Mechanisms of Neuromodulation by a Nonhypophysiotropic GnRH System Controlling Motivation of Reproductive Behavior in the Teleost Brain

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Abstract. Fine tuning of the nervous system in response to intrinsic and extrinsic cues is necessary for successful reproductive behavior. Gonadotropin releasing hormone (GnRH) was originally identified as a hypophysiotropic hormone that facilitates the release of gonadotropins from the pituitary. Although later studies reported their presence, the nonhypophysiotropic GnRH systems, which consist of two groups located in the terminal nerve (TN) and the midbrain tegmentum, respectively, has long been overshadowed by the hypophysiotropic GnRH system. By taking advantage of the teleost brains in which all three GnRH systems are well developed, the anatomical and electrophysiological properties of all three groups of GnRH neurons have been studied. However, despite our increasing endocrinological knowledge, we know very little about the manner of information flow by nonhypophysiotropic neuromodulatory GnRH neurons in the brain. In this article, we will review recent advances in the studies of nonhypophysiotropic GnRH neurons from cellular to behavioral levels. We will first discuss general features of the information processing by peptides and then introduce our recent approaches toward the understanding of the excitation-secretion coupling mechanism of single GnRH neuron using our newly developed primary culture system of isolated TN-GnRH3 neurons. We also introduce autocrine/paracrine regulation of TN-GnRH3 neurons by NPFF peptides for synchronization among them. In addition, we highlight recent advances in the neuromodulatory action of GnRH peptide on the information processing of sensory neuronal circuits and reproductive behavior. These multidisciplinary approaches will greatly advance our understanding of the complex action of GnRH peptides in relation to the brain control of reproduction.

Key words: Excitation-secretion coupling, GnRH, Neuromodulation, Reproductive behavior, Sensory modulation

Reproduction is a pivotal process for the continuation and evolution of life, and every organism exists as the result of reproduction. To increase their fitness, animals should be able to flexibly control their physiological status in regard to reproductions in response to various intrinsic and extrinsic cues, including photoperiods, climate changes, social interactions, stress and nutrition, which are detected by corresponding sensory systems.

In vertebrates, it has been well established that the hypothalamo-pituitary-gonadal (HPG) axis plays an important role in the control of reproductive functions, such as control of the menstrual cycle, gonadal maturation and mating behavior, etc. GnRH was originally identified as a hypophysiotropic hormone that is produced in the preoptic area and facilitates the release of gonadotropins (luteinizing hormone and follicle stimulating hormone) from the pituitary. The release of GnRH is regulated by gonadal steroid feedback mechanisms. A growing body of evidence recently suggests the importance of the kisspeptin neuron as a direct target of steroid feedback regulation of GnRH secretions, and the study of these neuronal systems is one of the hottest topics in reproductive neuroendocrinology [1]. However, biological information processing, in general, is a so-called ‘open system’ that concurrently receives various internal and external environmental cues and feeds the processed output back to various organs.

From this point of view, it is interesting and important to study nonhypophysiotropic and extrahypophysiotropic neuromodulatory GnRH neurons, a kind of “stepchild for reproductive biology” who has been overshadowed by its sister, the hypophysiotropic GnRH system. In many vertebrates, the extrahypophysiotropic GnRH system consists of up to two groups of GnRH neurons in the brain that are located in the terminal nerve (TN) and the midbrain tegmentum, although not all vertebrates have three GnRH systems [2, 3]. In many teleosts, all three GnRH systems are well developed, and it has been established that the paralogous genes for GnRH (\(\text{gnrh1}, \text{gnrh2} \text{and } \text{gnrh3}\)) are expressed separately in the three brain regions [2–4]; GnRH1 neurons are expressed in the preoptic area (POA), GnRH2 neurons are expressed in the midbrain tegmentum (TEG) and GnRH3 neurons are expressed in the TN. Although there are some teleost species that lack one of the three paralogs (e.g., \(\text{gnrh1}\) has been lost in salmon, goldfish and zebrafish, while \(\text{gnrh3}\) has been lost in eel and catfish [3]), three different GnRH systems can be anatomically defined in these species as well. In such cases, the remaining \(\text{gnrh}\) gene is expressed and is suggested to compensate functionally for the lost gene. Thus, it is suggested that the axonal projection pattern of each GnRH system, instead of its gene expression pattern, is closely related to its function(s). Accumulating evidence enables us to assume that this principle applies to vertebrates in general. The POA-GnRH1 neurons of tele-
osts directly project their axons to the pituitary [5] and are therefore apparently “hypophysiotropic.” On the other hand, the axons of the extrahypothalamic GnRH neurons (i.e., TEG-GnRH2 and TN-GnRH3 neurons) project widely throughout the brain but not to the pituitary [4, 6]. In addition, it was recently demonstrated by using GFP-transgenic medaka that GFP-expressing TEG-GnRH2 and TN-GnRH3 neurons show intrinsic regular firing activity in contrast to the irregular and spontaneous episodic electrical discharges of hypophysiotropic POA-GnRH1 neurons [7]. Thus, it has been suggested that these extrahypothalamic GnRH systems function as a modulator of the excitability of other neurons in wide areas of the brain, depending on their pacemaker activity, rather than as a regulator of gonadotropin release from the pituitary. These morphological and electrophysiological characteristics may be shared by many neuromodulatory neurons, such as other peptidergic neurons and the monoaminergic (histaminergic, serotonergic, noradrenergic, etc.) neurons. Therefore, to get a comprehensive picture of the brain control of reproduction, especially from their behavioral aspects, we should examine the manner of information flow of neuromodulatory GnRH neurons and compare it with that of neuroendocrine GnRH neurons (= direct regulatory mechanisms of the HPG axis).

In this short review, we will first discuss fundamental features of the information processing by neuromodulatory peptides, in general, then introduce experimental advantages of teleost non-hypophysiotropic GnRH neuronal systems (TN-GnRH3 neurons) for understanding the information processing by neuromodulatory GnRH neurons and then highlight recent advances in neuromodulatory action of GnRH peptides toward the understanding of the brain control of reproduction. Readers of this review are also suggested to refer to the recent reviews on the electrophysiological properties of medaka GnRH neurons [8] and a previous review on the properties of TN-GnRH3 neurons [9].

**Information Processing by Peptides in the Brain as a Biological Digital-analog Converter**

There are some fundamentally different features in the neuromodulatory peptidergic neurons compared with the other neurons that use classical neurotransmitters in the brain. The information processing by these “classical neurotransmitter neurons” is basically an orchestra of spikes conducting in billions of axons, which trigger zillions of tiny synaptic signals every second, from one neuron to another in neuronal networks, and the integration of these stochastic signals yields certain outcomes relevant for certain neural circuits. On the other hand, many peptidergic neurons are believed to act as modulators of ion channels and neurotransmission in the brain networks.

The “classical neurotransmitters” are released from axon terminals by a Ca²⁺-dependent exocytosis from small clear synaptic vesicles (SCVs), which are generally localized in presynaptic terminals. A typical presynaptic terminal contains several thousands of SCVs, but at any one time, only a few are ready to be released when a spike reaches the presynaptic terminal. Numerous studies concerning the neurotransmitter release from synaptic vesicles has been performed since the excellent studies of “quantal” acetylcholine release from neuromuscular junctions by Katz [10]. Since then, we can now count the number of released synaptic vesicles by recording the excitatory postsynaptic potentials (EPSPs) that are evoked spontaneously (miniature EPSPs) or by presynaptic stimulations (evoked EPSPs). It is rather rare that more than one vesicle is released per one spike. Typically, the release probability of a single synaptic vesicle by a single spike is between 0.1 and 0.3 at any given synapse in the guinea pig hippocampal neurons and 0.07 to 0.44 in the goldfish Mauthner neurons [11]. In general, when spikes occur in a cluster, the release probability increases progressively towards one [11].

Peptides are also released by a Ca²⁺-dependent exocytosis, but they are packaged in ultrastructurally different vesicles, large dense-core vesicles (LDCVs). Differences in the mechanisms underlying regulation of release of LDCVs and SCVs have been extensively reviewed elsewhere [12]. There is accumulating experimental evidence for the involvement of various proteins in the synaptic vesicle exocytosis, and similar molecular events are believed to underlie exocytosis of LDCVs.

Leng and Ludwig [13] reported an interesting estimation of stimulus–secretion coupling of peptide release from the hypothalamic magnocellular oxytocin and vasopressin neuronal systems. They estimated the amount of hormone secreted for a given spike activity and then calculated the release probability of vasopressin-containing vesicles by a single spike using morphometry and radioimmunoassay (RIA) data by taking advantage of the rodent vasopressin system; direct projection of single long axons to the pituitary and measurable release of plasma vasopressin in the medium enable simultaneous in vivo recording of firing activity and RIA measurement of plasma vasopressin concentrations. They estimated that the release probability of vasopressin-containing vesicles at each release site is only -0.0025! These estimations imply that the exocytosis of a LDCV is a surprisingly rare event; it typically requires 400 spikes to induce peptide release at any given release site. These calculations suggest that the peptides are not effective and faithful mediators of information flow in the brain at brief time scales.

Another important difference between the synaptic and peptidergic release is their release site. Although recent evidence indicates that extrasynaptic vesicular release can sometimes occur in the somatodendritic regions of neurons, the synaptic vesicles are preferentially released from presynaptic sites [14]. In contrast, LDCVs generally are not localized to synapses; some are found in synapses, but these vesicles are also distributed in soma, dendrites and axonal varicosities as well as nerve endings [15]. All of these regions can release peptides. Thus, the peptides are supposed to diffuse for some distance to reach receptors on the neurons that are not only near the site of release but also in relatively distant locations (so-called “action at a distance” or “volume transmission” [14]). Actually, it has been reported that the GnRH receptors are not necessarily located in immediate proximity to the GnRH immunoreactive fibers in the extrahypothalamic brain regions [16–18]. In addition, the peptides released by such infrequent release events as described above are considered to work at very low concentrations because they have nanomolar affinity for their receptors compared with the micromolar affinity for “conventional neurotransmitters” [14]. The range of the peptide action is limited by membrane-bound
peptidases and dilution over distance because peptides are not recycled, and replenishment is possible only through de novo synthesis. However, the dynamics of enzymatic degradation in the extracellular space of distinct brain areas and the cerebrospinal fluid and the real diffusion distances of peptides are largely unknown. There are also mechanisms to terminate the activation of receptors, such as desensitization and receptor internalization [19].

The differences described so far now appear to be widely recognized; however, there are clearly massive qualitative differences between the rate of release of SCVs and those of peptide-containing LDCVs. A typical presynaptic terminal contains thousands of SCVs, each containing “classical neurotransmitters” such as amino acids. The high release probability of a single SCV by a single spike and the rapid clearance mechanisms of “conventional neurotransmitters” from the synaptic cleft by their corresponding transporters and degradative enzymes allow a relatively tight association between spike activity and postsynaptic information flow. Thus, the conventional neurotransmitters transmit direct point-to-point messages from one particular neuron to another; they carry a message that matters only at a particular time and a particular place. By contrast, the release of a peptide-containing vesicle is a comparatively rare event for any neuron but has potentially widespread, long-lasting, and profound consequences. This is because the released peptides activate G-protein coupled receptors to trigger second messenger cascades, which often modulate the response of target neurons to the classical transmitters. Thus, the peptidergic neuromodulation is like a public announcement; the message lasts at least for a while (the duration varies continuously according to the time and dose) and will be received by a large number of neurons that have specific peptide receptors. We can explain such long-lasting effects of peptidergic information processing by using the analogy of a digital-to-analog converter. A digital-to-analog converter is an electronic circuit device that converts a digital code (0 or 1) to a smoothly varying analog signal (current, voltage or charge). This information flow is analogous to those of the excitation-secretion coupling mechanism and long-lasting properties of peptides. To be more specific, a train of postsynaptic potentials is integrated as slowly varying intracellular Ca^{2+} concentrations. The elevations in the intracellular Ca^{2+} concentrations induce the peptide release. The released peptides diffuse widely and activate their specific G-protein coupled receptors in the target neurons. These multistep integrations underlie the longlasting property of peptidergic effects. Therefore, for better understanding of the properties of such a digital-to-analog converter in living organisms, we should analyze their performance at the single neuron level: (i) the soma in the brain, as well as the distribution of widely branched neuronal processes that may release peptides and are embedded in the other types of neurons and glial cells. Therefore, to overcome these difficulties in the analysis of the excitation-secretion coupling of peptidergic neurons, we recently established a primary culture system of fish TN-GnRH3 neurons [23]. The whole brain of an adult dwarf gourami was dissected out, and the TN-GnRH3 neurons were sucked out from the brain with the aid of a suction pipette and plated on a coverslip in culture medium (Fig. 1A).


One of the best studied nonhypothalamic GnRH neurons with characteristic electrophysiological and anatomical features is the TN-GnRH3 neuron. The TN is a cranial nerve whose functions have not yet been fully characterized, and the majority of TN neurons produce GnRH3 peptide. The brain of a tropical freshwater teleost, the dwarf gourami (Colisa lalia), has several advantages for studying GnRH neurons at a single neuron level: (i) the somata of TN-GnRH3 neurons are large (20–40 μm in diameter), and (ii) they form a tight cell cluster consisting of five to 12 neurons without intercalating glial cells on the ventral surface of the brain. Therefore, we can easily identify unlabeled TN-GnRH3 neurons under a microscope with differential interference contrast optics (Fig. 1A). By using a whole brain in vitro preparation of the dwarf gourami, we succeeded in recording regular spontaneous electrical activities (pacemaker activities) from these GnRH neurons and analyzed ionic currents underlying the pacemaker activities [see 9 for details]. The pacemaker activity of TN-GnRH3 neurons is considered to reflect the physiological conditions of the animal and may be modulated by neural inputs of various sensory modalities [20]. Changes in the pacemaker activity, in turn, are suggested to alter the release of GnRH peptides from extensively branched axons and simultaneously modulate neuronal excitability in wide brain areas. Such neuromodulation may finally lead to long-lasting changes in animal behaviors including sexual motivation and arousal state [21]. Therefore, analysis of the excitation-secretion coupling of TN-GnRH3 neurons, which may lead to subsequent neuromodulation, will be the key to understanding the central regulation of reproductive functions of animals.

As we discussed above, despite intensive studies on the excitation-secretion coupling of neurotransmission, we know very little about the relationship between the firing activity and peptide release. Ishizaki et al. [22] conducted a static incubation of brain slices containing the cell bodies and fibers of TN-GnRH3 neurons and midbrain tegmentum-GnRH2 neurons to examine by using RIA whether GnRH peptides are actually released in the brain. Glutamate application significantly increased bulk GnRH release from slices in a dose-dependent manner. In accordance with this, glutamate applications increased the firing frequency of pacemaker activity of single TN-GnRH3 neurons. These results suggested that the increase in frequency of the pacemaker activity leads to an increase in the amount of GnRH released from TN-GnRH3 neurons. However, we cannot estimate the actual manner of excitation-secretion coupling of TN-GnRH3 neuron at a single neuron level.

The study of peptidergic neurons has been severely hampered because of their small number and the scattered distribution of the soma in the brain, as well as the distribution of widely branched neuronal processes that may release peptides and are embedded in the other types of neurons and glial cells. Therefore, to overcome these difficulties in the analysis of the excitation-secretion coupling of peptidergic neurons, we recently established a primary culture system of fish TN-GnRH3 neurons [23]. The whole brain of an adult dwarf gourami was dissected out, and the TN-GnRH3 neurons were sucked out from the brain with the aid of a suction pipette and plated on a coverslip in culture medium (Fig. 1A). The isolated TN-GnRH3 neurons could be cultured for up to 2 weeks. Cultured TN-GnRH3 neurons grew both axon- and dendrite-like processes on a flat substratum, and these processes were phenotypically similar to those found in situ (Fig. 1B). Unlike the neurons in situ, the
cultured neurons had somewhat depolarized resting membrane potentials and had no spontaneous firing activity. However, the membrane excitability of these cultured neurons was normal, and they showed subthreshold spontaneous oscillation of membrane potential and could be induced to fire in tonic patterns (Fig. 1C).

By using this isolated culture of TN-GnRH3 neurons and conventional whole brain in vitro preparations, we are now studying the cellular and molecular mechanisms underlying the GnRH peptide release from different cellular compartments of a single GnRH neuron and its relationship with the electrical activities. Our observations using a fluorescent exocytotic indicator dye, FM1-43, indicate that both somatic and terminal release of GnRH peptides occur in the TN-GnRH3 neuron (Fig. 1D). Fine structural evidence for somatic exocytosis could be observed in our previous electron microscopic observations [9, 24]. Similar stimulus-evoked exocytosis from the somatodendritic area has been reported in invertebrate serotonergic neurons [25] and oxytocin and vasopressin neurons of the magnocellular nucleus [26]. Therefore, somatodendritic release may be one of the general features of neuromodulatory neurons, and the different release pattern of neuroactive peptides according to the cellular compartments may reflect different intracellular Ca\(^{2+}\) mobilization dynamics. In addition, many peptidergic neurons also produce conventional neurotransmitters. For example, molecular biological evidence that suggests a glutamatergic transmission of TN-GnRH3 neurons has recently been published [27]. Although this phenomenon is often described as being ‘coreleased,’ the release of peptide-containing LDCVs is regulated rather independently of release from SCVs. Studies by Bean and Roth [28] indicate that in the case of colocalization of neuropeptide and dopamine, the peptide is often released at higher firing rates and particularly under burst firing patterns. However, while exocytosis of synaptic vesicles requires a rise of intracellular Ca\(^{2+}\) concentrations in the proximity of the Ca\(^{2+}\) channels at synapses, the peptide release is triggered by a global increase in intracellular Ca\(^{2+}\) [26]. In addition, differential changes of Ca\(^{2+}\) mobilization will occur depending on the local volume of intracellular space and the localization of Ca\(^{2+}\) mobilization machinery [12]. Therefore, even though a peptidergic neuron fires in the same pattern and frequency, local increases in Ca\(^{2+}\) at the terminal and the axonal varicosities tend to trigger neurotransmitter release, while a more diffuse rise in intracellular Ca\(^{2+}\) favors peptide release from the somatodendritic compartments. To test these possibilities, our culture of isolated TN-GnRH3 neurons should be useful.

In addition, in contrast to the neurotransmitter release from synapse, which occurs at a specialized release site (the active zone), it has been widely recognized that peptides are released from LDCVs from sites other than such active zones [14]. However, our observations thus far suggest that somatic release occurs at somewhat spatially restricted areas. Similar localized exocytotic events have been reported in the catecholamine release from chromaffin cells by electrochemical study [29] and in the peptide release from pituitary cells [30]. These phenomena probably reflect the so-called “compound exocytosis,” such as exocytotic events in which vesicles undergo fusion with each other as well as with the plasma membrane. In most cases, compound exocytosis occurs sequentially, with deeper-lying vesicles fusing, after a delay, with vesicles that have already fused with the plasma membrane [31]. By using functional imaging and electrophysiological techniques, the culture system of isolated TN-GnRH3 neurons will enable us to describe what excitation-secretion coupling of GnRH peptidergic neuron is like, whether GnRH release occurs at specialized zones and whether a different manner of GnRH release from different neuronal compartments occurs.

**Autocrine/paracrine Regulation of TN-GnRH3 Neuronal Activities for Synchronization Among GnRH Peptidergic Neurons**

Cell clustering is a prominent morphological feature of TN-GnRH3 neurons. In electron microscopic studies, it has been reported that the somata of TN-GnRH3 neurons of the dwarf gourami are closely apposed to each other without intervening glial cells, and frequent occurrence of coated vesicles lining the plasma...
membranes of the cell bodies and dendrites of the TN-GnRH3 neurons suggested exocytotic activities in the somatodendritic areas [24, 32]. Confocal micrograph images of labeled GnRH neurons (labeled with sGnRH immunohistochemistry in Fig. 2A and with intracellularly introduced biocytin in Fig. 2B) further support that fine neuronal processes containing GnRH3 peptide emerging from the soma (somatic process) surround the adjacent soma of TN-GnRH3 neurons. These structural features of TN-GnRH3 neurons suggest the existence of chemical interactions among them in the cluster.

The possible autocrine/paracrine regulation of pacemaker frequency by GnRH peptide in TN-GnRH3 neurons was first demonstrated electrophysiologically [33]. Bath application of GnRH3 peptide (so-called salmon-type GnRH, sGnRH) biphaseally modulated their pacemaker activity, resulting in a transient decrease and subsequent increase in the pacemaker frequency. This was suggested to function as an autocrine/paracrine positive feedback regulation of pacemaker activity by GnRH3 released from the TN-GnRH3 neurons in the cluster. This suggestion was later confirmed by demonstration of the expression of GnRH receptor mRNA in the TN-GnRH3 neurons using single-cell reverse transcriptase-polymerase chain reactions after whole cell patch-clamp recording [34].

Recently, the members of a class of neuropeptides containing the C-terminal Arg-Phe-NH2 (RFamide) have been highlighted as direct/indirect regulators of reproduction [35]. Interestingly, Saito et al. [36] found that FMRFamide-like peptide is likely involved in autocrine/paracrine negative feedback regulation of TN-GnRH3 neurons. It has been previously reported morphologically that TN-GnRH3 neurons are immunoreactive to a molluscan cardioexcitatory peptide (FMRFamide) [37]. Bath application of FMRFamide decreased the frequency of pacemaker activity of TN-GnRH3 neurons, and this decrease was suppressed by a blockade of the G-protein coupled receptor pathway by GDP-β-S. In addition, it was suggested that the FMRFamide-like peptide released from TN-GnRH3 neurons induced an increase in K+ conductance, caused hyperpolarization of membrane potentials and then inhibited pacemaker activity of TN-GnRH3 neurons [36].

From a phylogenetic analysis of the identified and unidentified RFamide peptides in vertebrates, Osugi et al. [38] suggested that there are at least five RFamide groups [Kisspeptin group, prolactin-releasing peptide (PrRP) group, pyrogulamylated Arg-Phe-amide peptide (QRFP/26RFa) group, Pro-Gln-Arg-Phe-amide (PQRFa/NPFF) group and Leu-Pro-Leu/Gln-Arg-Phe-amide (LPXRFa/RFRP) group]. Among these RFamide peptides, Saito et al. [36] demonstrated by immunohistochemistry that the FMRFamide-like peptide in TN-GnRH3 neurons of the dwarf gourami is actually NPFF. Oehlmann et al. [39] reported that the mRNA expression of zebrafish homolog of a PQRFa (mammalian NPFF) group (zF-PQRF) was localized in the TN-GnRH3 neurons. Actually, NPFF mimicked the effects of FMRFamide peptide on TN-GnRH neurons, and the inhibitory effect of NPFF on pacemaker frequency was blocked by RF9, a potent and selective antagonist for mammalian NPFF receptors [36]. Thus, it was suggested that the activation of K+ conductance by a FMRFamide-like peptide (γNPFF) released from the TN-GnRH3 neurons causes hyperpolarization of their own (autocrine) and/or neighboring neurons (paracrine) and then inhibition of pacemaker activity in TN-GnRH3 neurons [36]. Thus, there are both positive and negative autocrine/paracrine feedback regulations of pacemaker frequency in TN-GnRH3 neurons. These bidirectional feedback modulations of pacemaker activity by GnRH/NPFF peptides released from a somatodendritic region or recurrent axon varicosities may contribute to the synchronization of neuronal activities among the cluster of TN-GnRH3 neurons. These mechanisms will facilitate simultaneous release of the peptides and prevent supramaximal firings and depletion of the peptide contents of TN-GnRH3 neurons. A model of the bidirectional modulation mechanism has been proposed in the oxytocin neurons, in which oxytocin released from the dendrites has a positive feedback effect by mobilizing Ca2+ from the intracellular stores, while endocannabinoids have a cumulative inhibitory effect by suppressing the afferent input to the cells [40]. A temporal difference of the kinetics of these two pathways may enable bidirectional auto/paracrine modulations. Such temporally different modulation of ion channels may be involved in the differential autocrine/paracrine regulation of pacemaker activity.
paracrine regulation of pacemaker activity in TN-GnRH3 neurons. There is also a report on the synchronization of pacemaker activity among TN-GnRH3 neurons via gap junctional electrical coupling [41]. The functional significance of differential regulation of pacemaker activity in both directions is not known at present and deserves future study.

GnRH Peptides Modulate Sensory Information Processing Depending on the Reproductive Status

We have thus far discussed the characteristics of extrahypothalamic nonhypophysiotropic GnRH neurons. But how does the GnRH peptide that is released from these neurons modulate the information processing of target neurons to control reproduction, especially reproductive behaviors? Recently, many researchers are interested in the forebrain olfactory and visual systems as target systems of GnRH neuropeptide regulation because of the projection of TN-GnRH3 neurons [4, 37, 42–44] and localization of GnRH receptors [16–18].

Several studies have been performed to examine the possible neuropeptide regulation of GnRH in the olfactory system. Eisthen et al. [43] reported that GnRH modulates the sensitivity of olfactory receptor neurons of mudpuppies by modulating their Na+ and K+ channel properties. These effects appeared to be seasonal, with more animals responding to GnRH during the courtship and mating season. A later study reported that GnRH modulates natural odorant responses of axolotl as measured by electro-olfactogram [45]. Recently, we examined the effect of GnRH on the synaptic transmission from mitral to granule cells in the goldfish olfactory bulb by measuring electrically evoked in vitro field potentials (Fig. 3) [46]. GnRH enhanced the amplitude of field potentials that were evoked by retrograde stimulation of either the lateral or medial olfactory tract that conveys food (amino acids) or pheromonal information, respectively [47]. Furthermore, our data suggested that the increased amplitude of the field potential results from changes in the presynaptic release of glutamate from the mitral cell. Several studies have reported that the expression level of GnRH receptors in the olfactory bulb depends on the reproductive status or sex of the animals [48–50]. Therefore, GnRH possibly modulates the olfactory responsiveness for wide categories of odorants by modulating synaptic transmissions in the olfactory bulb in response to the expression levels of GnRH receptors, depending on the reproductive status.

GnRH peptide also appears to have neuromodulatory effects on visual information processing. The axons of TN-GnRH3 neurons innervate the retina and optic tectum [42, 44, 51]. GnRH positively modulates the efficiency of synaptic transmission from the retinal efferent axons to the periventricular neurons of the optic tectum in the rainbow trout [52]. Another study reported that GnRH applications induced a depolarization of horizontal cells, increased their response to small spots and elicited light adaptive formation of horizontal cell spinules in vitro by stimulating the dopaminergic interplexiform cells [51]. Maaswinkel and Li [53] found that olfactory stimulation with amino acids increased behavioral visual sensitivity of zebrafish, but this effect was eliminated after disruption of the TN projections to the retina or after the destruction of dopaminergic interplexiform cells. A later study by Huan et al. [54] suggests that the TN system has a modulatory effect on firing activity of retinal ganglion cells by regulating D1 dopaminergic receptor-coupled Ca2+ currents. Thus, the TN system and dopaminergic interplexiform cells are the main candidates for the olfactory modulation of visual sensitivity. Although the functional relationship between the TN-GnRH3 and olfactory systems and
whether GnRH or NPFF in the TN affect activity of dopaminergic interplexiform cells are not known, projections from the olfactory bulb to the TN-GnRH3 neurons [20] may support the existence of TN-mediated olfactory modulation of retinal information processing. Interestingly, the odorant-induced increase in behavioral visual sensitivity is observed only at dawn when the zebrafish is ready for mating [53]. Therefore, the neuromodulation by the TN probably contributes to some aspects of reproductive behavior such as territoriality and mate choice [55].

In addition to the olfactory and visual systems, a recent study by Maruska and Tricas [56] suggests that GnRH also modulates the processing of auditory information in the brain. They found abundant GnRH-immunoreactive axons in the auditory processing regions of the midbrain and hindbrain of the soniferous damselfish, *Abudelfataf abdominialis*. In vivo extracellular recording of single-neuron responses in the torus semicircularis showed that exogenous application of GnRH peptides caused a long-lasting decrease in spike frequency in response to both artificial auditory stimulus and complex natural sounds. GnRH also decreased response latency and increased auditory thresholds in a stimulus-dependent manner. This is the first report that GnRH peptides primarily cause an inhibitory action on the central neurons. Interestingly, the cell numbers and axonal densities of nonhypothalamic GnRH2 and GnRH3 neurons of the soniferous damselfish also changed depending on the seasons (comparatively lower during the protracted spring-summer spawning seasons).

Although further studies are necessary for understanding the mechanisms of GnRH-induced neuromodulation, all of these studies suggest that GnRH likely modulates sensory responsiveness of various modalities and that the modulatory action is probably further regulated by the expression levels of GnRH peptide and/or its receptor, depending on the animal’s reproductive state.

### Neuromodulatory Actions of GnRH Peptide as a Regulator of Reproductive Behavior

Reproductive behaviors of vertebrates are mainly regulated by a coordination of endocrine and neural systems, and many steroid hormones and neuropeptides, such as GnRH among others, are involved in the control of reproductive behavior. Due to the limitation of drug delivery methods and their promiscuous and long-lasting interactions with the receptors, we should pay extra attention to experimentally distinguish at the behavioral level whether the GnRH peptide acts directly on the brain or stimulates the production of sex steroids via the pituitary gland. Therefore, in this section, we will review some recent reports concerning the regulatory action of GnRH-3 peptides on reproductive behavior with no discrimination of GnRH molecular species. For a discussion from the endocrinological point of view, the reader is referred to a recent excellent review [57].

We previously reported that TN-GnRH neurons are probably responsible for controlling motivational or arousal state of the animal in general, including some repertoires of reproductive behavior, such as nest-building [2, 9, 21]. The repertoire of reproductive behavior in the dwarf gourami is illustrated in Fig. 4. The male dwarf gourami actively builds a spawning nest by bubbling from the mouth (nest building) when paired with a sexually mature female. The male sometimes leads the partner female to the bubble nest (leading to nest). When she is receptive, she approaches the male (approaching) and thrusts her mouth towards him (thrusting). The thrusting induces the male “clasping” of the female, and they spawn under the bubble nest. The frequencies of the behavioral repertoires were counted before and after electrolytic lesions of the clusters of TN-GnRH3 neurons. The behavioral analysis suggested that the TN-GnRH3 neurons are involved in the maintenance of the threshold for the initiation of reproductive behavior (“motivational state”). Metaphorically speaking, the TN-GnRH neurons may lower the “motivational barrier” for triggering a sequence of reproductive behavior repertoires, while their lesions may heighten it. Modified from Yamamoto et al. [21].
nest-building [58], aggression [58, 59] and spawning behavior [60] are influenced by GnRH3 peptide. A growing body of evidence suggests that such GnRH-mediated modulation of reproductive behavior probably involves modulation of relevant sensory information processing.

With regard to the behavioral significance of the regulation of pacemaker frequency in TN-GnRH3 neurons, Ramakrishnan and Wayne [61] recently reported some simple but interesting behavioral experiments, in which they tested a hypothesis that social cues from conspecifics modulate electrical activity of TN-GnRH3 neurons. They showed, by using female GnRH3-GFP transgenic medaka, that 24-h exposure of female medaka to male visual and chemosensory cues suppressed the electrical activity of female TN-GnRH3 neurons. They further found that a visual cue alone could decrease the spontaneous electrical activity of TN-GnRH3 neurons. In medaka, the visual cue is important for reproductive behavior because females observing male mating behavior choose males that court and spawn better. A previous morphological study suggested that TN-GnRH3 neurons receive multimodal (olfactory, visual and somatosensory) inputs [20]. Thus, the spontaneous pacemaker activity of TN-GnRH3 neurons appear to be closely related to the cues (visual cues in this case) related to the motivation for the sexual behavior. In addition to GnRH3, GnRH2 peptide is also reported to influence reproductive behaviors without physiologically regulating pituitary gonadotropins. The administration of GnRH2 peptide facilitated reproductive behavior and inhibited short-term food intake in female musk shrews [62] and goldfish [63].

As partly mentioned above, various environmental and internal states regulate neuromodulatory GnRH neuronal systems. In Indian major carp (Cirrhinus mrigala), for example, GnRH immunoreactivity in the TN changes seasonally, peaking during the prespawning season [64]. In chum salmon (Oncorhynchus keta), GnRH gene expression is elevated when prespawning salmon migrate upstream [50]. Furthermore, in the cichlid fish (Cichlasoma dimerus), the photoperiod controls GnRH3 contents in the TN, aggressive behavior and pituitary hormonal levels of the male [59].

In addition to the nonhypophysiotropic GnRH systems, a series of excellent studies by Fernald et al. [65–67] has suggested that male African cichlid fish, Astatotilapia burtoni, show reversible changes in size and gnrh1 mRNA expression of POA-GnRH1 neurons in response to the social status of an individual male. They showed that when adult male fish become socially dominant by acquiring a territory, the size of POA-GnRH1 neurons and their gnrh1 mRNA expressions increased significantly. Conversely, losing a territorial fight resulted in a decrease in cell size [65, 66]. A later study by Burmeister et al. [67] suggested that social signals affect GnRH1 signaling via activation of the immediate early gene egr-1. They removed the dominant territorial male from an aquarium, which, within minutes, led to striking changes in body color and behavior of subordinate male that were indicative of his dominance. This phenotype transition was accompanied by the activation of egr-1 around the POA-GnRH1 neurons. Interestingly, once male established their dominance, they did not show any egr-1 activity, although they exhibited dominance behaviors similar to those of climbing up males. Although TEG-GnRH2 and TN-GnRH3 neurons were not activated by this social status transition in this species, similar transcriptional changes may be involved in the pathway for neuromodulatory action by GnRH2 and GnRH3 peptides in response to long-lasting changes in the animal’s environmental, social and internal states.

Conclusions

GnRH is definitely an essential peptide in vertebrate reproduction. Without it, there is no gonadal maturation and no reproduction. Despite the progress in our understanding of the regulatory mechanisms of the HPG axis, we are still far from reaching firm conclusions regarding the nonhypophysiotropic actions of GnRH peptide. There are so many questions to be answered before we understand the control mechanisms of reproduction by such nonhypophysiotropic GnRH systems. First of all, we need to know about the differential excitation-secretion coupling mechanism of GnRH peptide release from the soma and dendrite for better understanding of the information flow in a single GnRH neuron. We believe that multidisciplinary studies using electrophysiology, functional imaging and quantitative analysis of the phenomena by computational methods using our isolated culture of TN-GnRH3 neurons may be one of the most powerful approaches for this purpose. Second, we should also examine the physiological action of GnRH peptides that are released from the neuromodulatory GnRH2/3 neurons against the sensory organs (olfactory epithelium and retina) or the brain regions involved in sensory information processing (olfactory bulb and optic tectum) to understand how GnRH peptide modulates the information flow in these areas. The third challenge is behavior. Although recent advances in behavioral neuroendocrinology have revealed functional significance of GnRH peptides in the control of certain aspects of reproductive behavior, we still do not know what neuronal activity triggers the release of GnRH peptide from nonhypophysiotropic GnRH neurons and induces reproductive behavior. Future in vivo electrophysiological recording of GnRH neuronal activities from freely-moving animals will enable us to understand the functional relationship between the GnRH-induced neuromodulation and reproductive behavior. Furthermore, as we mentioned above, it seems that neuromodulation by GnRH peptides is also dependent on the expression level of GnRH peptide itself and/or its receptors in response to the animal’s environmental, social and internal states. Therefore, the release and binding of GnRH peptide to receptors in the target brain area will be regulated on several different time scales (e.g., seasonal, diurnal and short-term social interactions such as dominance, mate choice, courtship or parental care).

The teleosts exhibit much wider species diversity than any other classes of vertebrates and can be found in nearly all aquatic environments, from high mountain streams to the deepest oceans. Such species diversity should have caused a wide variety of environmental adaptation of peripheral and central nervous systems that control various behaviors including reproductive behavior. We should take advantage of such functional and morphological diversity of teleost nervous system as well as the three well-defined and anatomically distinct GnRH systems of the teleosts for studying the neural control of reproduction. Furthermore, in medaka, we can take advantage of various molecular genetic tools such as...
transgenic animals. Thus, we hope that future studies will greatly advance our understanding of complex regulatory mechanisms of neuromodulation by GnRH peptides, such as excitation-secretion coupling mechanisms, the functional significance of population activity of GnRH neurons as a cell cluster, the nature of behaviorally relevant GnRH neuronal activity and excitation-transcription coupling of GnRH and its receptors in the target neurons depending on the animal’s environmental, social and internal states.

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