Social cues from conspecifics alter electrical activity of gonadotropin-releasing hormone neurons in the terminal nerve via visual signals

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Ramakrishnan S, Wayne NL. Social cues from conspecifics alter electrical activity of gonadotropin-releasing hormone neurons in the terminal nerve via visual signals. Am J Physiol Regul Integr Comp Physiol 297: R135–R141, 2009. First published May 6, 2009; doi:10.1152/ajpregu.00143.2009.—There are multiple populations of gonadotropin-releasing hormone (GnRH) neurons in the brains of vertebrates. The population located in the hypothalamus/preoptic area is the best studied and is known to ultimately control reproduction. Teleost fish have an additional population of GnRH neurons in the terminal nerve (TN) associated with the olfactory bulbs, the physiological function of which is still unclear. Anatomical and physiological studies provide evidence that TN-GnRH neurons have extensive projections in the brain and modulate neuronal activity. Although there is anatomical evidence that the TN receives olfactory and optic sensory inputs, it is not known if sensory information is transmitted to TN-GnRH neurons to modulate their activity. In the present study, we tested the hypothesis that social cues from conspecifics modulate electrical activity of TN-GnRH neurons from the intact brain of female medaka fish (Oryzias latipes). We further investigated the potential roles of chemosensory and visual signals in mediating the social cue response. We used a transgenic line of medaka with TN-GnRH neurons genetically tagged with green fluorescent protein, allowing visualization of specific neurons and their projections in the brain and modulate neuronal activity. Previous work has shown that TN-GnRH neurons exhibit a pattern of tonic action-potential firing that is an intrinsic property of this cell type (36). In teleosts, these different GnRH neuron populations have also been shown to project to different regions of the brain (19, 33, 36, 46), suggesting that they have distinct functional roles.

We have previously demonstrated that social cues from conspecifics modulate the function of TN-GnRH neurons (1, 51). However, the role of the hypothalamic/POA GnRH neurons in regulating the functioning of the pituitary, and hence gonadal activation, has been studied extensively (11, 41). However, relatively little is known about the functions of the midbrain- and TN-GnRH neuron populations. Work in musk shrews has shown that the midbrain GnRH neurons may be coordinating reproduction with metabolic states of the animal (28, 29). In the case of the TN-GnRH neurons, a neuromodulatory role in reproduction has been suggested. For example, lesions to TN-GnRH neurons affected nest building in dwarf gourami (58). GnRH gene expression in TN-GnRH neurons increased during spawning migration in salmon (39), and damage to the terminal nerve impaired mating in hamsters (54). However, a definitive function for the TN-GnRH neurons has not yet been established.

Yamamoto and Ito (57) showed that the TN-ganglion received afferent inputs from both the olfactory system through the telencephalon and possible visual and somatosensory information through the nucleus tegmento terminalis in the midbrain. Further, morphological studies of biocytin-filled TN-GnRH neurons in dwarf gourami revealed projections into various brain regions, including the olfactory bulbs, the optic nerves, and to areas in which the hypophysiotropic GnRH neurons are located (36). This suggests that TN-GnRH neurons could receive environmental signals and transmit them to other neural systems, thereby providing a modulatory role in relation to a dynamic external environment (2).

Given this neuroanatomical evidence potentially linking sensory systems to TN-GnRH neurons, we hypothesized that environmental stimuli—in this case, social cues—modulate the cell physiology of TN-GnRH neurons. We chose the teleost medaka (Oryzias latipes) for our experiments because this fish is sensitive to visual and behavioral social cues, which have been shown to affect body size (25), mate preference (21), and oviposition (14). Importantly, lines of transgenic medaka were generated in which TN-GnRH neurons express green fluorescent protein (GFP) under the control of specific GnRH promoters (38, 52). This allows visualization of specific neurons in the intact living brain for single-cell electrophysiological analysis. Previous work has shown that TN-GnRH neurons exhibit a pattern of tonic action-potential firing that is an intrinsic property of this cell population (36, 52). Internal modulators (e.g., GnRH peptide, endogenous opiate) have been shown to alter the beat frequency of TN-GnRH neurons (1, 51). However, the impact of environmental signals has mostly been studied in hypophysiotropic GnRH neurons with respect to changes to GnRH neuron morphology, GnRH mRNA and protein levels, and secretion (3, 32,
Here, we show for the first time that visual social cues modulate spontaneous action potential firing of GnRH neurons.

MATERIALS AND METHODS

Animals

Medaka fish (O. latipes) of the d-r strain were used to generate a stable transgenic line in which the GnRH3 promoter drives expression of GFP in TN-GnRH neurons (gift of Dr. Kataaki Okubo, National Institute for Basic Biology, Okazaki, Japan) (38, 52). Fish were fed twice daily with live brine shrimp and flake food (TetraMin, Blacksburg, VA), and maintained at a temperature of 28°C. Medaka are long-day photoperiod breeders ((31) personal observations), so fish were maintained in full-spectrum light under constant long-day photoperiod (14:10-h light-dark cycle). Lights-on was at 0900 in all experiments. Prior to dissection, fish were anesthetized by immersion in MS-222 (150 mg/l). All chemicals were purchased from Sigma Chemical (St. Louis, MO). All procedures were carried out in accordance with the Animal Care and Use Committee of the University of California at Los Angeles.

Experimental Design

Medaka fish were maintained in three separate tanks (20.8 m³): a female-only holding tank of stock medaka isolated from males for at least 1 wk; a control tank containing female medaka fish (n = 6); and an experimental tank with male fish (n = 6). Opaque cardboard was placed between tanks to avoid visual signals from adjacent tanks. Females from the stock tank were placed in either the control or the experimental tank at 1000 (1 h after lights-on) for 24 h, after which they were killed. After the death of a female from the stock, the tank was replenished, thereby constantly maintaining n = 6 fish in the stock tank.

Effect of combined visual and chemosensory cues on female TN-GnRH neuron electrical activity. To determine whether social cues modulate TN-GnRH neuron electrical activity, a test female was taken from the stock tank 1 h after lights on, and placed in a net enclosure (2.5 m³) within the male tank (Fig. 1A). After 24 h of male exposure, the female was killed, and whole-cell patch-clamp recordings of TN-GnRH neuron electrical activity were made from the excised intact brain. As a control, the following day, a test female from the stock tank was placed in a net in the control female tank and TN-GnRH neuron electrical activity was examined following 24 h of female exposure. The net prevented physical contact between the fish but did not hinder visual or chemosensory signals. In a pilot study, we found that 2 h of exposure to conspecifics did not produce a significant response; therefore, the duration of exposure was increased to 24 h. A total of n = 6 female fish were examined in both groups. The numbers of eggs laid by the test female during the 24-h exposure period were

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**Fig. 1.** Experimental design of the social cue experiments. Male and female symbols represent male and female fish, respectively. Test females from the stock tank are indicated in black. Dashed lines in the corners of the tanks represent the net (A–C); solid lines in the corner of the tanks represent the transparent container (D). A: test females were exposed to both visual and chemosensory cues from males or females. B: test females were exposed to male- or female-primed holding water. C: test females were placed in tanks with males or female controls with an opaque barrier, exposing them to only chemosensory but no visual inputs. D: test females were placed in transparent containers, exposing them to only visual but not chemosensory cues from males or female controls.
monitored. Twenty percent of the water in the tanks was changed every day. However, there was no water change on the day that the test female was placed in the novel tank. After killing the animal, 50% of the water in the tank was replaced with fresh water to dilute putative chemosensory cues from the test female. After killing the test female, the ovary was removed and fixed for histological analysis, and the brain was removed for electrophysiological analysis.

Effect of holding water on female TN-GnRH neuron electrical activity. To determine whether chemosensory cues are the primary social signal modulating TN-GnRH neuron electrical activity, we exposed the test females for 24 h to male- or female-primed holding water in the absence of the fish themselves. As before, animals were maintained in three groups with n = 6 fish in each tank: female stock tank; control female tank; experimental male tank (Fig. 1B). On the day of the experiment, a test female from the stock tank was placed within a net enclosure (2.5 m³) in the male tank 1 h after lights on. All of the male fish were taken out of the tank and placed in a temporary chamber just prior to introduction of the test female. Following 24 h of exposure to the male-primed holding water, test females were killed. Male fish were then transferred back to their tank, and 50% of the water was changed. As a control, test females were exposed to holding water that had contained females. After euthanization and dissection of the intact brain, TN-GnRH neuron electrical activity was monitored from test females that were exposed to either male- or female-primed holding water.

Effect of chemosensory but not visual cues on female TN-GnRH neuron electrical activity. It is possible that putative chemosensory signals released in the holding water could get degraded over a 24-h period, thereby affecting the response of the TN-GnRH neurons. To ensure continuous exposure to chemosensory cues in the absence of visual inputs, we used the following paradigm. The experimental setup was the same as the first social cue experiment (see Effect of combined visual and chemosensory cues on female TN-GnRH neuron electrical activity) with three groups of animals (n = 6 each), and the test females from the stock tank undergoing 24-h exposure within male or female tanks (Fig. 1C). However, an opaque barrier was placed in the tank during the exposure period, preventing visual access but allowing a free flow of water around the barrier. The fish in the experimental (male) and control (female) tanks were housed in net enclosures (2.5 m³) to prevent them from swimming around the barrier. TN-GnRH neuron electrical activity from n = 6 test females from the stock tank was examined under both conditions.

Effect of visual but not chemosensory cues on female TN-GnRH neuron electrical activity. To determine whether visual cues are the primary social signal modulating TN-GnRH neuron electrical activity, we used the following paradigm. As before, animals were maintained in three groups with n = 6 fish in each tank: female stock tank; control female tank; experimental male tank (Fig. 1D). A small transparent container (1.8 m³) was placed in the experimental and control tanks. On the day of the experiment, the container was filled with 90% fish water from the stock tank and 10% fresh fish water. A test female from the stock tank was placed in the container within the male tank 1 h after lights on. This allowed visual access, but no chemosensory exchange. Following 24-h exposure to the male visual cues, test females were killed. The transparent container was then drained of water and cleaned in preparation for the next experiment. As a control, test females were placed in a small transparent container (also 1.8 m³) within the female control tank for exposure to female visual cues. After euthanization and dissection of the intact brain, TN-GnRH neuron electrical activity was monitored from test females that were exposed to either male or female visual cues.

Electrophysiology

Electrophysiological recordings on TN-GnRH neurons were carried out as previously described (52). During recordings, aerated fish saline continuously bathed the brain and was perfused through the chamber at a rate of ~200 μl/min. The saline solution (9) contained 134 mM NaCl, 2.9 mM KCl, 2.1 mM CaCl₂, 1.2 mM MgCl₂, and 10 mM HEPES. Osmolarity was adjusted to 290 mOsm with glucose, and the pH was adjusted to 7.8 with NaOH. The internal solution for the whole-cell patch pipette (26) contained 112.5 mM potassium gluconate, 4 mM NaCl, 17.5 mM KCl, 0.5 mM CaCl₂, 1 mM MgCl₂, 5 mM MgATP, 1 mM EGTA, 10 mM HEPES, 1 mM GTP, 0.1 mM leupeptin, and 10 mM phosphocreatine. Osmolarity was adjusted to 290 mOsm by titrating the final volume of water, and pH was adjusted to 7.2 with KOH.

The recording chamber was placed under an upright microscope (BX50W; Olympus, Melville, NY) equipped with a ×40 water-immersion objective (0.8 numerical aperture; Olympus). Infrared (IR) differential contrast optics and an IR camera (OL-1500; Olympus) allowed the visual selection of GFP-expressing neurons in the presence of both ultraviolet and bright-field illumination. Patch pipettes were guided to the cell of interest with a micromanipulator (MP-285; Sutter Instruments, Novato, CA). Whole cell recordings of membrane potential (Vm) and action potentials were obtained using an Axopatch 200B amplifier (Axon Instruments, Foster City, CA) in current-clamp mode and digitized with an ITC-18 computer interface (Instrutech, Port Washington, NY). Recordings were monitored online using both AxoGraph software (Axon Instruments) and PowerLab data acquisition and analysis instrumentation and software (ADInstruments, Colorado Springs, CO) and stored offline for subsequent AxoGraph data analysis of interspike Vm, spike frequency, interspike interval, action potential amplitude, half-maximal spike width, and spike rise time. Data were collected if series resistance were <35 M Ohms and interspike Vm was at least ~40 mV.

Histological Analysis of Ovaries

Ovaries from the initial social cue experiment were fixed in Bouin’s fixative (70% saturated picric acid; 25% of a 40% formaldehyde solution; and 5% acetic acid added just prior to use) overnight at 4°C. They were then rinsed in 0.1 M PBS (pH 7.4) and cryoprotected in 20% sucrose (in PBS) at 4°C for 24 h prior to sectioning. Gonads were frozen in Tissue-TEK optimum cutting temperature compound (VWR, West Chester, PA) embedding medium over dry ice and stored at −20 °C. Horizontal sections (20 μm) were cut on a cryostat. A hematoxylin-and-eosin procedure (10) was used to stain the sections, which were mounted on slides using DPX mountant, for histological analysis. Slides were then observed using a dissecting microscope (American Optical, Buffalo, NY) at ×40, ×100, and ×400 magnification.

Data Analysis

Values are shown as the means ± SE. Electrophysiology parameters were analyzed by Student’s t-test. Statistics were performed using Instat Statistical Analysis (GraphPad Software, La Jolla, CA). P values < 0.05 were significant.

RESULTS

Effect of Combined Visual and Chemosensory Cues on Female TN-GnRH Neuron Electrical Activity

Representative electrophysiological recordings from the TN-GnRH neurons of female fish exposed to males (n = 6 cells from 6 fish) and control females (n = 6 cells from 6 fish) are shown in Fig. 2A. TN-GnRH neurons of female fish exposed to males compared with those exposed to control females showed a significant hyperpolarization of interspike membrane potential, a decrease in action potential firing frequency, and an increase in spike amplitude with no changes in spike width (Fig. 2B). These results indicate that exposure to

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novel males inhibits female TN-GnRH neuron electrical activity compared with exposure to females. We also examined the ovaries of the test females to see whether a 24-h exposure to males altered ovarian histology. No differences were observed between the ovaries of females exposed to males vs. those exposed to females. Normal oocyte production and maturation were seen in all tissue examined. In the absence of physical contact with a male, there was little to no egg laying in any of the test females.

**Effect of Holding Water on Female TN-GnRH Neuron Electrical Activity**

There was no effect of male/female holding water on electrical activity of TN-GnRH neurons of test females (experimental, male holding water: $n = 8$ cells from 6 fish; control, female holding water: $n = 6$ cells from 6 fish). Specifically, there were no significant differences in the interspike membrane potential (in millivolts; experimental: $-45.91 \pm 1.89$, control: $-48.894 \pm 1.45$), spike frequency (in Hertz; experimental: $5.0 \pm 1.8$, control: $4.14 \pm 0.59$), action potential amplitude (in millivolts; experimental: $70.77 \pm 5$, control: $73.57 \pm 7.3$), spike width (in milliseconds; experimental: $2.6 \pm 0.2$, control: $2.5 \pm 0.1$) and spike rise time (in milliseconds; experimental: $5.3 \pm 0.7$, control: $6.1 \pm 1$).

**Effect of Chemosensory but not Visual Cues on Female TN-GnRH Neuron Electrical Activity**

Representative electrophysiological recordings from the TN-GnRH neurons of female fish exposed to males ($n = 7$ cells from 7 fish) and control females ($n = 6$ cells from 6 fish) in the absence of visual cues are shown in Fig. 3A. Once again, no significant differences were seen between the TN-GnRH neuron electrical activities of the two groups with respect to interspike membrane potential, spike frequency, amplitude of action potentials, and spike width (Fig. 3B) and spike rise time. This indicates that chemosensory cues alone are insufficient to induce the social-cue response shown in Fig. 2.

**Effect of Visual but not Chemosensory Cues on Female TN-GnRH Neuron Electrical Activity**

Representative electrophysiological recordings from the TN-GnRH neurons of female fish exposed to males ($n = 6$ cells from 6 fish) and control females ($n = 6$ cells from 6 fish) in the absence of chemosensory cues are shown in Fig. 4A. TN-GnRH neurons of female fish exposed to males compared with those exposed to control females showed a significant hyperpolarization of interspike membrane potential, a decrease in action potential firing frequency, and an increase in spike amplitude with no changes in spike width (Fig. 4B). Exposure to male or female visual cues elicited a similar response as that of social cues shown in Fig. 2.

**DISCUSSION**

Previous work has shown that social cues alter the number of GnRH projections, the number and size of GnRH neurons (8, 47, 53), and levels of proGnRH protein (48). These studies examined GnRH neurons located in the hypothalamus and preoptic area. To our knowledge, this is only the second study to investigate the effect of social cues (22) on the electrical activity of any population of GnRH neurons and the first to identify visual cues as the relevant signal regulating electrical activity of TN-GnRH neurons.

TN-GnRH neurons from females showed a significant inhibition of electrical activity when exposed to male social cues compared with female social cues. Specifically, exposure to...
combined visual and chemosensory cues from males led to hyperpolarization of membrane potential and decreased frequency of action potential firing. Females exposed to just male-primed holding water did not show this depression in electrical activity. We determined that this was not due to degradation of chemosensory signals, as we also placed females in tanks with the males but in the absence of visual inputs. Further work showed that exposure to just male visual signals replicated the inhibitory effects of the original social-cue response. The functional significance of this inhibitory visual-cue response remains to be determined.

Previous work in male cichlid fish indicates that sex hormones can mediate the effects of social cues on GnRH neuron physiology. Greenwood and Fernald (22) determined that nonterritorial reproductively regressed cichlid fish and territorial reproductively active cichlids showed differences in their electrical properties of hypothalamic GnRH neurons in response to current injection. However, it is unclear whether a steroid-based mechanism could explain the relatively rapid effect of male social/visual cues on inhibiting the electrical response of the female TN-GnRH neurons in the present study. In the social cue experiment, there was no obvious effect of male exposure on female gonadal function, with the caveat that we did not investigate sex steroid secretion.

Social cues have been shown to affect electrical activity of nonsensory neurons in fish (22), birds (24, 44), rodents (7, 42), and primates, including humans (17, 43, 45, 50). Our data show that visual cues from conspecifics in the absence of chemosensory inputs were sufficient to alter female TN-GnRH neuron activity to a similar extent as seen with exposure to “complete” social cues. The importance of visual cues in medaka is demonstrated by studies showing that females observing male mating behavior chose males that courted and spawned better (20). Furthermore, there is evidence from previous studies in fish that chemosensory signals alone do not necessarily regulate behaviors or neural activity. For example, work in medaka indicated that males did not show a preference for female-holding water over regular water (14). Also, stimulation of goldfish olfactory epithelium with identified sex pheromones did not alter the activity of neurons in the TN (18). It may be that while chemosensory inputs are important, they need to be in the appropriate context, and the visible presence of the male might be essential for modulating TN-GnRH neuron activity. It is also possible that the males are not releasing the entire cohort of pheromones when they cannot actually see the female. All of our experimental paradigms examined TN-GnRH neuron physiology after 24-h exposure to various social cues. It may be that chemosensory cues alone have maximal effects on TN-GnRH neurons at a time point prior to 24 h. Chemosensory information to the TN-GnRH neurons may also serve as modulators without causing significant changes in the type of electrical activity monitored in the present study.

Functional Significance of the Change in Electrical Activity

The TN-GnRH neurons have a beating pattern of action potential firing ranging from 1 to 6 Hz (36, 52). It has been suggested previously that the frequency of firing of these neurons would vary between fish based on their physiology and external sensory inputs (2). Here, we showed social/visual signals altering interspike Vm and the firing frequency of the TN-GnRH neurons. In most excitable cells, depolarization of membrane potential leads to increased frequency of action potential firing, while hyperpolarization leads to depressed firing frequency. In both dwarf gourami and medaka TN-GnRH neurons, the frequency of action potential firing is voltage dependent (36, 52). Therefore, it is likely that the male-induced hyperpolarization of Vm led to decreased firing frequency. These changes in firing frequency presumably transmit different neural signals to target cells or within neural networks.

The role of frequency modulation in beating or tonically spiking neurons has been addressed in different model systems. Tonically active neurons in the primate striatum respond to external stimuli and are thought to detect motivationally relevant events (4). Such tonic pathways receiving and responding to sensory information are also present in respiratory centers and the preganglionic vagal motoneurons (27). In rodents, neurons of the locus ceruleus show a similar range of tonic firing as seen in our study (12). Alterations in their tonic-firing frequency affected transmitter release, changed facilitation to sensory responses, and modulated the functional connections in their target networks (12). Anatomical evidence shows that TN-GnRH neurons project to the olfactory bulb, the optic tectum, the hypothalamus, and the spinal cord (36). Thus, the alteration in TN-GnRH neuron tonic firing frequency could be affecting numerous functional circuits in terms of their responses to sensory stimuli, baseline responses, network connectivity, and rate of secretion.

TN-GnRH neurons have been studied in detail with regard to their firing properties and morphological projections (2). However, their role in relation to physiology and behavior remains unclear. The present data indicate that social cues alter the
electrical activity of TN-GnRH neurons. But there was no indication that these changes in TN-GnRH neurons modulated medaka reproduction. In the present study, we investigated a limited number of physiological and behavioral outputs of the reproductive system (ovarian histology and egg laying). We cannot discount the possibility that changes in the electrophysiology of TN-GnRH neurons control aspects of physiology and behavior that were not monitored in our experiments. Suggested roles of TN-GnRH neurons in regulating physiology and behavior are largely circumstantial, and without direct evidential support. Earlier studies indicate that various brain regions receiving TN-GnRH neuron projections are modulated by both GnRH peptide and unidentified TN neurons. It has been reported that GnRH peptide modulates olfactory neuron responses in association with the mating season in mudpuppies (15, 59), and that GnRH modulates the peripheral olfactory system in axolotls (40). Cells in the TN have been shown to mediate the olfactory modulation of visual sensitivity in zebrafish (34). Studies have also shown a role for GnRH in the visual system. In fish, two different GnRH receptor subtypes are expressed on the retinal cells (23), and morpholino knockdown of GnRH disrupts eye development (55). GnRH altered dopaminergic effects on horizontal cells (6) and has been implicated in the control of light adaptation in the retina of an insect (5). GnRH has also been shown to facilitate postsynaptic currents in the optic tectum evoked by stimulating retinal fibers in fish (30). Altered GnRH release from TN-GnRH neurons could thus be modifying olfactory and/or retinal sensory inputs that can possibly affect a number of different physiological systems.

**Perspectives and Significance**

The work described here shows that the visual system, specifically visual cues from conspecifics, plays an important role in regulating the physiology of TN-GnRH neurons in female medaka. Previous studies suggested that TN-GnRH neurons play an important role in development and function of the visual system. Taken together, this opens up the possibility of a reciprocal or feedback relationship between the visual system and TN-GnRH neurons—that visual cues modulate the activity of a set of neurons that then modulate the function of components of the visual system. This physiological connection between visual signals and modulation of neural activity expands our understanding of the role of TN-GnRH neurons as an important neuromodulator that links the external environment to the central nervous system. The present findings provide further insight into the functions of GnRH, an ancient and conserved family of peptides that appears to serve important roles in general neural regulation, as well as specialized control of reproduction (49).

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