Neuropeptide RFRP inhibits the pacemaker activity of terminal nerve GnRH neurons

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The terminal nerve gonadotropin releasing hormone (TN-GnRH) neurons show spontaneous pacemaker activity whose firing frequency is suggested to regulate the release of GnRH peptides and control motivation for reproductive behaviors. Previous studies of the electrophysiological properties of TN-GnRH neurons reported excitatory modulation of pacemaker activity by auto/paracrine and synaptic modulations, but inhibition of pacemaker activity has not been reported to date. Our recent study suggests that NPFF, a type of Arg-Phe-amide (RFamide) peptide expressed in TN-GnRH neurons themselves, inhibits the pacemaker activity of TN-GnRH neurons in an auto/paracrine manner. In the present study, we examined whether RFRPs (other RFamides), which are produced in the hypothalamus, modulate the pacemaker activity of TN-GnRH neurons as candidate inhibitory synaptic modulators. Bath application of RFRP2, among the three teleost RFRPs, decreased the frequency of firing of TN-GnRH neurons. This inhibition was diminished by RF9, a potent antagonist of GPR147/74, which are candidate RFRP receptors. RFRP2 changed the conductances for Na⁺ and K⁺. The reversal potential for RFRP2-induced current was altered by inhibitors of the TRPC3 channel (La³⁺ and 2APB) and by a less selective blocker of voltage-independent K⁺ channels (Ba²⁺). By comparing the current-voltage relationship in ACSF to that under each drug, the RFRP2-induced current was suggested to consist of TRPC channel-like current and voltage-independent K⁺ current. Therefore, synaptic release of RFRP2 from hypothalamic neurons is suggested to inhibit the pacemaker activity of TN-GnRH neurons by closing TRPC channels and opening voltage-independent K⁺ channels. This novel pathway may negatively regulate reproductive behaviors.
[Keywords]

RFRP, GnRH, peptide, dwarf gourami, neuromodulation
Almost all jawed vertebrates possess terminal nerve (TN) gonadotropin-releasing hormone (GnRH) neurons. Although the hypothalamic hypophysiotropic GnRH neurons facilitate gonadotropin release from the pituitary, the cell bodies of TN-GnRH neurons are located in between olfactory bulb and telencephalon and project their axons throughout the brain but not the pituitary (Yamamoto et al., 1995). TN-GnRH neurons show endogenous pacemaker activity, and their firing frequency is suggested to depend on the physiological state of the animal (Oka and Matsushima, 1993). The resulting release of GnRH peptide may modulate neuronal excitability in many brain areas (Abe and Oka, 2006, 2011). Such modulation may lead to long-lasting changes in the motivational and arousal state for certain animal behaviors (Yamamoto et al. 1997; Abe and Oka 2011). Therefore, understanding the regulatory mechanisms of pacemaker activity by other neurons is important for understanding their neuromodulatory functions of these neurons.

Previous studies suggested that the quantity of sGnRH (salmon GnRH; GnRH3) peptide released is closely related to the firing rate of the TN-GnRH neurons (Ishizaki et al., 2004). GnRH peptide has been reported to modulate the opening of ion channels such as K⁺ channels in the dorsal root ganglia (Adams and Brown, 1980) and the activity of voltage-gated sodium currents in the olfactory epithelium (Eisthen et al., 2000), as well as ionotropic glutamate receptors in the optic tectum (Kinoshita et al., 2007) and hippocampus (Yang et al., 1999). Taken together, these cellular physiological and behavioral studies suggest that the firing rate of TN-GnRH neurons regulates the release of sGnRH peptide, changings the excitability of the target neurons and therefore
modulating animal’s behavior by controlling motivational or arousal state (Oka, 2002; Abe and Oka, 2006).

Our previous studies described the regulatory mechanism of excitatory modulation by sGnRH of pacemaker activity in an auto/paracrine manner (Abe and Oka, 2000) as well as synaptic facilitation by glutamate and GABA released by neurons afferent to the TN-GnRH neurons (Kiya and Oka, 2003; Nakane and Oka, 2010). On the other hand, Saito et al. (2010) reported that NPFF, a member of the Arg-Phe-amide (RFamide) family of neuropeptides, inhibits the pacemaker activity of TN-GnRH neurons in an auto/paracrine manner. However, inhibition of pacemaker activity by synaptic inputs has not been reported in any vertebrate to date.

Recently, various RFamide peptides have been recognized as novel peptides involved in the central regulation of reproduction. Among the RFamides, the relationship between the RFamide related peptide (RFRP) neurons and the hypophysiotropic GnRH1 neurons recently attracts much attention (Ducret et al., 2009), when RFRP was shown to be one of the negative regulators of gonadotropin (FSH and LH) release (Clarke et al., 2009; Pineda et al., 2010). Moreover, neuroanatomical evidence demonstrates that RFRP neurons in the hypothalamus project to the close vicinity of TN-GnRH neurons in medaka (Matsumoto et al., 2006). The metastin (=kisspeptin) immunoreactive neurons and fibers described therein were recently identified as RFRP-immunoreactive. However, we do not know whether or how RFRP affects activity of non-hypophysiotropic or modulatory GnRH neurons. In the present study, we examined the effects of RFRP peptide on the pacemaker activity of TN-GnRH neurons by using whole cell patch clamp recording. We show that RFRP inhibits the pacemaker activities of TN-GnRH neurons by changing K+ and Na+ conductances, which may arise from
the closure of TRPC channels and the opening of voltage-independent (leak) $K^+$
channels.
[Methods]

Adult male and female dwarf gouramis (*Colisa lalia*; \( n = 200 \) fish), ~4 cm in standard length, were purchased from a local dealer. Each aquarium containing ~20 fish was maintained at 27°C on a 12:12 light:dark cycle. The fish were fed with worms daily.

All procedures were performed in accordance with the guideline principles for the care and use of experimental animals established by the Physiological Society of Japan and the University of Tokyo.

For the experiments, the fish were chilled by immersing them in crushed ice and quickly killed by decapitation. After careful removal of ventral meningeal membranes, the olfactory bulbs and telencephalon were separated from the rest of the brain, and the dorsal telencephalon was manually cut out with razor blades in an artificial cerebrospinal fluid (ACSF) consisting of (in mM) 140 NaCl, 5.0 KCl, 1 MgCl₂, 1.5 CaCl₂, 10 HEPES, and 10 glucose (pH 7.4 adjusted with NaOH). This ‘brain block’ containing the olfactory bulb and ventral telencephalon was then placed ventral side up in a hand-made recording chamber and continuously perfused with ACSF.

All the experiments were performed at room temperature (20–24°C). Patch pipette solution contained (in mM) 130 KCl, 3 MgCl₂, 10 HEPES, 0.4 EGTA, and 2 Na₂-ATP (pH 7.4 adjusted with KOH). Patch electrodes were made of borosilicate glass (G-1.5, Narishige, Tokyo, Japan) using a Flaming-Brown micropipette puller (P-97; Sutter Instruments, Novato, CA). The tip resistance of patch electrodes in ACSF was 4–7 MΩ. Because the liquid junction potential was so small and negligible (c.a. 1.2 mV), we did not compensate for it. In our preparations, TN-GnRH neurons of dwarf gouramis form a morphologically distinct neuronal cluster immediately beneath the ventral meningeal membrane (Oka and Ichikawa 1991), and they can be easily identified under a dissecting
microscope or microscopes equipped with differential interference optics (Abe and Oka 2006, 2009; Oka 2002; Oka and Matsushima 1993). The patch pipette was visually guided to the cluster of TN-GnRH neurons, located on the ventral surface of the transitional area between the olfactory bulb and the telencephalon under an upright microscope (E-600FN, Nikon, Tokyo, Japan) equipped with a 40× water-immersion objective lens (0.8 numerical aperture), infrared (IR) differential interference contrast optics, and an IR-CCD camera (C3077-78, Hamamatsu photonics, Hamamatsu, Japan).

All recordings were performed with an Axopatch 200B patch-clamp amplifier (Molecular Devices, Sunnyvale, CA). Whole cell voltage- and current-clamp recordings were digitized (2 k and 10 kHz, respectively) and stored on a computer using the Digidata 1322A and pCLAMP 9.2 software (Molecular Devices, Sunnyvale, CA). After gigaohm seal formation and “break in” for whole cell recording, characteristic spontaneous pacemaker activities were confirmed in current-clamp mode. The membrane resistance and capacitance of these neurons measured 83.0 ± 25.3 MΩ and 184.1 ± 53.2 pF, respectively (n = 5). During the whole recording periods of ramp stimulation experiment procedures, the baseline changes of the current responses were also monitored at 1 kHz using MiniDigi 1A and pCLAMP 9.2 software (Molecular Devices, Sunnyvale, CA).

We assessed the quality and stability of all recordings by applying hyperpolarizing step pulses (from -60 mV to -80 mV, 1.5s) before each ramp protocol to confirm that the membrane conductance had not changed. Statistical analyses were performed with Kyplot5 (Kyence, Tokyo, Japan) and Igor Pro 6 (WaveMetrics Inc., Lake Oswego, OR).

Drugs were dissolved in ACSF and used as follows: RF9 (10 μM, Sigma-Aldrich, St. Louis, MO), tetrodotoxin (TTX; 0.75 μM, Sigma-Aldrich, St. Louis, MO), 4-aminopyridine (4-AP; 5 mM, Wako, Osaka, Japan), Tetraethylammonium (TEA;
20mM, Sigma-Aldrich, St. Louis, MO), LaCl₃ (100 μM, Wako, Osaka, Japan), and 2-aminoethoxydiphenyl borate (2-APB; 100μM, Merck KGaA, Darmstadt, Germany).

Peptides corresponding to medaka RFRP1 (SLDLESFNIRVTPTSSKLNPIIPTAKPLHANMPLRF-NH₂), RFRP2 (VSNSSPNMPQRF-NH₂), and RFRP3 (SVREASPVLPQRF-NH₂) were synthesized by GL Biochem. (Shanghai, China) and used at 0.01 to 2.5 μM. Prior to RFRP applications, bovine serum albumin (0.1 %, 1 ~ 2 min; Sigma-Aldrich, St. Louis, MO) was perfused over the cells to prevent nonspecific binding of the peptides.

All data in this report are presented as means ± SE and the statistical significance levels were set to p < 0.05.
[Results]

Morphological and basic electrophysiological characteristics of TN-GnRH neurons have previously been reported in detail elsewhere (Abe and Oka 2006; Oka 2002; Oka and Ichikawa 1991; Oka and Matsushima 1993).

RFRP2 inhibits the pacemaker activity of TN-GnRH neurons

The regular spontaneous pacemaker activity of TN-GnRH neurons was inhibited by bath application of RFRP2 peptide. Fig. 1A illustrates the inhibition of pacemaker activity by RFRP2 (1 μM). In normal ACSF, TN-GnRH neurons showed regular pacemaker activity (6.3 ± 0.4 Hz, n = 5). The firing frequency of this pacemaker activity was gradually decreased by bath application of RFRP2, and firing was completely inhibited about 2 min after the onset of RFRP2 application. This inhibitory effect of RFRP2 on the pacemaker activity was accompanied by hyperpolarization of membrane potentials (-20.7 ± 0.3 mV, n = 5). After the end of RFRP2 perfusion, the pacemaker activity of TN-GnRH neuron showed an almost complete recovery to its original level.

Many vertebrates possess three forms of RFRP (RFRP1-3), and the three kinds of RFRP peptides in teleosts are processed from the same precursor molecule (Hinuma et al., 2000). To identify the RFRP peptide that inhibits the pacemaker activity of TN-GnRH neurons, we compared the normalized decrease in firing frequency of pacemaker activity by each kind of RFRP peptide at a concentration of 1 μM (Fig. 1B).

The normalized decrease in firing frequency was defined as

\[ \text{Frequency}_{(RFRP+)} / \text{Frequency}_{(RFRP-)} \times 100 \, \% \]

The frequency was calculated from the number of spikes during 0.4 min periods, counted
around 1.5 min before and after the onset of RFRP application (RFRP- and RFRP+, respectively). In Fig.1B, lower normalized frequency indicates stronger inhibition of the pacemaker activity. Among the three types of RFRPs, only RFRP2 application significantly decreased the firing frequency of TN-GnRH neurons [100.1 ± 2.0 % (vehicle; n = 5) versus 102.8 ± 1.8 % (+RFRP1; n = 6; n.s.), 33.6 ± 12.7 % (+RFRP2; n = 9; P < 0.001), and 83.3 ± 8.1 % (+RFRP3; n = 7; n.s.); Dunnet’s multiple comparison test]. We therefore, focused on the effects of RFRP2 in the analysis below.

Next, the normalized decrease in firing frequency of pacemaker activity was plotted against the concentration of RFRP2 (Fig. 1C). This concentration response curve (Fig. 1C solid line) can be well fitted by the equation

\[ R = \frac{[\text{RFRP2}]^n}{[\text{EC}_{50}^n + [\text{RFRP2}]^n]} \]

where R is the normalized decrease in the firing frequency. The EC50 of RFRP2 was determined to be 839 nM, and n was 2.07. Since the n value for Hill’s equation indicates co-operativity, n = 2.07 here demonstrates that the ligand binding reaction is positively cooperative. Once RFRP2 is bound to the receptor, its affinity for the other RFRP2 molecules may increase. For NPFF binding to GPR74 (NPFFR2), on the other hand, Hill’s n is c.a. 1, which suggests that NPFF does not show co-operativity (Gouarderes et al., 1997, Zeng et al., 2003). RFRPs are thus considered to be different from NPFF.
in not only the binding affinity but also the mode of binding, although they do bind to
the same receptor.

**RFRP2 directly inhibits the pacemaker activity of TN-GnRH neurons via specific receptors**

To elucidate possible mechanisms underlying the RFRP2-induced decrease in firing
frequency, we next examined whether RFRP2 directly inhibits pacemaker activities of
TN-GnRH neurons after blockage of action potential-dependent synaptic inputs from
other neurons by TTX application. In the presence of TTX (0.75 μM), RFRP2
reversibly induced hyperpolarization of the membrane potential (-21.3 ± 1.9 mV, n = 6;
Fig. 2). This result suggests that RFRP2 directly modulates the pacemaker activity of
TN-GnRH neurons.

To further understand the possible mechanisms of RFRP2-induced inhibition of
pacemaker activity, we next examined the effect of RF9, a potent antagonist of
GPR147/74, which have been identified as candidate NPFF and RFRP receptors
(Simonin et al., 2006). Pretreatment with RF9 (10 μM) blocked the inhibitory action of
RFRP2 peptide (1 μM) on the pacemaker activity of TN-GnRH neurons (Fig. 3A and B).
In normal ACSF, TN-GnRH neurons showed a regular beating discharge pattern
(5.5 ± 0.4 Hz; Fig. 3Ba). Prior perfusion of RF9 alone did not affect firing activity
In the presence of RF9 (10 μM), the inhibitory effect of RFRP2 (1 μM) was clearly diminished (Fig. 3Bc). The decrease in firing frequency was significantly diminished by RF9 [Fig. 3C; 71.4 ± 6.0 % (n = 6) by RFRP2 with RF9 vs. 100.1 ± 2.0 % (n = 5) by vehicle; n.s.]. RF9 alone did not decrease the firing frequency of TN-GnRH neurons [98.0 ± 2.9 %, n = 6, vs. vehicle; n.s.]. These results suggest that the inhibitory effects of RFRP2 on pacemaker activity of TN-GnRH neurons are mediated by GPR 147/74.

**RFRP2 modifies multiple ion conductances in TN-GnRH neurons**

To examine the ionic mechanisms of RFRP2-induced hyperpolarization of TN-GnRH neurons, we recorded changes in the membrane current at a holding potential of -60 mV in the presence of TTX using whole-cell patch-clamp recordings (Fig. 4A). Perfusion of a supramaximal concentration of RFRP2 (2.5 μM) reversibly induced outward current responses (183.9 ± 21.5 pA; n = 6). Before and during RFRP2 application (Fig. 4A dashed vs solid boxes), we recorded currents in response to slow voltage ramps (Fig. 4B) to determine which ionic permeability contributes to the current changes induced by RFRP2. In this experiment, we depolarized TN-GnRH neurons from -60 to -10 mV for 1.5 s before applying a voltage ramp to inactivate the voltage-gated
calcium channels: the falling phase of the voltage ramp, from -10 to -110 mV during 1.5 s period, was used for plotting the current-voltage (I/V) relationship (dashed rectangle in Fig. 4B). The I/V relationships before RFRP2 (dotted curves) and during RFRP2 perfusion (solid curves) under various extracellular ionic concentrations are shown in Fig. 4C. From the intersection of two I/V curves, we determined the reversal potential for the RFRP2-induced current. In normal ACSF ([K+]o = 5 mM), the recorded reversal potential was -38.0 ± 2.0 mV (n = 6; Fig. 4Cb and D). This value is far from the theoretical equilibrium potentials for K+ (-82.0 mV) and Na+ (+89.6 mV) calculated using the Nernst equation, and instead suggests that RFRP2 changes permeability for multiple ionic species. We therefore, identified the ions comprising the RFRP2-induced current by changing the extracellular concentrations for K+, Na+ and Ca2+.

At different extracellular K+ concentrations ([K+]o; 1 and 20 mM), the reversal potentials for RFRP2-induced currents were -51.0 ± 3.7 (Fig. 4Ca, n = 5) and -13.0 ± 0.7 mV (Fig. 4Cc, n = 5), respectively. Similarly, a decrease in extracellular Na+ concentration shifted the reversal potentials of RFRP2-induced currents (-59.7 ± 2.9 and -49.0 ± 1.5 mV in 46 and 70 mM [Na+]o; n = 5 for both; Fig. 4Cd and e, respectively). When these reversal potentials were plotted as a function of [K+]o or [Na+]o, the relations could be well fitted by straight lines predicted from the Nernst equation for K+ or Na+.
(Fig. 4D; 58 mV per log unit changes, $r^2 = 0.95$ and 0.99 for K$^+$ (○) and Na$^+$ (■), respectively). However, changing extracellular Ca$^{2+}$ concentrations did not shift the reversal potential (-38.0 ± 2.0 and -40.7 ± 2.7 mV in 1.5 and 6 mM [Ca$^{2+}$]o, n = 6 and 5, n.s. by Student’s t-test; Fig. 4Ca and f, and ◊ in Fig. 4D), suggesting that RFRP2 changes permeability for both K$^+$ and Na$^+$, but not for Ca$^{2+}$.

**RFRP2 modulates both TRPC and voltage-independent K$^+$ currents.** Because RFRP2 changed the permeability of both Na$^+$ and K$^+$, we searched for ionic current(s) that are sensitive to RFRP2 application. Subtraction of the ramp current responses recorded during RFRP2 application from those obtained before the application (Fig. 5Aa, dotted and solid curves, respectively) yielded an I/V relationship for the net RFRP2-sensitive current (Fig. 5Ab). The obtained I/V relationship exhibited a region of negative slope conductance from -110 to -70 mV, outward rectification, and a reversal potential at ~ -40 mV. This I/V relationship suggests that RFRP2 blocks an inward current at the physiological membrane potential (~40 ~ -60 mV) of TN-GnRH neurons.

To elucidate the identity of this RFRP2-sensitive current, we next recorded current responses before and during RFRP2 application in the presence of La$^{3+}$ (an antagonist of TRPC1, 3, 6 and 7 and agonist of TRPC4 and 5; 100 μM) or 2-APB (an antagonist of
TRPC3, 4, 5 and 6; 200 μM). Figures 5C and D show subtraction of the current responses recorded before RFRP2 application from those obtained during the application in the presence of La\(^{3+}\) and 2-APB, respectively. The addition of La\(^{3+}\) shifted the reversal potential of the RFRP2-sensitive current from -38.0 ± 2.0 to -83.4 ± 3.4 mV (n = 8; Fig. 5C and F), and the addition of 2-APB resulted in a shift of -90.6 ± 7.2 mV (n = 5; Fig. 5D and F). These plots showed nearly linear I/V relationships with polarities that reversed around the theoretical equilibrium potential for K\(^+\) (-82.0 mV). In addition, we compared the RFRP2-induced current changes at a holding potential of -60 mV (Fig. 5G). La\(^{3+}\) and 2-APB significantly decreased the amplitude of RFRP2-induced changes in the holding current (183.9 ± 21.5 pA in normal ACSF vs. 76.0 ± 16.2 pA in La\(^{3+}\) or 71.2 ± 13.3 pA in 2-APB; n = 6, 8 and 5; p < 0.001 and p < 0.01, respectively). These results suggest that RFRP2 in the presence of La\(^{3+}\) or 2-APB increased the magnitude of a K\(^+\) current that has no voltage dependence, because RFRP2 still induced changes in the holding current and its reversal potential was near the theoretical equilibrium potential for K\(^+\). To confirm the presence of a voltage-independent K\(^+\) current, we next recorded current responses in response to the same voltage ramp in the presence of 0.1 mM Ba\(^{2+}\), which serves as a less-selective blocker of the voltage-independent K\(^+\) currents (= leak K\(^+\) currents). In this experiment, the reversal
potential for the RFRP2-induced current was changed from -38.0 ± 2.0 mV to -12.9 ± 5.5 mV (Fig. 5E and F), and the subtraction of the current responses recorded during RFRP2 application from those obtained before the application exhibited a constant inward current region from -110 to -60 mV (Fig. 5E). The shape of the I/V relationship resembles that of the TRPC current (Qiu et al., 2010). Ba²⁺ also reduced the RFRP2-induced increase in the holding current (Fig. 5G). However, prior application of 4-AP (5 mM), a classical blocker of voltage dependent K⁺ channels, did not change the reversal potential of RFRP2 induced current (-43.3 ± 2.5 mV; Fig. 5B and F, n = 7), although 4-AP did decrease the RFRP2-induced current changes at a holding potential of -60 mV (Fig. 5G). Furthermore, when TEA (20 mM), another blocker of voltage dependent K⁺ channels, was added in either the extracellular or the intracellular solution, the reversal potential for the RFRP2-induced current did not shift (-41.8 ± 7.8 mV with TEA and 5mM 4AP in the extracellular solution, n = 4; -27.1 ± 6.4 mV with TEA in the intracellular solution, n = 6; both n.s. by Dunnet’s test). These results suggest that RFRP2 opens voltage-independent K⁺ current(s). Taken together, we conclude that RFRP2 induces both the closure of TRPC current and the opening of voltage-independent K⁺ currents.
In the present study, we found that RFRP (particularly RFRP2) reversibly inhibits the pacemaker activities of TN-GnRH neurons by simultaneously closing TRPC current and opening voltage-independent K⁺ currents via GPR147/74. RFRP was discovered during the search for a human RFamide using the rat genome database (Hinuma et al., 2000). Previous studies suggested that RFRP3 inhibits the electrical activities of hypothalamic GnRH1 neurons in mice (Ducret et al., 2009). Our recent anatomical observation that RFRP immunoreactive fibers densely project near the TN-GnRH neurons in medaka (Matsumoto et al., 2006) led us to our present electrophysiological investigation to determine whether RFRP affects the pacemaker activity of teleost non-hypophysiotropic TN-GnRH neurons. To the best of our knowledge, this is the first report that hypothalamic reproduction-related peptide, RFRP, affects the firing activity of non-hypophysiotropic TN-GnRH neurons. Our results suggest that RFRP2 inhibits the TRPC current and activates voltage-independent K⁺ currents. First, La³⁺ and 2-APB, antagonists of TRPC, diminished the change in the permeability of multiple ions in response to RFRP2. Both La³⁺ and 2-APB reduced the increase in the holding current by RFRP2. Because RFRP2 increased the outward current, we deduce that RFRP2 also inhibits TRPC-mediated inward currents. Zhang et al. (2008) reported that kisspeptin, another member of RFamide family of peptides, opens TRPC channels in rat GnRH1 neurons. Together, this study and our present results suggest that kisspeptin and RFRP reciprocally open / close TRPC current, because both kisspeptin and RFRP change the permeability of Na⁺ and K⁺ but not Ca²⁺, and their effect was blocked by TRPC antagonists. In the presence of La³⁺ or 2-APB, RFRP2 still induced an outward current with
a reversal potential close to the equilibrium potential for K⁺. This residual RFRP-induced outward current was probably comprised of voltage-independent (= leak) K⁺ current(s), because Ba²⁺, a blocker of voltage-independent K⁺ currents (Lesage, 2003), shifted the reversal potential of RFRP-induced currents and diminished the increase in the holding current by RFRP2. In addition, 4-AP, a classical voltage-dependent K⁺ current blocker, did not inhibit the effect of RFRP2. Therefore, we suggest that RFRP2 decreased TRPC current and increased voltage-independent K⁺ currents. This is supported from the fact that: i) the sum of the increase in RFRP2-induced holding current in the presence of La³⁺ and Ba²⁺ was approximately the same as that in normal ACSF, and ii) the shape of the I/V relationship of RFRP2-induced current in normal ACSF was similar to the sum of those in La³⁺ and Ba²⁺.

How does RFRP modulate voltage-independent K⁺ and TRPC currents? It has been suggested that RFRP receptors (GPR147/74) are coupled to Gᵢ proteins (Mollereau et al., 2002, Fukusumi et al., 2006), and some voltage-independent K⁺ (KCNK) channels are opened through the activation of Gᵢ by noradrenalin (Xiao et al., 2009). As to the signaling mechanisms for the K⁺ conductance modulation, it is interesting to note that serotonin also enhances the opening of leak K⁺ channels called TREK through Gᵢ-coupled receptor signaling (Honore, 2007). Here, the physiological level of cAMP is inhibitory to the opening of TREK and the activation of the Gᵢ-coupled receptor diminishes the level of cAMP, which results in the opening of TREK channels, leading to hyperpolarization. Similar mechanisms may apply to the upregulation of voltage-independent K⁺ currents by RFRP. In addition, activation of Gᵢ inhibits phospholipase C (PLC; Watkins et al., 1994). Because TRPC channels are known to be activated by PLC (Rasolonjanahary et al., 2002), RFRP2 may inhibit TRPC current via
the G\textsubscript{i} signaling pathway.

A previous study suggested that TN-GnRH neurons produce NPFF, another type of RFamide, and inhibit the activities of TN-GnRH neurons in an auto / paracrine manner (Saito et al., 2010). Liu et al. (2001) and Clarke et al. (2009) reported that both NPFF and RFRP activate GPR 147/74. However, our dose-response analysis of RFRP2 action on pacemaker activity suggests that RFRP works at higher concentration than NPFF (Saito et al, 2010). Human RFRPs have lower affinity for NPFFR2 than for NPFFR1 (Liu et al., 2001). In addition, although NPFF causes changes in the K\textsuperscript{+} conductance, RFRP2 causes changes in multiple ionic conductances. Both GPR147/74 are coupled to G\textsubscript{i}, and activation of the G\textsubscript{i} pathway can explain both activation of the voltage-independent K\textsuperscript{+} current and inhibition of the TRPC current. In the case of NPFF receptors, both the NPFF and its super-agonist, PFR(Tic)amide can bind to the same NPFFR1, but they induce different biological effects (Chen et al., 2006). In addition, PFR(Tic)amide is effective at a higher concentration than NPFF and is suggested to cause augmented receptor activation by changing the structures of NPFF receptors. Thus, it may be possible that the increase in K\textsuperscript{+} conductance can be induced by both RFRP and NPFF, but RFRP might overactivate GPR147/74.

A question the arises: what is the physiological significance of the effect of RFRP? As the name implies, TN-GnRH neurons release the peptide GnRH3, and the released GnRH3 is suggested to modulate activities of target neurons (Abe and Oka, 2011). Thus, TN-GnRH neurons are probably responsible for controlling the motivational or arousal state of the animal, including sexual behavior (Yamamoto et al., 1997). In addition, it has been suggested that the firing frequency of TN-GnRH neurons plays an important role in controlling the release of GnRH3 peptide (Ishizaki et al., 2004).
On the other hand, it has been reported in the rat that the somata of RFRP neurons are located in the dorsomedial nucleus of the hypothalamus and their fibers project widely in the brain (Kriegsfeld et al., 2006). Because RFRP was originally discovered as an inhibitor of gonadotropin release (Tsutsui et al., 2000), many researchers have focused on the mechanisms of RFRP actions on pituitary gonadotropes and the effects of RFRP in its projection areas in the CNS and neural inputs to the RFRP neurons have not yet been investigated. In our previous study, Saito et al. (2010) reported that NPFF, which is also a ligand of GPR147/74, inhibits the pacemaker activity of TN-GnRH neurons in an auto- and paracrine manner. Interestingly, the functional role of NPFF peptide in teleosts has not been examined, although NPFF has been reported as one of the mediators of nociceptive stimulus in mammals (Roumy and Zajac, 1998). Fujita et al. (1991) performed an in vivo extracellular recording of TN neuronal activity in goldfish and reported that tail pinching (= nociceptive stimulus) induced a decrease in firing frequency of TN-GnRH neurons. Our previous and present studies demonstrate that the pacemaker activity of TN-GnRH neuron is inhibited by both NPFF and RFRP. In medaka, RFRP immunoreactive cell bodies are located in the hypothalamus, and they densely project their axons near the TN-GnRH neurons (Matsumoto et al., 2006). Taken together, it may be possible that RFRP neurons in the hypothalamus function as an integrator of nociception and reproductive status. Here, the changes in the activity of ionic current, whose gating is controlled at subthreshold membrane potentials by TRPC currents or leak K⁺ currents, probably contributes to the regulation of the activity of TN-GnRH neurons continuously. Our present results may at least partially explain the physiological significance of RFRP at the single cell level, which is believed to serve as an interface between the sensory inputs and the motivation for
reproductive behaviors. Furthermore, the results of the previous studies suggest that
immobilization stress increases RFRP mRNA expressions in rats (Johnson et al., 2007;
Kirby et al., 2009) and in bird brains (Calisi et al., 2008). Further, RFRP neurons
express glucocorticoid receptors and receive stress information via glucocorticoids
released from the adrenal cortex (Kirby et al., 2009). Moreover, intracerebroventricular
(icv) injection of RFRP decreases sexual behavior in mammals (Johnson et al., 2007).
Taken together with these studies, we hypothesize that RFRP may play a pivotal role in
the transmission of stress information from the hypothalamus to TN-GnRH neurons
as a negative motivational signal for sexual behavior. Therefore, it should be
an interesting future topic to analyze whether activation of RFRP neurons modulates
the pacemaker activity of TN-GnRH neurons and changes sexual behaviors.

[Acknowledgements]

We thank Drs. M. K. Park, Yasuhisa Akazome, Shinji Kanda, Ryo Nakane and Takafumi
Kawai (The University of Tokyo) for helpful technical advice and discussion, and
Dr. Heather Eisthen for critical reading of the manuscript. We also thank Ms. Miho
Kyokuwa for their excellent care of the fish used in this study.

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This work was supported by Grants-in-Aid from the Japan Society for the Promotion of
Science (20247005 to Y.O.) and Ministry of Education, Culture, Sports, Science and
Technology (23770072 to H.A. and 20021012 to Y.O.) and PROBRAIN of Japan (to Y.O.).

[References]


16. Ishizaki M, Iigo M, Yamamoto N, Oka Y. Different modes of gonadotropin-releasing hormone (GnRH) release from multiple GnRH systems


33. Roumy M, Zajac JM. Neuropeptide FF, pain and analgesia. *Eur J Pharmacol* 345:


41. Yang SN, Lu F, Wu JN, Liu DD, Hsieh WY. Activation of gonadotropin-releasing hormone receptors induces a long-term enhancement of excitatory postsynaptic currents mediated by ionotropic glutamate receptors in the rat hippocampus.
42. Zeng Z, McDonald TP, Wang R, Liu Q, Austin CP. Neuropeptide FF receptor 2 (NPFF2) is localized to pain-processing regions in the primate spinal cord and the lower level of the medulla oblongata. *J Chem Neuroanat* 25: 269-278, 2003

Figure 1. RFRP2 inhibits the pacemaker activity of TN-GnRH neurons
A: Bath application of RFRP reversibly inhibited the pacemaker activity of TN-GnRH neurons. B: Among the three RFRP peptides, RFRP1-3, only RFRP2 showed a significant decrease in the normalized firing frequency (Dunnet’s multiple comparison test; *** : p < 0.001). C: Dose-response relationship between RFRP2 concentration and normalized frequency during RFRP2 application. Numbers in parentheses near the filled circles represent the numbers of neurons tested for each RFRP2 concentration. The concentration-response curve was fitted with the Hill equation, and the EC$_{50}$ was 839 nM.

Figure 2. RFRP2 directly inhibits the pacemaker activity of TN-GnRH neurons.
RFRP2 hyperpolarized the membrane potential of TN-GnRH neuron in the presence of 0.75 μM TTX. After washout, the membrane potential of TN-GnRH neuron recovered to the pre-treatment level (dotted line).

Figure 3. RF9 (a potent antagonist of GPR147/74, candidate NPFF and RFRP receptors) diminishes the inhibitory effect of RFRP2.
A: In whole cell current clamp recording, prior application of RF9 (10 μM) diminished the inhibitory effect of RFRP2 (1 μM) on pacemaker activity. B: In ACSF, TN-GnRH neurons show a beating discharge pattern (a). Prior application of RF9 alone did not change the firing activity (b). In the presence of RF9, RFRP2 did not show any inhibitory effect on the firing frequency of pacemaker activity (c). C: RFRP2-induced decrease in the normalized frequency was significantly diminished by RF9 (Dunnet’s multiple
Figure 4. RFRP2 changes the membrane permeability for Na\(^+\) and K\(^+\) ions.

A: In voltage clamp recording (holding potential = -60 mV), the holding current was increased by RFRP2 perfusion in the presence of 0.75 \(\mu\)M TTX. B: A voltage ramp protocol was applied before (dotted rectangle in A) and during (solid rectangle in A) the application of RFRP2. C: I/V relationships are plotted using the falling phase of the voltage ramp protocol (dotted rectangle in B), while the extracellular concentration of each ion is changed. The I/V relationships before RFRP2 (dotted curves) and during RFRP2 perfusions (solid curves) under various extracellular ionic concentrations are shown. The ionic composition of normal ACSF is [K\(^+\)] = 5 mM, [Na\(^+\)] = 140 mM and [Ca\(^{2+}\)] = 1.5 mM. D: The reversal potentials of RFRP2-induced currents are plotted as logarithmic functions of the extracellular ion concentrations (○ for K\(^+\), ■ for Na\(^+\) and ◊ for Ca\(^{2+}\)). The lines indicate the slopes for K\(^+\) (solid line) or Na\(^+\) (dashed line) that were calculated from the Nernst equation.

Figure 5. RFRP2 modulates TRPC and voltage-independent K\(^+\) currents.

A: I/V relationship of the RFRP2-induced current in normal ACSF. (a) Current responses of a TN-GnRH neuron before (dotted line) and during (solid line) RFRP2 application. (b) Subtracted current response during and before RFRP2 applications is used to generate the I/V relationship of net RFRP2-sensitive current. B: The I/V relationship of RFRP2-sensitive current in the presence of 4-AP (5 mM), a blocker of voltage-dependent K\(^+\) channels. It is not changed by 4-AP. C, D: The I/V relationships of RFRP2-sensitive currents in the presence of La\(^{3+}\) (100 \(\mu\)M; C) or 2-APB (200 \(\mu\)M; D), comparison test; *** : p < 0.001).
antagonists of TRPC1, 3, 6 and 7 (La$^{3+}$) or TRPC 3, 4, 5 and 6 (2-APB). Note that the shape of the I/V relationships indicates no clear rectification in the presence of La$^{3+}$ or 2-APB. E: I/V relationship of RFRP2-sensitive current in the presence of Ba$^{2+}$ (100 μM), a less selective blocker of voltage-independent K$^+$ channels. Note that the reversal potential becomes more depolarized to ~ -20 mV and the shape of the I/V relationship exhibits a constant inward current region from -110 to -60 mV, resembling the TRPC current. F: The reversal potentials for RFRP2-sensitive current are more depolarized, in comparison with normal, in the presence of Ba$^{2+}$ or are more hyperpolarized in the presence of La$^{3+}$ or 2-APB (Dunnet’s multiple comparison test; ** : p < 0.01, *** : p < 0.001). G: RFRP2-induced outward shift in holding currents is sensitive to 4-AP, Ba$^{2+}$, La$^{3+}$, and 2-APB. Each current difference is calculated by subtracting the holding current before application of RFRP2 from that measured during RFRP2 application (Dunnet’s multiple comparison test; * : p < 0.05, ** : p < 0.01).
A

Voltage (mV)

0 5 10 Time (min)

100 80 60 40 20 0

Normalized frequency (%)

Vehicle RF9 RFRP2 RF9 RFRP2

***

RF9 (10µM) RFRP2 (1µM)

B

a) ACSF

Voltage (mV)

0 5 10 Time (sec)

ACSF

b) RF9 (10µM)

Voltage (mV)

0 5 10 Time (sec)

b) RF9 (10µM)

c) RF9 (10µM) + RFRP2 (1µM)

Voltage (mV)

0 5 10 Time (sec)

c) RF9 (10µM) + RFRP2 (1µM)

C

Normalized frequency (%)

Vehicle RF9 RFRP2 RFRP2 + RF9

(5) (6) (5) (9)