABA<sub>A</sub> response is a common
property shared by GnRH neurons in general, although the
anatomy and function are clearly different between TN-GnRH
neurons and hypothalamic GnRH neurons, and GABA<sub>A</sub> re-
sponse of the third GnRH system in the tegmentum (midbrain
GnRH neurons) remains to be investigated. It is interesting to
note that TN-GnRH neurons and hypothalamic GnRH neurons
have been reported to share common embryonic origins, the
olfactory placode (Abraham et al. 2008; Okubo et al. 2006;
Wierman et al. 2004). In this context, the report of Kaneko et
al. (2004) that the primary olfactory neurons of the rat and
mouse accumulate [Cl<sup>-</sup>] and have depolarized E<sub>Cl</sub> value is
very suggestive. A depolarizing response to GABA<sub>A</sub> receptor

![Diagram](http://jn.physiology.org/)

**FIG. 6.** Bumetanide suppresses the excitatory response in-
duced by GABA<sub>A</sub> receptor activation. A: recordings obtained in
the cell-attached loose patch-clamp configuration. Muscimol
puffer-applied at 5 μM in 3-min interval (arrowheads) evoked
volleys of action currents. Bumetanide (50 μM) was bath-
applied during the period indicated by the solid bar above the
trace. The traces indicated by the arrows a and b are shown in
B on a faster time scale. B: volleys of action currents evoked by
muscimol. Note that the number of action currents decreased
during the bath application of bumetanide (b) compared with
that before the drug application (a). C: the firing rate histogram
(2-s bin) constructed from the data in A. Bath application of
bumetanide (50 μM) decreased the number of spikes evoked by
muscimol, and this inhibition could be partially reversed after
washout (n = 3 cells from 3 fish).
activation has also been shown in peptidergic neurons, such as the hilar NPY neurons in the mouse (Fu and van den Pol 2007) and endocrine cells in the pituitary of frogs (Le Foll et al. 1998) and rats (Zemkova et al. 2008). Thus it may be possible that the depolarizing GABA_A response may be more generally associated with certain neuronal and endocrine functions, which have not been elucidated yet.

\[ \text{Mechanisms underlying the accumulation of [Cl}^{-}\text{]} \]

In general, Cl\textsuperscript{−} homeostasis in most brain cells is controlled by NKCC1 and a specific-specific potassium-coupled chloride co-transporter (KCC2), two electrically neutral cation/chloride co-transporters (Gamba 2005). NKCC1 promotes intracellular accumulation of Cl\textsuperscript{−}, and KCC2 activity leads to reduction of [Cl\textsuperscript{−}]i. Bumetanide (50 μM), blocker for NKCC transporters, substantially suppressed the excitatory GABA_A response in TN-GnRH neurons. This suggests that NKCC is one of the factors responsible for the maintenance of high [Cl\textsuperscript{−}]i, and the excitatory GABA_A receptor response in TN-GnRH neurons of the dwarf gourami. It has been shown that NKCC is also one of the factors responsible for high [Cl\textsuperscript{−}]i in the mouse hypothalamic GnRH neurons (DeFazio et al. 2002) and the rodent primary olfactory neurons (Kaneko et al. 2004).

\[ \text{Effects of excitatory GABA actions on the pacemaker activity of TN-GnRH neurons} \]

Puffer application of low-dose muscimol (5 μM) slightly depolarized the membrane potential, thereby increasing pacemaker frequency of TN-GnRH neurons. However, higher-dose muscimol (50 μM) rapidly increased the pacemaker frequency and then inhibited the pacemaker potential, probably because the persistent depolarization after the increase of action potentials inactivated the voltage-gated Na\textsuperscript{+} channels. In addition, higher-dose muscimol caused a large decrease in the membrane resistance, which may shunt the ionic conductances responsible for the generation of spontaneous pacemaker activity.

GABA has been reported to induce similar bidirectional effects (excitatory - inhibitory) in other neurons or endocrine cells, such as rodent hypothalamic GnRH neurons (DeFazio et al. 2002; Yin et al. 2008) and endocrine cells in the pituitary (Le Foll et al. 1998; Zemkova et al. 2008). It may be speculated that the inhibition of firing in higher-dose muscimol functions as a low-pass filter that prevents excessive excitation of the neurons. Because muscimol was locally puffer-applied to the cell body, it is not certain that the inhibition of pacemaker activity observed in the present study really occurs physiologically in response to the synaptic activation. Further experiments such as electrical stimulation of GABAergic afferent fibers may be necessary.

\[ \text{Functional considerations} \]

As shown in the present study, GABA_A receptor activation showed excitatory effect on the pacemaker activity of TN-GnRH neurons in the adult dwarf gourami (in response to low-dose muscimol), which may result in the same effect on the pacemaker activity as glutamate (Kiya and Oka 2003). Previous morphological study has revealed possible multimodal sensory inputs to the TN-GnRH neurons (Yamamoto and Ito 2000), and these may be the source of glutamatergic and GABAergic inputs to the TN-GnRH neurons. Therefore the GABAergic synaptic inputs may increase the pacemaker frequency by relaying the stimulatory sensory inputs from the environment and cause simultaneous release of GnRH peptides in various brain regions. Because glutamate and GABA have redundant excitatory effects on TN-GnRH neurons, it is possible that there is functional segregation between glutamatergic and GABAergic afferents. In the morphological study mentioned in the preceding text, afferent neurons in the adjacent region of TN-GnRH neurons could not be detected because of the technical limitations (Yamamoto and Ito 2000). Therefore it may also be possible that GABAergic afferent neurons in the local circuit with TN-GnRH neurons. Therefore anatomical identification of the sources of GABAergic afferent neurons will be an interesting future topic.

The fact that GABA, a major inhibitory neurotransmitter in the brain, has a depolarizing effect in TN-GnRH neurons may suggest that some other neurotransmitters may mediate hyperpolarizing synaptic transmission in place of GABA. Previous studies demonstrated that hyperpolarizing response is induced by applying the agonist of group 3 metabotropic glutamate receptors (mGluRs) (Kiya and Oka 2003) and FMRFamide (T. H. Saito, R. Nakane, H. Abe, and Y. Oka, unpublished observation) in the dwarf gourami. Previous intracellular recording studies showed that electrical stimulation of the olfactory nerve and the medial olfactory tract caused an IPSP-like hyperpolarizing response that inhibited the pacemaker activity of TN cells in carp (Fujita et al. 1985) and that electrical stimulation of the central and peripheral nerve trunk of the TN ganglion induced similar hyperpolarizing response in neurons within the TN ganglion in elasmobranchs (White and Meredith 1987), although it is not clear whether the neurons recorded in their studies are GnRH neurons (TN-GnRH neurons) or not. These hyperpolarizing responses showing relatively fast time course may counterbalance the depolarizing response mediated by glutamate, GABA, etc.

\[ \text{In summary, our study demonstrates that GABA_A receptor activation depolarizes TN-GnRH neurons of the adult dwarf gourami and increases the frequency of their pacemaker activity. This excitatory effect of GABA is suggested to arise from high [Cl\textsuperscript{−}], and Cl\textsuperscript{−}-accumulating co-transporter, NKCC, is one of the factors responsible for this. The excitatory effect of GABA may have certain function in the sensory transmission to TN-GnRH neurons. The physiological significance of this excitatory effect of GABA in neuromodulatory TN-GnRH neurons is an interesting future problem to be solved.} \]

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