Electrophysiological Analysis of the Inhibitory Effects of FMRFamide-Like Peptides on the Pacemaker Activity of Gonadotropin-Releasing Hormone Neurons

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Saito TH, Nakane R, Akazome Y, Abe H, Oka Y. Electrophysiological analysis of the inhibitory effects of FMRFamide-like peptides on the pacemaker activity of gonadotropin-releasing hormone neurons. J Neurophysiol 104: 3518–3529, 2010. First published October 20, 2010; doi:10.1152/jn.01027.2009. Gonadotropin-releasing hormone (GnRH) neurons in the terminal nerve (TN) show endogenous pacemaker activity, which is suggested to be dependent on the physiological conditions of the animal. The TN-GnRH neurons have been suggested to function as a neuromodulatory neuron that regulates long-lasting changes in the animal behavior. It has been reported that the TN-GnRH neurons are immunoreactive to FMRFamide. Here, we find that the pacemaker activity of TN-GnRH neuron is inhibited by FMRFamide: bath application of FMRFamide decreased the frequency of pacemaker activity of TN-GnRH neurons in a dose-dependent manner. This decrease was suppressed by a blockade of G protein–coupled receptor pathway by GDP-β-S. In addition, FMRFamide induced an increase in the membrane conductance, and the reversal potential for the FMRFamide-induced current changed according to the changes in [K+]out as predicted from the Nernst equation for K+.

INTRODUCTION

The terminal nerve (TN) gonadotropin-releasing hormone (GnRH) neurons are present in almost all jawed vertebrates. Unlike the hypothalamic hypophysiotropic GnRH neurons that project to the pituitary (in teleosts) and facilitate gonadotropin release, the TN-GnRH neurons project throughout the brain but not to the pituitary (Yamamoto et al. 1995) and show endogenous pacemaker activity, the frequency of which is suggested to be dependent on the physiological conditions of the animal (Oka and Matsushima 1993). Electrophysiology studies of the TN-GnRH neurons have suggested that they are involved in the regulation of motivational or arousal states for certain behaviors (Wirsig and Leonard 1987; Yamamoto et al. 1997). Concerning the pacemaker activity of TN-GnRH neurons, radioimmunoassay study using semi-intact brain preparations of the dwarf gourami suggested that the increase of pacemaker frequency by depolarization stimulates GnRH peptide release from TN-GnRH neurons in an intensity-dependent manner (Ishizaki et al. 2004). In addition, it has been known for a long time that GnRH peptides modulate K+ channels (Adams and Brown 1980) and Ca2+ channels (Elmslie et al. 1990) of neurons in the sympathetic ganglia and ionotropic glutamatergic transmission in the optic tectum (Kinoshiba et al. 2007) and hippocampus (Yang et al. 1999). Thus it is hypothesized that the pacemaker frequency of TN-GnRH neurons regulates the GnRH peptide release, changes the excitability of target neurons, and causes long-lasting changes in animal behaviors (Abe and Oka 2007; Oka 2002).

As to the regulation of pacemaker activity of TN-GnRH neurons, we have already shown that the pacemaker activity of TN-GnRH neurons is modulated by salmon GnRH (sGnRH), which is the same molecular species of GnRH peptide produced by TN-GnRH neurons themselves (Abe and Oka 2000). Combined with an electron microscopic study suggesting the occurrence of somatodendritic release of sGnRH (Oka and Ichikawa 1991), it has been suggested that TN-GnRH neurons have auto/paracrine regulatory mechanisms of their pacemaker activities (Abe and Oka 2007; Oka 2002). On the other hand, it has been reported that some peptidergic neurons are also immunoreactive to multiple peptides (Brown and Bourque 2004). In the TN-GnRH neurons, it has been reported that they are also immunoreactive to FMRFamide peptide in the dwarf gourami (Wirsig-Wiechmann and Oka 2002), and FMRFamide modulates the firing pattern of retinal ganglion cells in goldfish (Stell et al. 1984, 1987). Thus we were interested in the possible auto/paracrine effects of FMRFamide-like peptides corelease with sGnRH on the pacemaker activity of TN-GnRH neurons.

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In this study, we first show that the pacemaker activity of the TN-GnRH neuron is directly (not via other neurons) inhibited by FMRFamide, and this inhibition is caused by the hyperpolarization caused by activation of K+ conductance in the TN-GnRH neurons. However, because FMRFamide has been shown not to be the endogenous peptide in vertebrate brains, we next tried to determine the identity of endogenous FMRFamide-like peptides. As a candidate for endogenous FMRFamide-like peptides that localize in TN-GnRH neurons, there is
a report in zebrafish (Oehlmann et al. 2002) that a peptide homologous to mammalian PQRFamide (NPFF/NPAF), a member of RFamide family, is localized in TN-GnRH neurons. This suggests that the unidentified FMRFamide-like peptide in TN-GnRH neurons of the dwarf gourami may be a homolog of zebrafish PQRFamide. Therefore we performed cloning and sequence analysis of the PQRFamide gene in dwarf gourami. We performed immunohistochemical preabsorption experiments, and the results suggested that FMRFamide-like peptide in TN-GnRH neurons of the dwarf gourami is NPFF. In fact, we show here that the pacemaker activity of TN-GnRH neurons is also inhibited by NPFF and that this inhibition is blocked by RF9, a potent and selective antagonist for mammalian NPFF receptors.

Because there has been no neurotransmitter/neuromodulator candidate that inhibits the pacemaker activity of TN-GnRH neurons, this finding is the first report on the presence of an inhibitory peptidergic neuromodulator that affects the pacemaker activity of TN-GnRH neurons.

METHODS

All procedures were performed in accordance with the guidelines of the Physiological Society of Japan and the University of Tokyo for the Use and Care of Experimental Animals.

Electrophysiology

For electrophysiological studies of TN-GnRH neurons, we used whole brain or brain block in vitro preparations of the dwarf gourami (Colisa lalia, a freshwater tropical fish; see Abe and Oka 2007). Because TN-GnRH neurons of the dwarf gourami form a morphologically distinct neuronal cluster immediately beneath the ventral meningeal membrane (Oka and Ichikawa 1991), they can be easily identified under a dissecting microscope or microscopes equipped with differential interference optics (Abe and Oka 2007, 2009; Oka 2002; Oka and Matsuhashima 1993). This gives an obvious experimental advantage over peptidergic neurons of other vertebrates, because they are small and scattered, so that it has been difficult to study the physiology of single vertebrate peptidergic neurons.

Adult male and female dwarf gouramis (n = 75 fish), ~4 cm in standard length, were purchased from a local dealer. Each fish tank containing ~20 fish was maintained at 27°C and 12-h light, 12-h dark cycle. The fish were fed with worm once a day until used. For the experiments, they were chilled by immersing them in crushed ice and were quickly killed by decapitation. After careful removal of ventral meningeal membrane, thick brain slices of the forebrain (~1 mm thickness) containing TN-GnRH neurons were manually cut out with razor blades in an artificial cerebrospinal fluid (ACSF) consisting of (in mM) 124 NaCl, 5.0 KCl, 1.3 MgSO4, 2.4 CaCl2, 10 HEPES, 10 glucose, and 0.1% BSA (pH 7.4 adjusted with NaOH). All the experiments were performed at room temperature (20–24°C). Patch pipette solution contained (in mM) 70 K-gluconate, 40 KCl, 3 MgCl2, 10 HEPES, 10 glucose, and 0.1% BSA (pH 7.4 adjusted with NaOH). The AC SF was ~2–4 MΩ. The patch pipette was visually guided to the cluster of TN-GnRH neurons, which were located on the ventral surface of the transitional area between the olfactory bulb and the telencephalon under an upright microscope (E-600FN, Nikon) equipped with a 40× water-immersion objective (0.8 numerical aperture), infrared (IR) differential interference contrast optics, and an IR-CCD camera (C3077-78, Hamamatsu photonics, Hamamatsu, Japan). The brain block preparation was continuously superfused with an oxygenated ACSF solution. After gigaohm seal formation and “break in” for the whole cell recording mode, characteristic spontaneous pacemaker activities were confirmed in the current-clamp mode. The membrane resistance and capacitance in the whole cell recordings were 79.9 ± 3.2 (SE) MΩ and 257.1 ± 21.2 (SE) pF, respectively. Recordings were performed with a patch-clamp amplifier, MultiClamp 700A (Molecular Devices, Sunnyvale, CA), and whole cell voltage- and current-clamp recordings were digitized (2 kHz) and stored on a computer using the Digidata 1322A and pCLAMP 9.2 software (Molecular Devices). Statistical analyses were performed with Kxplot (version 5, Kyence) and Clampfit (Molecular Devices). Drugs were used as follows: FMRFamide (0.1–10 μM, Sigma), GDP-β-S (2 mM, Sigma), TTX (0.75 μM, Sigma). Peptides corresponding to medaka NPFF (ESVLHQPQRF-COH,) were synthesized by Hayashi-kasei (Osaka, Japan) after cloning and were used at 10 nM to 1 μM. All data in this report are presented as means ± SE.

Molecular cloning of the dwarf gourami using LsoGen (Nippon Gene). One hundred micrograms of isolated total RNA was further purified to recover polyA+ RNA using Oligoex-DT Super (Takara, Shiga, Japan). One microgram of polyA+ RNA was applied to the SMART RACE kit (Clontech Laboratories, Palo Alto, CA) according to the manufacturer’s protocol for 5’ and 3’ first-strand cDNA synthesis. Degenerate PCR primers were designed based on 14 amino acids (PQQWSMAYPQRF) conserved among medaka, zebrafish, and green spotted pufferfish putative NPFF genes. 5'-rapid amplification of cDNA (RACE) was carried out using dwarf gourami brain cDNA library constructed with the SMART RACE Kit. Primary amplification was performed with a universal primer mixture (UPM), complimentary to the adaptor sequence provided with the kit and degenerate primers (antisense, 5’-TTCCRAAYCTTCGNGNAC-NGCCAT-3’). Nested PCR was performed using a primer (NUP) complementary to the adaptor sequence provided with the kit and degenerate nest primer (antisense, 5’-CAAGCATGTCCCATADYATGTGCCNNGG-3’). PCR conditions were as follows: denaturation at 94°C for 5 min, followed by 5 cycles at 94°C for 30 s and 72°C for 2 min, 5 cycles at 94°C for 30 s and 70°C for 30 s, 72°C for 2 min, and 25 cycles at 94°C for 30 s, 64°C for 30 s, and 72°C for 2 min. The PCR products were gel purified using Gel Extraction Kit (Qiagen, Hilden, Germany), and ligated into pGEM-T vector (Promega, Madison, WI). Three independent positive clones were sequenced to confirm the sequence information. Based on the determined sequence of 5′ portion of NPFF cDNA, the specific primers for 3’-RACE were designed (sense 1: 5’-TCTTCCAGACCAAGGCGGTCTGGAC-3’ and sense 2: 5’-ATCTCCTGACGTCCGGTCTAGACG-3’). Primary and nested PCRs were performed using sets of primers, UPM and sense 1, and NUP and sense 2, respectively. The PCR conditions were the same as that of T-RACE. The PCR products were processed for sequence analysis similarly to T-RACE.

Immunohistochemistry

Adult male and female dwarf gouramis (n = 6 fish), ~4 cm in standard length, were used for immunohistochemistry. The fish were deeply anesthetized by immersing them in a 0.02% MS-222 (3-aminobenzoic acid ethyl ester) solution and were quickly killed by decapitation. The fish brains were dissected out and fixed with 4% paraformaldehyde in 0.1 M PBS (pH 7.4) for 2–3 days at 4°C. The fixed specimens were immersed in 30% sucrose in 0.1 M PBS for 3–6 h, embedded in 5% agarose (type IX-A; Sigma) and 20% sucrose in 0.1 M PBS, and frozen in n-hexane (~60°C). Serial frontal sections were cut at 30 μm thick on a Leica CM3050 S cryostat (Leica Microsystems, Wetzlar, Germany) and mounted on adhesive slides. A rabbit polyclonal antibody to FMRFamide (Cat. FA 1134, Enzo Life Sciences, Plymouth Meeting, PA) was used. The sections were incubated overnight with the antibody that had been diluted 15,000-fold.
with 0.1 M PBS containing 0.3% Triton X-100 (PBST) and 10% normal goat serum. The sections were washed twice with 0.1 M PBST and immersed in 0.3% H₂O₂ in methanol for 30 min to block endogenous peroxidase activities. The sections were washed twice with 0.1 M PBST and reacted with biotin-labeled anti-rabbit IgG (1:200) for 2 h. Subsequently, the sections were washed twice with 0.1 M PBST. Avidin-biotin-horseradish peroxidase complex solution [ABC solution: Sigma (Vectastain), ABC elite kit] was prepared and incubated for 60 min, and the sections were treated with ABC solution for 60 min. The sections were washed again three times with PBST, after which 3,3′-diaminobenzidine (DAB) and hydrogen peroxide were added for visualization of the peroxidase reaction. The reactions were carried out at room temperature unless otherwise noted. To test the possibility that anti-FMRFamide antiserum cross-reacts with PQRamide (NPFF), sections were incubated in anti-FMRFamide antiserum that had been preabsorbed overnight at 4°C with synthetic medaka NPFF. The sections were coverslipped with a mounting medium and were observed under a Leica DMS000B microscopy equipped with a Leica DFC310 FX CCD camera (Leica Microsystems).

RESULTS

Morphological and basic electrophysiological characteristics of TN-GnRH neurons were previously reported in detail (Abe and Oka 2007; Oka 2002; Oka and Ichikawa 1991; Oka and Matsushima 1993). TN-GnRH neurons can be identified easily by their characteristic anatomy (location, soma size, and morphology) and regular intrinsic pacemaker activities (Oka and Matsushima 1993).

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

**FIG. 1.** FMRFamide inhibits the pacemaker activity of terminal nerve–gonadotropin-releasing hormone (TN-GnRH) neurons. **A:** in whole cell current-clamp recording from a TN-GnRH neuron, bath application of FMRFamide inhibited its pacemaker activity. **B:** in artificial cerebrospinal fluid (ACSF), the TN-GnRH neuron showed a beating discharge pattern (a). Bath application of FMRFamide (10 µM) inhibited pacemaker activity (b). After washout, the firing activity recovered (c). C: time course of instantaneous frequency (the reciprocal of interspike interval) of the pacemaker activity plotted from the data in A. D: dose–response relationships between the FMRFamide concentration and the normalized pacemaker frequency during the FMRFamide applications. Numbers in parentheses near the filled squares represent the numbers of neurons tested for each FMRFamide concentration. The concentration–response curve was fitted with the Hill’s equation, and an EC₅₀ was 2.85 µM.

**FMRFamide inhibits the pacemaker activity of TN-GnRH neurons**

The pacemaker activities of TN-GnRH neurons were inhibited by bath application of FMRFamide. Figure 1A shows an example of the inhibition of pacemaker activity by FMRFamide (10 µM). In ACSF, TN-GnRH neuron showed a slow regular beating discharge pattern (5.2 ± 0.2 Hz, n = 5; Fig. 1Ba). During the bath application of FMRFamide, the firing frequency of pacemaker activity was gradually decreased, and the firing was completely blocked about 20 s after the onset of the application (Fig. 1Bb). The time course of this inhibition of the frequency of pacemaker activity is plotted in Fig. 1C. After washing out FMRFamide with normal ACSF, pacemaker frequency almost recovered to its original level (Fig. 1, Bc and C). Thus FMRFamide reversibly inhibited the pacemaker activity of TN-GnRH neurons. This FMRFamide-induced decrease in firing frequency showed a clear dose dependence (Fig. 1D). Here, the normalized decrease in firing frequency of pacemaker activity was plotted against the concentration of FMRFamide. The normalized firing frequency was defined as

\[
\text{Frequency}_{(+ \text{FMRFamide})} / \text{Frequency}_{(\text{before application})} \times 100 \%
\]

The frequency here indicates the number of spikes during 20-s periods, counted immediately before and during the application of FMRFamide (70–90 s after the onset of FMRFamide application). The lower value for the normalized frequency indicates the stronger effects of FMRFamide on firing activity.
tion–response curve (Fig. 1D, solid line) could be well fitted with an equation

$$ R = \frac{[\text{FMRFamide}]^n}{EC_{50} + [\text{FMRFamide}]^n} $$

where $R$ is the normalized firing frequency. The $EC_{50}$ was 2.85 μM, and $n = -0.36$.

**FMRFamide inhibits the pacemaker activity via G protein–coupled receptors**

To analyze the mechanisms underlying this inhibition of pacemaker activity, we examined the effects of FMRFamide under synaptically isolated conditions. In the presence of TTX, FMRFamide clearly hyperpolarized the membrane potential of TN-GnRH neurons (14.9 ± 1.1 mV, $n = 15$; Fig. 2). This result suggests that FMRFamide directly modulates the pacemaker activity of TN-GnRH neurons.

We next examined whether the activation of the G protein–coupled receptor pathway was involved in this inhibitory effect. To examine this possibility, we diazylated the recorded neuron with GDP-β-S, a GDP derivative that is a competitive inhibitor of many G protein–mediated processes, by including it in the patch pipette solution. After that, we examined the effect of bath application of FMRFamide under the same conditions as in the control. To ensure the diffusion of GDP-β-S into the cytoplasm of the TN-GnRH neuron, we started data collection 10 min after the whole cell recording was established, which is based on our previous experiments (Abe and Oka 2000). Figure 3A shows the pacemaker activity of a cell recorded with a large-tip patch pipette (~2 MΩ) containing 2 mM GDP-β-S in the pipette solution. In ACSF, this neuron showed clear beating discharge pattern (Fig. 3Ba). The firing frequency in this condition (4.4 ± 0.5 Hz, $n = 6$) was not significantly different from those in the control (4.1 ± 0.7 Hz, $n = 8$). After diffusion of GDP-β-S into the TN-GnRH neuron, the inhibitory effect of FMRFamide (5 μM) on the firing frequency of pacemaker activity was diminished. Figure 3Bb (90 s after the onset of FMRFamide perfusion) was recorded during similar time periods of FMRFamide-induced decrease in the pacemaker activity (corresponding to Fig. 1Bb). The time course of changes in the frequency of pacemaker activity is plotted in Fig. 3C. The decrease in normalized firing frequency was significantly diminished by the intracellular application of GDP-β-S (Fig. 3D; 16 ± 7% in control using a normal pipette solution, $n = 8$ vs. 53 ± 8% when GDP-β-S was added to the pipette solution, $n = 6$; $P < 0.05$, two-tailed alternate Welch $t$-test). From these results, we suggest that the activation of a G protein–coupled receptor mediates this inhibition of the pacemaker activity by FMRFamide in TN-GnRH neurons.

**FMRFamide activates K⁺ conductances in TN-GnRH neurons**

To study the ionic mechanisms that cause the FMRFamide-induced hyperpolarization of TN-GnRH neurons, we measured changes in the membrane conductance before and during the application of FMRFamide (10 μM). To measure the conductance changes, we repetitively applied brief (200 ms) negative current pulses (~0.2 nA) at an interval of 10.5 s (Fig. 4A, top trace) and compared the membrane potential changes before (Fig. 4A, dotted line) and during (Fig. 4A, solid line) application of FMRFamide in the presence of 0.75 μM TTX. The current and voltage traces on an expanded time scale are shown in Fig. 4B. According to Ohm’s law ($V = IR$), we calculated the membrane conductance from each voltage response and represented mean membrane conductance at each period (as an averaged value from 5 trials). The membrane conductance was increased by bath application of FMRFamide (9.4 ± 1.2 nS before application of FMRFamide vs. 13.8 ± 1.9 nS during application of FMRFamide, $P < 0.001$, $n = 7$; Fig. 4C), although the membrane conductance in the control ACSF increased by 10.2 ± 0.3 nS (Fig. 4D). In this experimental condition, we compared the membrane conductance between the control and the FMRFamide application group at different membrane potentials, because FMRFamide hyperpolarized the membrane potential. The membrane conductance measurements at different membrane potentials could be affected by a differential availability or activation of voltage-dependent ion channels. To exclude these influences, we performed membrane conductance measurements in the presence of FMRFamide at the same membrane potential as the control resting membrane potential by injecting appropriate constant current, and we found that the membrane conductance was also increased (data not shown). These results suggest that FMRFamide activates certain type(s) of conductance. Therefore to examine which ion permeability underlies the conductance increase by FMRFamide application, we measured the reversal potential of FMRFamide-

![FIG. 2. FMRFamide directly inhibits the pacemaker activity of TN-GnRH neurons. FMRFamide hyperpolarized the membrane potential of TN-GnRH neurons in the presence of 0.75 μM TTX. After washout, the membrane potential of TN-GnRH neurons recovered to the pretreatment level (shown as dotted line).](http://jn.physiology.org/10.1152/jn.00636.2009)
induced currents from the intersection of current–voltage relationships before and during the FMRFamide application, which was obtained from the current responses during the voltage-clamp recording using a slow voltage ramp from −10 to −110 mV; duration = 1.5 s) protocol. The applied voltage ramp is shown in the inset of Fig. 4D, and the falling phase of the voltage ramp (dotted rectangle in Fig. 4D, inset) was used for plotting the current–voltage relation. In this experiment, we depolarized the TN-GnRH neurons from −60 to −10 mV for 1.5 s before applying voltage ramp to inactivate voltage-gated calcium channels. From the current responses obtained before and during the FMRFamide application, we determined the reversal potential of FMRFamide-induced current from the intersection of two current–voltage curves (Fig. 4D, arrow). The increased slope of the current–voltage curve during FMRFamide application (Fig. 4D, solid line) also suggests that certain kind(s) of conductance(s) are activated by FMRFamide application. The recorded reversal potential was −87.7 ± 3.0 mV in normal ACSF solution ([K+]out = 5 mM, n = 6), and this value closely corresponded to the theoretical equilibrium potential for K+ (−89.0 mV) according to the Nernst’s equation. We next examined the effect of changes in extracellular K+ concentrations ([K+]out; 2.5 and 15 mM in addition to the normal concentration of 5 mM) on the reversal potentials of FMRFamide-induced currents. The reversal potentials of the FMRFamide-induced currents in 2.5 and 15 mM [K+]out were −110.1 ± 1.8 (n = 6) and −59.6 ± 4.5 mV (n = 5), respectively (Fig. 4E). When these reversal potentials were plotted as a function of [K+]out, the relation could be well fitted by a line predicted from the Nernst’s equation for K+ (dotted line in Fig. 4E; 58 mV per log unit changes in [K+]out). It indicates that the conductance(s) activated by FMRFamide are based on permeability highly selective for K+. Therefore we concluded that FMRFamide activates K+-selective conductance via G protein–coupled pathway, directly (not by synaptic inputs) hyperpolarizes TN-GnRH neurons, and inhibits the pacemaker activity of TN-GnRH neurons.

Cloning and sequence analysis of PQRFamide (NPFF/NPAF) cDNA in dwarf gourami

As mentioned in Introduction, it is suggested that TN-GnRH neurons of the dwarf gourami express unidentified FMRFamide-like peptides that are probably homologous to zebrafish PQRFamide (NPFF/NPAF). To examine this possibility, we cloned PQRFamide cDNA from dwarf gourami. As shown in Fig. 5A, the dwarf gourami NPFF/NPAF cDNA was 660 or 830 bp in length, without polyA tail. The difference in length is because of the different polyA addition sites. The cDNA consisted of an open reading frame (ORF) of 387 bp encoding a precursor protein of 128 amino acids, flanked by 5′ and 3′ UTRs of 156 and 84 or 293 bp, respectively. The first 15 amino acids were predicted to constitute the putative signal peptide.
Comparison among the deduced amino acid sequences of NPFF/NPAF shows a relatively low identity, although the mature peptides of NPFF and NPAF and C-terminal cleavage and amidation site (GR and GRK or GKK) are well conserved among species (Fig. 5). Therefore we decided to use medaka NPFF peptide for the immunological and electrophysiological analysis of NPFF in the dwarf gourami TN-GnRH neurons.

**NPFF immunoreactivity in TN-GnRH neurons was abolished by the preabsorption of anti-NPFF antiserum with NPFF**

To examine the possibility that unidentified FMRFamide-like peptides in TN-GnRH neurons of dwarf gourami are QORFamide, we performed a series of immunohistochemical experiments. In agreement with the previous work (Wirsig-Wiechmann and Oka 2002), TN-GnRH neurons are immunoreactive to the anti-FMRFamide antiserum. Figure 6A shows the distribution of FMRFamide-immunoreactive (FMRFamide-ir) fibers in the rostroventral telencephalon. The immunoreaction of the FMRFamide-ir fibers was almost completely abolished when the anti-FMRFamide antiserum had been preabsorbed with medaka NPFF peptide (10 µM, overnight at 4°C; Fig. 6B) but not with NPAF peptide (data not shown). The results of the immunohistochemical experiments, together with the results of cloning, strongly suggest that the endogenous FMRFamide-like peptide in TN-GnRH neurons is NPFF. Therefore we used NPFF to examine its inhibitory effects on TN-GnRH neurons pacemaker activities in the following sections.

**NPFF inhibits the pacemaker activity of TN-GnRH neurons**

The pacemaker activities of TN-GnRH neurons were inhibited by a bath application of NPFF instead of FMRFamide. Figure 7A shows an example of the inhibition of pacemaker activity by NPFF (100 nM). In ACSF, TN-GnRH neuron showed a slow regular beating discharge pattern (3.6 ± 0.5 Hz, n = 6; Fig. 7Aa). During the bath application of NPFF, the firing frequency of pacemaker activity was gradually de-
creased, and the firing was completely blocked about 150 s after the onset of the application (Fig. 7A). The time course of this inhibition of pacemaker activity is plotted in Fig. 7C. After washing out NPFF with normal ACSF, the pacemaker frequency partially recovered (Fig. 7, A and C). Thus NPFF reversibly inhibited the pacemaker activity of TN-GnRH neurons. This NPFF-induced decrease in firing frequency showed a clear dose dependence (Fig. 7D).

Fig. 5. cDNA and deduced amino acid sequences of the dwarf gourami NPFF/NPAF gene and its comparison with vertebrate NPFF/NPAF genes. A: the nucleotide sequence and the deduced amino acid sequence of dwarf gourami NPFF/NPAF. The putative signal peptide is underlined, and predicted mature peptides (NPFF and NPAF) are boxed. The putative polyadenylation signals are underscored. Bold letter at position of 627 represents alternative polyadenylation site. The nucleotide sequence of dwarf gourami NPFF/NPAF has been submitted to the GenBank and is available under the Accession No. AB571483. B: comparison of amino acid sequences of NPFF/NPAF precursors among different species. The amino acid residues conserved in all species are shaded in black, and those conserved in 80 and 60% of the species are shaded in gray and light gray, respectively. Sequences for human (AF005271), mouse (BC107295), zebrafish (AB193140), and torafugu (AB193132), NPFF/NPAF peptides were retrieved from the GenBank. Medaka NPFF/NPAF (ENSORLG00000004908) was obtained from Ensembl medaka genome database. Stickelback and green spotted puffer NPFF/NPAFs (partial sequences) were also deduced from genomic data downloaded from Ensembl Genome Browser. The NPFF and NPAF regions are underlined. Gaps indicated by hyphens are introduced in some sequences to maximize alignment.

Fig. 6. FMRFamide immunoreactivity in the fibers was abolished by the preabsorption of anti-FMRFamide antiserum with NPFF. A: frontal section of the telencephalon of dwarf gourami. Dense FMRFamide immunoreactive fibers were distributed in the rostroventral telencephalon. B: FMRFamide immunoreactivity in these fibers was abolished by the preabsorption of anti-FMRFamide antiserum with synthetic medaka NPFF. The arrows indicate the location of cell cluster of TN-GnRH neurons. Scale bars, 100 μm.
RF9, a potent and selective NPFF receptor antagonist, blocked the inhibitory effects of NPFF and FMRFamide on the pacemaker activity of TN-GnRH neurons

To know the receptors mediating NPFF-induced inhibition of pacemaker activity, we next examined the effect of RF9, a potent and selective antagonist of NPFF receptors. RF9 (10 μM) potently blocked the inhibitory effect of NPFF (100 nM) on the pacemaker activity of TN-GnRH neurons (Fig. 8A). In ACSF, TN-GnRH neurons showed a clear beating discharge pattern (Fig. 8Ba; 2.2 ± 0.5 Hz, n = 6). In the presence of RF9, the inhibitory effect of NPFF (100 nM) on the firing frequency of pacemaker activity was clearly diminished. Figure 8Bb (90 s after the onset of NPFF perfusion) was recorded during similar time periods of the NPFF-induced decrease in the pacemaker activity (corresponding to Fig. 7Bb). The time course of changes in the frequency of pacemaker activity is plotted in Fig. 8C. The decrease in normalized firing frequency was significantly diminished by RF9 (Fig. 8D; 38 ± 15% in control using a normal ACSF, n = 6, vs. 82 ± 9% in the presence of RF9, n = 6; P < 0.05, 2-tailed alternate Welch t-test). From these results, we suggest that the receptors that are similar to mammalian NPFF receptors mediate this inhibitory effect of NPFF on the pacemaker activity of TN-GnRH neurons.

RF9 (20 μM) also blocked the inhibitory effect of FMRFamide (5 μM) on pacemaker activity (Fig. 8D; 16 ± 8% in control using a normal ACSF + FMRFamide, n = 8 vs. 69 ± 9% in RF9 + FMRFamide, n = 6; *P < 0.01, 2-tailed alternate Welch t-test). These results suggest that the inhibitory effects of FMRFamide shown in the previous sections (Figs. 1–4) are also mediated by the receptors that are similar to mammalian NPFF receptors.

**DISCUSSION**

In this study, we showed that the pacemaker activity of TN-GnRH neurons is inhibited in a dose-dependent manner by FMRFamide-like peptides. FMRFamide was first isolated and characterized from the nervous system of molluscs (Price and Greenberg 1977). Recently, FMRFamide-like peptides have been isolated in many vertebrate species, and they have been considered to serve neuroendocrine or neuromodulatory functions (Bechtold and Luckman 2007). FMRFamide-like immunoreactivity in the TN neurons has been described in various vertebrate species including bichir, dwarf gourami, frogs, and shrews, and their axons have been stained by FMRFamide antiserum (Malz and Kuhn 2002; Muske and Moore 1988; Rastogi et al. 2001; Wirsig-Wiechmann and Oka 2002; Wright and Demski 1996). Walker and Stell (1986) reported that GnRH and FMRFamide, both of which have been reported to be the immunoreactive molecules in TN neurons, modulate the firing activity of retinal ganglion cells of goldfish, Carassius auratus. They reported that bath application of FMRFamide could be excitatory, inhibitory, or even biphasically inhibitory–excitatory, whereas the actions of sGnRH to the retinal ganglion cells are usually excitatory. However, the physiological mechanisms of these excitatory and/or inhibitory actions were

![Figure 7](http://jn.physiology.org/)

**Fig. 7.** NPFF inhibits the pacemaker activity of TN-GnRH neurons. A: in a whole cell current-clamp recording from a TN-GnRH neuron, bath application of NPFF (100 nM) inhibited the spontaneous pacemaker activity. B: in ACSF, the TN-GnRH neuron showed a beating discharge pattern (a). Bath application of NPFF (100 nM) inhibited the pacemaker activity (b). After washout, the firing activity partially recovered (c). C: time course of instantaneous frequency (the reciprocal of interspike interval) of the pacemaker activity plotted from the data in A. D: dose–response relationships between the NPFF concentration (10 nM, 100 nM, and 1 μM) and the normalized pacemaker frequency during the NPFF applications.
not studied by these authors and have not been studied up to now. In this study, we showed that bath application of FMRFamide is inhibitory to the spontaneous firing activity of TN-GnRH neurons. These results newly suggest that FMRFamide-like peptides, which were shown in the immunocytochemical study in TN-GnRH neurons to coexist with GnRH (Wirsig-Wiechmann and Oka 2002), may act as an inhibitory auto/paracrine factor.

As to the neuromodulatory actions of FMRFamide peptides, there have been several reports on the excitatory or inhibitory modulations of ion channels via activation of G protein-coupled receptors (N- and L-type Ca$^{2+}$ currents, Edmonds et al. 1990 and Kavaliers 1987; K$^+$ currents, Buttner et al. 1989; Ducret et al. 2009; Michel et al. 2002; and Walsh and Byrne 1989; hyperpolarization-activated Cl$^-$ current, Buttner and Siegelbaum 2003). In this study, we showed that FMRFamide decreases the excitability of TN-GnRH neurons through activation of certain kind(s) of K$^+$ conductance(s). The GDP-β-S did not completely block the inhibitory action of FMRFamide, it is also possible that there are some components of FMRFamide actions that do not depend on the G protein-coupled receptor activations. In this respect, it is interesting to note that two kinds of cation-permeable ionotropic FMRFamide receptors [FMRFamide-activated Na$^+$ channel (FaNaC) in invertebrates and acid-sensing ion channels (ASICs) in vertebrates] have recently been reported (Lingueglia et al. 2006). However, the results of the present voltage-clamp experiments (see Fig. 4) strongly suggest that the increase of K$^+$ permeability via the activation of G protein-coupled receptors is the predominant component underlying the conductance changes that lead to the inhibitory effect of FMRFamide.

In the first half of this study, we used a synthetic molluscan FMRFamide peptide to study its effects on the TN-GnRH neurons, and the value of EC$_{50}$ of FMRFamide-induced inhibition of pacemaker activity was relatively high (2.85 µM). Walker and Stell (1986) reported that a similarly high FMRFamide concentration was necessary to affect membrane potentials of the goldfish retinal ganglion cells (the threshold dose was 1.5 µM). These results suggest that the endogenous FMRFamide-like peptide in the retina and TN-GnRH neurons (this study) may be different from the molluscan FMRFamide peptide, although the TN-GnRH neurons have been reported to be immunoreactive to the anti-FMRFamide antiserum (Wirsig-Wiechmann and Oka 2002). Indeed, a large number of RFamide peptides, defined by their conserved carboxyl-terminal arginine (R) and phenylalanine (F) residues, have been quite recently identified in the nervous systems of animals in all major vertebrate phyla (Bechold and Luckman 2007). From a phylogenetic analysis of the identified and putative RFamide peptides in vertebrates, Osugi et al. (2006) suggested that there are at least five RFamide groups in vertebrates [Kisspeptin group; prolactin-releasing peptide (PrRP) group; pyroglutamylated Arg-Phamide peptide (QRFP) group, e.g., 26RFa; Pro-Gln-Arg-Phe-
amide (PQRFa) group, e.g., neuropeptide FF (NPFF); and Leu-Pro-Leu/Gln-Arg-Phe-amide (LPXRFa) group, e.g., RFamide-related peptides (RFRPs)]. Among these RFamides, the mRNA expression of zebrafish homolog of PQRFamide (mammalian NPFF) group (zfPQRF) has been reported to be localized in the TN-GnRH neurons (Oehlmann et al. 2002). This localization corresponds to the anterior part of FMRFamide-like immunoreactive neurons in zebrafish embryo (Pinelli et al. 2000). Immunoreactivities to the sGnRH and FMRFamide-like antigen are confined to the TN-GnRH neurons in dwarf gourami as well (Wirsig-Wiechmann and Oka 2002). Therefore it is plausible that anti-FMRFamide antiserum recognizes an unidentified FMRFamide-like peptide that is similar to zebrafish PQRFamide in dwarf gourami TN-GnRH neurons.

It has been reported that NPFF, NPAF, and other related peptides derive from a common PQRFamide precursor in several mammalian species (Perry et al. 1997; Vilim et al. 1999). Among these peptides, NPFF has been well studied. NPFF is expressed in hypothalamus, brain stem, and spinal cord (Vilim et al. 1999), and it has been suggested that NPFF is involved in nociception, the modulation of opioid-induced analgesia, and other physiological processes (Panula et al. 1999; Roumy and Zajac 1998). In teleosts, NPFF and NPAF may also derive from common PQRFamide precursor, considering the amino acid sequences (Fig. 5B). In addition, mass spectrometry showed the existence of mature NPFF and NPFF peptides in the brain of medaka (Suehiro et al. 2009). Unlike mammals, however, it has been reported in zebrafish that the mRNA expression of PQRFamide is localized only in TN-GnRH neurons and not in the caudal part of the brain (Oehlmann et al. 2002).

In this study, we cloned a PQRFamide gene encoding both NPFF and NPAF in dwarf gourami brain. The predicted mature peptides of NPFF and NPAF showed high identities with that of other vertebrates, especially medaka and zebrafish. The amino acid sequences of NPFF are almost identical between dwarf gourami and medaka except one N-terminal residue (Fig. 5B). FMRFamide immunoreactivity in the fibers was almost abolished when the anti-FMRFamide antiserum had been preabsorbed with NPFF (10 μM). In the brain of dwarf gourami, only the TN-GnRH neurons were immunoreactive to FMRFamide, and no other FMRFamide-ir cell bodies were observed (Wirsig-Wiechmann and Oka 2002). Therefore FMRFamide-ir fibers are considered to originate from TN-GnRH neurons. We also showed that the pacemaker activity of TN-GnRH neuron is inhibited by NPFF, which is effective at lower concentration (10 nM–1 μM) than FMRFamide (1–10 μM, EC50 = 2.85 μM).

The inhibitory effect of NPFF was blocked in this study by RF9 (10 μM), a potent and selective antagonist for mammalian NPFF receptors, which was recently developed (Simon et al. 2006). Two G protein–coupled receptors for NPFF have been identified and referred to as NPFF receptor 1 (NPFF-R1) and NPFF receptor 2 (NPFF-R2) (Bonini et al. 2000; Zajac 2001). NPFF-R2 shows a somewhat higher binding affinity for PQRFamides (NPFF, NPAF etc), whereas NPFF-R1 is more responsive to RFRPs (Liu et al. 2001). It has been shown that RF9 selectively antagonize NPFF-R1 and NPFF-R2 at doses ≥10 μM (Simon et al. 2006). Therefore it is suggested that the inhibitory effect of NPFF in dwarf gourami is mediated by the NPFF receptors similar to mammalian NPFF-R1 and/or NPFF-R2. Vyas et al. (2006) reported that FMRFamide exhibits relatively low-binding affinities with human NPFF-R2 than NPFF. Similar difference of binding affinities with endogenous FMRFamide-like peptide receptors can explain the difference of EC50 of the inhibitory effects of FMRFamide and NPFF on pacemaker activity. Therefore we suspect that NPFF, which coexist with GnRH in TN-GnRH neurons of dwarf gourami, may act as an inhibitory auto/paracrine factor (Abe and Oka 2000, 2007), and the activation of NPFF receptors decreases the excitability of TN-GnRH neuron membrane by activating certain kind(s) of K+ conductance(s).

What is the physiological significance of such modulations by FMRFamide-like peptide (possibly NPFF) of the pacemaker activity of TN-GnRH neurons? It has been shown that the TN-GnRH neurons project widely in the brain and sensory organs and function as a neuromodulator through the neuromodulatory effect of sGnRH peptide, which probably regulates the motivational or arousal state of the fish (Abe and Oka 2007). If we assume that FMRFamide-like peptide is also released from the TN-GnRH neurons, it may function as a central or peripheral neuromodulator, as has been suggested in the axolotl olfactory organ (Park et al. 2003). In addition, it has been shown that the firing frequency plays an important role in controlling peptide release of sGnRH peptide from the TN-GnRH neurons (Ishizaki et al. 2004). It has also been reported that sGnRH peptide, which is synthesized by the TN-GnRH neurons, is released not only from the axonal varicosities but also from the somatodendritic area (Abe and Oka 2009). Our previous electrophysiological study suggests that this somatodendritic release induces transient decrease followed by sustained increase of pacemaker activity via auto/paracrine mechanisms (mainly positive feedback) (Abe and Oka 2000). This action is considered to be mediated by GnRH receptors that are expressed in the TN-GnRH neurons themselves (Hajdu et al. 2007). If we assume that the FMRFamide-like peptide acts in a similar manner, it may be possible that the TN-GnRH neurons negatively regulate the pacemaker activity of their own (negative feedback) by releasing FMRFamide-like peptides from somatodendritic region or recurrent collaterals to their own or neighboring cell regions to prevent supramaximal firing activities. A similar mechanism has been proposed in the oxytocin neurons, in which the release of oxytocin from a single neuron decreases excitatory postsynaptic potential (EPSP) of the oxytocin neuron (Hirasawa et al. 2004). Therefore it will be an interesting future topic to analyze physiological mechanisms of this peptidergic neuromodulation induced by FMRFamide-like peptide and to study the possible differential regulation of the pacemaker activity by sGnRH and FMRFamide-like peptide (possibly NPFF).

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No conflicts of interest, financial or otherwise, are declared by the authors.

REFERENCES


