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Sex Steroids and Communication Signals in Electric Fish: A Tale of Two Species

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Key Words

Estrogen · Testosterone · Dihydrotestosterone · Electric fish · *Apteronotus* · EOD · Pacemaker neurons · Species differences

Abstract

Weakly electric fish are good model animals to study the evolution of interspecific and sexual differences in communication signals. This is because the neural circuits producing these signals are simple and conserved among related species while the signals are highly species-specific, sexually-dimorphic, and under hormonal control. Here we focus on two related species of weakly electric gymnotiform fish that emit a wave-type discharge. These species differ in the direction of the sexual dimorphism of their electric organ discharge (EOD) frequencies and their propensity to produce aggressive communication signals called 'chirps'. Brown ghost *(Apteronotus leptorhynchus)* males produce high frequency EODs while females produce low frequency EODs. When presented with an EOD mimic, males chirp frequently, while females seldom chirp. By contrast, black ghost *(A. albifrons)* males discharge at lower EOD frequencies than females, and there is no sex difference in chirping in this species. Accordingly, non-aromatizable androgens raise EOD frequency in brown ghosts, but lower it in black ghosts. Androgens induce chirping in female brown ghosts, but do not increase the propensity to chirp in female black ghosts. Thus, the difference in sexually-dimorphic communication signals between these two species can be explained by differences in their responses to sex steroids. Future studies will elucidate how the neural circuits generating these signals are differentially sensitive to steroids in these species.

Introduction

Gonadal steroid hormones are highly conserved among vertebrates where their primary role is to coordinate reproduction [Norman and Litwack, 1987]. An essential aspect of successful reproduction is mating with conspecifics and avoiding interspecific matings. To that end, many species have evolved species-specific communication signals that aid in reproductive isolation. It is paradoxical that while gonadal steroids are so highly conserved among vertebrates, the signalling behaviors that they trigger are not. An intriguing question, then, is how neural circuits generating reproductive signals have evolved in different species, especially closely-related species, to respond differently to the same hormonal stimulus.

This question is most easily addressed in species with well-defined hormonally-sensitive communication signals in which the neural circuits underlying those signals are

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understood and electrophysiologically accessible. In this paper we highlight the electromotor system of weakly electric fish as a superb system for this kind of analysis.

Brown and Black Ghosts Show Differences in the Direction and Extent of Sexual Dimorphisms

Brown ghosts *(Apteronotus leptorhynchus)* and black ghosts *(A. albifrons)* are in the family Apteronotidae, one of the most phylogenetically derived and successful families of gymnotiforms [Alves-Gomes et al., 1995; Albert et al., 1998; Dunlap et al., 1998]. Brown and black ghosts are the apteronotids most extensively studied in the laboratory because they are easily obtainable in the pet trade. However, fortuitous the choice of these species, they are interesting in that they show species differences in the direction and extent of sexual dimorphisms and in their responses to hormones.

The brown ghost is very sexually dimorphic in its body size and EOD frequency. Males are larger than females and have longer, more muscular snouts which are used for butting and biting each other during aggressive encounters. Male brown ghosts have higher EOD frequencies than females [Hagedorn and Heiligenberg, 1985; Meyer et al., 1987; Dulka and Maler, 1994; Zucker, 1997; Dunlap et al., 1998] (fig. 1). This is a case of ‘reverse sexual dimorphism’ in that males have lower EOD frequencies than females in the other wave-type genera that have been studied (i.e. *Sternopygus*, *Eigenmannia*), and this represents a phylogenetically older pattern [Alves-Gomes et al., 1995; Alves-Gomes, 1998; Dunlap and Zakon, 1998b]. However, unlike the ‘reverse’ sexual dimorphism observed in some bird groups in which females are larger, initiate courtship, and aggressively defend territories while males incubate the eggs (Hohn, 1969), brown ghost males show aggressive and reproductive behaviors typical of other piscine species.

The sexual dimorphism in EOD frequency likely signals the fish’s sex and, perhaps, its ‘quality’ or reproductive state. In a tank with fish of both sexes a dominance hierarchy is established among males: the largest male, who has the highest EOD frequency, becomes dominant [Hagedorn and Heiligenberg, 1985]. All the females in the tank mate with this male on different nights. It has not been explicitly tested whether this situation is the result of female choice or male-male competition. In either case, since the largest male has the highest EOD frequency, it is not known whether an assessment of the dominant male either by other males or by females is based on EOD frequency, other characteristics of the EOD such as amplitude or curvature of the electric field lines, or an independent measure of body size utilizing other sensory cues.

Black ghosts also show a pronounced sex difference in size and but less difference in body shape than brown ghosts (fig. 1). EOD frequency is sexually dimorphic in this species as well, but in the opposite direction from brown ghosts: male black ghosts have lower EOD frequencies than females [Dunlap et al., 1998]. As mentioned above, this is the typical pattern among other gymnotiform wave-species. However, EOD frequency appears to be less sexually dimorphic in black ghosts than in brown ghosts. One must be cautious in judging the extent of sexual dimorphisms in non-breeding fish in captivity. This is true of black ghosts which attain a large size in the wild (H. Zakon, pers. observ.). However, black ghosts show greater overlap in EOD frequencies of the two sexes even when fish of both species have comparable levels of plasma androgens (~5 ng/ml 11 ketotestosterone, ~10 ng/ml testosterone) and similar gonadosomatic indices [Dunlap et al., 1998c].

Chirping: Social Signals for Aggression and Courtship

Like other wave-type gymnotiforms, apteronotids modulate their EOD frequency during social encounters to signal motivational state. A number of EOD modulations have been described for apteronotids but we will only discuss ‘chirping’. Brown and black ghosts generate ‘pings’ or ‘chirps’ [Larimer and Macdonald, 1968; Bullock, 1969] which are transient increases in EOD frequency accompanied by a decrease in EOD amplitude. In a tank of freely-behaving brown ghosts, males make short chirps (~5–30 ms duration) at each other and at females during aggressive encounters [Hagedorn and Heiligenberg, 1985; Dunlap, unpubl. observ.]. Females seldom chirp under these conditions.

Male brown ghosts initially make short chirps at females during the initiation of courtship [Hagedorn and Heiligenberg, 1985] but, as courtship commences, the duration and intensity of the male’s chirps increases; courtship chirps may be over 200 ms long with frequency excursions of more than 200 Hz [Murray, 1995; J.T. Nickla and J. Dulka, pers. commun.].

Courtship chirping by the male is hypothesized to put the female into sexual receptivity and induce ovulation [Hagedorn and Heiligenberg, 1985]. During egg-laying, the female brown ghost lays on her side and expels a single large egg, which she does repeatedly at intervals of a few minutes, eventually laying 5–30 eggs in a night [Hagedorn and Heiligenberg, 1985]. As the male swims about the female

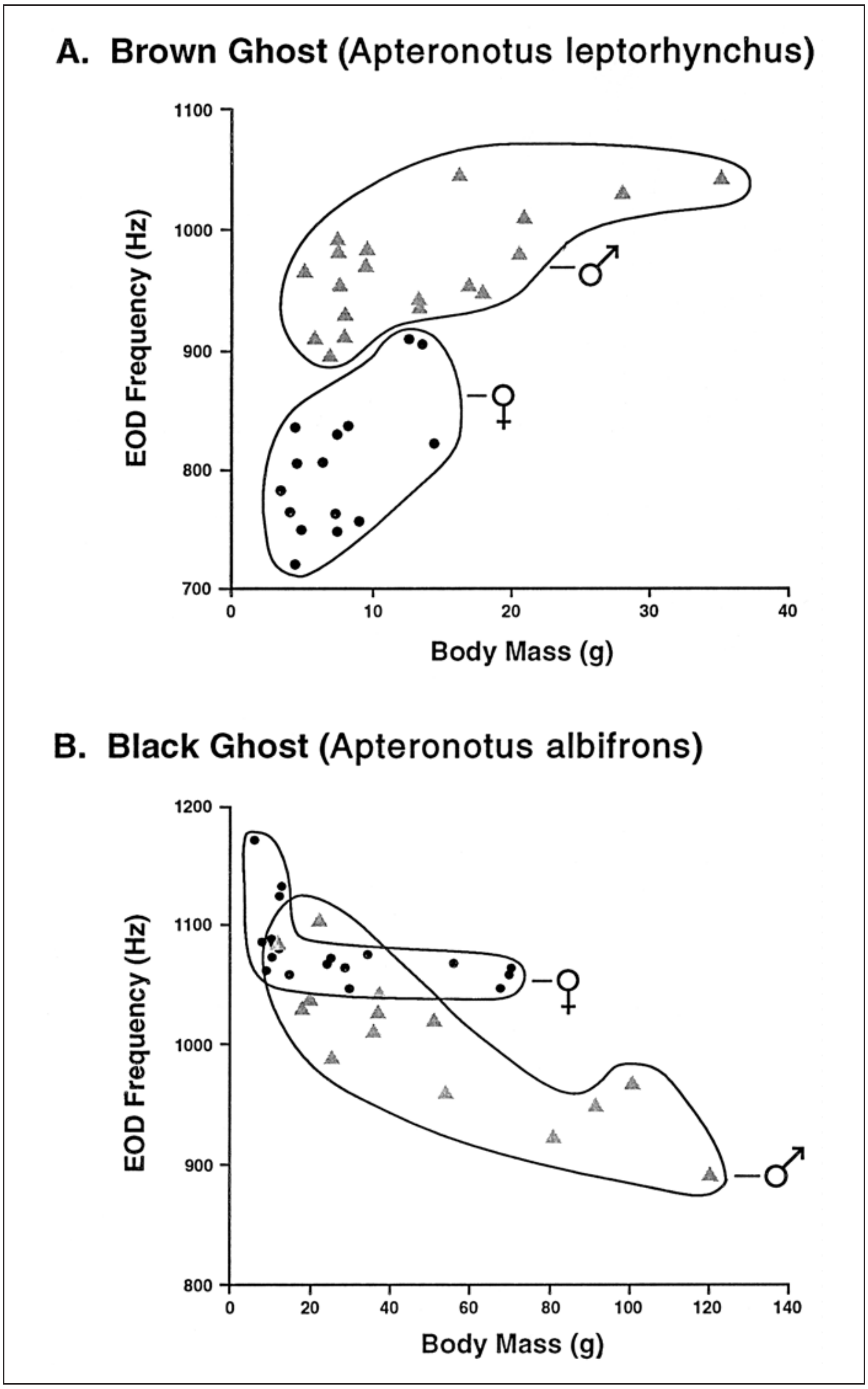

Fig. 1. EOD frequency as a function of body mass for both sexes of (**A**) the brown ghost *(A. leptorhynchus)* and (**B**) the black ghost *(A. albifrons).*

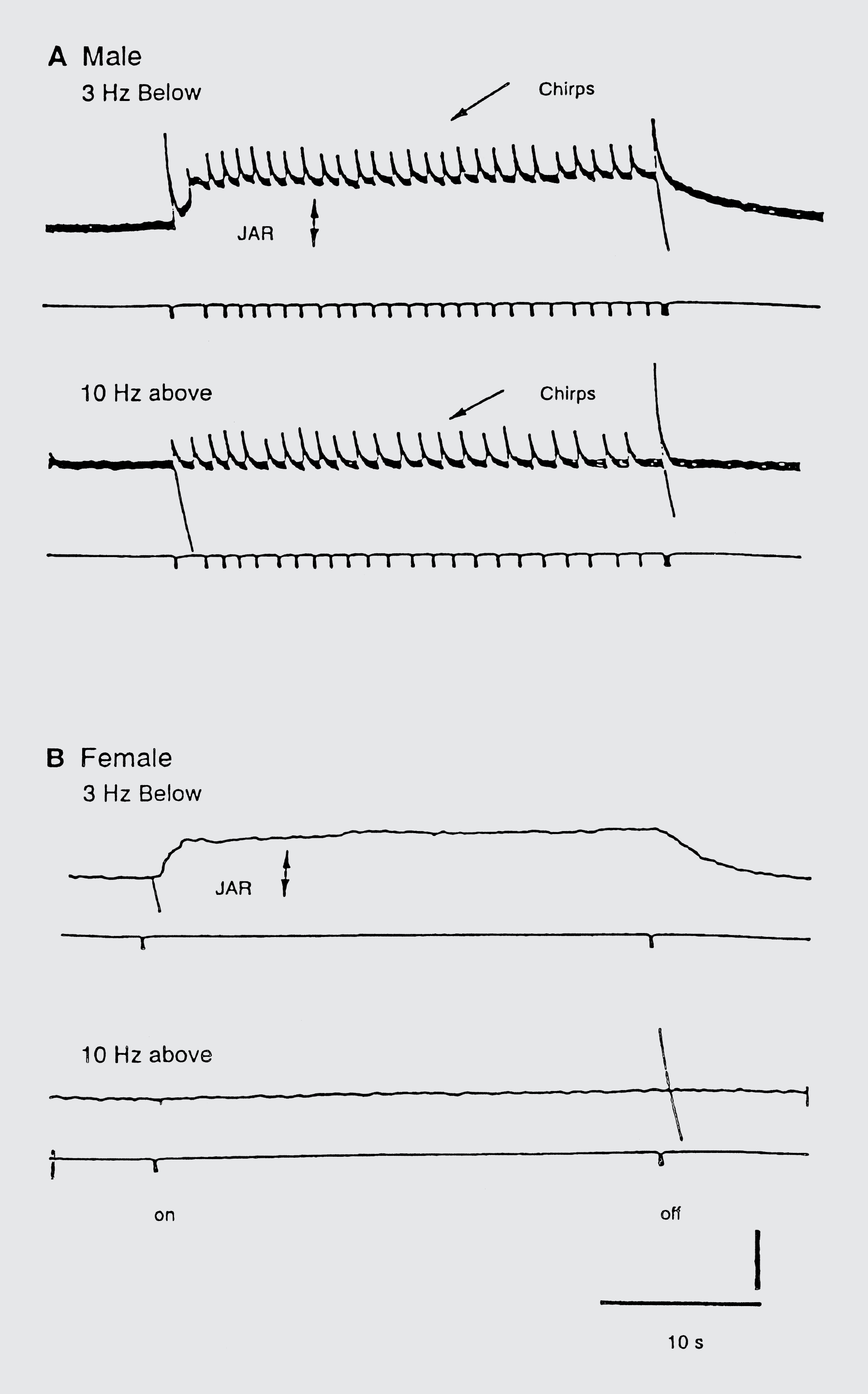

Fig. 2. Sex difference in chirping in brown ghosts. Male brown ghosts (**A**) chirp to an EOD mimic of either 3 Hz below or 10 Hz above its own EOD frequency and perform a jamming avoidance response (JAR), which is a rise in their baseline EOD frequency during the presentation of a mimic EOD a few Hz below their own. Females (**B**) do not chirp, but only show a JAR to 3 Hz below their own EOD frequency. From Dulka and Maler [1994].

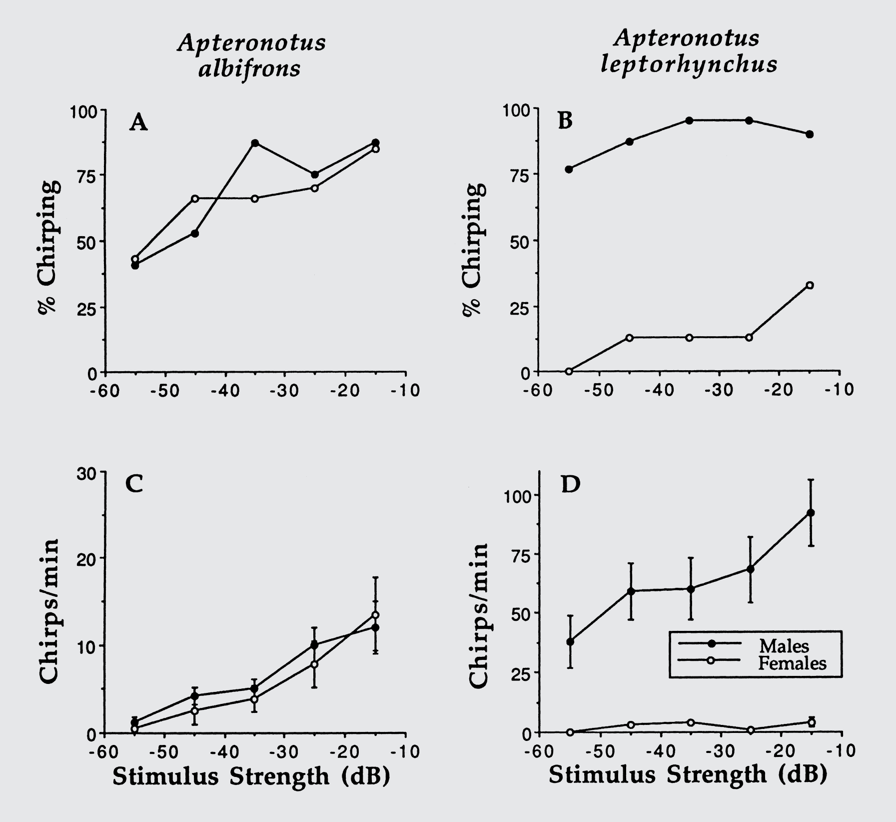

Fig. 3. Species- and sex-differences in chirping. Black ghost *(A. albifrons)* (**A**, **C**) show no sex difference in the percent of individuals chirping or the number of chirps produced to an EOD mimic 3 Hz below each fish's EOD frequency over a range of amplitudes (–55 dB = 0.07 mV/cm, –15 dB = 7 mV/cm). A sex difference in these parameters is very evident in brown ghosts *(A. leptorhynchus)* (**B**, **D**). From Dunlap et al. [1998].

making long-duration intense courtship chirps, the female draws the male toward her with a series of small short chirps (2–15 ms) each time she is about to expel an egg [Murray, 1995; J. Dulka, pers. commun.]. Thus, while chirping is most robust in the male, it is evident in reproductive females although the duration of the chirps are quite different.

Nothing is known about electrical signalling during courtship and breeding in black ghosts.

Aggressive chirping is more easily elicited than courtship chirping and has, therefore, been most frequently studied. A typical procedure to study aggressive chirping is to place a fish into a tube to keep it stationary so its EOD can be recorded, and then present it an EOD mimic (typically a sine-wave) via electrodes on either side of the tube for a period of 30 s or a minute. Despite this highly unnatural stimulus geometry, when a fish is given an EOD mimic near its own EOD frequency in a 'chirp chamber', it chirps in response, the number of chirps depending on stimulus amplitude (fig. 2, 3). Quite clearly, male brown ghosts chirp significantly more than females at all stimulus amplitudes [Dye, 1987; Zupanc and Maler, 1993; Dulka and Maler, 1994; Dunlap et al., 1998] (fig. 2, 3). Although the stimulus configuration is highly unnatural under these circumstances, this sex difference in chirping is confirmed in staged encounters in which a freely-swimming fish is given access to another fish behind a plastic mesh-screen [Dunlap and Zakon, 1998a].

Similar to brown ghosts, black ghosts chirp when presented with an EOD mimic. However, there is no sex difference in chirping: male black ghosts chirp less than male brown ghosts, but female black ghosts chirp more than female brown ghosts to comparable stimuli.

Thus, black and brown ghosts differ in the direction and extent of sex differences in EOD frequency and 'aggressive' chirping to sine-wave stimuli.

Steroid Hormones, EOD Frequency and Chirping

The profile of plasma steroids in both species is typical for teleosts. Males of both species have higher plasma levels of the androgen 11 ketotestosterone (11 KT) than females [Zucker, 1997; Dunlap et al., 1998]. Both sexes have similar levels of testosterone (T) in both species [Dunlap et al., 1998]. Female brown ghosts have higher levels of estrogen (E_2) than males; E_2 has not been measured in black ghosts.

In keeping with the lower EOD frequency of female brown ghosts, E_2 treatment lowers EOD frequency of gonadally-intact or gonadectomized fish of either sex [Meyer et al., 1987; Schaefer and Zakon, 1996]. Implantation of intact or gonadectomized brown ghosts with T also lowers EOD frequency. This effect is blocked by the aromatase inhibitor fadrozol, even in gonadectomized fish, suggesting that aromatization occurs in the brain [Zucker, 1997]. Treatment of intact females with aromatase inhibitor raises EOD frequency suggesting that ongoing aromatization of T to E_2 is responsible for the low EOD frequency in intact females. Treatment of fish with the non-aromatizable androgen 11 KT raises EOD frequency, again, as predicted from the direction of the sex difference in EOD frequency. Interestingly, DHT is reported to have no effect on EOD frequency in this species [Meyer et al., 1987; Dulka, 1997; but see Zucker, 1997]. This is true even when it induces chirping in the same fish (see below) and at blood levels that have large effects on EOD frequency in other gymnotid species [Dulka et al., 1995].

T and DHT induce chirping in females with about the same potency. However, while T- or DHT-implanted females chirp significantly more (~2–4 chirps/min) than intact or control-implant females ($\ll$1 chirp/min), their level of chirping is still much less than observed in an intact male stimulated at approximately the same stimulus intensity (>40–60 chirps/min) [Dulka et al., 1995; Dulka, 1997; Dunlap et al., 1998] (fig. 3).

One interpretation of this is that some presently unknown factor acts in concert with androgens to induce full masculinization. Precedent for this occurs in *Xenopus* in which full masculinization of the sexually-dimorphic larynx cannot be induced in adulthood when females are treated with androgen implants, but does occur with transplantation of testes [Watson and Kelley, 1992; Watson et al., 1993]. Another interpretation of these results is that the nervous systems of the two sexes are organized differently and therefore respond differently to androgens. It is possible, for example, that the female CNS is 'programmed' to respond to androgens by producing female-typical chirps that occur during egg-laying rather than a male-typical level of chirping as occurs during aggressive encounters [Dulka, 1997]. This would be an intriguing result since fish brains are much more influenced by steroid hormones in an activational than organizational fashion [Zakon, 1999]. These hypotheses could be tested by comparing the responses of castrated males and females to androgen replacement and testis transplantation.

Despite its influence on basal EOD frequency, 11 KT has little ability to induce chirping in females in a 'chirp chamber' [J. Dulka, pers. commun.]. Paradoxically, in a study utilizing staged encounters between two male brown ghosts, the chirp rates of males correlated with endogenous levels of 11 KT [Dunlap et al., 1998a]. In the latter study, no other steroids were measured so it is possible that 11 KT levels covaried with those of other steroids, such as T and DHT (T and 11 KT levels usually do covary in fish including gymnotiforms [Zakon et al., 1991] and that these steroids, rather than 11 KT, actually influence chirp rates.

The effects of E_2 on chirping have not yet been investigated. It will be interesting to determine whether E_2 actively inhibits chirping, whether it induces female-like chirps such as those produced during egg-laying, or whether it has no effect.

The lack of effect of DHT on EOD frequency and its potent action on chirping and the opposite effects of 11 KT on these parameters in brown ghosts suggest that the brain regions underlying these behaviors possess different isoforms of androgen receptors. Indeed, distinct isoforms that have these sensitivities have been discovered [Pasmanik and Callard, 1988; Sperry and Thomas, 1999]. Thus, one might predict that different isoforms of androgen receptors might be found in the cells responsible for controlling EOD frequency and those that generate chirping (see below).

Black ghosts resemble other gymnotiform species in which treatment with androgens lowers EOD frequency: their EOD frequency is lowered by T, DHT, and 11 KT [Dunlap et al., 1998]. In keeping with the lack of sex difference in chirping in this species, these same hormones had no effect on chirping in gonadectomized fish of either sex, even when blood levels of implanted fish were comparable to or exceeded slightly brown ghosts (see below).

The EOD-Generating Circuitry

The EOD is controlled by a midline nucleus in the ventral medulla called the pacemaker nucleus. This nucleus comprises only three neuronal cell types. About 100 pacemaker neurons, which are endogenously-active oscillatory neurons, are electrotonically-coupled to each other and to a second cell type called relay cells. The relay cells, which are driven by the pacemaker neurons, are the output neurons of the nucleus sending their axons down the spinal cord. In addition, there are small newly-discovered interneurons which seem to be glycinergic cells that receive electrotonic inputs, presumably from pacemaker or relay cells, and make chemical synapses on both cell types [Turner and Moroz, 1995; G.T. Smith, Y. Lu, and H. Zakon, unpubl. observ.]. The function of this cell is unknown although likely roles are to tonically influence EOD frequency or act like a 'brake' helping to terminate chirps.

The axons of the relay neurons innervate spinal electromotoneurons (EMNs) [Bennett et al., 1967]. In all other gymnotiforms, the EMNs make cholinergic synapses on myogenically-derived electrocytes. The Apteronotidae are unique in that the myogenic electric organ degenerates during their juvenile development and is replaced by a neurogeneic electric organ. In the older juveniles and adults, the axons of the EMNs fasciculate in the periphery and comprise the electric organ. They possess specialized nodes of Ranvier that underlie this function [Waxman et al., 1972; Kirschbaum, 1983].

Intracellular recordings of PMN neurons in in vitro slice preparations show that they fire spontaneously at rates related to the EOD frequency of the fish from which they came. In other words, PMN neurons fire at lower frequencies in females than in males. The firing rate of the PMN neurons, then, is a sexually-dimorphic and individually distinct character.

The EMNs do not spontaneously fire in most other groups of gymnotiforms; in the Apteronotidae, however, they are spontaneously active at high rates [Schaefer and Zakon, 1996]. This is likely because it is more effective for the PMN to entrain EMNs that are spontaneously active at or close to EOD frequency rather than to have to depolarize them cycle by cycle when they must maintain such unusually high firing rates.

Modulations of the basic EOD frequency are generated by inputs to the pacemaker nucleus from the prepacemaker nucleus (PPn) and the sublemniscal prepacemaker nucleus which densely cover relay and pacemaker neurons with glutamatergic chemical synapses. The input from one division of the PPn, the PPnC (for 'chirping'), is responsible for chirping. Activation of this input depolarizes relay cells via AMPA receptors and thereby transiently increases EOD frequency during a chirp [Kawasaki and Heiligenberg, 1988, 1989, 1990; Metzner, 1993; Juranek and Metzner, 1997]. Presumably, chirp duration is determined by the length of time that the input from the PPnC is active; this suggests that the PPnC input behaves differently in males and females during spawning since males produce chirps of tenfold longer duration than females.

How Do Hormones Modulate EOD Frequency?

Since steroids influence EOD frequency it is reasonable to imagine that the pacemaker neurons possess nuclear steroid receptors. In the only study on the distribution of androgen receptors in the brown ghost brain, none were found in the pacemaker nucleus [Zucker, 1997]. However, it is not certain whether these cells are truly devoid of nuclear androgen receptors, whether there are different isoforms of the receptor, or whether they are present but below the level of detection.

Physiological observations suggest that the pacemaker neurons are influenced by steroids. When fish are implanted with either 11 KT or E_2 for two weeks and the pacemaker nucleus is removed and put in slice, the pacemakers of 11 KT-implanted fish fire at higher frequencies and those from E_2-implanted fish fire at lower frequencies than controls [Schaefer and Zakon, 1996]. This implies that neurons in the pacemaker nucleus are altered by steroid treatment.

In the Apteronotidae, the EMNs are oscillators and they are influenced by hormones as well. Fish can be given a spinal cord transection, which removes the descending input from the pacemaker nucleus onto the EMNs, and then implanted with steroid hormones. EMNs in 11 KT-treated fish fire at a higher frequency when recorded in a slice preparation two weeks after hormone implantation, and those in E_2-implanted fish fire at a lower frequency [Schaefer and Zakon, 1996]. This indicates that hormones influence the EMNs without the mediation of the PMN, and suggests that the EMNs themselves are targets of hormone action.

How do steroids alter the firing frequency of the pacemaker neurons and EMNs? The first stage of answering this question is to identify their ion currents. Recent studies indicate that pacemaking activity in the PMN is initiated by the interaction of the depolarizing effects of a persistent TTX-sensitive Na^+ current and a low-threshold T-type Ca^{2+} current, and the hyperpolarizing influence of a rapid K^+ current [Dye, 1991; G.T. Smith and H. Zakon, unpubl. observ.]. The next step is to study the amplitudes and dynamics of these currents under voltage clamp to see how they differ in cells firing at different frequencies, and how these currents are modulated by steroids.

How Do Hormones Modulate Chirping?

Steroids might modulate chirping by acting either on neurons within the PPn or its inputs. Besides its inputs from thalamic electrosensory areas, the PPn receives strong input from hypothalamic and limbic areas [Wong, 1997]. One source of input to this nucleus is a substance P-like input, presumably from cells in the lateral hypothalamus. This input is believed to influence chirping behavior because substance P like-immunoreactivity (SPl-ir) levels are low in females and high in males [Dulka et al., 1995; Weld and Maler, 1992] and injection of substance P into the region of the PPn elicits chirping [Weld et al., 1991]. The PPn also possesses high levels of tachykinin receptors (substance P is in the tachykinin family) [Weld et al., 1994].

Implantation of females with T or DHT increases the level of SPl-ir in the PPn [Dulka et al., 1995]. Interestingly, neither of these steroids brings the level of labeling up to that observed in intact males, which agrees with the inability of these steroids to fully masculinize female chirping behavior. It will be intriguing to determine how the levels of SPl-ir are influenced in parallel with chirping behavior in the experiments outlined above to test for organizational effects vs. additional factors acting together with steroids in generating these sex differences.

Androgen receptors were not detected in the brown ghost lateral hypothalamus [Zucker, 1997], although they are observed there in *Sternopygus* [S. Gustavson and H. Zakon, pers. observ.]. Interestingly, androgen receptors were noted in the central posterior nucleus of the thalamus. The central posterior nucleus is adjacent to the PPn and considered to be functionally continuous with it [Zupanc and Heiligenberg, 1992]. The central posterior nucleus is found in many teleosts where it is involved in a variety of reproductive behaviours [Demski and Dulka, 1986]. It is possible that the PPn is an 'extension' of this nucleus in weakly electric fish. The extent to which steroidal modulation is directly on neurons in the PPn or via its inputs must still be resolved.

Nothing is known about SPl-ir in black ghosts. Does the lack of a sex difference in chirping in black ghosts correspond to a lack of a sex difference in the density of the SPl-ir input to the PPn?

Questions for the Future

We have come a long way in understanding how the simple circuitry that controls EOD frequency is modified by steroids. A deeper understanding on the cellular level will entail a careful analysis of the ion currents in pacemaker cells, relay cells, and EMNs, and the role of the small interneurons. A key point for a comparative analysis is to understand how hormones 'push' the EOD frequency of brown and black ghosts in opposite directions. For example, do androgens regulate a depolarizing current (Na^+, Ca^{2+}) in brown ghosts and a hyperpolarizing current (K^+) in black ghosts, or do they act on the same current but increase it's magnitude in one species and decrease it in the other?

A comparative analysis of the neural and hormonal mechanisms that control chirping in brown and black ghosts holds great promise. Besides understanding how sex differences in chirping come about in brown ghosts, we must also learn how they do not occur in black ghosts. Furthermore, we have no idea of the function of chirping in black ghosts. Perhaps the lack of sex difference in black ghosts relates to a change of function of chirping. Learning about the social and reproductive behavior of this species will provide a context for interpreting the physiology.

From an evolutionary perspective, one advantage of working with brown and black ghosts is that they are closely related and their phylogenetic relation to other gynotiforms is well-described. This allows for comparative work that will indicate how many different apteronotid species show the basal gymnotiform pattern of male EOD frequency lower than female, or how many show the brown ghost pattern of 'reverse' sexual dimorphism of EOD frequency. Has 'reverse' sexual dimorphism evolved once or multiple times in the Apteronotidae?

Our hope is that with detailed knowledge of the neurons involved in each behavior, the ion channels and neurotransmitter receptors of each neuron, the type of steroid receptors and which channels they modulate, and a complete gymnotiform phylogeny, we will be able to understand on a very reductionistic level how sexual dimorphisms have evolved and how the same steroids come to activate very different behaviors in closely related species.

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