GnRH suppresses excitability of visual-processing neurons in the optic tectum

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Animals change their behavior in response to sensory cues in the environment as well as their physiological status. For example, it is generally accepted that their sexual behavior is modulated according to seasonal environmental changes or the individual’s maturational/reproductive status, and neuropeptides have been suggested to play important roles in this process. Some behavioral modulation arises from neuropeptide modulation of sensory information processing in the central nervous system, but the neural mechanisms still remain unknown. Here, we focused on the neural basis of neuropeptide modulation of visual processing in vertebrates. The terminal nerve neurons that contain gonadotropin releasing hormone 3 (TN-GnRH3 neurons) are suggested to modulate reproductive behavior and have massive projections to the optic tectum (OT), which plays an important role in visual processing. In the present study, to examine whether GnRH3 modulates retino-tectal neurotransmission in the OT, we analyzed the effect of GnRH3 electrophysiologically and morphologically. We found that field potentials evoked by optic tract fiber stimulation, which represent the retino-tectal neurotransmission, were modulated postsynaptically by GnRH3. Whole-cell recording from the postsynaptic neurons in the retino-tectal pathway suggested that GnRH3 activates BK channels and thereby suppresses the membrane excitability. Furthermore, our improved morphological analysis using fluorescently
labeled GnRH peptides showed that GnRH receptors are localized mainly around the cell bodies of the postsynaptic neurons. Our results indicate that TN-GnRH3 neurons modulate the retino-tectal neurotransmission by suppressing the excitability of projection neurons in the OT, which underlies the neuromodulation of behaviorally relevant visual information processing by the neuropeptide GnRH3.
Animals modify their behavior in response to their physiological status as well as sensory cues in the surrounding environment. For example, for successful reproduction, during the breeding season, sensory information processing in sexually mature animals changes in order to facilitate detection, attraction, or acceptance of their partners, and to enable performance of sexual behaviors. Such sexual behavior in many animals is modulated according to seasonal environmental changes or their maturational/reproductive status, and neuropeptides have been suggested to play important roles in such modulation (Argiolas and Melis 2013; Dornan and Malsbury 1989). Fish provide a good model for investigating the mechanisms of neuropeptide action on behaviorally relevant sensory processing systems. For example, male goldfish not only use chemical but also visual cues when chasing females during the breeding season (Thompson et al. 2004), and sailfin mollies show seasonal variation in mate choice preferences based on visual cues (Heubel and Schlupp 2008). Such changes in behaviors are suggested to arise from neuropeptide modulation of sensory information processing in the central nervous system, but the neural mechanisms still remain unknown. Here, we focused on the neural basis of peptidergic neuromodulation of visual processing in the optic tectum. In non-mammalian vertebrates including teleosts, the non-geniculate system through the optic tectum plays essential roles in visual
information processing (Karten and Shimizu 1989; Kinoshita and Ito 2006; Springer et al. 1977; Yager et al. 1977).

The terminal nerve neurons that contain gonadotropin releasing hormone 3 (TN-GnRH3 neurons) are suggested to receive information concerning reproductive status (Onuma et al. 2005; Zempo et al. 2013) and modulate reproductive behaviors (Yamamoto et al. 1997). These neurons also have massive projections to the optic tectum in teleosts (Amano et al. 2002; Oka and Matsushima 1993; von Bartheld and Meyer 1986). Therefore, we hypothesized that TN-GnRH3 neurons play an important role in modulating visual information processing according to reproductive status.

TN-GnRH3 neurons release the peptide GnRH3, and GnRH3 has been shown to modulate activity in the olfactory bulb (Kawai et al. 2010), in olfactory receptor neurons (Eisthen et al. 2000), and in retinal circuits (Umino and Dowling 1991). Here, we examined whether GnRH3 modulates the retino-tectal synaptic transmission by analyzing field potentials and the membrane excitability of visual information processing neuron in the dwarf gourami, a teleost fish that has frequently been used for the study of neuromodulatory GnRH neurons (Abe and Oka 2002; Ishizaki et al. 2004; Wirsig-Wiechmann and Oka 2002).

Our field potential analysis revealed that GnRH3 modulates the retino-tectal
neurotransmission postsynaptically. Furthermore, we showed that GnRH3 suppresses excitability of the stratum periventricular (SPV) neurons, which have been reported as postsynaptic neurons for the retino-tectal neurotransmission (Kinoshita et al. 2005; Vanegas et al. 1974a; Vanegas et al. 1971), by activating large conductance Ca\(^{2+}\)-activated K\(^+\) (BK) channels. Finally, we examined the localization of GnRH receptors in the optic tectum morphologically using an improved method that employs GnRH peptides labeled with fluorescence (GnRH-fluoroprobe). In brain slices treated with GnRH-fluoroprobe, the fluorescent signals were observed mainly near the cell bodies of the SPV neurons. The results of the present electrophysiological and morphological analyses suggest that GnRH3 modulates the retino-tectal neurotransmission by suppressing the excitability of postsynaptic neurons in the optic tectum.
[Material and Methods]

Animals

Adult male dwarf gouramis (*Trichogaster lalius*), ~5 cm in standard length, were purchased from a local dealer. Each aquarium containing ~20 fish was maintained at 27°C on a 14:10 light:dark cycle. All fish were fed worms daily. All procedures were performed in accordance with principles for the care and use of experimental animals established by the Physiological Society of Japan and the University of Tokyo.

Slice preparation

Fish were anesthetized by chilling in ice. We quickly killed them by decapitation and isolated the whole brain. The brain was put in an artificial cerebrospinal fluid (ACSF) consisting of (in mM) 134 NaCl, 2.9 KCl, 1.2 MgCl₂, 2.1 CaCl₂, 10 HEPES, and 10 glucose (pH 7.4 adjusted with NaOH). We embedded the brain in a 3.6% low melting temperature agar (Agarose type IX-A, Sigma-Aldrich, St. Louis, MO) solution prepared with ACSF. 400 μm sagittal sections were cut in ACSF containing choline chloride instead of NaCl using a Vibratome 3000 (Leica Biosystems, Germany). The brain slices were incubated in ACSF on ice for one hour and brought back to room temperature before the experiments.
Electrophysiology

For field potential recordings, a slice was placed in a chamber filled with ACSF containing 0.1 % BSA to prevent nonspecific binding of peptides to the plastic and glassware. This ACSF was perfused gravitationally at 1-2 ml/min. Recording electrodes were made of borosilicate glass (GD-1.5; Narishige, Tokyo, Japan) using a Flaming-Brown micropipette puller (P-97; Sutter Instruments, Novato, CA). The borosilicate electrode filled with 0.1 % agar in 2 M NaCl was placed 100μm under the surface of the brain slice for recording in the superficial tectal layers (Stratum opticum, SO, and Stratum fibrosum et griseum superficiale, SFGS), where the retino-tectal synapses are formed on the distal dendrites of SPV neurons and the largest synaptic responses were recorded (Vanegas 1983; Vanegas et al. 1971), ~ 200 μm away from the stimulating electrode. The field potential was amplified by AVH-11(x2,000, Nihon Kohden, Tokyo, Japan) and recorded using a Digidata1322A and pClamp8.2 software (Molecular Devices, Sunnyvale, CA). Electrical stimuli were applied using an electronic stimulator (SEN-3301; Nihon Kohden, Tokyo) through an isolation unit (SS-201J; Nihon Kohden). The bipolar stainless steel electrode consisted of a pair of electrolytically polished stainless steel insect pins, approximately 10 μm apart and 1 μm in tip diameter, which were lacquer-coated, leaving 20- to 30-μm uncoated bared tips.
For stimulation of the optic tract fibers, we placed the bipolar stainless steel stimulating electrode in the superficial tectal layers, the main layers of optic tract fiber projections (SO and SFGS). Electrical pulse stimuli of 0.1 ms duration at 0.5–1 mA were applied every 30 second. Synthetic GnRH3 peptides were purchased from GL Biochem (Shanghai, China). 5 min after the start of recording, GnRH3 was applied for 4 min. We used the following drugs depending on experiments; CNQX: 6-cyano-7-nitroquinoxaline-2,3-dione (Abcam, Cambridge, UK), Antide (Sigma Aldrich, St. Louis, MO, US), IBX: iberiotoxin (Abcam, Cambridge, UK).

For whole-cell recording, the internal solution consisted of (in mM): K-gluconate 100, KCl 30, EGTA 0.05, MgCl₂ 4, HEPES 10, K₂ATP 4, and Na₂GTP 0.5. The junction potential was -12 mV, and membrane potentials were adjusted using this value. The tip resistance of patch electrodes in ACSF was 10-15 MΩ. Recordings were performed using an Axopatch 200B patch-clamp amplifier (Molecular Devices, Sunnyvale, CA). Whole-cell current- and voltage-clamp recordings were digitized at 10 kHz and stored on a computer using a Digidata 1322A and pCLAMP 9.2 software (Molecular Devices, Sunnyvale, CA). For analysis of membrane excitability, we used the minimum response that elicited at least one action potential during current injections of three different intensities (0.01, 0.015, or 0.02 nA).
Luciferase assay

HEK293T cells 70-80% confluently were cultured in 100 mm dishes. The culture medium contained 5% FBS (Life Technologies, Carlsbad, CA), 4 mM L-glutamine (Wako, Japan), and 4.5 g Dulbecco’s Modified Eagle Medium (Nissui Pharmaceutical CO., LTD, Japan) in 500 ml water. 30 μl polyethylenimine (PEI, Polyethylenimine "Max", Polysciences, Inc., Warrington, PA) and 1.5 ml Opti-MEM (Life Technologies, Carlsbad, CA) were mixed and incubated for 5 min. We then added the following constructs into another 1.5 ml Opti-MEM; each GnRH receptor 5.5 μg (gift from Dr. Okubo, (Okubo et al. 2003; Okubo et al. 2001)), 9 μg firefly luciferase vector (pGL4.33[luc2P/SRE/Hygro] Vector, Promega, Madison, WI) and 0.5 μg renilla luciferase vector (pGL4.74[hRluc/TK] Vector, Promega, Madison, WI). After incubation in PEI solution, both Opti-MEM solutions were mixed and incubated for 20 min at room temperature. We then replaced the culture medium with mixed transfection solution and incubated the dish at 37°C overnight. The transfected HEK cells were then subcultured into 24 well plates in a culture medium containing 1% FBS. 18 hours later, 1 μM GnRH2 or 1 μM fluorescence probe in the culture medium containing 1% FBS (vehicle) or the vehicle only were applied for 6 hours. GnRH-fluoroprobes
[pGlu-His-Trp-Ser-His-D-Lys(\(\text{N}^\epsilon\)-FAM)-Trp-Tyr-Pro-Gly\(\text{NH}_2\) (GnRH-FL) and

\(\text{pGlu-His-Trp-Ser-His-D-Lys(\(\text{N}^\epsilon\)-TAMRA)-Trp-Tyr-Pro-Gly-NH}_2\) (GnRH-TAMRA)]

were designed based on a previous report (Hazum et al. 1980). Probes were synthesized

using a standard Fmoc-based solid-phase peptide synthesis (Oishi et al. 2008). Cells

were collected and the luminescence measured by LUMAT LB9507 (Berthold

Technologies, Germany) according to the manual for Dual-Luciferase Reporter Assay

System (Promega).

Analysis of the localization of GnRH receptors in HEK cells

For morphological observation, HEK cells were cultured on poly-L-lysine coated glass

(Matsunami glass Ind., Ltd., Japan) in 60 mm dishes. The GnRH receptor (1 \(\mu\)g / 60 mm

dish) and EGFP-N1 (0.4 \(\mu\)g / dish, Takara, Japan) were transfected to HEK293 cells

with polyethylenimine (2.88\(\mu\)l / dish) as described previously. After 2 overnight

incubations, each glass covered with cells was put in a 24 well plate filled with

serum-free medium. 30 min later, HEK cells were incubated with 1 \(\mu\)M

GnRH-fluoroprobe or plain serum-free medium for 30 min at 37°C. Then, we washed

cells with PBS twice and examined them under a confocal laser-scanning microscope

LSM-710 (Carl Zeiss, Germany) at 1-\(\mu\)m optical section.
Analysis of the localization of GnRH receptors with fluoroprobes

200 μm thick frontal slices were used for the analysis. Brain sections were cut as described above and incubated at room temperature for 1 hour after slicing. To block non-specific binding, slices were treated with 0.1% BSA/ACSF for 30 minute and then the fluorescence probe solution or vehicle control was applied for 1 hour. The GnRH-fluoroprobe [pGlu-His-Trp-Ser-His-D-Lys($N^\epsilon$-FAM)-Trp-Tyr-Pro-Gly-NH$_2$ (GnRH-FL)] was designed based on a previous report (Hazum et al. 1980). We chose GnRH2 as the backbone of GnRH-fluoroprobe, because GnRH2 shows the highest affinity for each GnRH receptor in fish for GnRH1-3 (Illing et al. 1999; Okubo et al. 2003; Okubo et al. 2001). The probe was synthesized using a standard Fmoc-based solid-phase peptide synthesis (Oishi et al. 2008). After application of the GnRH-fluoroprobe, the brain slices were fixed with 4% PFA/PBS at 4°C overnight. The slices were then incubated for 6-8 h with a mouse antiserum against fluorescein, diluted 1:1,000 in the blocking solution. For analysis of localization of GnRH receptors with fluoroprobes, the signals were intensified by sequentially incubating the slices in biotinylated anti-mouse IgG (Vector Laboratories, Burlingame, CA) for 6-8 h, the ABC kit (Vector Laboratories, Burlingame, CA) in PBST for 30-45 min, and
streptavidin-Alexa555 for 4h. Nuclei were counter-stained by Hoechst 33342 (Life Technologies, Carlsbad, CA). The slices were then mounted on slides and coverslipped with CC/Mount (Diagnostic BioSystems, Pleasanton, CA). The slides were examined and photographed using a confocal laser-scanning microscope LSM-710 (Carl Zeiss, Germany), and the resulting images were analyzed using ImageJ software with the MBF ImageJ plug-ins (Tony Collins, McMaster University). All images were captured in the same exposure condition, and we used the same brightness and contrast values for ImageJ in all three groups.

Statistics

Statistical analyses were performed using the Kyplot5 software (Kyence, Tokyo, Japan) and the Igor Pro 6 software (WaveMetrics Inc., Lake Oswego, OR) with the Taro tool (an Igor macro set written by Dr. Taro Ishikawa, Jikei University School of Medicine). Different statistical tests were used for different experiments, as described in the Results and Figure legends. All data in the present study are presented as mean ± S.E.M.
[Results]

GnRH3 modulates retino-tectal neurotransmission.

In non-mammalian vertebrates, retino-tectal neurotransmission is important for visual processing (Karten and Shimizu 1989; Kinoshita and Ito 2006). It has been proposed that synaptic transmission from optic tract fibers to the deep tectal (SPV) neurons (retino-tectal neurotransmission) is essential for visual processing in the optic tectum (Kinoshita and Ito 2006; Vanegas 1974; Vanegas et al. 1974b). We examined whether GnRH3 modulates the retino-tectal neurotransmission by stimulating optic tract fibers and recording the evoked local field potential (LFP) from the synaptic region of SPV neurons (Vanegas et al. 1974b). Because the tectal neurons form distinctive layers and show characteristic LFPs in each layer, field potential recordings have often been used in analyses of retino-tectal neurotransmission (Vanegas 1983; Vanegas et al. 1971). The field potential evoked by electrical stimulation is shown in Fig.1A, B. The first large positive peak indicates an artifact produced by stimulation and almost masks the presynaptic responses. The subsequent large negative wave most probably represents the postsynaptic response of the SPV neurons in the optic tectum (Kinoshita and Ito 2006; Vanegas et al. 1974a). This synaptic transmission is likely monosynaptic (Kinoshita and Ito 2006; Vanegas et al. 1974b). Because the negative waves were almost completely blocked by 20μM CNQX (Fig. 1A), they probably reflect
glutamatergic neurotransmission. Thus, the negative waves are at least suggested to contain monosynaptic glutamatergic current components of SPV neurons in response to the optic tract fiber stimulation. It should be noted, however, that the negative waves have also been suggested to contain regenerative population spike components (see Vanegas 1983 as well as the Discussion, below). Then, the evoked LFPs were recorded and compared with those in the presence of GnRH3. Fig. 1B shows representative traces before, during and after GnRH3 application. GnRH3 application increased the rise time from 10% to 90% of peak amplitude (Fig. 1C) and the negative wave duration measured at half maximum amplitude (Fig. 1D). These effects were diminished by antide, which is a GnRH receptor antagonist (Fig. 1C; Vehicle: 91.8 ± 3.9 %, GnRH3: 134.0 ± 15.9 %, GnRH3 + Antide: 93.9 ± 4.2 %; Steel’s test, *: p < 0.05, Fig. 1D; Vehicle: 95.9 ± 1.4 %, GnRH3: 115.8 ± 4.1 %, GnRH3 + Antide: 98.1 ± 3.1 %; Steel’s test, **: p < 0.01). On the other hand, the peak amplitudes of the field potentials were not significantly affected by GnRH3 application (Fig. 1E; Vehicle: 98.8 ± 1.0 %, GnRH3: 92.6 ± 6.0 %, GnRH3 + Antide: 102.2 ± 7.4 %; Steel’s test, n.s.). Overall, our results indicated that GnRH3 modulated the shape of LFPs evoked by stimulation of optic fibers, which is suggested to be mediated by the GnRH receptors (see below).
GnRH3 modulates the LFP postsynaptically

We used paired pulse ratio to determine whether GnRH3-induced modulation is a presynaptic or postsynaptic effect. Comparing the paired pulse ratio (PPR: the ratio of amplitude of the responses to the first and the second stimulation) before and after treatment allows one to determine whether the effect is pre- or postsynaptic (Manabe et al. 1993); if the PPR changes after treatment, the modulation is suggested to be presynaptic, and vice versa. Fig. 2A shows the LFP in response to paired stimulation of retino-tectal fibers at an interval of 150 ms (Kawai et al. 2010). Fig. 2B shows the time course of the PPR. Given that the PPRs appear not to change before and after GnRH application, the results suggest that GnRH3-induced modulation is mainly postsynaptic.

GnRH3 suppresses excitability of visual processing neurons in the optic tectum

In the optic tectum, the SPV neurons receive direct synaptic inputs from optic tract fibers and project to the other brain regions. SPV neurons are thought to play a principal role in visual information processing in the teleost optic tectum (Kinoshita et al. 2005; Kinoshita and Ito 2006; Vanegas 1974; Vanegas et al. 1974b). The data presented in Figs. 1 and 2 suggest that GnRH3 modulates these postsynaptic SPV neurons. For further analysis of the postsynaptic mechanism induced by GnRH3, we examined effects of GnRH3 on the excitability of the SPV neurons using whole-cell patch clamp.
recording. We generated action potentials in SPV neurons by applying a series of current-steps (50 msec, 0.01~0.02 nA) through a recording whole-cell pipette. Figures 3A-C show representative action potentials of a SPV neuron before (A), during (B), and after (C) application of GnRH3. As illustrated in Figure 3B the number of action potential decreased during application of GnRH3. The normalized response for each condition is plotted in Fig. 3D. The vertical axis indicates the response normalized by the spike number evoked by the minimum current step before GnRH3 application. GnRH3 markedly suppressed the ability of spike generation in the SPV neurons (Before: 100%, GnRH3: 45.3 ± 17.3 %, Washout: 107.1 ± 7.1 %; ANOVA and Tukey Kramer’s test, n = 7 in each group, **: p < 0.01). The result clearly demonstrates that GnRH3 suppressed the excitability of SPV neurons in the optic tectum.

GnRH3 activates large conductance Ca²⁺-activated K⁺ channel

To analyze the ionic mechanisms underlying GnRH3-induced modulation, we compared the current-voltage relationships of SPV neurons with or without GnRH3 application. We applied ramp stimulation (Fig. 4A inset) and analyzed the current-voltage relationship before, during, and after GnRH3 application under voltage clamp (Fig. 4A). The results indicate that the current increased during GnRH3 application (black solid line) and recovered after washout (gray solid line) to the level
before application (black dotted line). The reversal potential (calculated by the point of intersection of black solid and dotted lines) was $-85.6 \pm 2.4 \text{ mV (n = 7)}$, which was near the calculated reversal potential for potassium, $-97 \text{ mV}$. Therefore, this result suggests that GnRH3 modulates one or more $K^+$ conductance. Because GnRH receptors are coupled to $G_{q/11}$-protein (Naor et al. 1995; Stojilkovic et al. 1994a), GnRH3 is expected to modulate the current-voltage relationships via modulation of $K^+$ conductance(s) activated by intracellular Ca$^{2+}$ increase. To examine whether Ca$^{2+}$-activated $K^+$ channels are modulated by GnRH3, we analyzed the GnRH3-induced current recorded in plain ACSF or in ACSF containing iberiotoxin (IBX), a blocker of large conductance Ca$^{2+}$-activated $K^+$ channel, BK channel (Waring and Turgeon 2009). Fig. 4B shows the GnRH3-induced current ([current during GnRH3 application] – [current before GnRH3 application]). The GnRH3-induced current (black dots) was almost completely blocked by IBX application (gray dots). This result suggests that GnRH3 suppresses the excitability of SPV neurons by activating BK channels.

Next, we examined whether blockage of BK channel diminishes the GnRH3-induced modulation of the field potential. We performed the field potential recording as shown in Fig 1B while blocking BK channels by perfusing IBX during the entire recording (Fig. 5). When GnRH3 was applied in the presence of IBX, the GnRH3-induced
modulation of the field potential was nullified (Fig. 5A); as shown in Figs. 5B and 5C, the differences in rise time and the duration measured at half maximum amplitude were not significant (Fig. 5B; Vehicle: 100.6 ± 0.2 % vs. GnRH3: 98.3 ± 1.9 %; Student’s t-test, n.s., Fig. 5C; Vehicle: 99.0 ± 2.0 % vs. GnRH3: 103.0 ± 3.6 %; Student’s t-test, n.s.). This result strongly suggests that GnRH3 modulates the retino-tectal neurotransmission by activating BK channels of the SPV neurons postsynaptically.

The localization of GnRH receptors in optic tectum

Finally, we examined the localization of GnRH receptors in SPV neurons in brain slices using a slight modification of a method involving fluorescently-labeled GnRH peptides (GnRH-fluoroprobe) developed for dissociated cells (Hazum et al. 1980; Hazum and Nimrod 1982; Lloyd and Childs 1988). Because technical difficulties so far have prevented analysis of the localization of GnRH receptors by immunohistochemistry or in situ hybridization in the brain, we here examined the localization of GnRH receptors in the optic tectum by an improved method using GnRH-fluoroprobe. We first demonstrated that our synthesized fluoroprobe selectively binds to GnRH receptors using a luciferase assay and morphological binding assay (see Methods section for more information, images not shown). We used fluorescein-labeled GnRH for brain slices because the fluorescein signals can be amplified using
immunohistochemical method. GnRH-fluoroprobe was applied for 1 hour and was amplified following the methods as described in Material and Methods. Fig. 6 shows GnRH-fluoroprobe signal (A), Hoechst nuclear counter-stain (B), and overlay (C, D) in the optic tectum. As shown in Fig. 6C and D, the GnRH-fluoroprobe signals were observed to surround the cell bodies of the SPV neurons (Figs. 6Aa, Ca, Da). Vehicle application did not result in specific binding (Figs. 6Ab, Cb, Db). The binding of the fluoroprobe was competitively inhibited by a high-dose (10 μM) of non-labeled GnRH3 peptide (Figs. 6Ac, Cc, Dc). The binding of fluoroprobes on synaptic terminals was hard to detect, indicating that the concentration of GnRH receptors in the terminals is very low (Maruska and Tricas 2007). The results of the GnRH-fluoroprobe experiment provide morphological supports for the hypothesis that GnRH3 modulates postsynaptic neurons, the SPV neurons, indicated by our electrophysiological results.
In the present study, we showed that GnRH3 modulates the retino-tectal neurotransmission postsynaptically. GnRH3 increased the rise time and the duration measured at half maximum amplitude of the field potentials evoked by the optic tract fiber stimulations. GnRH3 also suppressed the excitability of SPV neurons by activating large conductance Ca\(^{2+}\)-activated K\(^+\) channels, BK channels. Our morphological analysis using a GnRH-fluoroprobe indicated that SPV neurons express GnRH receptors.

**GnRH3 suppresses the excitability of the SPV neurons and modulates LFPs by activating BK channels via GnRH receptors**

We first analyzed field potentials which represent monosynaptic neurotransmission from optic tract fibers to SPV neurons in the optic tectum (Vanegas et al. 1974a; Vanegas et al. 1974b). The results of analysis showed that GnRH3 modulates the field potential, and our PPR analysis indicates that the modulatory effects are postsynaptic. We then showed that GnRH3 suppresses the excitability of SPV neurons by activating BK channels. It has been generally accepted that currents that are induced by BK channels decrease the number of action potentials and thereby suppress the excitability of neurons (Arias-Garcia et al. 2013; Sun and Dale 1998).
We next analyzed the distribution of GnRH-fluoroprobe binding sites as a way to examine the localization of GnRH receptors. Previous immunohistochemical studies in teleost brains indicate that TN-GnRH3 neurons have massive projection to the deep layers (SAC: stratum album central, and SGC: stratum griseum centrale) and sparse projection to the surface layers in the optic tectum (Maruska and Tricas 2007; Oka and Ichikawa 1990). On the other hand, the optic tract fibers mainly project to the superficial layers (SO and SFGS, where we recorded LFPs), and also sparsely to SAC and SGC (Maruska and Tricas 2007; von Bartheld and Meyer 1987). Our analysis of the localization of GnRH receptors in the optic tectum using GnRH-fluoroprobe showed that GnRH receptors are expressed in SPV neurons. Taken together, these results suggest that TN-GnRH3 neurons mainly act on the cell bodies of the SPV neurons in the deep layer of the optic tectum.

Fig. 7 schematically shows the hypothetical model (A), the GnRH3-induced modulation of the field potential (B), and SPV neuron action potentials (C). We illustrated the schematic diagram in Fig. 7B by referring to Vanegas (Vanegas 1983), who suggested that the long dendrite of the SPV neuron possesses an ability to generate regenerative action potentials, and Rall & Shepherd (Rall and Shepherd 1968) who also suggested the presence of active dendrites of the mitral cell. We further assumed that the
LFP evoked by optic nerve stimulation represents superimposition of EPSPs and back-propagated dendritic spikes. The dotted and solid lines indicate the LFP before and during GnRH3 application, respectively. Although the difference in the anatomical localization of GnRH-receptors (somatic region of SPV neurons in deep layer) and recording site of the LFPs (synaptic region in superficial layer) may appear puzzling at first sight, the above-mentioned interpretation of the LFP can reasonably explain the present experimental results. Actually, Vanegas (see p59, 60, Vanegas 1983; Fig.1, Vanegas et al. 1974a) suggested that the negative component of the LFP evoked by the stimulation of optic nerve in fish optic tectum was the superimposition of extracellularly recorded EPSPs and regenerative population spikes. He also suggested that the rise time and duration of the LFP evoked by optic tract fiber stimulation change as shown in Fig. 7B (present study), when the number of action potentials of SPV neurons was experimentally decreased.

In summary, considering our electrophysiological and morphological results, we propose a model of GnRH3-induced modulation of the retino-tectal neurotransmission (Fig. 7A). First, GnRH3 activates BK channels in SPV neurons, suppressing the excitability of those neurons (Fig. 7C). Then the number of synaptically driven action potentials in SPV neurons evoked by optic tract fiber stimulation is decreased because
GnRH3 suppresses excitability. As a result, the rise time and the duration of the LFP increase (Fig. 7B).

Possible intracellular mechanisms of GnRH3-induced modulation

Here, we discuss possible intracellular mechanisms of GnRH3-induced modulation. Although one previous study in rainbow trout examined GnRH-induced modulation of retino-tectal neurotransmission (Kinoshita et al. 2007), the study did not analyze the mechanisms in detail. The results of the present study suggest the following mechanisms. It has been reported that GnRH receptors are G_{q/11} protein coupled receptors (Naor et al. 1995; Stojilkovic et al. 1994a). The G_{q/11} type G-proteins are coupled to the phospholipase C-mediated signaling pathway (Naor et al. 1995; Stojilkovic et al. 1994a; Stojilkovic et al. 1994b). By activating this pathway, GnRH3 induces intracellular Ca^{2+} increase due to release from IP_{3}-sensitive Ca^{2+} stores (Abe and Oka 2002; Karigo et al. 2014). This intracellular Ca^{2+} increase is considered to activate BK channels. On the other hand, phospholipase C-mediated signaling pathways also induce diacyl glycerol production (Naor et al. 1995). Diacyl glycerol can be converted to arachidonic acid, and arachidonic acid in turn activates BK channels in mouse vomeronasal neurons (Zhang et al. 2008). One or both of these pathways,
intracellular Ca\textsuperscript{2+} increase and/or arachidonic acid modulation, may operate in SPV neurons and suppress the excitability of SPV neurons via activation of BK channels. In the presence of an intracellular solution with high EGTA (1 mM) and no GTP, GnRH\textsubscript{3} did not induce a K\textsuperscript{+} current (data not shown), which strongly supports our hypothesis that GnRH\textsubscript{3} activates BK channels by causing an increase in intracellular Ca\textsuperscript{2+}. Similar modulation of BK channels by GnRH has also been reported in previous studies of gonadotrophs (Sikdar et al. 1989; Waring and Turgeon 2009). Previous studies of modulation of neuronal excitability by GnRH have focused on facilitation caused by inhibition of the K\textsuperscript{+} M-current (Brown 1988). Thus, to our knowledge, the present study is the first one showing the inhibition of the neuronal excitability by GnRH\textsubscript{3}.

The expected physiological functions of GnRH\textsubscript{3}-induced modulation

The retino-tectal neurotransmission plays a central role in visual processing in non-mammalian vertebrates (Karten and Shimizu 1989; Kinoshita and Ito 2006). Tectal neurons in vertebrates receive visual inputs in the surface layer and somatosensory inputs in the deep layer, integrate them, and use this input to select appropriate behaviors (Butler and Hodos 2005; Finlay et al. 1978; Llinás et al. 1976). Our present study contributes to our understanding of GnRH\textsubscript{3}-induced modulation of visual
processing in the optic tectum. We demonstrated that GnRH3 suppresses the excitability of postsynaptic neurons via BK channels, thereby modulating the retino-tectal neurotransmission. This modulation of visual information processing could have several effects. For example, it is possible that habituation of visual information processing (Northmore and Gallagher 2003) is prevented by suppressing the excitability of projection neurons. GnRH3-induced modulation may expand the dynamic range of optic tectum response to visual input, as is the case in modulation of auditory system via BK channels (Kurt et al. 2012). Animals generally need visual cues for the success of reproduction and change visual preferences seasonally (Heubel and Schlupp 2008; Satou et al. 1994; Thompson et al. 2004). Previous studies also suggest that TN-GnRH3 neurons change their firing activity when animals receive visual cues from the opposite sex (Okuyama et al. 2014; Ramakrishnan and Wayne 2009). Therefore, GnRH3-induced modulation of synaptic transmission from optic tract fibers to SPV neurons should play an important role in the neuromodulation of behaviorally relevant visual information processing during the breeding season.

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[References]


Figure 1. GnRH3 modulates the field potential of retino-tectal neurotransmission

A: Field potentials evoked by electrical stimulation of optic tract fibers (average of three traces) before (solid black line) and during 20 μM CNQX (dotted gray line) application. The first large positive peak represents an artifact, and the subsequent large negative peak indicates the postsynaptic response of the SPV neurons. B: Field potentials evoked by electrical stimulation of optic tract (average of three traces) before (dotted black line), during (solid black line), and after GnRH3 application (solid gray line). C~E: Rise time (C), duration measured at half maximum amplitude (D), and peak amplitude (E) in vehicle, GnRH3 (100 nM) or GnRH3 (100 nM) plus antide (1 μM). All values were normalized by dividing the average of three trials during GnRH3 application (two minutes after application) to the average of three trials measured 2.5 minutes after the start of recording. In the box plot, bottom and top bars are minimum and maximum of each group respectively, the box is the quartile area, and the middle horizontal bar shows the median. Data were analyzed using Steel’s multiple comparison test (*: p < 0.05, **: p < 0.01).

Figure 2. GnRH3 modulates field potentials postsynaptically
A: Field potentials in response to paired stimulation of retino-tectal fibers (average of three traces) before (dotted line) and during GnRH3 (solid line). B: Time course of the paired pulse ratio (n = 6). In the box plot, bottom and top bars are minimum and maximum of each group respectively, box is quartile area, and the horizontal bar in the middle shows the median.

**Figure 3.** GnRH3 suppress the excitability of SPV neurons

A-C: The action potential responses of SPV neurons evoked by current injection (bottom inset) before (A), during (B), and after application of 1 μM GnRH3 (C). D: The normalized response before, during, and after application of 1 μM GnRH3. The vertical axis indicates the response normalized by the minimum spike number before GnRH3 application. n = 7 for each group. Data were analyzed using ANOVA and Tukey Kramer’s test (**: p < 0.01). Error bars indicate S.E.M. before: 1min before GnRH3 application, during: 2 min after GnRH3 application, after: 5 min after finishing GnRH3 application.

**Figure 4.** GnRH3 modulates the large conductance Ca\(^{2+}\)-activated K\(^+\) channel

A: Current-voltage relationship before (dotted line), during (black line), or after
application of 1 μM GnRH3 (gray line) during the falling phase of the ramp stimulation (dotted rectangle in inset). Inset shows the entire ramp protocol. Inset scale: 50 mV, 1.5 ms. before: 1 min before GnRH3 application, during: 2 min after GnRH3 application, after: 5 min after finishing GnRH3 application. B: GnRH3-induced current calculated by subtracting the current before GnRH3 application from the current during GnRH3 application. n = 7 for 1 μM GnRH3: black dot, n = 4 for 1 μM GnRH3 with 100 nM Iberiotoxin (IBX): dark gray dot. The vertical bar through each symbol indicates the S.E.M.

Figure 5. GnRH3 modulates retino-tectal neurotransmission via large conductance Ca\(^{2+}\)-activated K\(^+\) channel

A: Field potentials evoked by electrical stimulation of the optic tract (average of three traces) in the presence of 100 nM Iberiotoxin (IBX) before (gray dotted line) and during 100 nM GnRH3. B, C: Normalized rise time (B) and duration measured at half maximum amplitude (C) during application of vehicle or GnRH3 (100 nM) in the presence of IBX. There was no significant statistical difference between vehicle and GnRH3 in B, C (n = 5, Student’s t-test). before: 1 min before GnRH3 application, during: 2 min after GnRH3 application. In the box plot, bottom and top bars are...
minimum and maximum of each group respectively, box is quartile area, and the
horizontal bar in the middle indicates the median.

**Figure 6. GnRH receptors are mainly localized in SPV neurons**

A: Fluorescent signals of GnRH-fluoroprobe (GnRH-FL), B: signals of Hoechst nuclear
stain, C: overlay images, and D: the magnified overlay images of the area indicated in
white squares in C, in the presence of 1 μM GnRH-fluoroprobe (a), vehicle (b), and 1
μM GnRH-fluoroprobe plus 10 μM GnRH3 (c). Each photograph is representative from
three trials. In the overlay images, magenta is GnRH-fluoroprobe, and green is Hoechst.

Scale bar: 20 μm (C), 10 μm (D)

**Figure 7. Hypothetical model of GnRH3-induced modulation of retino-tectal
neurotransmission**

A: Experimental arrangement and schematic image of GnRH3-induced modulation of
retino-tectal neurotransmission. B: Schematic diagram of LFP illustrated following
Venegas (Vanegas 1983) and Rall & Shepherd (Rall and Shepherd 1968). Dotted line:
field potential before GnRH3 application. Solid line: field potential during GnRH3
application. The negative component of the LFP evoked by the stimulation of optic
nerve in the optic tectum is based on the assumption that the LFP is the superimposition of extracellularly recorded EPSPs and regenerative population spikes. C: Illustration of the result of whole-cell recording of SPV neurons evoked by current injection showing that the excitability of SPV neurons is suppressed by GnRH3.
**Vehicle GnRH3** (100nM) + **Antide** (1μM)

**Normalized duration measured at half maximum amplitude (%)**

**Normalized rise time (%)**

**Normalized amplitude (%)**
A

B

Paired Pulse Ratio

during/before GnRH3

GnRH3 100nM

Before

GnRH3

Voltage (mV)

-0.4

0.0

0.2

0.4

150ms
**Figure 1.**

- **A** before -60 mV
- **B** GnRH3
- **C** washout

**D**

<table>
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<th>Normalized response (%)</th>
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<tbody>
<tr>
<td>before</td>
<td><strong>100</strong></td>
</tr>
<tr>
<td>GnRH3</td>
<td><strong>80</strong> ± 10</td>
</tr>
<tr>
<td>washout</td>
<td><strong>60</strong> ± 10</td>
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Significance levels: **p < 0.001**
A) 

Voltage (mV)

Current (pA)

Before

GnRH3 1µM

Washout

B) 

Current (pA)

Voltage (mV)

GnRH3 1µM

GnRH3 1µM + IBX 100nM

Other text and figures as shown in the image.
Figure A: Voltage traces showing the effect of Iberiotoxin 100nM and GnRH3 on voltage response.

Figure B: Bar graph showing normalized rise time percentage before and during GnRH3 application.

Figure C: Bar graph showing normalized duration measured at half maximum amplitude before and during GnRH3 application.
A a  GnRH-FL  b  control  c  GnRH-FL + GnRH3

B a  Hoechst  b  c

C a  overlay  b  c

D a  b  c